



The D₃-creatine dilution method non-invasively measures muscle mass in mice

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Abstract

Developing accurate methods to quantify age-related muscle loss (sarcopenia) could greatly accelerate development of therapies to treat muscle loss in the elderly, as current methods are inaccurate or expensive. The current gold standard method for quantifying sarcopenia is dual-energy X-ray absorptiometry (DXA) but does not measure muscle directly—it is a composite measure quantifying “lean mass” (muscle) excluding fat and bone. In humans, DXA overestimates muscle mass, which has led to erroneous conclusions about the importance of skeletal muscle in human health and disease. In animal models, DXA is a popular method for measuring lean mass. However, instrumentation is expensive and is potentially limited by anesthesia concerns. Recently, the D₃-creatine (D₃Cr) dilution method for quantifying muscle mass was developed in humans and rats. This method is faster, cheaper, and more accurate than DXA. Here, we demonstrate that the D₃Cr method is a specific assay for muscle mass in mice, and we test associations with DXA and body weight. We evaluated the D₃Cr method compared to DXA-determined lean body mass (LBM) in aged mice and reported that DXA consistently overestimates muscle mass with age. Overall, we provide evidence that the D₃Cr dilution method directly measures muscle mass in mice. Combined with its ease of use, accessibility, and non-invasive nature, the method may prove to more quickly advance development of preclinical therapies targeting sarcopenia.

KEYWORDS

aging, mice, sarcopenia, skeletal muscle

Abbreviations: BM, bone mass; CT, computed tomography; D3Cr, D3-Creatine; DXA, dual x-ray absorptiometry; FBM, fat body mass; LBM, lean body mass; MM, muscle mass; MRI, magnetic resonance imaging.

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1 | INTRODUCTION

Skeletal muscle makes up the largest fraction of tissue in the body (Wang et al., 1992), though the accurate quantification of skeletal muscle mass remains elusive. Accurate quantitation of skeletal muscle is vital for studying sarcopenia, an age-associated syndrome defined as the gradual loss of muscle mass resulting in functional decline, disability, and loss of independence. Quantification of muscle size with imaging using computed tomography (CT) and magnetic resonance imaging (MRI) is available; however, these methods are expensive, slow, and impractical in large cohort studies. Both methods are powerful tools for the characterization of body composition, though they have drawbacks such as ionizing radiation and necessary special expertise to infer muscle mass (Meganck & Liu, 2017). These limitations provide a rationale for alternative muscle mass quantification methods.

Dual x-ray absorptiometry (DXA) has emerged as the most commonly used method for body composition analysis. DXA uses a three-compartment categorization method: Bone and fat mass are separately quantified through the differential absorption of high and low photon energies, and then, lean mass (all remaining tissues excluding fat and bone) is differentially calculated. The method's popularity results from its relatively quick and easy measurement, low radiation dose, and precision. However, its use is often limited due to instrumentation availability and cost. Although DXA is used as a surrogate for muscle, it does not measure muscle mass.

A method for the non-invasive measurement of muscle mass was recently reported (Clark et al., 2014). The D₃-creatine (D₃Cr) dilution method is non-invasive, accurate, and has the potential to become the new gold standard for quantifying muscle mass in preclinical models (Stimpson et al., 2012). Isotopically labeled creatine had been previously employed to determine creatine pool size in humans, with positive correlations to functional capacity and risk of injurious falls disability, and mortality (Cawthon et al., 2019, Cawthon et al., 2021, Cawthon et al., 2022). This correlation is noticeably absent in the literature of similar studies using DXA (Schaap et al., 2013). The D₃Cr method has also been reported to accurately measure age-associated muscle wasting in rats (Stimpson et al., 2012) but has remained undeveloped in mice, the most commonly used preclinical model.

In our study, we assess the use of the D₃Cr dilution method to quantify muscle mass in mice. We tested a single dosage (2mg/kg) of methyl-D₃ labeled creatine and repeatedly collected urine post-IP to determine appropriate sampling time windows. Mass spec analysis of urinary creatinine enrichment allowed for quantification of skeletal muscle mass, which was able to be directly compared with total body weight and respective DXA results.

The D₃ method relies on the assumption that urine creatinine is solely derived from the total body creatine pool (about 98% of which is sequestered in the sarcomere) (Clark et al., 2014). To accurately measure creatine and creatinine ratios (Cr/Crn) in urine, it is important to identify all potential exogenous sources of these metabolites, as dietary creatinine can confound the quantification of

urine creatinine enrichment with deuterium. Mice in our study were all purchased from Jackson Laboratories and reared on a low-fat diet (Envigo Teklad #2918) free of animal products or potential dietary Cr/Crn. Therefore, all Crn detected in urine of mice in this study is derived from endogenous production or administered D₃Cr, primarily metabolized in muscle.

To determine whether our dosage of D₃Cr was sufficient to measure muscle mass, we carried out a pilot study in young C57BL/6J mice (5 months old, males and females, Jackson Laboratories #000664). These initial studies used a single intraperitoneal (IP) injection of D₃-creatine at 2mg/kg (mpk) of body weight. This dosage was chosen based on prior studies that mice demonstrate increased creatinuria and findings that higher doses (>4mpk) of injected creatine were necessary to elevate plasma creatine levels (AA & AE, 1958). Unfasted mice received a single injection of D₃Cr, and urine was collected at 0, 2, 6, 12, 24, and 48h post-IP. Sequential urine collections were used to determine the optimal window to capture urinary D₃Crn enrichment, which reflects the percentage of converted D₃-labeled creatine to creatinine in the total creatine pool. Similar to previous reports in rats (Stimpson et al., 2012), urinary creatinine enrichment was highest at 6h post-IP, and steady state enrichment was achieved by 24h (Figure 1a). Steady state enrichment was still observed at 48h, though it is undetermined within our study how long this steady state may last in mice. Consistent with prior literature, we assume that once steady state enrichment is achieved, the enrichment of urine D₃-creatinine will match the enrichment of intramyocellular D₃Cr as a result of the irreversible conversion of creatine to creatinine, reported to be ~2% per day (Heymsfield et al., 1983).

Total creatine pool size of each pilot sample was calculated from D₃-creatinine enrichment at 24h post-intraperitoneal (IP) injection (Figure 1b) using a previously reported formula for determining pool size based on the enrichment of a tracer, assuming that 98% of total body creatine is sequestered in skeletal muscle (Andrews et al., 1998). From the creatine pool size, skeletal muscle mass was derived by dividing creatine pool size by 4.3g/kg (the average concentration of creatine in wet muscle mass (Andrews et al., 1998), Figure 1c). Both creatine pool size and muscle mass are plotted with and without spillage correction, calculated using Cr/Crn values at 2, 6, and 12h (Shankaran et al., 2018). Due to the insubstantial contribution to total muscle mass, spillage correction was not calculated in further studies.

After deducing the appropriate sampling window, we explored whether the D₃Cr method could be used to detect age-associated muscle loss in a cross-sectional analysis of mice ranging from 5 to 25 months of age. Reports in humans demonstrate that the D₃Cr method is sufficient to observe age-associated muscle loss (Pirker & Katzenschlager, 2017) but has been widely unexplored in murine models. As per our initial pilot studies, mice were purchased from Jackson Laboratories and reared on Cr/Crn-free diets. Unfasted mice were subjected to a single IP injection of 2mpk body of D₃Cr followed by urine collections at 24h post-IP injection. Respective body composition was determined by DXA in the same week.

It is well established that mice on multiple genetic backgrounds gain weight as they age (Pappas & Nagy, 2019; Evans et al., 2021). This is unlike humans who tend to reach a plateau of total body weight while concurrently losing muscle at later ages (Alley et al., 2008). Here, we demonstrate age-associated changes in body composition using a

cross-sectional study design with muscle quantification performed both by DXA and D₃Cr dilution method. Consistent with prior studies in mice, we observed that mice had higher body weights with increasing age ($p=0.0003$, one-way ANOVA) (Figure 2a) and increased percentages of DXA-determined fat body mass (FBM) ($p=0.0033$,

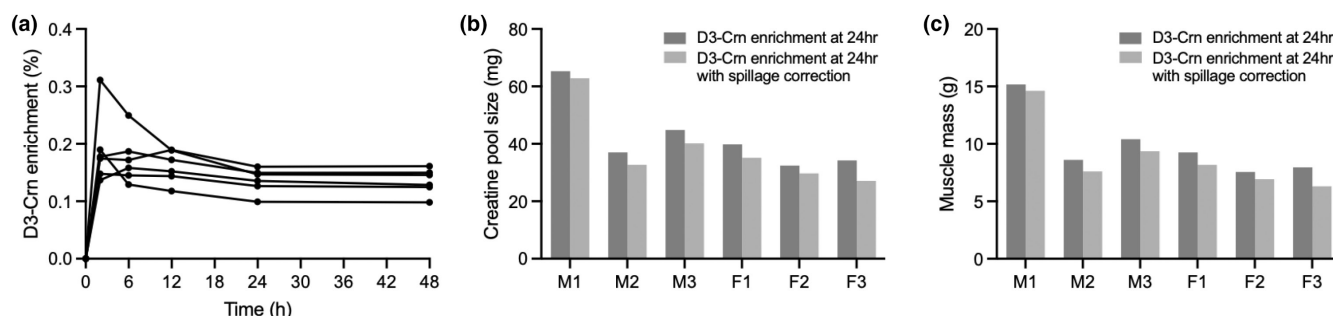


FIGURE 1 Creatine pool size and muscle mass can be calculated from mouse urine by D₃-creatinine enrichment following an IP injection of D₃-labeled creatine. (a) D₃-creatinine enrichment values at varying timepoints from individual urine samples of young mice following an IP injection of D₃-labeled creatine. (b) Creatine pool size (mg) values of individual mice calculated using their respective D₃-creatinine enrichment at 24 h. Bars represent creatine pool sizes either uncorrected or corrected with spillage effects observed at 0, 2, 6, and 12 h post-IP. (c) Muscle mass (g) values of individual mice calculated using their respective D₃-creatinine enrichment at 24 h. Bars represent creatine pool sizes either uncorrected or corrected with spillage effects observed at 0, 2, 6, and 12 h post-IP. "M#" individual male mice, "F#" individual female mice.

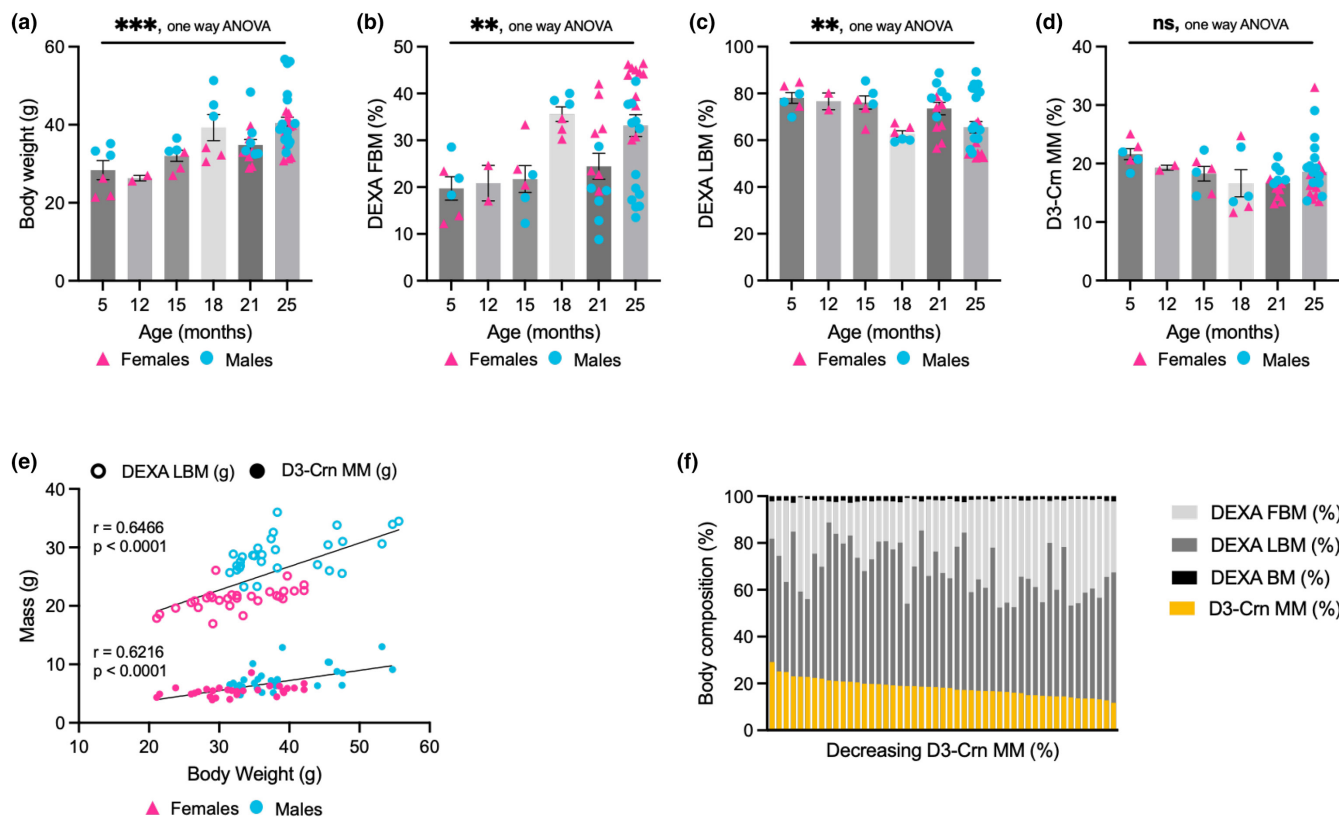


FIGURE 2 Cross-sectional analysis of aged mice revealed lower DXA-determined lean mass and increased fat mass independent of D₃Cr determined skeletal muscle mass. (a) Body weights of mice, grouped by age. (b) DXA-determined fat body mass (FBM) percentages, grouped by age. (c) DXA-determined lean body mass (LBM) percentages, grouped by age. (d) D₃Cr dilution method calculated muscle mass (D₃Cr MM) percentages, grouped by age. (e) Both DXA LBM and D₃-Crn MM correlate significantly with total body weight. (f) Decreasing skeletal muscle mass determined by D₃-Crn contrasted with DEXA in mice of varying ages. BM, bone mass, FBM, fat body mass, LBM, lean body mass, D₃-Crn MM, skeletal muscle mass as determined by D₃Cr dilution method. Significance – ns $p>0.05$, * $p<0.05$, ** $p<0.005$, *** $p<0.0005$.



one-way ANOVA) (Figure 2b). Despite their increased total body weight, aged mice had lower lean body mass (LBM) percentages ($p=0.0094$, one-way ANOVA) (Figure 2c) indicating an age-associated loss of LBM, typically attributed to muscle mass (Volpi et al., 2004). However, excluding fat, DXA cannot distinguish between non-muscle and muscle (Shepherd et al., 2017). We explored whether the D_3 Cr method would demonstrate decreases in skeletal muscle mass, consistent with their DXA scans. Interestingly, D_3 Cr determined muscle mass (D_3 Cr MM) was not statistically altered with increasing age (5, 12, 15, 18, 21, 25 m.o., $p=0.1950$, one-way ANOVA) (Figure 2d).

Both DXA determined LBM and D_3 Cr muscle mass were significantly correlated with body weight ($R^2=0.4181$ ($p<0.0001$), $R^2=0.3864$ ($p<0.0001$)), respectively (Figure 2e), regardless of age or sex. Evaluation of LBM by DXA showed a larger total mass than D_3 Cr MM, possibly reflecting non-muscle tissue as a component of LBM, as previously suggested. Correlation between DXA LBM and D_3 Cr MM was significant, though skewed largely by sex (Figure S1A–C); further analyses were performed by separating males and females and produced statistically insignificant results ($p=0.1137$ and 0.5290 , respectively). This finding demonstrates conflicting data for which method may be accurately determining skeletal muscle mass. Weights of individual hindlimbs muscles taken at the time of dissection were found to be statistically unchanged between age groups (Figure S1E), supporting data derived from the D_3 Cr method. Additionally, the magnitude of signal detected in both tested methods is worth emphasizing—DXA attributes 60%–80% LBM per mouse, while as D_3 Cr was ~15%–30% MM, more consistent with relative proportions of muscle mass in many mammals (Figure 2f). This suggests that the majority of signal in LBM is not derived from skeletal muscle, and the age-associated loss being quantified may be loss of another tissue type, unexplored within our study.

Our work highlights the overestimation of DXA-estimated muscle mass by direct comparison with D_3 Cr derived skeletal muscle mass. We demonstrate the use of the D_3 Cr dilution method in mice and apply mass spec analysis to confirm creatinine enrichment and steady state, though there are limitations to its use, and these should be considered when determining which method to use to calculate muscle mass. The D_3 Cr dilution method directly analyzes whole body muscle mass, which is made up of varying fiber types, but the formula used for the calculation of creatine pool size (and skeletal muscle mass) assumes 100% glycolytic (type II) fibers. This assumption negates differing creatine uptake rates in oxidative (type I) fibers and impedes studies in aging mice which are characterized to have altered fiber quality with age (Giacomello et al., 2020). The D_3 Cr dilution method may determine total body muscle mass but cannot reflect age-associated changes in muscle quality. Further limitations lie in our cross-sectional study design, as each animal undergoes individualized rates of aging. Our study design used both sexes and increasing cohort size with age, assumed to have increased variability (>21 months). Our study allowed for timely assessment of the D_3 Cr dilution method across ages, but further longitudinal studies are necessary to more accurately address the nature of sarcopenia phenotypes in aging mice. In conclusion, we demonstrate an alternative

method for non-invasively measuring total skeletal muscle mass in mice with direct comparisons to field standards. It will be important to use this methodology in tandem with other methods for future validation studies as well as measuring muscle mass in future pre-clinical studies of sarcopenia, utilizing longitudinal studies to better understand how muscle changes with age.

2 | METHODS

2.1 | D3-creatine

D3-creatine was purchased from Cambridge Laboratories (DLM-1302-0.25) (LOT#PR-30122) (PSO#22A-0183). Solution was made to 0.6 mg/mL in PBS for a final delivery of 2 mg/kg of body weight.

2.2 | Mice

Studies included the use of male and virgin female C57BL/6J mice (Jackson Laboratories #000664) aged between 20 and 99 weeks of age. All mice were communally housed and aged-matched with ad libitum access to water and diet (Envigo Teklad #2918) in a pathogen and temperature-controlled room with a 12 h light–dark cycle beginning at 06:00 AM. All procedures were conducted in accordance with NIH Guidelines for Care and Use of Animals and were approved by the Institutional Animal Care and Use Committees at Buck Institute for Research on Aging.

Mice: Our N for young mice (5 months) was 6 (3 M/3F), for adult mice (12, 15, and 18 months) was 6 (3 M/3F), and for aged mice (21 months) was 13 (6 M/7F) and 22 (25 months) (11 M/11F), respectively.

2.3 | Exogenous creatine

Mice were maintained on a low-fat chow diet (Envigo Teklad #2918) free of animal products or exogenous creatine to prevent cross contamination of measured creatine.

2.4 | Body composition testing

Dual-energy x-ray absorptiometry (DXA) scans were used to quantify body composition in anesthetized (2% isoflurane) immobilized mice by InAlyzer 2S (Micro Photonics). DXA scans were performed during the same week as D_3 -Cr injections.

2.5 | D3-creatine dilution protocol

The day of the study, experimental mice were weighed before being administered a single dose of 2 mg/kg body weight D_3 -creatine by intraperitoneal injection. Following the injection, mice were placed



back into their cage with ad libitum access to food and water. Urine samples were collected 2, 6, 12, 24, and 48 h post-dosing and stored at -80°C .

2.6 | Assessment of urinary D3-creatinine

Mouse urine was stored at -20°C until analyzed for D₃-creatine within the Nutrition Science and Toxicology Department / Hellerstein Laboratory at UC Berkeley. Urine samples were processed for D₃-creatinine enrichment, as well as creatine and creatinine concentrations to determine Cr/Crn ratio using methods described in detail elsewhere (Clark et al., 2014; Shankaran et al., 2018). Briefly, samples and standards were mixed with an internal standard of D5-labeled Cr and Crn, proteins were precipitated by the addition of acetonitrile followed by centrifugation, and aliquots of the supernatant were diluted in acetonitrile for liquid-chromatography mass-spectrometry (LC/MS) analysis. Mass spectrometry was performed on a Sciex 6500 QTRAP (AB Sciex LLC, Framingham, MA) operating in multiple reaction monitoring mode. For determining the Cr and Crn concentration from which the Cr/Crn ratio was calculated, transitions (132.1/44.1) and (114.1/44.1) were monitored. Quantitation was performed using an external standard curve using an internal standard mixture of D5 labeled Cr and Crn following transitions (137.1/47.1) and (119.1/47.1). For determining the enrichment of D₃-Crn, quantitation was performed using a standard curve for D₃-Crn and D₃-Cr enrichment that spanned from 0% to 0.577% and measured multiple reaction monitoring transitions (114.1/44.1) corresponding to the M0 peak of Crn and 117.1/47.1 which corresponds to D3-Crn.

2.7 | Statistical analysis

Statistical analysis across all age groups was assessed by one-way ANOVA, while unpaired analyses between specific age groups were assessed by t-test. XY plot analyses were assessed by simple linear regression, and correlation values were calculated using Pearson's Correlation test. For gait analyses, XY plot analyses were assessed either by simple linear regression or second order polynomial (quadratic) nonlinear regression [Step cycle]. Significance is reported as follows: ns $p > 0.05$, * $p < 0.05$, ** $p < 0.005$, *** $p < 0.0005$.

AUTHOR CONTRIBUTIONS

SM, WE, and MH involved in conceptualization and supervision. LW, SS, MH, WE, and SM drafted the manuscript. LW, NE, and SS involved in analysis and visualization. EG, SG, EN, SS, AD, LP, MCM, and CP involved in experimental procedure. SE and NS provided operational support. All the authors have read and agreed to the final version of the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest. Drs Hellerstein and Evans are listed as co-inventors on the filed patents for the D₃-creatine dilution method; however, they do not derive any income related to commercial use of this method nor do they control the intellectual property.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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