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Randomized Control Trials

# Short-term intermittent fasting and energy restriction do not impair rates of muscle protein synthesis: A randomised, controlled dietary intervention☆

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SUMMARY

*Background:* Intermittent fasting (IF) is an effective energy restricted dietary strategy to reduce body and fat mass and improve metabolic health in individuals with either an overweight or obese status. However, dietary energy restriction may impair muscle protein synthesis (MPS) resulting in a concomitant decline in lean body mass. Due to periods of prolonged fasting combined with irregular meal intake, we hypothesised that IF would reduce rates of MPS compared to an energy balanced diet with regular meal patterns.

*Aims:* We assessed the impact of a short-term, ten days, alternate day fasting or a continuous energy restricted diet to a control diet on integrated rates of skeletal MPS in middle-aged males with overweight or obesity.

*Methods:* Twenty-seven middle-aged males with overweight or obesity (age: 44.6 ± 5.4 y; BMI: 30.3 ± 2.6 kg/m$^2$) consumed a three-day lead-in diet, followed by a ten-day controlled dietary intervention matched for protein intake, as alternate day fasting (ADF: 62.5 energy (En)%, days of 25 En% alternated with days of 100 En% food ingestion), continuous energy restriction (CER: 62.5 En%), or an energy balanced, control diet (CON: 100 En%). Deuterated water ($D_2O$) methodology with saliva, blood, and skeletal muscle sampling were used to assess integrated rates of MPS over the ten-day intervention period. Secondary measures included fasting plasma glucose, insulin, and gastrointestinal hormone concentrations, continuous glucose monitoring, and assessment of body composition.

*Results:* There were no differences in daily rates of MPS between groups (ADF: 1.18 ± 0.13, CER: 1.13 ± 0.16, and CON: 1.18 ± 0.18 %/day, $P > 0.05$). The reductions in body mass were greater in ADF and CER compared to CON ($P < 0.001$). Lean and fat mass were decreased by a similar magnitude across groups (main time effect, $P < 0.001$; main group effect, $P > 0.05$). Fasting plasma leptin concentrations decreased in ADF and CER ($P < 0.001$), with no differences in fasting plasma glucose or insulin concentrations between groups.

*Conclusion:* Short-term alternate day fasting does not lower rates of MPS compared to continuous energy restriction or an energy balanced, control diet with matched protein intake. The prolonged effects of IF and periods of irregular energy and protein intake patterns on muscle mass maintenance remain to be

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**List of abbreviations and their definitions:**

| | |
|---|---|
| ADF | Alternate day fasting |
| AUC | Area under the curve |
| ANOVA | Analysis of variance |
| BM | Body mass |
| BMI | Body mass index |
| CON | Energy balance control diet |
| CER | Continuous energy restriction |
| CV | Coefficient of variation |
| D2O | Deuterated water |
| DXA | Dual-energy X-ray absorptiometry |
| FSR | Fractional synthesis rate |
| IF | Intermittent fasting |
| MPE | Mole percent excess |
| MPS | Muscle protein synthesis |
| VAS | Visual analogue scale |
| CGM | Continous glucose monitoring |
| GLP-1 | Glucagon like protein-1 |
| LM | Lean mass |
| FM | Fat mass |
| MPB | muscle protein breakdown |
| MVPA | Moderate and vigorous physical activity |
| REE | Resting energy expenditure |
| PA | Physical activity |
| PYY | Peptide-YY |

## 1. Introduction

A sustained energy deficit, achieved through a reduction in energy intake and/or an increase in energy expenditure, is a prerequisite for the loss of body mass (BM). However, BM loss during dietary energy restriction results in a concomitant loss of lean mass (LM) [1,2] which reduces muscle strength, impairs mobility, and increases the risk of developing chronic metabolic disease. Therefore, a critical health issue during diet-induced weight loss is how to maximise the loss of fat mass (FM) while concomitantly preserving LM.

Maintenance of muscle mass is regulated through the balance between the rates of muscle protein synthesis (MPS) and breakdown (MPB). Food intake, in particular dietary protein, induces a postprandial rise in circulating (essential) amino acids (leucine, valine, and isoleucine) thereby increasing delivery of protein-derived amino acids to skeletal muscle tissue and the activation of mTORC1signalling [3], which stimulates MPS [4–7]. In addition, resistance exercise, with the subsequent mechano-transduction, and the anabolic hormones growth hormone and testosterone can directly increase rates of MPS. Periods of restricted energy intake decrease rates of MPS in both the fasted and fed state [8–13]. Consequently, a chronic reduction in energy intake is accompanied by a substantial decline in LM [14–16].

Intermittent fasting (IF) and continuous energy restriction (CER) are popular nutritional strategies to rapidly reduce BM and improve metabolic health. CER involves reducing daily energy intake by 15–40 % energy (En%) while meal frequency remains unchanged. In contrast, IF involves continuous or interrupted periods of fasting or severe energy restriction (one to several days per week) combined with non-fasting periods without restrictions [17]. Common IF strategies include alternate day fasting (ADF), periodic fasting, or intermittent energy restriction [18]. Short-term IF induces rapid loss of BM and FM, improves skeletal muscle insulin sensitivity, and enhances metabolic parameters associated with increased risks of obesity, diabetes, and cardiovascular disease [18,19]. The health benefits of IF are likely due to reductions in BM, and in this regard are similar to those accrued after CER. However, IF protocols may confer additional health benefits through fasting-mediated effects on energy utilisation (e.g., lipolysis and ketogenesis) and autophagy [20]. Studies that have directly compared the effects of IF and CER have reported little difference in BM and FM reductions or improvements in metabolic health [21–26]. Aside from the positive effects of IF on losses in BM and FM, concurrent decreases in LM have been reported following IF protocols [27,28]. Protein meal timing and a balanced distribution of protein-containing meals throughout the day supports greater rates of MPS and net balance over 24 h [29–31]. Therefore, an uneven protein intake distribution pattern, such as during IF, may impair MPS and contribute to muscle mass loss. However, it is unclear if prolonged fasting periods and irregular energy/protein intake affect MPS during IF, beyond the effect of inducing a negative energy balance.

In the present study we compared the effects of ten days of IF (in the form of ADF), CER, or an energy-balanced diet, all with matched protein intakes, on rates of skeletal MPS in middle-aged males with overweight or obesity. We hypothesised that ten days of restricted energy intake (ADF and CER) would reduce rates of MPS compared with a control diet, with lower rates of MPS following ADF compared with CER.

## 2. Methods

### 2.1. Participants

Recreationally active (engaging in sports or structured exercise $\leq$ three days/week and not participating in any structured resistance exercise program, or taking <10,000 steps/day), middle-aged males (aged 35–55 y) with an overweight or obese status (BMI 25–35 kg/m$^2$) volunteered to participate in this parallel, randomised controlled trial. The study was prospectively registered at Australian New Zealand Clinical Trials Registry (ACTRN12619000757112) and was conducted at Australian Catholic University's (ACU) Melbourne campus between May 2019 and March 2020.

This investigation was part of a larger clinical trial investigating the effect of IF dietary strategies on muscle protein metabolism (four conditions, $n = 44$ in total, Supplemental Fig. 1). COVID-19 restrictions in Victoria, Australia, prevented reaching the target

total sample size ($n = 44$) and recruitment ceased at $n = 41$ participants, with $n = 37$ completing the intervention. Participants were recruited using targeted campaigns of flyers/posters displayed at the university, databases from previous studies, and via a research participant recruitment company (Trialfacts, Melbourne, Australia). All participants were informed of the purpose of the study, the experimental procedures, and possible risks before providing written informed consent to participate. The study was approved by the ACU Human Research Ethics Committee (2018-291HC) and human experimentation and procedures followed were in accordance with the ethical standards outlined in the Helsinki Declaration of 1975 as revised in October 2013 for use of human subjects and tissue.

### 2.2. Pretesting and dietary intervention

After study entry, participants visited the laboratory in an overnight fasted (>10 h) state for a baseline assessment of resting energy expenditure (REE; using the final 15 min of 25 min measurement) using a calibrated TrueOne 2400 metabolic cart (TrueOneRMR, Parvo Medics, Sandy, USA) and measurements of height, BM, and blood pressure (supine, in triplicate with 1 min intervals using ProBP 3400, Welch Allan Inc, Skaneateles Falls, USA). BM was measured every study visit in a fasted state, wearing light clothing and no shoes, using the same scale. Body composition was assessed by dual-energy X-ray absorptiometry (DXA; GE Lunar iDXA Pro and enCORE software Version 16, General Electric, Boston, USA). In our laboratory, DXA scans are reproducible with coefficients of variation (CV) of <1.5 %. Participants were deemed healthy based on their responses to a medical questionnaire and screening results (HbA1c<6.5 %). The experimental protocol is presented in Fig. 1.

Following pretesting, randomisation was performed using a computerised random-number generator executed by an independent person and sealed opaque envelopes (block randomisation, $n = 4$). Due to the nature of the study all investigators could not blinded to conditions. Participants were invited within four weeks of pretesting to collect their individualised prepacked meals for a three-day lead-in, control diet and have a continuous glucose monitor (CGM, Freestyle Libre 2, Abbott, Chicago, USA) fitted into the subcutaneous fat tissue of the upper arm and an accelerometer worn around the waist (ActiGraph GTX3+, Pensacola, USA; worn during waking hours only). All participants were instructed to refrain from strenuous physical activity and alcohol consumption and maintain a consistent sleep routine three days before pretesting and until the end of the study. An accredited practicing dietitian calculated the energy and macronutrient composition for the three-day controlled, energy-balanced, standardised diet (100 energy (En)%, 55 En% carbohydrate, 30 En% fat, and 15 En% protein) and ten-day intervention diet based upon the individuals' energy requirements (REE multiplied by an activity factor of 1.4 to reflect a sedentary light activity lifestyle [32]). After three days of the lead-in diet, participants commenced a ten-day controlled dietary intake period, receiving all weighed and packed food items according their individual requirements and intervention, comprising continuous energy restriction (CER; 62.5 En%), alternate day fasting (ADF; 25 En % food consumption on days 1, 3, 5, 7 and 9, alternated with 100 En % food consumption on days 2, 4, 6, 8 and 10), or an energy-balanced, control diet (CON; 100 En%). Protein intake was set at 1.0 g/kg/day for all groups (see Table 2 for diet composition data), with a protein distribution in CON: 33-32-35 %, CER: 23-30-49 %,

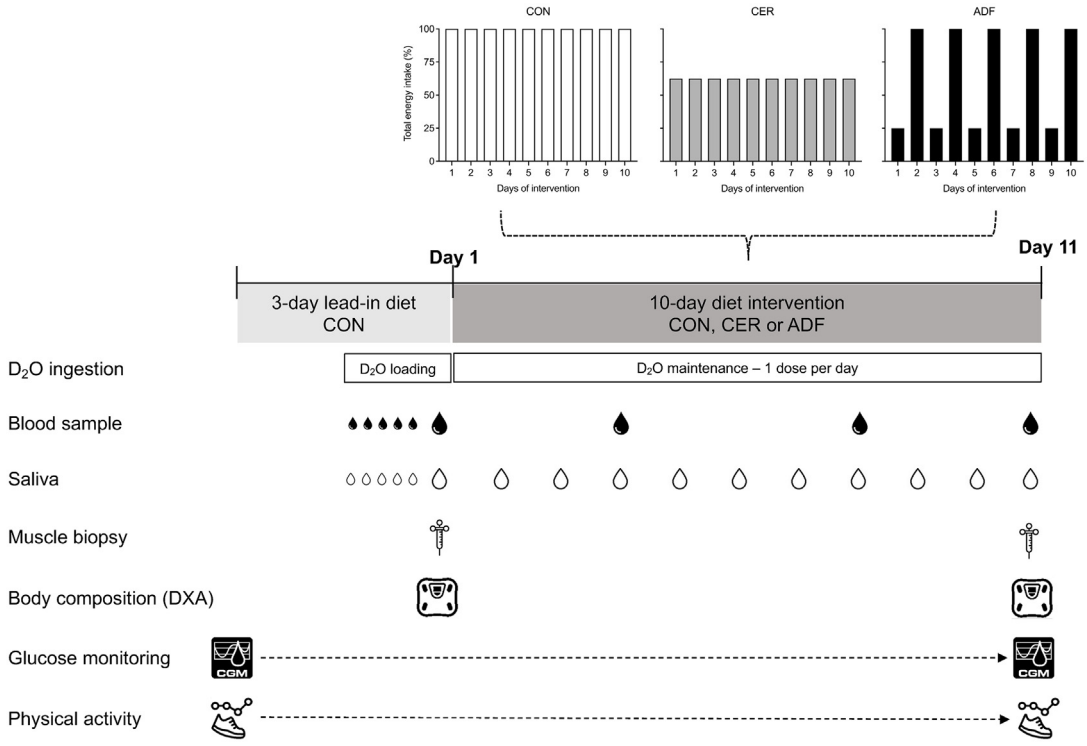



**Fig. 1.** Overview of the 13-day study protocol where 27 middle-aged males with overweight or obesity completed a three-day lead in diet followed by a ten-day intervention diet. Integrated rates myofibrillar fractional synthesis rates were measured over the ten-day intervention period using deuterated water ($D_2O$) methodology with repeated saliva, blood, and muscle samples. Secondary measurements included continuous glucose and physical activity monitoring, body composition (DXA) scans, and the assessment of fasting glucose, insulin, and gastrointestinal hormone concentrations. ADF: alternate day fasting, CER: continuous energy restriction, CGM: continuous glucose monitoring, CON: control, D2O: deuterated water, DXA: Dual-energy x-ray absorptiometry.

**Table 1**
Participant demographics and blood profiles of 27 middle-aged males with overweight or obesity randomised to a ten-day control (CON, $n = 8$), continuous energy restriction (CER, $n = 10$), or alternate day fasting (ADF, $n = 9$) diet following a three-day lead-in, control diet.

| | CON ($n = 8$) | | CER ($n = 10$) | | ADF ($n = 9$) | |
|---|---|---|---|---|---|---|
| | Pre | Post | Pre | Post | Pre | Post |
| Age (y) | 42.3 ± 5.8[a] | N/A | 48.9 ± 2.9[b] | N/A | 41.9 ± 4.5[a] | N/A |
| Body weight (kg) | 96.6 ± 7.3 | 95.4 ± 7.4 | 95.8 ± 9.6[a] | 92.9 ± 9.9[b]‡ | 91.6 ± 8.9[a] | 88.9±9[b]‡ |
| BMI (kg/m$^2$) | 30.3 ± 2.3 | 29.8 ± 2.4 | 31.1 ± 2.7 | 29.9 ± 2.8 | 29.3 ± 2.5 | 28.2 ± 2.6 |
| Systolic blood pressure (mmHg) | 128 ± 6 | 126 ± 9 | 126 ± 10 | 121 ± 8 | 120 ± 7 | 121 ± 6 |
| Diastolic blood pressure (mmHg) | 81 ± 3 | 78 ± 6 | 79 ± 9 | 75 ± 6 | 77 ± 8 | 75 ± 6 |
| Heart rate (bpm) | 63 ± 7 | 59 ± 7 | 65 ± 11 | 63 ± 11 | 65 ± 9 | 60 ± 8 |
| HbA1c (%) | 5.4 ± 0.3 | 5.4 ± 0.4 | 5.4 ± 0.3 | 5.5 ± 0.3 | 5.6 ± 0.5 | 5.5 ± 0.5 |
| Cholesterol (mmol/L) | 4.8 ± 0.4[a] | 4.4 ± 0.5[b]‡ | 5.8 ± 1.1[a] | 4.7 ± 0.9[b]‡ | 5.4 ± 0.6[a] | 4.7 ± 0.8[b]‡ |
| HDL cholesterol (mmol/L) | 1.2 ± 0.5 | 1.0 ± 0.4 | 1.6 ± 1.9 | 0.9 ± 0.3 | 1.0 ± 0.3 | 1.0 ± 0.3 |
| LDL cholesterol (mmol/L) | 2.7 ± 0.6[a] | 3.2 ± 1.5 | 3.8 ± 1.1[b] | 3.2 ± 0.9 | 3.5 ± 0.4 | 3.2 ± 0.7 |
| Triglycerides (mmol/L) | 2.1 ± 1.0[a] | 1.8 ± 0.6[b]‡ | 2.1 ± 1.0[a] | 1.2 ± 0.3[b]‡ | 2.0 ± 1.2[a] | 1.1 ± 0.4[b]‡ |

BMI: body mass index. Pre: morning of day 1 of the intervention diet (except for blood pressure, morning before the start of intervention diet), Post: morning of day 11, following ten days of the intervention. Data is shown as mean ± SD. Different letters show differences between groups or from pre to post‡ (within-groups) using ANOVA, $P < 0.05$.

**Table 2**
Dietary composition and physical activity data of 27 middle-aged males with overweight or obesity randomised to a ten-day control (CON, $n = 8$), continuous energy restriction (CER, $n = 10$), or alternate day fasting (ADF, $n = 9$) diet following a three-day lead-in control diet.

| | CON ($n = 8$) | | CER ($n = 10$) | | ADF ($n = 9$) | | | |
|---|---|---|---|---|---|---|---|---|
| | Lead-in | Intervention | Lead-in | Intervention | Lead-in | Intervention average | Intervention fed day | Intervention fasting day |
| **Diet composition** | | | | | | | | |
| Energy, kJ/day (% from calculated energy requirements) | 11637 ± 782 | 11665 ± 736 (100 ± 2) | 11150 ± 1039[a] | 7014 ± 600[b] (63 ± 1) | 11668 ± 822[a] | 7373 ± 498[b] (63 ± 1) | 11806 ± 789[a] (101 ± 1) | 2941 ± 238[b]* (25 ± 1) |
| Protein, g (En%) | 97 ± 7 (14 ± 1) | 98 ± 7 (14 ± 1) | 97 ± 10 (15 ± 1)[a] | 98 ± 10 (24 ± 2)[b] | 93 ± 9 (14 ± 2)[a] | 94 ± 9 (22 ± 2)[b] | 117 ± 11[a] (17 ± 2) | 70 ± 6[b]* (41 ± 4) |
| Carbohydrate, g (En%) | 382 ± 31 (56 ± 1) | 384 ± 31 (56 ± 1) | 363 ± 35[a] (55 ± 1)[a] | 211 ± 19[b] (51 ± 2)[b] | 386 ± 32[a] (56 ± 1)[a] | 224 ± 19[b] (52 ± 1)[b] | 367 ± 31[a] (55 ± 1) | 67 ± 9[b]* (39 ± 3) |
| Fat, g (En%) | 94±8[a] (30 ± 1) | 94 ± 6 (30 ± 1) | 90±9[a] (30 ± 0)[a] | 48±5[b] (25 ± 1)[b] | 95±8[a] (30 ± 1)[a] | 53±5[b,c] (27 ± 1)[b,c] | 91 ± 8[a] (28 ± 1) | 16 ± 3[b]* (20 ± 3) |
| **Physical activity** | | | | | | | | |
| Time spent in sedentary (%) | 69 ± 7 | 66 ± 10 | 67 ± 5 | 66 ± 7 | 66 ± 10 | 62 ± 11 | 61 ± 12 | 65 ± 11 |
| Time spent in light PA (%) | 27 ± 5 | 30 ± 6 | 30 ± 7 | 30 ± 7 | 30 ± 8 | 33 ± 11 | 34 ± 12 | 31 ± 11 |
| Time spent in MVPA (%) | 3 ± 3 | 3 ± 2 | 3 ± 2 | 4 ± 1 | 4 ± 3 | 5 ± 2 | 5 ± 3 | 5 ± 2 |
| Steps (n/day) | 10505 ± 3540 | 11442 ± 2918 | 12274 ± 3099 | 13465 ± 2872 | 13077 ± 5334 | 13486 ± 5061 | 14460 ± 4986[a] | 12102 ± 4623[b]* |
| Valid wear time (min) | 908 ± 48 | 906 ± 87 | 923 ± 49 | 949 ± 58 | 958 ± 91 | 918 ± 50 | 932 ± 55 | 883 ± 92 |

MVPA: moderate and vigorous physical activity, PA: physical activity. Data are expressed as mean ± SD. Different letters show differences between groups (comparisons were made for all groups during lead-in and intervention) and from the lead-in to intervention diet (within-group comparison), using ANOVA. In ADF, eating vs fasting days were compared using paired samples t-tests, * differences between fasting and fed days. Statistical significance was set at $P < 0.05$.

and ADF: 100 % at breakfast on fast days and 23-32-44 % of total protein intake on fed days. The macronutrient composition of the diet was calculated using FoodWorks© (Version 8, Xyris Software, Brisbane, Australia). All food items were individually weighed and packed by research staff and contained food items for breakfast (bread, oats, sultanas, almonds, peanut butter, honey, milk, and yoghurt), lunch (bread, wraps, tuna, chicken breast, cheese, tomatoes, and yoghurt), snacks (muesli bars, dried apricots, apple and orange juice), and dinner (ready-made meals chicken tajine or minced beef burrito mix (Dineamic, Keysborough, Victoria, Australia); frozen vegetables, rice, and couscous). The CER and ADF (on fasting days) diets contained high-protein products such as egg white, parmesan cheese, drinking chocolate powder and milk, and whey protein isolate powder (85.5 g/100 g protein, Bulk Nutrients, Grove, Tasmania, Australia) to meet protein targets during energy restriction. An example of each diet is shown in Supplemental Table 1. All participants received a personalised handbook containing a daily overview of food items to be consumed and checked off. Participants were requested to consume their breakfast at 0800 h, lunch at 1400 h, and dinner at 2000 h every day, and complete questions on their daily sleeping habits and patterns of physical activity.

### 2.3. Experimental protocol

An 11-day deuterated water ($D_2O$) protocol was applied to measure integrated rates of MPS, comprising a 'loading day' (on day three of the lead-in diet) during which eight doses of 50 mL of 70MPE $D_2O$ (Sigma Aldrich) were ingested, and a daily 50 mL dose ingestion during the subsequent ten days. Saliva samples for the assessment of body water $^2H$ enrichments were collected daily at home before meal ingestion, by chewing on a cotton swab for 1 min to saturate the cotton swab with saliva before being placed into collection tubes (Salivette Sarstedt AG&CO). Tubes were refrigerated at home and brought into the lab (on days 1, 4, 8, and 11) for processing together with empty $D_2O$ bottles to determine compliance. Subsequently, saliva collection tubes were spun at 1800 *g* for 10 min to extract the saliva into a sample tube, which was aliquoted and frozen at −80 °C until analysis. Fasting blood samples (~15 mL) were collected every laboratory visit to assess plasma [$^2H$]-alanine

enrichments, circulating metabolites (HbA1c levels, total, HDL, and LDL cholesterol, triglycerides, plasma glucose and insulin concentrations), and hormone concentrations (ghrelin, leptin, peptide-YY (PYY), and glucagon like protein-1 (GLP-1). Blood samples were collected in EDTA-containing tubes, a small sample of whole blood was taken for HbA1c and a lipid panel analysis (Cobas b 101, Roche Diagnostics Ltd, Basel, Switzerland) and the remaining sample was centrifuged at 1800 *g* for 10 min at 4 °C. Blood samples for gastrointestinal hormone analyses were collected in EDTA tubes and 40 μL of a protease inhibitor (200 mM AEBSF, Pefabloc, Roche) and 20 μL DPP-IV inhibitor (Sigma Aldrich, USA) was added per 2 mL whole blood and centrifuged at 3000 *g* for 10 min at 4 °C. For ghrelin analyses, 15 μL HCL was added to 300 μL plasma before freezing. Aliquots of plasma were stored at −80 °C until further analysis. On day 1 and 11, a skeletal muscle biopsy was taken to measure muscle protein-bound $^{2}$H-alannine enrichment levels for the calculation of muscle protein fractional synthesis rates (FSR, %/day). Muscle biopsies were obtained from the middle region of the *M. medialis vastus lateralis*, 15 cm above the patella and ~4 cm below entry through the fascia, using the percutaneous Bergström needle biopsy technique [33]. Biopsy samples were dissected carefully from any visible non-muscle material. The muscle samples were immediately frozen in liquid nitrogen and stored at −80 °C until further analysis.

### 2.4. Physical activity and continuous glucose monitors analyses

Physical activity data using ActiGraph GT3X + accelerometers were obtained in 1-min epoch data files and average sedentary (<100 counts per min, cpm), light-intensity (100–1951 cpm), and moderate-vigorous physical activity (MVPA) intensity (≥1952 cpm) activity time were calculated on valid (≥10 h) days [34]. Wear time validation was performed to exclude non-wear periods (continuous 0 counts for >90 min) and participants' self-reported sleep and wake times were used to remove sleep time from analysis. As a result, all participants had a minimum of two days sufficient wear time (>10 h) for the lead-in period and a minimum of nine days for the intervention period.

Analysis of the CGM data were performed using the continuously stored data (not including the day of monitor insertion) with the GLU package in R (version 4.0.5, Shake and Throw) [35] (https://github.com/MRCIEU/GLU). During the intervention, participants were instructed to swipe their glucose reader over the CGM sensor at least every 8 h to retrieve the data. As a result, only full days of data were included in the daily analysis, with a minimum of two days of complete data being included for the lead-in (ADF: $n = 7$, CER: $n = 8$, CON: $n = 6$) and intervention period (ADF: $n = 7$, CER: $n = 10$, CON: $n = 7$). Fasting and fed days were separately calculated for the ADF intervention ($n = 5$ complete fast and fed data). Mean, SD, and CV were calculated and compared between lead-in and intervention periods using 24 h complete data. Total area under the curve (AUC) was calculated for 24 h periods using the trapezoid method with a baseline of zero.

### 2.5. Plasma and muscle analyses

For glucose analysis, thawed plasma samples were measured in duplicate using an YSI 2900 analyser (YSI Life Sciences, Yellow Springs, OH, USA), and plasma insulin concentrations were measured with a commercially available ELISA (Alpco Ltd., Windham, NH, USA), with CVs of 1.1 % and 5.3 %, respectively. Plasma gut hormone analyses were performed using human metabolic hormone magnetic bead panels (MILLIPLEX® MAP, Millipore, Merck, Germany) on the Limenix MagPix (Millipore, Sigma, Burlington, USA) for the analytes PYY, GLP-1, ghrelin, and leptin. Analyses of body water $^{2}$H, plasma [$^{2}$H]-alanine, and myofibrillar protein-bound [$^{2}$H]-alanine enrichments were conducted, as previously described [36].

### 2.6. Calculations

Myofibrillar protein FSR was calculated using the standard precursor-product method:

$$FSR\ (\%/day) = \frac{Em2 - Em1}{E_{precursor} \cdot t} \cdot 100\%$$

where $E_{m1}$ and $E_{m2}$ are the myofibrillar protein-bound [$^{2}$H]-alanine enrichments (in MPE) on days 11 and 1, respectively; $E_{precursor}$ represents weighed mean body water $^{2}$H-enrichments (in MPE, corrected by a factor of 3.7 based on the deuterium labelling during *de novo* alanine synthesis) or plasma free [$^{2}$H]-alanine enrichments; and $t$ represents the time between biopsies on days 1 and 11.

### 2.7. Statistical analysis

All data are expressed as mean ± SD, and, where appropriate, the 95 % CI. One-way analysis of variance (ANOVA) was used to compare differences between groups for the primary outcome, FSR. One-way ANOVA was used to determine differences in baseline characteristics and secondary outcomes including diet composition, levels of physical activity, and changes in body mass and body composition. Two-way ANOVA with time as within-group factor and intervention as between-group factor was used to compare differences over time (from lead-in to intervention period) in secondary outcomes including diet composition, physical activity levels, 24 h glucose variability and total AUC, body composition, HbA1c, lipids, plasma glucose, insulin, gastrointestinal hormones, and amino acid enrichments. In the case of a significant interaction between time and intervention, separate analyses were performed to determine time-effects for each group (one-factor repeated measures ANOVA) with Bonferroni post-hoc tests to locate these differences and between-group effects for each time-point (unpaired students t-test). A test of normality (Shapiro-Wilk) was performed for primary and secondary outcomes. In case of non-normal distributed data, independent sample Kruskal-Wallis tests were performed. Based upon previous work applying similar methodology investigating the effect of ten days of 40 % energy restriction on integrated MPS rates [10], a sample size of $n$=10 per group, with a 10 % dropout rate, was calculated using an unpaired two-sided t-test (α of 0.05, power of 95 %, and calculated effect size of 1.7), to detect statistical differences in FSR following energy restriction when compared to CON. A difference in integrated MPS (FSR, primary outcome) between CON and CER of 0.4 %/day (25 % relative difference between interventions) was estimated following ten days of CER with a SD of ~0.23 %/day. Moreover, based upon previous work applying similar study measurements [8,10,12,37], a sample size of ten subjects per group was considered feasible to be able to successfully complete this type of intervention study. Statistical significance was set at $P < 0.05$. All calculations were performed using the statistical software program SPSS (version 26.0, IBM Corp., Armonk, USA).

## 3. Results

### 3.1. Participants, dietary adherence, and physical activity

Participant characteristics are presented in Table 1. No differences in baseline demographics were observed except for age, which was

higher in CER compared to CON (+7 y [+2, +12 y]) and ADF (+7 y [95 % CI: +1, +12 y]; both, $P < 0.05$). During the ten-day intervention, diastolic blood pressure and heart rate decreased across all groups (main time effect, $P < 0.05$), with no differences between groups. Decreases in plasma cholesterol (CON: −0.4 ± 0.1 mmol/L, CER: 1.1 ± 0.2 mmol/L, ADF: 0.7 ± 0.2 mmol/L) and triglycerides (CON 0.3 ± 0.3 mmol/L, CER: 0.8 ± 0.6 mmol/L, and ADF: 0.9 ± 0.8 mmol/L) were observed across all groups (main time effect, $P < 0.001$, main group effect, $P > 0.05$).

Diet composition and physical activity data are presented in Table 2. Energy content of the lead-in diet averaged 11,467 ± 899 kJ [11,112,11,823 kJ] with no differences between interventions. As prescribed, the energy content and macronutrient composition in the intervention period did not differ from the lead-in diet in CON (−29 ± 265 kJ [−250, +193 kJ]; $P = 0.77$). In CER, total energy content decreased by −37 % [−36.5, −37.6 %] compared to the lead-in diet, and as a result of the maintained protein intake of 1.0 g/kg/day (+9 ± 1 En%), the carbohydrate (−4±1 En%) and fat contribution (−5±1 En%) to total energy intake decreased (all, $P < 0.001$). Total energy intake in ADF was −37 % [−36.4, −37.2 %] lower when compared to the lead-in diet, with an increase in protein contribution to total energy intake (+8 ± 1 En%) and decrease in carbohydrate (−5±1 En%) and fat (−3±1 En%) contribution (all, $P < 0.001$). Mean energy and macronutrient intake was lower on fasting vs fed days (all, $P < 0.001$). Protein content averaged 1.3 g/kg/day on fed days and 0.8 g/kg/day on fasting days, resulting in a difference of 23 ± 3 En% between fasting and fed days ($P < 0.001$). There was no difference between ADF and CER for energy, protein, or carbohydrate content, while fat intake was lower in CER (25±1En %) compared to ADF (27 ± 1 En%, $P = 0.01$).

Time spent in physical activity intensities, step counts, and total wear time did not differ between groups in the lead-in and intervention period. The proportion of time spent in sedentary activities decreased from the lead-in to intervention period across all groups (main time effect, $P = 0.02$), with no differences between groups (main group effect, $P = 0.74$). There was no change in other physical activity intensity and step counts from lead-in to intervention in any of the groups. In ADF, total step count on fasting days was lower when compared to fed days ($P = 0.02$), and the time spent in sedentary activities tended to be lower on fed days ($P = 0.07$), while time spent in light intensity activities higher ($P = 0.05$) compared to fasting days.

### 3.2. Body composition

Body composition data are presented in Fig. 2. BM (from DXA, Fig. 2A) decreased across all interventions ($P$-interaction = 0.08, main time effect, $P < 0.001$) by −2.5 ± 0.8 kg [−3.1, −1.9 kg] in ADF, −2.4 ± 1.0 kg [−3.1, −1.7 kg] in CER, and −1.3 ± 0.7 kg [−1.8, −0.7 kg] in CON, with no differences between groups (main group effect, $P = 0.47$). The %BM change did not differ between groups (*data not shown*). LM (Fig. 2B) and fat mass (Fig. 2C) decreased during the intervention (main time effect, both $P < 0.001$), with no differences between interventions in LM (−1.3 ± 0.7 kg [−1.7, −0.8 kg] in ADF, −1.0 ± 1.2 kg [−1.7, −0.3 kg] in CER, and 0.2 ± 0.5 kg [−0.5, +0.2 kg] in CON; $P = 0.11$) and FM loss (−1.3 ± 0.5 kg [−1.6, −0.9 kg] in ADF, −1.4 ± 0.7 kg [−1.8, −1.0 kg] in CER, and 1.1 ± 0.4 kg [−1.3, −0.8 kg] in CON; $P = 0.80$). No changes were observed in relative total BM, LM, and FM changes (*data not shown*). The change in trunk LM was greater in CER compared to CON ($P = 0.04$), but no differences in appendicular LM change were observed between interventions ($P = 0.06$); Supplemental Fig. 2). Total BM (from the scale, Supplemental Fig. 3) changed during the intervention period ($P$-interaction<0.001), with a greater change in BM in ADF and CER compared to CON (both, $P < 0.001$). The changes in BM were greatest between day 1 and day 4 in ADF compared to CON ($P = 0.03$) and within day 1 to day 8 in ADF and CER compared to CON ($P < 0.001$). No changes in BM between groups were observed in the lead-in period (*data not shown*).

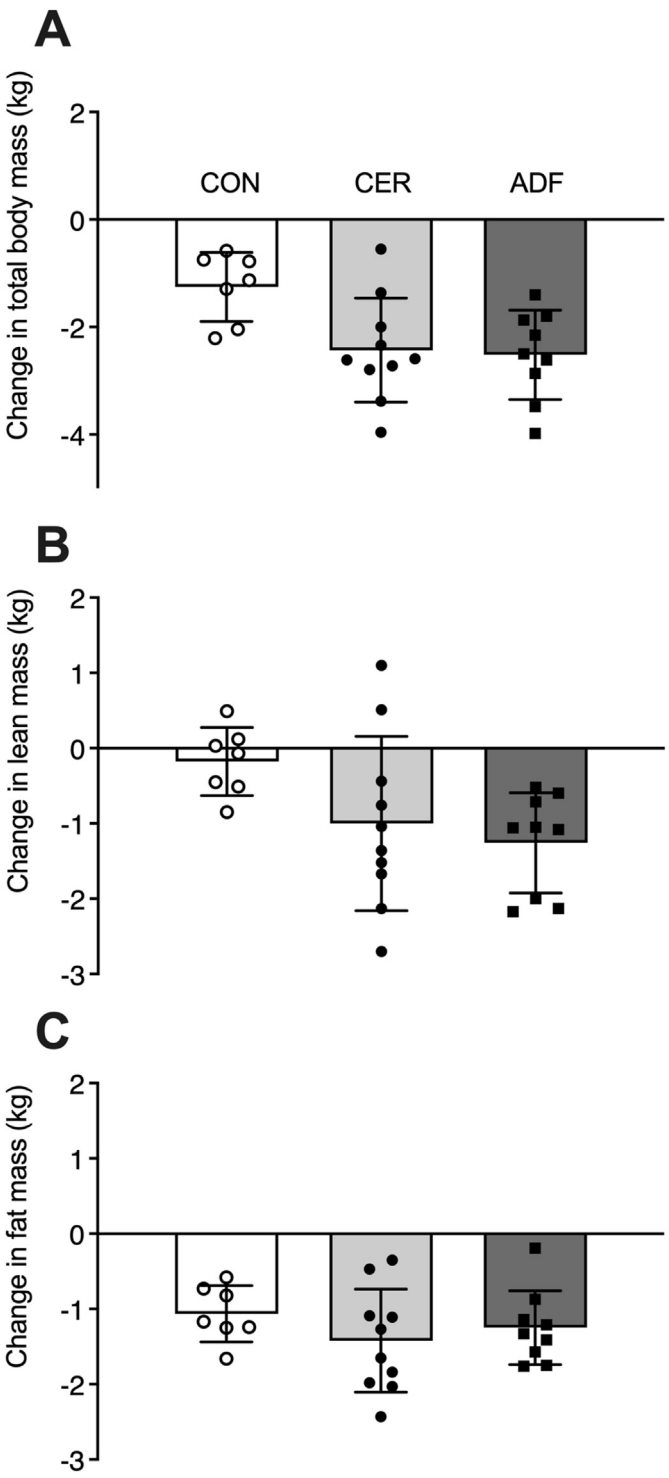



**Fig. 2.** Dual-energy x-ray absorptiometry (DXA) derived estimate changes in body composition (kg) of **A**: total body mass, **B**: lean mass, and **C:** fat mass, in middle-aged males with overweight or obesity randomized to a ten-day control (CON, $n = 8$*), continuous energy restriction (CER, $n = 10$), or alternate day fasting (ADF, $n = 9$) diet. **Data for n = 7 in CON is shown.* Values represent individual values (dots) and mean ± SD (bars). Data were analysed by one-way ANOVA to locate differences between groups. No differences were observed between groups, $P > 0.05$.

### 3.3. Glucose, insulin, and gastrointestinal hormone concentrations

Fasting plasma glucose and insulin concentrations during the ten-day intervention are presented in Fig. 3. No difference in fasting plasma glucose concentrations (Fig. 3A) were observed (*P*-interaction = 0.49). Fasting plasma insulin concentrations (Fig. 3B) during the intervention period differed between groups (*P*-interaction = 0.01), with a change in fasting insulin concentrations from day 1 to day 11 in ADF (time effect, $P = 0.004$), while no change over time was observed in the other intervention groups (both, $P > 0.05$). No differences between groups in fasting insulin concentrations were observed on any of the days of the intervention. Fasting plasma gastrointestinal hormone concentrations are presented in Fig. 4. No differences between groups in fasting levels at start of the intervention (day 1) in any of the hormones were observed. Fasting plasma ghrelin (Fig. 4A), GLP-1 (Fig. 4C), and PYY (Fig. 4D) concentrations did not change throughout the intervention (all, *P*-interaction>0.05). Fasting plasma leptin concentrations (Fig. 4B) differed between groups (*P*-interaction<0.001), with an overall decrease in plasma leptin levels in ADF and CER (time effect, $P < 0.001$), while no changes were observed in CON ($P > 0.05$). Fasting leptin concentrations were lower in ADF when compared to CON on day 8 ($P = 0.02$).

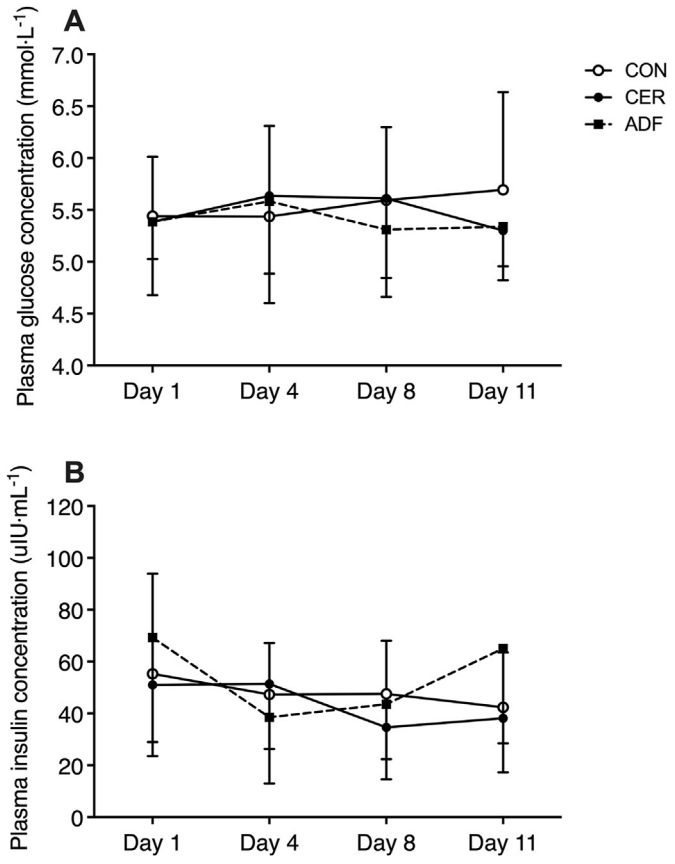



**Fig. 3.** Fasting plasma glucose (**A**; mmol/L) and insulin (**B**; uIU/mL) in middle-aged males with overweight or obesity randomised to a ten-day control (CON, $n = 8$), continuous energy restriction (CER, $n = 10$), or alternate day fasting (ADF, $n = 9$) diet. Values represent mean ± SD. Data were analysed by repeated measures (time x treatment) ANOVA. One-way ANOVA were used to locate difference between groups and Bonferroni post-hoc test to locate differences over time. **A**: *P*-interaction = 0.49; main time effect, $P = 0.66$; main group effect, $P = 0.90$. **A**: *P*-interaction = 0.01; individual time effect, $P < 0.05$ for ADF. No differences were observed between groups on any timepoint, $P > 0.05$.

### 3.4. Continuous glucose monitoring

Mean 24 h glucose profiles are shown in Fig. 5. There were no differences in mean, SD, total AUC, and CV of 24 h glucose concentrations between groups during the lead-in period (Supplemental Table 2). During the intervention, mean, and total AUC of 24 h glucose concentrations decreased (*P*-interaction<0.05), with no differences between groups. In ADF, total AUC, mean, SD, and CV of 24 h glucose concentrations were lower on fasting days when compared to fed days ($P < 0.05$).

### 3.5. Rates of myofibrillar protein synthesis

Body water $^2$H, plasma [$^2$H]-alanine, and myofibrillar protein-bound [$^2$H]-alanine enrichments are presented in Supplemental Fig. 4. Body water $^2$H and plasma [$^2$H]-alanine enrichments increased during the intervention (main time effect, $P < 0.001$), with no differences between groups. No differences in myofibrillar protein-bound [$^2$H]-alanine enrichments were observed between groups. Integrated rates of MPS are presented in Fig. 6. FSR based upon body water $^2$H enrichments (ADF: 1.18 ± 0.13, CER: 1.13 ± 0.16, and CON: 1.18 ± 0.18 %/day; Fig. 6A) and plasma [$^2$H]-alanine enrichments (ADF: 1.19 ± 0.14, CER: 1.11 ± 0.12, and CON: 1.26 ± 0.22 %/day; Fig. 6B) did not differ between interventions ($P > 0.05$).

### 3.6. Appetite, satiety, and fatigue

Qualitative questions, using digital visual analogue scales (VAS), on appetite, satiety, and fatigue are presented in Supplemental Table 3. No differences in VAS were observed in any of the questions on appetite, hunger, and fatigue, except for satiety ('*How full do you feel right now*?') which was lower in CER when compared to ADF on days 8 and 11 ($P < 0.05$), and tiredness ('*How tired do you feel right now?*') which decreased throughout the intervention in ADF (*P*-interaction<0.05) and was lower on day 8 compared to CER ($P < 0.05$), while no changes were observed in CER and CON.

## 4. Discussion

Popular IF diets, characterised by irregular meal patterns and prolonged periods of fasting, may negatively impact muscle protein metabolism beyond the effect of energy restriction. In contrast to one of our original hypotheses, we observed no differences in daily MPS rates following ten days of ADF and CER compared to an energy balanced diet in males with overweight or obesity.

### 4.1. The impact of IF and CER on skeletal MPS rates

Periods of fasting (12–72 h) reduce whole-body and leg protein synthesis rates [38,39] while concomitantly increasing rates of protein breakdown [40,41]. However, to date, the MPS response to multiple days of IF has not been determined. In the present study, we applied an IF strategy, ADF, whereby participants consumed a large breakfast (containing ~3000 kJ and ~70 g protein) on fasting days, followed by 24 h of total food abstinence until the next day's breakfast. ADF is commonly practised by 0–40 En% food consumption on fasting days alternated with *ad libitum* intake on 'eating' days. We report that intermittent 24 h fasting periods with matched protein intakes do not lower integrated MPS rates over ten consecutive days of ADF. In our study protocol, we matched protein intake on both fasting and fed days to isolate the cumulated effect of prolonged fasting and energy restriction in the absence of changes in absolute protein intake. The matched protein intake enabled us to determine the effect of ADF compared to more

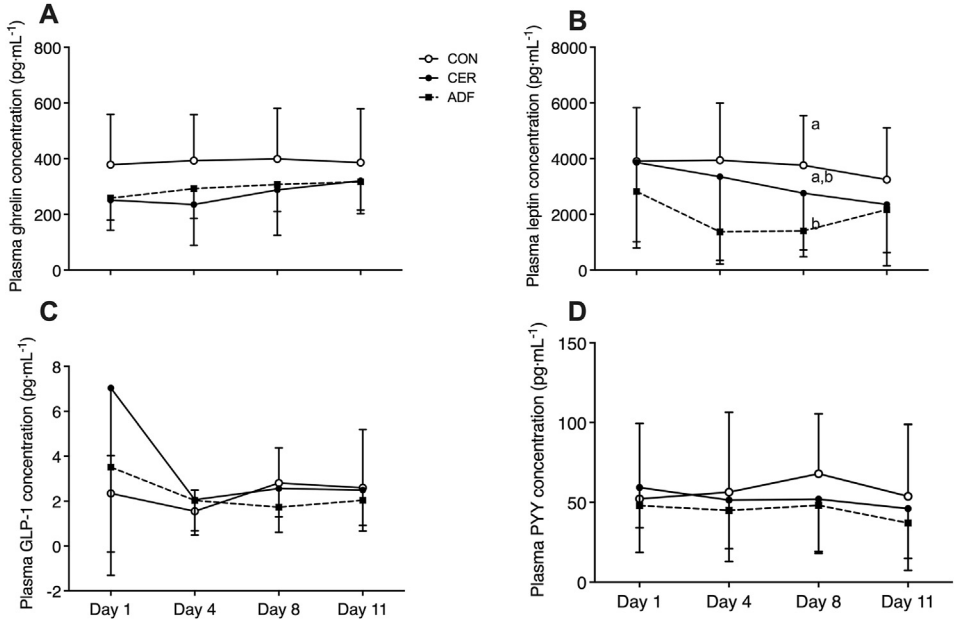



**Fig. 4.** Fasting plasma ghrelin (**A**), leptin (**B**), GLP-1 (**C**), and PYY (**D**) concentrations (pg/mL) in middle-aged males with overweight or obesity randomised to a ten-day control (CON, $n = 8$*), continuous energy restriction (CER, $n = 10$), or alternate day fasting (ADF, $n = 9$*) diet. **GLP-1 and PYY analyses are reported for n = 6 in CON and n=8 in ADF.* Values represent mean ± SD. Data were analysed by repeated measures (time x treatment) ANOVA. One-way ANOVA were used to locate difference between groups and Bonferroni post-hoc test to locate differences over time. **A**: *P*-interaction = 0.40; main time effect, $P = 0.008$; main group effect, $P = 0.19$. **B**: *P*-interaction<0.001; individual time effect, $P < 0.001$ for CER and ADF; between group difference ADF vs CON on Day 8, $P = 0.02$. **C**: *P*-interaction = 0.31; main time effect, $P = 0.02$; main group effect, $P = 0.89$. **D:** *P*-interaction = 0.06; main time effect, $P = 0.18$; main group effect, $P = 0.97$. Different letters show differences between groups, $P < 0.05$.

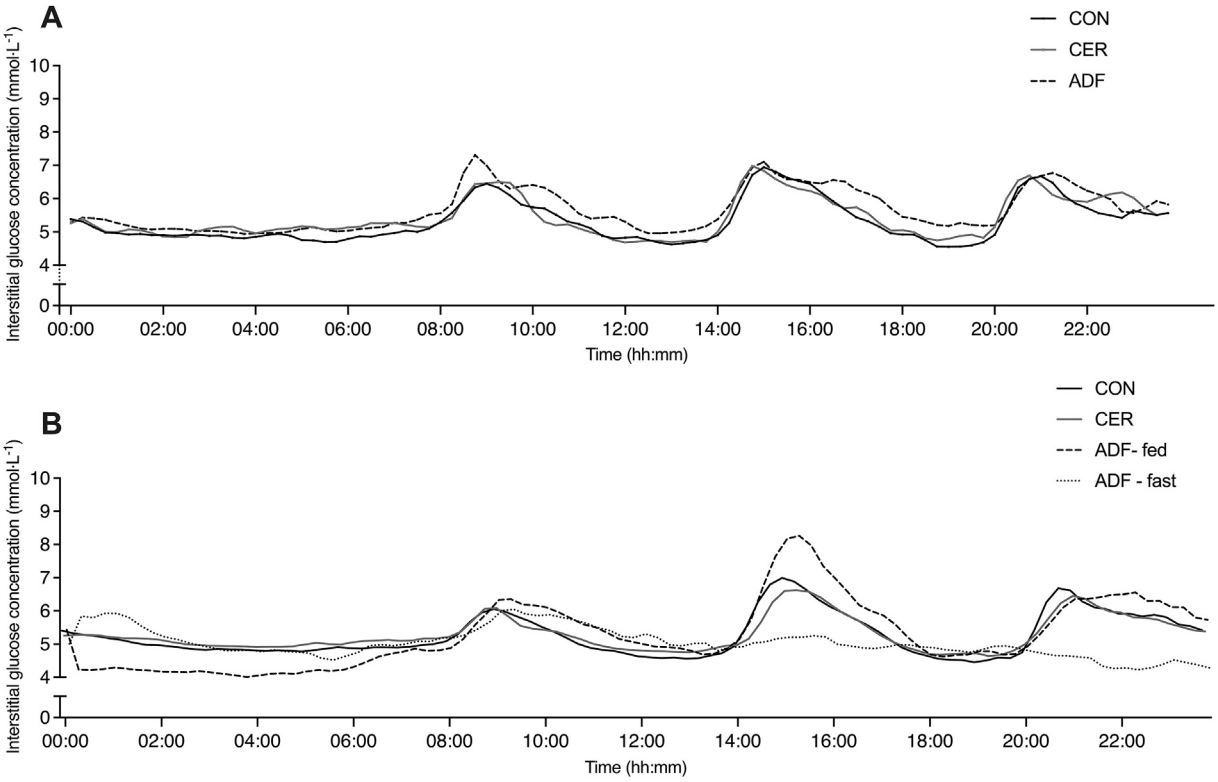



**Fig. 5.** Interstitial 24 h glucose concentrations (mmol/L) in middle-aged males with overweight or obesity during a three-day lead in diet (**A**) followed by a ten-day (**B**) control (CON, $n = 8$), continuous energy restriction (CER, $n = 10$), or alternate day fasting (ADF, $n = 9$) diet. Values represent means.

traditional CER strategies, though, such a compensation in total protein intake would be unlikely during 'free-living' IF strategies, thereby resulting in lower protein intake levels on fasting days. In addition, we hypothesised that ten days of restricted energy intake would impair MPS compared with an energy-balanced, control diet. Previous short-term CER studies that observed declines of

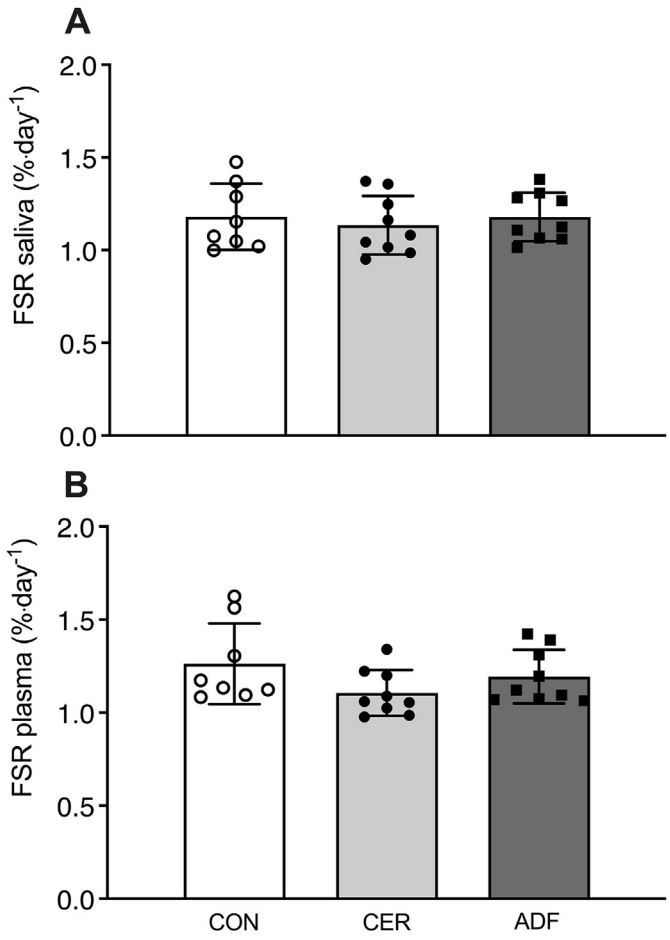



**Fig. 6.** Integrated myofibrillar protein fractional synthetic rates (FSR, %/day) using deuterated water methodology and mean body water $^{2}H$ (**A**) or plasma [$^{2}H$]-alanine (**B**) enrichments as precursor, in middle-aged males with overweight or obesity during a ten-day control (CON, $n = 8$), continuous energy restriction (CER, $n = 9$), or alternate day fasting (ADF, $n = 9$) diet. Dots represent individual values, bars represend mean± SD. Data were analysed with one-way ANOVA. No differences were observed between groups, $P > 0.05$.

15–20 % in rates of MPS [10–13], provided higher protein intake levels (1.2–2.4 g/kg/day) than the amount we provided in our cohort of middle-aged males with overweight or obesity. Besides the total amount of protein, the distribution of protein intake throughout the day is an important determinant to attenuate the decrease in MPS following energy restriction [12]. Here, the uneven protein distribution in CER, with a skewed absolute protein intake towards the evening meal, did not affect MPS compared to the even protein distribution in CON. Recent work from Justesen et al., reported similar findings, showing no differences in MPS following three days of skewed or even protein distribution with comparable protein intake levels in healthy, older adults [42]. To the best of our knowledge, the present study is the first to show that ten days of isonitrogenous energy restriction in either an ADF or CER pattern does not impact MPS.

### 4.2. The effect of IF and CER on metabolic health outcomes

To compare the short-term health effects between ADF and CER, we included several parameters of metabolic health as secondary outcome measurements. No effects were observed in blood pressure, total cholesterol, and triglycerides following ADF or CER. Fasting plasma insulin levels decreased following ADF only, though this was likely an acute effect of the 24 h fasting period prior to the laboratory visits on days four and eight. Furthermore, indices of glycaemic control improved across all groups, with no differences between ADF or CER. Although in ADF, total AUC, mean, SD, and CV of 24 h glucose concentrations were lower on fasting days compared to fed days, this did not induce an overall effect, which is in line with previous work, reporting no differences in the glucose-enhancing effects following short-term IF and CER [21–26,43]. As we included normoglycaemic individuals, improving glycaemic control was not the primary objective of the study, nor it is likely that a short period of energy-balanced IF would affect glycaemic control in ten days. However, reducing the daily eating windows can effectively improve glucose homeostasis; we recently showed that ten days of an 8 h compared to 12 h eating window improves glycaemic control in a comparable population [44]. Lastly, no differences in feelings of hunger and appetite were observed between groups, whereas a decrease in fasting leptin levels was observed following both energy restriction diets (ADF and CER).

### 4.3. Body composition and physical activity levels

In the present study, we provided all meals based upon REE requirements and self-reported physical activity levels. Nonetheless, reductions in BM were seen across all groups. The change in BM largely occurred in the first eight days of ADF and CER, while weight loss in the control diet mainly occurred in the last days of the intervention. It is likely that the meals we provided in the intervention diets were higher in dietary quality and nutritional content (i.e., dietary fibres) compared to participants' habitual food intake pattern, inducing weight loss later in the intervention period. Body composition measurements in previous investigations have shown losses of BM largely to be due to a reduction in FM following three weeks of CER, while ADF induced similar decreases in FM and fat free mass [43]. We observed reductions in LM and FM in a similar magnitude following ADF and CER. While these changes in body composition cannot be explained by any acute changes in rates of MPS, it is likely that an increase in MPB contributed to the reduction in LM. It is important to note that acute changes in muscle protein turnover rates reflect altered anabolic responses to dietary protein intake and/or physical activity, and, as such, do not directly translate to changes in LM. As physical activity sensitises the muscle to dietary protein and directly stimulates MPS, we instructed participants to maintain their level of habitual physical activity. Overall physical activity levels did not differ across intervention groups, but physical activity levels with ADF were modified by fasting periods, with a tendency for increased sedentary behaviour on fasting days compared to fed days. This increase in sedentary behaviour on fasting days is in line with participants reporting feelings of greater tiredness following ADF. Yet, it remains unclear to what extent a change in physical (in)activity levels during IF affect rates of MPS and, over the long-term, muscle mass.

### 4.4. Implications for the preservation of muscle health in clinical practise

There is a broad range of dietary IF strategies, where those with fewer meals per day or more prolonged fasting periods (e.g., '5:2' diet or periodic fasting) are likely have a greater effect on MPS than those with more frequent meals per day. We have recently shown that an isonitrogenous, energy-matched eight h time restricted eating pattern vs the same energy intake consumed over 12 h does not lower MPS [44]. It is possible that in uncontrolled IF protocols where protein intakes are not regularly optimised or chronically low (such as during fasting-mimicking diets), the MPS response to protein intake would be further impaired and, as such, negatively

affect muscle mass maintenance over the longer term. However, this hypothesis remains untested. The present investigation provides evidence that when maintaining adequate protein intake levels during short-term IF, MPS rates are not impaired, thereby highlighting the importance of protein-dense meals during IF practices. The absolute protein intake level sufficient to support muscle mass maintenance during IF strategies warrants further investigation.

In contrast to our hypothesis, no differences in the primary outcome, MPS rates, was observed following ten days of ADF or CER compared to an energy balanced, control diet. Yet, these findings are integral to inform future work in order to provide understanding of the effect of IF on muscle mass maintenance. Though, our study has some limitations. We provided a diet composed of standard products that were available from supermarkets as would be applied in daily practice, with the aim to meet an adequate overall protein intake. However, no analyses of the diets for any specific differences in (essential) amino acid content was performed, limiting our evaluation of between-group amino acid consumption differences beyond macronutrient composition. It should be noted that reduced energy availability or periods of prolonged fasting might also affect MPB. It is currently unknown what the impact of IF strategies is on rates of MPB (and net balance) as there is a paucity of data applying detailed methodology for assessing MPB in human intervention studies in response to IF and/or CER diets. Lastly, implementation of IF in individuals where anabolic resistance is present and muscle mass preservation is critical (i.e., individuals with obesity, older individuals with sarcopenia, patients with acute or chronic disease such as. diabetes mellitus, malnutrition, or chronic obstructive pulmonary disease, or individuals with reduced physical activity levels or increased sedentary behaviour), may have deleterious effects. While IF is an appealing dietary strategy to induce rapid weight loss and improve parameters of metabolic health, considerations in relation to muscle mass preservation should be a priority. Future studies examining IF protocols should include measurements of MPS and/or MPB and address muscle-specific changes in mass and strength to understand the consequences of IF on muscle health.

In conclusion, IF in the form of ADF, with matched, adequate protein intakes does not impair MPS rates when compared to an energy balanced diet or CER on a daily basis. The prolonged effects of IF and more extreme patterns of irregular energy and protein intake on muscle mass maintenance require future investigation.

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## Author contributions

IWKK, EBP, LJCvL, and JAH formulated the research question and designed the study. IWKK, EBP, MJW, BER, and RCH conducted the experimental trials. JMS and JPBG performed the stable isotope analyses. IWKK performed the (statistical) data analysis, data interpretation, and wrote the manuscript together with EBP, LJCvL, and JAH. IWKK and JAH had primary responsibility for final content. All authors read and approved the final content of the manuscript.

## Conflict of interest

None of the authors declared any conflict of interest.

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## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2024.09.034.

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