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Running head: Protein during weight loss in postmenopausal women

Abbreviations: 1-RM, one-repetition maximum; ANOVA, analysis of variance; BM, Body mass; BMI, body mass index; EBW, energy balance whey; ERW, energy restriction whey; EX, exercise; FSH, follicle-stimulating hormone; FSR, fractional synthetic rate; *i*AUC, incremental area under the curve; LBM, lean body mass, MPS, muscle protein synthesis; RDA, recommended dietary allowance; TG, triglycerides

Trial registry: clinicaltrials.gov (ID: NCT03326284)

Data described in the manuscript will be made available upon request pending

Abstract

Background: Diet-induced weight loss is associated with a decline in lean body mass, as mediated by an impaired response of muscle protein synthesis (MPS). The dose-response of MPS to ingested protein, with or without resistance exercise, is well characterised during energy balance but limited data exist under conditions of energy restriction in clinical populations.

Objective: To determine the dose-response of MPS to ingested whey protein following short-term diet-induced energy restriction in overweight, postmenopausal, women at rest and post-exercise.

Design: Forty middle-aged (58.6±0.4 years), overweight (BMI: 28.6±0.4), postmenopausal women were randomised to one of four groups: Three groups underwent 5 days of energy restriction (~800 kcal/d). On day 6, participants performed a unilateral leg resistance exercise bout before ingesting either a bolus of 15g (ERW15, n=10), 35g (ERW35, n=10) or 60g (ERW60, n=10) of whey protein. The fourth group (n=10) ingested a 35g whey protein bolus after 5 days of an energy balanced diet (EBW35, n=10). Myofibrillar fractional synthetic rate (FSR) was calculated under basal, fed (FED) and post-exercise (FED-EX) conditions by combining an L-[*ring*-$^{13}C_6$]phenylalanine tracer infusion with the collection of bilateral muscle biopsies.

Results: Myofibrillar-FSR was greater in ERW35 (0.043±0.003%/h, P=0.013) and ERW60 (0.042±0.003%/h, P=0.026) than ERW15 (0.032±0.003%/h), with no differences between ERW35 and ERW60 (P=1.000). Myofibrillar-FSR was greater in FED (0.044±0.003%/h, P<0.001) and FED-EX (0.048±0.003%/h, P<0.001) than BASAL (0.027±0.003%/h), but no differences were detected between FED and FED-EX (P=0.732) conditions. No differences in myofibrillar FSR were observed between EBW35 (0.042±0.003%/h) and ERW35 (0.043±0.003%/h, P=0.744).

Conclusion: A 35 g dose of whey protein, ingested with or without resistance exercise, is sufficient to stimulate a maximal acute response of MPS following short-term energy restriction in overweight, postmenopausal women, and thus may provide a per serving protein recommendation to mitigate muscle loss during a weight loss program. Trail registration: clinicaltrials.gov (NCT03326284).

Key words: Females, middle-aged, obesity, weight loss, muscle protein synthesis

1.0 Introduction

The worldwide prevalence of overweight and obese middle-aged (40-65 years) adults represents an increasingly important public health challenge within the discipline of human and clinical nutrition (1, 2). Accordingly, considerable attention has focused on optimising weight loss interventions that target this population demographic (3, 4). Specifically, the efficacy of complex weight loss interventions that combine non-pharmacological nutritional and exercise strategies have focussed on dietary protein manipulation with (5) or without (6-8) the inclusion of a structured resistance-based exercise training program to mitigate the counter-productive loss of lean body mass (LBM).

The efficacy of a diet-induced weight loss intervention depends, at least in part, on the retention of LBM during a period of energy deficit (9, 10). This notion is supported by clinical studies that report a clear association between muscle mass index, defined as the skeletal muscle mass:fat mass ratio, and metabolic disease risk, functional decline, and mortality (11, 12). The preponderance of evidence suggests that muscle atrophy during energy restriction is mediated by suppressed postabsorptive and postprandial rates of muscle protein synthesis (MPS) (13-16), although an upregulation in muscle protein breakdown during energy restriction also has been reported (17). Moreover, whereas similar basal rates of MPS have been observed between

obese and lean individuals (18), studies have reported a reduced postprandial response of MPS to protein ingestion in overweight/obese individuals *vs.* age-matched lean controls (19, 20). In addition, clinical studies have demonstrated an impaired muscle anabolic response to protein feeding and exercise training in postmenopausal women compared to older men and healthy young adults (21-25). Hence, these data provide compelling rationale for developing targeted dietary interventions aimed at mitigating muscle loss during diet-induced energy restriction specifically in postmenopausal women.

Accumulating evidence suggests that increasing the protein content of an energy-restricted diet represents an effective dietary intervention to mitigate muscle atrophy, and promote fat mass loss, during diet-induced weight loss in overweight and obese individuals (26, 27). Accordingly, a general consensus exists that the optimal daily protein intake to maintain muscle mass during weight loss is ~50% greater than the current recommended dietary allowance (RDA), ranging from 1.2-1.6 g protein⁄kg BM/d (26, 28). Nevertheless, acute metabolic studies that measure the response of MPS to protein feeding under conditions of energy restriction are warranted in overweight/obese individuals to refine this protein recommendation on a per-serving basis (29). Whereas the dose response of MPS to ingested protein has been characterized in young (30-33), middle-aged (34) and older (35, 36) men in energy balance, comparable studies have not been conducted in middle-aged women. Based on the apparent sexual dimorphism in response of MPS to protein feeding post menopause (23), intuitively the optimal protein dose for maximal stimulation of MPS in middle-aged and older adult men may not directly translate to age-matched postmenopausal women under conditions of energy restriction.

The specific objective of this proof-of-principle study was to examine the dose-response of MPS to ingested protein at rest (primary outcome) and during the acute (3 h) recovery period

following resistance exercise in a cohort of middle-aged, overweight, postmenopausal women following 5 days of diet-induced energy restriction. The whey protein doses (15 g, 35 g, 60 g) were selected to characterise a complete dose-response curve. In addition, to determine the influence of energy restriction on the MPS response to protein ingestion, we compared rates of MPS in postmenopausal women following ingestion of 35 g of whey protein during conditions of energy restriction and energy balance. Our primary hypothesis was that protein feeding would augment rates of MPS above basal fasting values in a dose-dependent manner (i.e., 15 g < 35 g < 60 g) following short-term energy restriction (primary outcome). Secondly, we hypothesized that rates of MPS would be augmented with resistance exercise compared to rest, regardless of protein dose. Finally, we hypothesized the MPS response to ingestion of 35 g whey protein would be attenuated following a period of energy restriction *vs*. energy balance in middle-aged, postmenopausal, women.

2.0 Methods

2.1 Subjects and ethical approval

Forty (n = 40) healthy, middle-aged (58.6 ± 0.4 y) women were recruited for this study (**Table 1**). Written informed consent was provided by all participants that were deemed healthy based on a screening interview and routine blood sample analyses. Volunteers were eligible to participate if they were aged 50-65 years, postmenopausal (defined as no menstrual bleeding for 6 months, follicle-stimulating hormone (FSH) concentration > 30 IU/L, oestrogen concentration < 50 pmol/L), non-smokers and recorded a body mass index (BMI) > 25. The study was conducted at the Department of Public Health, Aarhus University, Aarhus, Denmark between July 2017 and March 2018. The trial was registered at clinicaltrials.gov (ID:

NCT03326284) and conducted in accordance with standards of the local ethics committee of Central Denmark Region (1-10-72-56-17) and the Declaration of Helsinki.

2.2 Study design

A randomized, single blinded, parallel study design was conducted to determine the dose-response of myofibrillar fractional synthesis rate (FSR) to ingested whey protein at rest (FED) and post-exercise (FED-EX) following a 5-day period of energy restriction in middle-aged, overweight postmenopausal women. The response of myofibrillar FSR to a moderate dose (35 g) of ingested whey protein was also measured following a controlled 5-day period in energy balance to determine the influence of energy status on MPS rates. In total, 40 women were randomly assigned to one of four groups (**Figure 1**). Three groups underwent a 5-day energy restricted dietary intervention (ER, ~800 kcal/d; n = 30) and one group continued their habitual energy balanced diet (EB, ~1785 kcal/d, n = 10) prior to conducting an acute metabolic trial for measurement of myofibrillar FSR. Metabolic trials (**Figure 2**) were identical in design except for administering 15 g (ERW15; *n*=10), 35 g (ERW35; *n*=10 and EBW35; *n*=10) or 60 g (ERW60; *n*=10) of whey protein. Participants remained blinded to their assigned protein dose for the study duration. All trials included an acute bout of unilateral knee extension resistance exercise. Due to participant discomfort with the muscle biopsy procedure, we were unsuccessful in obtaining sufficient tissue from five participants and thus the measurement of plasma L-[*ring*-$^{13}C_6$]-phenylalanine enrichment and calculation of myofibrillar FSR are expressed as n = 10 (ERW15), n = 8 (ERW35), n=8 (ERW60) and n = 9 (EBW35), as displayed in **Figure** 1).

2.3 Screening visit

Eligible participants attended the laboratory after an overnight fast >1 wk prior to conducting the experimental trial. A blood sample was analysed for routine biomarkers of general

metabolic health and sex hormone concentrations. Women with concentrations of oestrogen <50 pmol/L, FSH < 30 IU/L, HbA1c > 7.3 mmol/mol, alanine transaminase > 45 U/L, and/or thyroid-stimulation hormone > 4.5×10^{-3} IU/L were excluded from participation. Body composition was determined using dual-energy x-ray absorptiometry (DXA; GE Lunar DXA scan, GE Healthcare, WI, USA) and a maximum strength test was conducted. At the screening visit the project coordinator performed a simple randomization procedure (participants drew lots from an opaque envelope) to allocate participants to one of the four treatments. The participants were blinded to the protein dose allocation.

2.4 Maximum strength testing

One-repetition maximum (1RM) for leg extension (Technogym-Selection line, Technogym, Italy) was estimated in accordance with the procedure described by (37). The test was conducted after a self-administered 10 min warm-up on an ergometer bike. Leg assigned to exercise was randomly selected, i.e., independent of dominance.

2.5 Diet and physical activity control

Participants commenced their assigned diets five days before the experimental visit. Energy-restricted groups (ERW15, ERW35 and ERW65) were provided with soups, shakes and meal replacement bars (Nutrilett, Orkla Health AS, Oslo, Norway) for consumption, and advised to consume 200 g of low-calorie water dense vegetables (i.e., cucumber, tomatoes, and lettuce) and > 2 L of water daily. Participants assigned to the energy balance group (EBW35) were instructed to replicate their habitual diet and register all food consumption using a diet registration mobile phone app (MADLOG mini, MADLOG Aps, Kolding, DK). Energy allowances in the energy balance group were set to provide sufficient energy to maintain energy balance as determined by using the Harris Benedict equation for estimation of basal metabolic rate, which was multiplied by a factor (1.4–1.5) corresponding to a moderate

physical activity level (38). The approximate energy requirements were as follows: 2057 ± 51 kcal/d (ERW15); 2024 ± 29 kcal/d (ERW35); 2098 ± 43 kcal/d (ERW60); 2026 ± 39 kcal/d (EBW35). Thus, the energy restricted diet would induce an estimated energy deficit of ~1200 kcal / d. Physical activity level during the experimental period was standardized by instructing participants to target a daily step count of 6,000–10,000 steps as quantified by a Yamax pedometer (Yamax PZ270 Power Walker Lite, Yamasa Tokei Keike Co., Ltd, Japan). Non-caloric drinks (e.g., black coffee and tea) were permitted *ad libitum* until 24-h prior to commencing the experimental day, whereas alcohol or caffeinated drinks were prohibited within 24-h of the experimental day. The participants were permitted only to drink water after 8:00 p.m. the evening before the experimental day.

2.6 Infusion protocol

Participants reported to the laboratory at 7:30 a.m. after an overnight fast. Body weight was measured and two catheters were inserted into an antecubital vein and a dorsal hand vein of the contralateral arm. A baseline blood sample was collected for determination of background phenylalanine enrichment before a primed (6.0 µmol/kg LBM), continuous (6.0 µmol/kg LBM/h) infusion of L-[*ring*-$^{13}C_6$]-phenylalanine (Cambridge Isotopes, Andover, MA, USA) was initiated. The cannulated hand was heated for arterialized blood sampling throughout the infusion protocol. At 90 min after starting the infusion, a muscle biopsy was obtained from the leg assigned to resistance exercise (FED-EX). Next, participants rested supine before performing a single bout (5 sets × 10 repetitions) of unilateral leg extension at 80% 1RM with 2 min rest between sets. If a participant could not complete a full set, the load was lowered by 5 – 10%. A muscle biopsy was then obtained from the contralateral resting leg (FED). Immediately after the muscle biopsy, participants ingested their assigned whey protein bolus

and then rested in a supine position for 3 h before two further muscle biopsies were obtained from the exercised (FED-EX) and non-exercised (FED) leg.

2.7 Protein beverages

Whey protein beverages (Lacprodan® HYDRO.REBUILD, Arla Foods Ingredients Group P/S, Viby J, DK) were administered immediately after collection of the second muscle biopsy obtained after exercise (**Table 2**). Beverage flavour was chocolate or mint based on personal preference. The volume of all beverages was 300 ml. To minimize perturbations in plasma isotopic enrichment, beverages were enriched with L-[*ring*-$^{13}C_6$]-phenylalanine. Based on previous observations of transient elevations in plasma $^{13}C_6$ phenylalanine enrichments following bolus ingestion of 40 g of whey protein (31), we adjusted the beverage enrichment of L-[*ring*-$^{13}C_6$]-phenylalanine as follows depending on the whey protein dose: 15 g protein dose: 10%; the 35 g dose: 8.5% and the 60 g dose: 6.25%.

2.8 Muscle biopsy and blood sampling

All blood samples were dispensed into pre-chilled coated (EDTA or lithium heparin) blood collection tubes. Serum-separator tubes were allowed to clot for 30 min before centrifugation (1,500 *g* for 15 min at 5°C). As described above, a total of four muscle biopsies (two from each leg; ~250 mg) were obtained from the *vastus lateralis* (~12–15 cm proximal to patella) under local anaesthesia (10 ml Xylocain® 10mg/ml, AstraZeneca, Sweden) using a 5 mm Bergström needle with manual suction. Muscle samples were snap frozen and stored at –80°C until further analysis.

2.9 Analytical procedures

2.9.1 Blood metabolite concentrations

Plasma amino acid concentrations and serum insulin concentrations were determined as described by Bornø and van Hall (39) and Christensen, et al. (40), respectively. Blood glucose

concentration was quantified using a HemoCue Glucose 201 RT Analyzer (HemoCue® AB, Ängelholm, Sweden) and plasma urea concentration was determined using absorption photometry (Cobas 6000, Roche, Basel, CH and Chemistry XPT System, Siemens Healthcare A/S, Ballerup, DK).

2.9.2 Stable isotope analysis

Plasma phenylalanine enrichments were determined as described previously (41). To isolate intramuscular free amino acids and myofibrillar proteins, muscle samples (25-35 mg wet weight) were homogenized by ceramic beads (lysing matrix D; FastPrep®-24 homogenizer, MP Biomedicals, Santa Ana, CA) in 1 mL of prechilled homogenization buffer (Tris 0.02 M [pH, 7.4]; NaCl 0.15 M; EDTA 2 mM, EGTA 2 mM, one protease inhibitor tablet per 10 mL buffer) and then centrifuged at 10,000 g for 15 min at 4°C. This process was repeated with the remaining pellet without the protease inhibitor tablet solubilized in the buffer. The two supernatants (~2 mL) were transferred to vials with 2 mL ice cold 100% acidic acid. The free amino acids were subsequently purified over columns with acidified cation exchange resin as described previously (42). Next, 1 mL NaOH (0.3 M) was added to the pellet from the homogenization process containing structural proteins, homogenized for 30 s and left in a heating block (50°C) for 2 × 30 min (vortexed in between) and centrifuged (10,000 g, 10 min, 4°C). Supernatants were transferred to vials suitable for hydrolysis. This process was repeated with the remaining pellet and supernatants merged. Perchloric acid (1 mL 2 M) was added to the supernatants containing myofibrillar proteins. Vials were vortexed and left on ice for 20 min. After centrifugation (3,000 g, 10 min, 4°C), supernatants were discarded and the pellets washed twice in EtOH (1 mL 70%), vortexed and centrifuged (3,000 g, 10 min, 4°C). The remaining pellets were vortexed in a mix of 2 mL HCl and 1 mL Dowex resin (Bio-Rad Laboratories, Hercules, CA), before overnight incubation (110°C). Subsequently, the

myofibrillar amino acids were purified over cation exchange resin columns using NaOH (2M) for elution. Amino acids were derivatized with N-acetyl-propyl as described previously (42). Finally, the derivatized samples were injected into a gas-chromatography combustion isotope ratio mass spectrometer (ThermoFisher Scientific, Hemel Hempstead, UK). For practical reasons, the muscle samples were analyzed at University of Birmingham and University of Nottingham. The analyses used the same protocols for sample preparation. Data was inspected visually and statistically to identify any effect of analysis-site. No effect of site was detected ($P > 0.05$).

2.10 Calculation of myofibrillar MPS

Myofibrillar FSR was calculated using the standard precursor equation:

$$FSR\ (\%\ \times\ h^{-1}) =\ \Delta E_{protein} \div\ E_{precursor} \times 1/\Delta time\ \times\ 100$$

Where $\Delta E_{protein}$ is the difference in tracer enrichment in the myofibrillar protein fraction between two biopsy samples, $E_{precursor}$ is the arterialized blood precursor defined as the area under the curve (AUC) for plasma enrichments of labelled phenylalanine over the 3-h incorporation periods. $\Delta time$ is the time interval between muscle biopsies.

2.11 Data presentation and statistics

A sample size of 32 (8 participants/group) was calculated a *priori* based on previous data from comparable studies with similar participant characteristics investigating the dose-response of myofibrillar FSR to ingested protein in older men (34, 35). This calculation was based on the assumption that the minimal detectable difference in FSR between protein dosages would be 0.01 %/h when the SD of the means was set to be 0.007 %/h. The 1-β error of probability was set at 0.8 and an α-level of < 0.05.

Statistical analysis of myofibrillar FSR data (primary endpoint) was conducted using a repeated measures mixed effects model with *protein dose* (ERW15, ERW35, ERW60) and *condition* (BASAL, FED, FED-EX) as independent variables in the fixed part of the model. Participants were included in the random part of the model. Data were analysed for main effects and any interaction between the two independent variables. Bonferroni *post hoc* tests were applied if statistical significance of interactions or main effects were reached. *Post hoc* analyses of main effects were performed independently of the other independent variable. To determine the influence of energy status on myofibrillar FSR, a similar mixed effects model was used with *energy status* (EBW35, ERW35) and *condition* (BASAL, FED, FED-EX) as independent variables in the fixed part of the model and participants in the random part. Other endpoints (insulin, urea, glucose, amino acid concentrations and phenylalanine enrichments) were analysed using a similar mixed model with *protein dose* and *time* as fixed effects, and participants as a random effect. Main effects (*protein dose, time*) and interactions, as well as post hoc analyses, were performed as described above. One-way analyses of variance (ANOVA) was used for data presented as incremental area under the curve (*i*AUC). *i*AUC was calculated with the baseline set as timepoint 0. Normality and homogeneity of data were checked by inspecting QQ-plots and plots of residuals versus the fitted values. Serum insulin concentrations were deemed heteroskedastic from visual inspection and consequently log-transformed before statistical analyses. Data are presented as means ± SEM unless otherwise stated. All statistical analyses were performed using STATA version 14.2 (StataCorp LP, Collage Station, TX, USA) and significance was set at an α-level of < 0.05.

3.0 Results

3.1 Diet, exercise and body weight

Total energy and macronutrient intakes were lower in the energy-restricted diet groups than the energy balance diet group (all $P < 0.05$, **Table 3**). Average daily step count was comparable between groups (ERW15: 7502 ± 454 steps; ERW35: 8953 ± 620 steps; ERW60: 7722 ± 470 steps; EBW35: 7718 ± 573 steps; $P > 0.05$). A decline in body weight was observed in all ERW groups during the 5-day energy restriction period (ERW15: –2.4 ± 0.2 kg; ERW35: –1.8 ± 0.2 kg; ERW60: –2.8 ± 0.3 kg; all $P < 0.001$), with no change in EBW35 (–0.2 ± 0.2 kg, $P = 0.32$). Weight loss was greater in ERW60 than ERW35 ($P = 0.03$). The total weight lifted throughout the exercise protocol was similar between groups (mean ± SD; ERW15: 655 ± 247 kg; ERW35: 679 ± 137 kg; ERW60: 771 ± 224 kg; EBW35: 776 ± 198 kg; ERW15 vs ERW35 vs ERW60, $P = 0.435$; ERW35 vs EBW35, $P= 0.221$)

3.2 Amino acid concentrations

Plasma phenylalanine concentration peaked at 60 min post protein ingestion for all groups, with the magnitude of increase greater in ERW35 (105 ± 3 μmol/L) and ERW60 (107 ± 4 μmol/L; than ERW15 (83 ± 3 μmol/L, both $P < 0.001$). Phenylalanine concentration returned to baseline at 3 h post protein ingestion in ERW15 and ERW35 but remained elevated in ERW60 (90 ± 4 μmol/L; $P < 0.001$; **Figure 3a**). The *i*AUC of phenylalanine concentration increased in a dose-dependent manner (all $P < 0.05$; **Figure 3b**), with no differences between ERW35 and EBW35 ($P = 0.99$).

Plasma leucine concentration peaked at 60 min post protein ingestion in ERW15 and ERW35 and 120 min post protein ingestion in ERW60 and remained elevated for the remainder of the experimental trial ($P < 0.001$; **Figure 4a**). The *i*AUC of leucine concentration increased

330 in a dose-dependent manner (all $P < 0.001$) and was greater in ERW35 than EBW35 ($P < 0.008$,
331 **Figure 4b**).

332 **3.3 Plasma glucose, serum insulin and urea concentrations**

333 A main effect of time was observed for glucose concentration after protein ingestion ($P = 0.03$;
334 **Supplementary Figure 1a**), but post hoc analyses showed no difference from baseline at any
335 time ($P > 0.05$). No time × dose interaction ($P = 0.39$) or differences in *i*AUC of plasma glucose
336 concentration was observed between groups ($P > 0.05$, **Supplementary Figure 1b**).

337 Serum insulin concentrations peaked 30 – 60 min after protein ingestion ($P < 0.01$) and
338 returned to baseline levels at 3 h post protein ingestion in ERW15 and ERW35 (**Figure 5a**).
339 The *i*AUC of serum insulin concentration was higher in ERW35 and ERW60 than ERW15 (P
340 < 0.05) and higher in ERW60 than in ERW35 ($P = 0.033$, **Figure 5b**). No differences in insulin
341 concentration were observed between ERW35 and EBW35 ($P = 0.756$).

342 The highest plasma urea concentrations were observed at 3 h post protein ingestion in
343 all groups (time effect: $P < 0.001$, **Supplementary Figure 2a**) and were greater in EBW35 (7.0
344 ± 0.3 mmol/L) and EBW60 (8.2 ± 0.3 mmol/L) compared to EBW15 (5.2 ± 0.3 mmol/L). No
345 differences in *i*AUC of plasma urea concentration (all $P > 0.05$; **Supplementary Figure 2b**

346 **3.4 Plasma phenylalanine enrichments**

347 A steady state in plasma L-(*ring*-$^{13}C_6$)phenylalanine was reached 30 min after initiating the
348 infusion (**Figure 6**). Despite enriching all protein beverages with tracer, a modest decline in
349 plasma L-(*ring*-$^{13}C_6$)phenylalanine enrichment was observed in EBW35, ERW35 and ERW60
350 post protein ingestion.

351 **3.5 Myofibrillar fractional synthetic rate**

352 A main effect of protein dose was observed across all conditions (BASAL, FED and FED-EX)

combined (P = 0.006)(**Figure 7**). Post hoc analysis revealed a greater response of myofibrillar FSR in ERW35 (32%, +0.010 ± 0.003%/h, P = 0.013) and ERW60 (29%, +0.009 ± 0.003%/h, P = 0.026) than ERW15, with no differences between ERW35 and ERW60 (P = 1.000). A main effect of condition was observed for all groups combined (P < 0.001), with myofibrillar FSR 63% greater in FED (+0.017 ± 0.004%/h, P < 0.001) and 79% greater in FED-EX (+0.021 ± 0.004%/h, P < 0.001) than BASAL, but no differences were detected between the FED and FED-EX (P=0.732) conditions. In addition, no protein dose × condition interaction was detected (P = 0.744) (**Figure 7**). Moreover, no main effects of diet (energy restriction *vs*. energy balance, P = 0.744) or diet × condition interaction (P = 0.996) were observed for myofibrillar FSR when EBW35 and ERW35 groups only were included in the statistical model. However, a main effect of condition (P < 0.001) was observed for this analysis as well (**Figure 7**).

4.0 Discussion

This clinical randomised controlled trial investigated the dose-response relationship between ingested whey protein and *in vivo* postprandial rates of MPS in middle-aged, overweight postmenopausal women under conditions of diet-induced weight loss. Utilizing a unilateral leg resistance exercise model, we measured the dose-response of myofibrillar FSR to ingested protein at rest (FED) and post-exercise (FED-EX) following 5 days of energy restriction. In addition, we examined the influence of energy status (i.e., energy balance *vs*. energy restriction) on basal and postprandial myofibrillar FSR in response to ingestion of a moderate (35 g) dose of whey protein. By design, a modest (~2 kg) decline in body weight was observed in all energy restricted groups, with body weight stable in the energy balance group. The primary study finding was a plateau in dose response of myofibrillar FSR to ingested protein at 35 g of whey protein, with no additional stimulation of MPS with the ingestion of 60 g of whey protein (ERW15 < ERW35 = ERW60) following 5 days of energy restriction in overweight,

postmenopausal women. A secondary finding was that resistance exercise failed to potentiate the acute response of myofibrillar FSR to increasing doses of ingested whey protein following energy restriction. Finally, the acute period of energy restriction did not modulate the postprandial response of myofibrillar FSR to ingestion of a moderate dose (35 g) of whey protein. Taken together, these data indicate that ingesting a 35 g dose of high-quality protein on a per meal/serving basis, with or without resistance exercise, is sufficient to stimulate a maximal postprandial response of MPS following an acute period of energy deficit in overweight, postmenopausal women. Thus, an appropriate practical recommendation for this important clinical sub-population is to ingest 35 g of high-quality protein per meal during a weight loss programme.

Current knowledge regarding the dose-response of MPS to ingested protein is primarily based on studies in healthy young and older adults in energy balance. A general consensus exists that the dietary protein induced stimulation of MPS is finite whereby, above a certain threshold protein dose, the fate of ingested protein-derived amino acids is primarily non-anabolic (i.e., oxidation) rather than incorporation into bound new muscle protein (43). For instance, previous studies observed a plateau in the dose-response of MPS to ingested protein at a 20 g dose in healthy young men under conditions of energy balance, with the 40 g protein dose conferring no additional stimulation of MPS (30, 31, 44). The opposing argument suggests the anabolic response to ingested protein is not limited by the maximal stimulation of protein synthesis (45). This viewpoint is evidenced by studies that conducted whole-body assessments of protein synthesis, i.e., aggregate protein synthesis rates across all body tissues combined, rather than tissue-specific (i.e., muscle) measurements of MPS (46, 47). In the present study, the maximal effective protein dose for stimulation of MPS was 35 g of whey protein in middle-aged, overweight, postmenopausal, women under conditions of short-term diet-induced energy

restriction. While the postprandial response of MPS was markedly greater in ERW35 and ERW60 than ERW15, we observed no differences in myofibrillar FSR between ERW35 and ERW60 groups. These data corroborate the findings of Robinson, et al. (34) that reported an upper limit to the stimulation of MPS with the ingestion of 36 g of beef protein in middle-aged men in energy balance. Although we did not perform a direct comparison between men and women, our results suggest that energy restricted middle-aged, overweight, postmenopausal, women respond similarly to protein feeding as their male counterparts in energy balance. Hence, taken together these data suggest that following 5 d of energy restriction, 35 g of whey protein is sufficient for the maximal stimulation of MPS in middle-aged, overweight postmenopausal woman.

The interaction of exercise training and increased dietary protein intake during a period of energy deficit represents an evidence-based strategy to mitigate the impaired response of MPS, and potential subsequent decline in muscle mass, associated with diet-induced weight loss in overweight women (48, 49). Consistent with this notion, a longitudinal study by Layman, et al. (5) demonstrated that the addition of a resistance-based exercise training program (2 d/wk resistance training + 5 d/wk walking) to a high protein diet (1.6 g/kg BM/d) promoted the loss of fat mass and retention of lean body mass in middle-aged women that undertook a 4-month weight loss trial. In addition, the impairment in basal myofibrillar FSR following 5 days of energy restriction in resistance-trained young adults was restored following a single bout of resistance exercise to levels observed at rest in energy balance (15). These authors also reported that protein ingestion increased MPS in a dose-dependent manner above rates observed at rest during energy balance (15). However, in the present study, and refuting our original hypothesis, we report no additive effect of resistance exercise on the postprandial response of MPS. Whereas myofibrillar FSR was greater in FED and FED-EX than BASAL

across dose groups, no statistical difference in MPS was observed between FED and FED-EX conditions. In contrast, previous dose-response studies, conducted under conditions of energy balance and utilizing the same unilateral exercise model as the present study, have demonstrated greater MPS rates in the exercised *vs*. rested leg in healthy young (31), middle-aged (34) and older (35) adults. Hence, we may deduce that 5 days in energy deficit is sufficient to inhibit the exercise-induced stimulation of MPS in middle-aged, postmenopausal woman that are less responsive to resistance exercise as an anabolic stimulus compared with their resistance-trained young adult counterparts (15, 23).

An alternative factor that may underpin the lack of exercise-induced stimulation of MPS may be the relatively short 3 h tracer incorporation period employed in the present study. Whereas protein ingestion alone elicits a rapid, but transient, stimulation of MPS, peaking 90–120 min post ingestion (50, 51), prior resistance exercise has been shown to sustain myofibrillar FSR over an extended 5 h postprandial period compared with feeding alone (30). Accordingly, previous reports of an exercise-induced increase in postprandial MPS in healthy young and older adults was measured over a 6 h incorporation period (52). Hence, it remains unclear whether the lack of exercise-induced increase in postprandial myofibrillar FSR was physiologically inherent to the studied cohort of overweight post-menopausal women under conditions of energy deficit, or merely an artefact of the tracer period for measurement of MPS.

The attenuated rate of MPS previously reported during energy restriction (14-16) has been proposed to represent an adaptive mechanism to conserve energy during weight loss. This notion is intuitive given that MPS is an energetically expensive metabolic process that requires ~4 moles of ATP to initiate the translation elongation step of MPS (53). Accordingly, studies in healthy, weight stable, young adults demonstrate an ~25% decrease in basal rates of MPS during the early (5–10 d) phase of an energy-restricted diet (13, 15, 16), with minimal changes

449 in muscle protein breakdown (16). Moreover, an extended period of energy restriction (21 days)
450 was shown to elicit a suppressed postprandial response of MPS to 20 g of ingested milk protein
451 (54) when daily protein intake was restricted to the RDA (0.8 g/kg BM/d). Hence, based on
452 acute metabolic studies in healthy young adults, the primary metabolic driver of LBM loss
453 during energy deficit appears to be phase dependent, with basal rates of MPS impaired during
454 the early phase of energy restriction, and the postprandial response of MPS attenuated during
455 later periods of energy restriction. Refuting our original hypothesis, we report no differences in
456 basal or postprandial (FED or FED-EX conditions) myofibrillar FSR between EBW35 and
457 ERW35 groups, despite the 2 kg decline in body mass in ERW35 following the diet period.
458 This counter-intuitive finding was likely attributed to differences in experimental design
459 between past (14, 15) and present studies. We utilized a parallel, between-subjects, design to
460 determine the influence of energy status on myofibrillar FSR, whereas previous studies
461 employed a more sensitive within-subject crossover design with participants serving as their
462 own control (14-16). Interestingly, previous studies in physically-active young adults have
463 demonstrated a high protein diet (1.6–2.4 g/kg/d) to be effective in preserving basal and
464 postprandial rates of MPS, and reducing loss of LBM during short-term energy restriction (54).
465 Hence, a follow up study that manipulates dietary protein intake during a longer-term (weeks –
466 months) period of energy restriction is warranted in a clinical population of overweight,
467 postmenopausal women.

468 A strength of the present study relates to novelty in terms of investigating the protein-
469 dose MPS response relationship under conditions of energy deficit in a clinically relevant,
470 homogenous sample of middle-aged, overweight, postmenopausal women. Moreover, fraction-
471 specific measurements of myofibrillar-FSR were conducted under basal, fed and exercised-fed
472 conditions, and thus provided comprehensive insight into postabsorptive, postprandial and

473 exercise-stimulated responses of MPS to energy restriction. However, we acknowledge several
474 limitations. First, for practical reasons, the trial was conducted as a single-blinded study. In this
475 regard, the investigators that performed the experimental trial and statistical analysis were not
476 blinded to group allocation. However, all sample analyss for the measurement of MPS (primary
477 endpoint) weres performed by blinded investigators, and thus the single blinded nature of the
478 trial was unlikely to bias study findings. Second, while measurements of MPS were conducted
479 under multiple conditions, i.e., resting and post-exercise, energy balance and energy restriction,
480 the study was powered based on previous dose-response studies conducted in energy balance.
481 Third, the energy restriction period was severe (~800 kcal/d) and short-term (5 days) and thus
482 direct translation of our findings to clinically relevant (20% energy deficit for weeks to months)
483 periods of weight loss must be considered with caution. Fourth, due to limited available muscle
484 tissue, it was not possible to use intracellular $^{13}C_6$ phenylalanine enrichments as the true
485 precursor in the calculation of myofibrillar FSR and instead plasma $^{13}C_6$ phenylalanine
486 enrichments were used for the calculation of MPS. Finally, we did not conduct measurements
487 of muscle protein breakdown alongside MPS. Hence, it was not possible to calculate the
488 response of net muscle protein balance to protein feeding during energy deficit. Interestingly,
489 previous studies have reported an increased stimulation of muscle protein breakdown following
490 10 days of moderate (20%) energy deficit (17), suggesting a mechanistic action of muscle
491 proteolysis in muscle mass loss during diet-induced energy restriction, at least over prolonged
492 periods of weight loss. Moreover, future studies are warranted to establish the dose-response of
493 MPS to ingested protein during weight loss in other clinical populations that experience muscle
494 loss, i.e., sarcopenic obese older adults, over chronic periods of diet-induced weight loss.
495 Deuterium oxide tracer methodology is ideally suited to the measurement of free-living,
496 integrated, rates of MPS over prolonged periods of weight loss (55), and thus once fully re-

established in the field of muscle protein metabolism, may be utilised in future studies to inform protein recommendations for muscle mass retention during weight loss in clinical populations.

5.0 Conclusion

We demonstrate that ingesting a 35 g dose of high-quality protein on a per meal/serving basis, with or without resistance exercise, is sufficient to stimulate a maximal postprandial response of MPS during a short-term period of weight loss in middle-aged, overweight, postmenopausal women. These results provide a foundation for devising refined protein recommendations on a per serving/meal basis for this clinical group during a weight loss programme.

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References

1. WHO. Country profiles on nutrition, physical activity and obesity in the 53 WHO European Region Member States. Methodology and summary. World Health Organization, 2013:18.
2. Hales CM, Carroll MD, Fryar CD, Ogden CL. Prevalence of obesity among adults and youth : United States, 2015–2016. In: National Center for Health S, ed. Hyattsville, MD, 2017.
3. Carbone JW, McClung JP, Pasiakos SM. Recent Advances in the Characterization of Skeletal Muscle and Whole-Body Protein Responses to Dietary Protein and Exercise during Negative Energy Balance. Adv Nutr. 2019/01/01 ed. School of Health Sciences, Eastern Michigan University, Ypsilanti, MI. Military Nutrition Division, US Army Research Institute of Environmental Medicine, Natick, MA., 2019:70-9.
4. Colleluori G, Villareal DT. Aging, obesity, sarcopenia and the effect of diet and exercise intervention. Exp Gerontol. 2021;155:111561. doi: 10.1016/j.exger.2021.111561.
5. Layman DK, Evans E, Baum JI, Seyler J, Erickson DJ, Boileau RA. Dietary protein and exercise have additive effects on body composition during weight loss in adult women. J Nutr. 2005/07/28 ed. Department of Food Science and Human Nutrition, University of Illnois, Urbana, 61801, USA. dlayman@uiuc.edu, 2005:1903-10.
6. Gwin JA, Church DD, Hatch-Mcchesney A, Howard EE, Carrigan CT, Murphy NE, et al. Effects of high versus standard essential amino acid intakes on whole-body protein turnover and mixed muscle protein synthesis during energy deficit: A randomized, crossover study. Clinical Nutrition. 2021;40(3):767-77. doi: 10.1016/j.clnu.2020.07.019.
7. Skov AR, Toubro S, Rønn B, Holm L, Astrup A. Randomized trial on protein vs carbohydrate in ad libitum fat reduced diet for the treatment of obesity. Int J Obes Relat Metab Disord. 1999;23(5):528-36. doi: 10.1038/sj.ijo.0800867.
8. Gwin JA, Church DD, Hatch-Mcchesney A, Allen JT, Wilson MA, Varanoske AN, et al. Essential amino acid-enriched whey enhances post-exercise whole-body protein balance during energy deficit more than iso-nitrogenous whey or a mixed-macronutrient meal: a randomized, crossover study. Journal of the International Society of Sports Nutrition. 2021;18(1). doi: 10.1186/s12970-020-00401-5.
9. Weinheimer EM, Sands LP, Campbell WW. A systematic review of the separate and combined effects of energy restriction and exercise on fat-free mass in middle-aged and older adults: implications for sarcopenic obesity. Nutr Rev. 2010/07/02 ed. Department of Foods and Nutrition, Purdue University, West Lafayette, Indiana 47907, USA., 2010:375-88.
10. Villareal DT, Chode S, Parimi N, Sinacore DR, Hilton T, Armamento-Villareal R, et al. Weight loss, exercise, or both and physical function in obese older adults. N Engl J Med. 2011/04/01 ed. Division of Geriatrics and Nutritional Science, Washington University School of Medicine, St. Louis, USA. dennis.villareal@va.gov, 2011:1218-29.
11. Wolfe RR. The underappreciated role of muscle in health and disease. Am J Clin Nutr. 2006/09/09 ed. University of Texas Medical Branch, Department of Surgery and

Shriners Burns Hospital, Metabolism Unit, Galveston, TX 77550, USA. rwolfe@utmb.edu, 2006:475-82.
12. Bigaard J, Frederiksen K, Tjonneland A, Thomsen BL, Overvad K, Heitmann BL, et al. Body fat and fat-free mass and all-cause mortality. Obes Res. 2004/08/05 ed. Institute of Cancer Epidemiology, The Danish Cancer Society, Strandboulevarden 49, DK-2100 Copenhagen, Denmark. janne@cancer.dk, 2004:1042-9.
13. Pasiakos SM, Vislocky LM, Carbone JW, Altieri N, Konopelski K, Freake HC, et al. Acute energy deprivation affects skeletal muscle protein synthesis and associated intracellular signaling proteins in physically active adults. J Nutr. 2010/02/19 ed. Department of Nutritional Sciences, University of Connecticut, Storrs, CT 06269, USA., 2010:745-51.
14. Hector AJ, Marcotte GR, Churchward-venne TA, Murphy CH, Breen L, von Allmen M, et al. Whey Protein Supplementation Preserves Postprandial Myofibrillar Protein Synthesis during Short-Term Energy Restriction in Overweight and Obese Adults 1 – 3. The Journal of nutrition. 2015;145(C):1-7. doi: 10.3945/jn.114.200832.1.
15. Areta JL, Burke LM, Camera DM, West DW, Crawshay S, Moore DR, et al. Reduced resting skeletal muscle protein synthesis is rescued by resistance exercise and protein ingestion following short-term energy deficit. Am J Physiol Endocrinol Metab. 2014/03/07 ed. Exercise and Nutrition Research Group, Health Innovations Research Institute, School of Medical Sciences, RMIT University, Melbourne, Australia;, 2014:E989-97.
16. Hector AJ, McGlory C, Damas F, Mazara N, Baker SK, Phillips SM. Pronounced energy restriction with elevated protein intake results in no change in proteolysis and reductions in skeletal muscle protein synthesis that are mitigated by resistance exercise. FASEB J. 2017/09/14 ed. Department of Kinesiology, McMaster University, Hamilton, Ontario, Canada. School of Physical Education and Sport, University of Sao Paulo, Sao Paulo, Brazil. Division of Physical Medicine and Rehabilitation, Department of Medicine, McMaster University, H, 2018:265-75.
17. Carbone JW, Pasiakos SM, Vislocky LM, Anderson JM, Rodriguez NR. Effects of short-term energy deficit on muscle protein breakdown and intramuscular proteolysis in normal-weight young adults. Appl Physiol Nutr Metab. 2014;39(8):960-8. doi: 10.1139/apnm-2013-0433.
18. Kouw IWK, van Dijk JW, Horstman AMH, Kramer IF, Goessens JPB, van Dielen FMH, et al. Basal and Postprandial Myofibrillar Protein Synthesis Rates Do Not Differ between Lean and Obese Middle-Aged Men. J Nutr. 2019;149(9):1533-42. doi: 10.1093/jn/nxz104.
19. Smeuninx B, McKendry J, Wilson D, Martin U, Breen L. Age-Related Anabolic Resistance of Myofibrillar Protein Synthesis Is Exacerbated in Obese Inactive Individuals. J Clin Endocrinol Metab. 2017;102(9):3535-45. doi: 10.1210/jc.2017-00869.
20. Murton AJ, Marimuthu K, Mallinson JE, Selby AL, Smith K, Rennie MJ, et al. Obesity appears to be associated with altered muscle protein synthetic and breakdown responses to increased nutrient delivery in older men, but not reduced muscle mass or contractile function. Diabetes. 2015;64(9):3160-71. doi: 10.2337/db15-0021.
21. Smith GI, Atherton P, Villareal DT, Frimel TN, Rankin D, Rennie MJ, et al. Differences in muscle protein synthesis and anabolic signaling in the postabsorptive state and in response to food in 65-80 year old men and women. PLoS One. School of

Medicine, Washington University, St. Louis, Missouri, United States of America., 2008:e1875.
22. Bamman MM, Hill VJ, Adams GR, Haddad F, Wetzstein CJ, Gower BA, et al. Gender differences in resistance-training-induced myofiber hypertrophy among older adults. J Gerontol A Biol Sci Med Sci. Geriatric Research, Education, and Clinical Center, VA Medical Center, Birmingham, Alabama, USA. mbamman@uab.edu, 2003:108-16.
23. Smith GI, Reeds DN, Hall AM, Chambers KT, Finck BN, Mittendorfer B. Sexually dimorphic effect of aging on skeletal muscle protein synthesis. Biol Sex Differ. Division of Geriatrics and Nutritional Science, Washington University School of Medicine, 660 South Euclid Avenue; Campus, Box 8031, Saint Louis, MO 63110, USA. mittendb@wustl.edu., 2012:11.
24. Smith GI, Mittendorfer B. Sexual dimorphism in skeletal muscle protein turnover. J Appl Physiol (1985). 2015/12/25 ed. Washington University, School of Medicine, St. Louis, Missouri. Washington University, School of Medicine, St. Louis, Missouri mittendb@wustl.edu., 2016:674-82.
25. Smith GI, Villareal DT, Sinacore DR, Shah K, Mittendorfer B. Muscle protein synthesis response to exercise training in obese, older men and women. Med Sci Sports Exerc. 2012/01/17 ed. Center for Human Nutrition, Division of Geriatrics and Nutritional Science, Washington University School of Medicine, St. Louis, MO 63110, USA., 2012:1259-66.
26. Leidy HJ, Clifton PM, Astrup A, Wycherley TP, Westerterp-plantenga MS, Luscombe-marsh ND, et al. The role of protein in weight loss and maintenance 1 – 5. American Journal of Clinical Nutrition. 2015;101:1320-9. doi: 10.3945/ajcn.114.084038.1320S.
27. Wycherley TP, Moran LJ, Clifton PM, Noakes M, Brinkworth GD. Effects of energy-restricted high-protein, low-fat compared with standard-protein, low-fat diets: a meta-analysis of randomized controlled trials. Am J Clin Nutr. 2012/10/26 ed. Sansom Institute for Health Research, Division of Health Sciences, University of South Australia, Adelaide, Australia. tom.wycherley@unisa.edu.au, 2012:1281-98.
28. Layman DK, Anthony TG, Rasmussen BB, Adams SH, Lynch CJ, Brinkworth GD, et al. Defining meal requirements for protein to optimize metabolic roles of amino acids. American Journal of Clinical Nutrition. 2015;101(6):1330S-8S. doi: 10.3945/ajcn.114.084053.
29. Murphy CH, Shankaran M, Churchward‐Venne TA, Mitchell CJ, Kolar NM, Burke LM, et al. Effect of resistance training and protein intake pattern on myofibrillar protein synthesis and proteome kinetics in older men in energy restriction. The Journal of Physiology. 2018;596(11):2091-120. doi: 10.1113/jp275246.
30. Moore DRDR, Robinson MJMJ, Fry JLJL, Tang JE, Glover EI, Wilkinson SB, et al. Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. American Journal of Clinical Nutrition. 2009;89(1):161-8. doi: 10.3945/ajcn.2008.26401.INTRODUCTION.
31. Witard OC, Jackman SR, Breen L, Smith K, Selby A, Tipton KD. Myofibrillar muscle protein synthesis rates subsequent to a meal in response to increasing doses of whey protein at rest and after resistance exercise. American Journal of Clinical Nutrition. 2014;99(1):86-95. doi: 10.3945/ajcn.112.055517.
32. Churchward-Venne TA, Pinckaers PJM, Smeets JSJ, Betz MW, Senden JM, Goessens JPB, et al. Dose-response effects of dietary protein on muscle protein synthesis during

recovery from endurance exercise in young men: a double-blind randomized trial. Am J Clin Nutr. 2020;112(2):303-17. doi: 10.1093/ajcn/nqaa073.
33. Macnaughton LS, Wardle SL, Witard OC, McGlory C, Hamilton DL, Jeromson S, et al. The response of muscle protein synthesis following whole-body resistance exercise is greater following 40 g than 20 g of ingested whey protein. Physiol Rep. 2016;4(15). doi: 10.14814/phy2.12893.
34. Robinson MJ, Burd NA, Breen L, Rerecich T, Yang Y, Hector AJ, et al. Dose-dependent responses of myofibrillar protein synthesis with beef ingestion are enhanced with resistance exercise in middle-aged men. Applied Physiology, Nutrition, and Metabolism. 2013;38(2):120-5. doi: 10.1139/apnm-2012-0092.
35. Yang Y, Breen L, Burd NA, Hector AJ, Churchward-Venne TA, Josse AR, et al. Resistance exercise enhances myofibrillar protein synthesis with graded intakes of whey protein in older men. The British journal of nutrition. 2012;108(10):1780-8. doi: 10.1017/S0007114511007422.
36. Holwerda AM, Paulussen KJM, Overkamp M, Goessens JPB, Kramer IF, Wodzig W, et al. Dose-Dependent Increases in Whole-Body Net Protein Balance and Dietary Protein-Derived Amino Acid Incorporation into Myofibrillar Protein During Recovery from Resistance Exercise in Older Men. J Nutr. 2019/02/06 ed. NUTRIM School of Nutrition and Translational Research in Metabolism. Top Institute Food and Nutrition (TIFN), Wageningen, The Netherlands. Central Diagnostic Laboratory, Maastricht University Medical Center+, The Netherlands., 2019.
37. Kemmler WK, Lauber D, Wassermann A, Mayhew JL. Predicting maximal strength in trained postmenopausal woman. J Strength Cond Res. 2006/12/30 ed. Institute of Medical Physics, University of Erlangen, Erlangen, Germany. wolfgang.kemmler@imp.uni-erlangen.de, 2006:838-42.
38. Nordic Nutrition Recommendations 2012 : Integrating nutrition and physical activity. 5 ed. Copenhagen: Nordisk Ministerråd, 2014.
39. Bornø A, van Hall G. Quantitative amino acid profiling and stable isotopically labeled amino acid tracer enrichment used for in vivo human systemic and tissue kinetics measurements. Journal of Chromatography B. 2014;951-952:69-77. doi: https://doi.org/10.1016/j.jchromb.2014.01.019.
40. Christensen B, Nellemann B, Larsen MS, Thams L, Sieljacks P, Vestergaard PF, et al. Whole body metabolic effects of prolonged endurance training in combination with erythropoietin treatment in humans: a randomized placebo controlled trial. Am J Physiol Endocrinol Metab. 2013/08/08 ed. Department of Endocrinology and Internal Medicine, NBG/THG, Aarhus University Hospital, Aarhus, Denmark;, 2013:E879-89.
41. Holm L, Reitelseder S, Dideriksen K, Nielsen RH, Bulow J, Kjaer M. The single-biopsy approach in determining protein synthesis in human slow-turning-over tissue: use of flood-primed, continuous infusion of amino acid tracers. Am J Physiol Endocrinol Metab. Institute of Sports Medicine Copenhagen, Department of Orthopedic Surgery, Bispebjerg Hospital and Center for Healthy Aging, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark; and Department of Biomedical Sciences, Faculty of Healt, 2014:E1330-9.
42. Bechshoeft R, Dideriksen KJ, Reitelseder S, Scheike T, Kjaer M, Holm L. The anabolic potential of dietary protein intake on skeletal muscle is prolonged by prior light-load exercise. Clinical nutrition (Edinburgh, Scotland). 2013;32(2):236-44. doi: 10.1016/j.clnu.2012.06.015.

43. Churchward-Venne TA, Holwerda AM, Phillips SM, van Loon LJC. What is the Optimal Amount of Protein to Support Post-Exercise Skeletal Muscle Reconditioning in the Older Adult? Sports Medicine. 2016;46(9):1205-12. doi: 10.1007/s40279-016-0504-2.
44. Cuthbertson D, Smith K, Babraj J, Leese G, Waddell T, Atherton P, et al. Anabolic signaling deficits underlie amino acid resistance of wasting, aging muscle. FASEB J. Division of Molecular Physiology, School of Life Sciences, University of Dundee, Dundee, Scotland., 2005:422-4.
45. Kim IY, Deutz NEP, Wolfe RR. Update on maximal anabolic response to dietary protein. Clin Nutr. 2018;37(2):411-8. doi: 10.1016/j.clnu.2017.05.029.
46. Kim IY, Schutzler S, Schrader A, Spencer HJ, Azhar G, Ferrando AA, et al. The anabolic response to a meal containing different amounts of protein is not limited by the maximal stimulation of protein synthesis in healthy young adults. Am J Physiol Endocrinol Metab. 2015/11/05 ed. Department of Geriatrics, Center for Translational Research in Aging and Longevity, Donald W. Reynolds Institute on Aging, University of Arkansas for Medical Sciences, Little Rock, Arkansas; and iykim@uams.edu. Department of Geriatrics, Center for Transla, 2016:E73-80.
47. Park S, Jang J, Choi MD, Shin YA, Schutzler S, Azhar G, et al. The Anabolic Response to Dietary Protein Is Not Limited by the Maximal Stimulation of Protein Synthesis in Healthy Older Adults: A Randomized Crossover Trial. Nutrients. 2020;12(11). doi: 10.3390/nu12113276.
48. Lockard B, Mardock M, Oliver J, Byrd M, Simbo S, Jagim A, et al. Comparison of Two Diet and Exercise Approaches on Weight Loss and Health Outcomes in Obese Women. International Journal of Environmental Research and Public Health. 2022;19(8):4877. doi: 10.3390/ijerph19084877.
49. Kim JE, O'Connor LE, Sands LP, Slebodnik MB, Campbell WW. Effects of dietary protein intake on body composition changes after weight loss in older adults: a systematic review and meta-analysis. Nutr Rev. 2016/02/18 ed. J.E. Kim, L.E. O'Connor, and W.W. Campbell are with the Department of Nutrition Science, Purdue University, West Lafayette, Indiana, USA. L.P. Sands is with the Department of Human Development, Virginia Polytechnic Institute and State University, Blacksbu, 2016:210-24.
50. Bohe J, Low JF, Wolfe RR, Rennie MJ. Latency and duration of stimulation of human muscle protein synthesis during continuous infusion of amino acids. J Physiol. 2001/04/18 ed. Metabolism Unit, Department of Surgery, University of Texas Medical Branch, Shriners Burns Hospital, Galveston, TX 77550, USA., 2001:575-9.
51. Atherton PJ, Etheridge T, Watt PW, Wilkinson D, Selby A, Rankin D, et al. Muscle full effect after oral protein: time-dependent concordance and discordance between human muscle protein synthesis and mTORC1 signaling. The American journal of clinical nutrition. 2010;92(5):1080-8. doi: 10.3945/ajcn.2010.29819.
52. Pennings B, Koopman R, Beelen M, Senden JM, Saris WH, van Loon LJ. Exercising before protein intake allows for greater use of dietary protein-derived amino acids for de novo muscle protein synthesis in both young and elderly men. Am J Clin Nutr. 2010/11/19 ed. Top Institute Food & Nutrition, Wageningen, Netherlands., 2011:322-31.
53. Browne GJ, Proud CG. Regulation of peptide-chain elongation in mammalian cells. Eur J Biochem. 2002;269(22):5360-8. doi: 10.1046/j.1432-1033.2002.03290.x.

54. Pasiakos SM, Cao JJ, Margolis LM, Sauter ER, Whigham LD, McClung JP, et al. Effects of high-protein diets on fat-free mass and muscle protein synthesis following weight loss: A randomized controlled trial. FASEB Journal. 2013;27(9):3837-47. doi: 10.1096/fj.13-230227.
55. Wilkinson DJ, Franchi MV, Brook MS, Narici MV, Williams JP, Mitchell WK, et al. A validation of the application of D(2)O stable isotope tracer techniques for monitoring day-to-day changes in muscle protein subfraction synthesis in humans. American journal of physiology Endocrinology and metabolism, 2014:E571-9.

Tables

Table 1. Participant characteristics

	ERW15 *(n = 10)*		ERW35 *(n = 10)*		ERW60 *(n = 10)*		EBW35 *(n = 10)*	
	Mean (SD)							
Age (yrs)	58.9	(5.3)	57.7	(5.4)	57.3	(3.9)	57.7	(5.4)
Total body mass (kg)	81.3	(10.0)	78.6	(6.7)	83.5	(9.0)	79.0	(8.8)
Lean body mass (kg)	45.0	(5.4)	42.5	(2.5)	45.0	(4.2)	44.2	(3.4)
Body fat (%)	41.4	(4.0)	42.6	(4.7)	42.7	(5.7)	40.8	(3.5)
BMI (kg/m^2)	28.4	(2.1)	28.2	(2.0)	29.2	(3.8)	28.7	(2.6)
1RM (kg)	17.1	(5.6)	18.4	(3.2)	19.3	(5.5)	19.8	(4.7)
Oestrogen concentrations (pmol/L)	26.3	(22.3)	26.3	(13.1)	21.7	(6.3)	22.8	(8.7)
FSH concentrations (IU/L)	89.2	(29.2)	77.7	(19.5)	68.5	(13.9)	71.2	(23.0)
Testosterone concentrations (nmol/L)	1.0	(0.7)	0.8	(0.2)	0.9	(0.4)	0.8	(0.4)
Plasma cholesterol concentration (mmol/L)	5.6	(0.8)	5.6	(0.7)	5.6	(0.9)	5.7	(0.7)
Plasma TG concentration (mmol/L)	1.0	(0.7)	1.1	(0.4)	1.3	(0.2)	1.1	(0.5)

All values are means ± SD. BMI, body mass index; 1-RM, one-repetition maximum; FSH, follicle-stimulating hormone; TG, triglycerides.

Table 2. Amino acid composition of protein beverages

Amino acid	Percent of total amino acids
Histidine	1.5%
Isoleucine	6.3%
Leucine	10.6%
Lysine	9.8%
Methionine	2.4%
Phenylalanine	2.7%
Threonine	7.0%
Tryptophane	1.3%
Valine	5.7%
ΣEssential amino acids	47.3%
Alanine	5.5%
Arginine	2.2%
Asparagine	10.4%
Cysteine	1.9%
Glutamic acid	18.0%
Glycine	1.5%
Proline	6.2%
Serine	4.6%
Tyrosine	2.4%
ΣNon-essetial amino acids	52.7%

Table 3. Energy and macronutrient intake in energy restricted and energy balanced diet groups

	ER (*n = 30*)		EB (*n = 10*)	
	Mean (SD)			
Absolute energy intake (kcal/d)	800	(–)	1790*	(352)
Relative energy intake (kJ/kg/d)	42	(5)	95*	(17)
Absolute CHO intake (g/d)	87	(–)	181*	(42)
Relative CHO intake (g/kg/d)	1.1	(0.1)	2.3*	(0.2)
Absolute PRO intake (g/d)	62	(–)	88*	(18)
Relative PRO intake (g/kg/d)	0.8	(0.1)	1.1*	(0.2)
Absolute fat intake (g/d)	22	(–)	66*	(15)
Relative fat intake (g/kg/d)	0.3	(0.0)	0.8*	(0.2)

All values are means ± SD. Data were analysed using a one-factor ANOVA. *significant difference *vs*. energy restricted groups for corresponding measurements ($P < 0.001$). ER, energy restricted diet group; EB, energy balanced diet group; CHO, carbohydrate; PRO, protein.

Figures

Figure 1. Flowchart of enrolment process.

Figure 2. Overview of study design and experimental trial. Blood samples were collected prior to initiation of L-(*ring*-$^{13}C_6$)phenylalanine infusion (–270 min; Baseline) and periodically thereafter during the experimental day. A single bout of unilateral leg resistance exercise was initiated 20 min prior to ingestion of whey protein beverage. Muscle biopsies were collected from the exercised leg (FED-EX) at –180, and 450 min timepoints and non-exercised leg (FED) at 0 min and 450 min timepoints. Assigned beverages containing either 15, 35 or 60 g of whey protein were ingested immediately after the muscle biopsy at 0 min.

Figure 3. Plasma phenylalanine concentration expressed over time (a) and as *i*AUC (b) in energy restricted and energy balanced groups. ERW15 (✕), energy-restricted diet with ingestion of 15 g whey protein; ERW35 (▽), energy-restricted diet with ingestion of 35 g whey protein; EBW35 (▲), energy balanced diet with ingestion of 35 g whey protein; ERW60 (○), energy restricted diet with ingestion of 60 g whey protein. A repeated measures mixed effects model was used for statistical analysis of data over time. Analysis of *protein dose* response: Main effect of time, $P < 0.001$; main effect of group (*protein dose*), $P < 0.001$; time × group interaction, $P < 0.001$. Analysis of energy status: Main effect of time, $P < 0.001$; main effect of group (ERW35 vs EBW35), $P < 0.856$; time × group interaction: $P < 0.452$. ⁎ significant difference from ERW15 at corresponding timepoint; #significant difference from ERW35 at corresponding timepoint. A one-way ANOVA was used for statistical analysis of data expressed as *i*AUC. *i*AUC analysis of *protein dose* response, $P < 0.001$. *i*AUC analysis of energy status (ERW35 & ERB35), $P = 0.752$. ⁎ significant difference from

ERW15; # significant difference from ERW35. Data are expressed as means ± SEM (n = 10 for all groups). EX, exercise.

Figure 4. Plasma leucine concentrations expressed over time (a) and as *i*AUC (b) in energy restricted and energy balanced groups. ERW15 (✕), energy restricted diet with ingestion of 15 g whey protein; ERW35 (▽), energy restricted diet with ingestion of 35 g whey protein; EBW35 (▲), energy balanced diet with ingestion of 35 g whey protein; ERW60 (○), energy restricted diet with ingestion of 60 g whey protein. A repeated measures mixed effects model was used for statistical analysis of data presented over time. Analysis of *protein dose* response, Main effect of time: $P < 0.001$; main effect of group (*protein dose*), $P < 0.001$; time × group interaction, $P < 0.001$. Analysis of energy status: Main effect of time, $P < 0.001$; main effect of group (ERW35 vs EBW35), $P < 0.001$; time × group interaction: $P = 0.002$. ⁎ significant difference from ERW15 at corresponding timepoint; # significant difference from ERW35 at corresponding timepoint. A one-way ANOVA was used for statistical analysis of data expressed as *i*AUC. *i*AUC analysis of *protein dose* response, $P < 0.001$. *i*AUC analysis of energy status (ERW35 & ERB35), $P < 0.001$. ⁎ significant difference from ERW15; # significant difference from ERW35. Data are expressed as means ± SEM (n=10 for all groups). EX, exercise.

Figure 5. Serum insulin concentrations expressed over time (a) and as *i*AUC (b) in energy restricted and energy balanced groups. ERW15 (✕), energy restricted diet with ingestion of 15 g whey protein; ERW35 (▽), energy restricted diet with ingestion of 35 g whey protein; EBW35 (▲), energy balanced diet with ingestion of 35 g whey protein; ERW60 (○), energy restricted diet with ingestion of 60 g whey protein. A repeated measures mixed effects model was used for statistical analysis of data presented over time. Analysis of *protein dose* response: Main effect of time, $P <$

0.001; main effect of group (*protein dose*), $P < 0.001$; time × group interaction, $P < 0.001$. Analysis of *energy status*: Main effect of time, $P < 0.001$; main effect of group (ERW5 & EBR35), $P = 0.988$; time × group interaction, $P = 0.936$. ⁎ significant difference from ERW15 at corresponding timepoint; # significant difference from ERW35 at corresponding timepoint. A one-way ANOVA was used for statistical analysis of data expressed as *i*AUC. *i*AUC analysis of *protein dose* response: $P < 0.001$. *i*AUC analysis of energy status (ERW35 & ERB35), $P = 0.756$. ⁎ significant difference from ERW15; # significant difference from ERW35. Data are expressed as means ± SEM (n = 10 for all groups). EX, exercise.

Figure 6. Arterialized plasma phenylalanine enrichment expressed over time in energy restricted and energy balanced groups. ERW15 (✕), energy-restricted diet with ingestion of 15 g whey protein; ERW35 (▽), energy-restricted diet with ingestion of 35 g whey protein; EBW35 (▲), energy balanced diet with ingestion of 35 g whey protein; ERW60 (○), energy restricted diet with ingestion of 60 g whey protein. A repeated measures mixed effects model was used for statistical analysis of data over time. Analysis of *protein dose* response: Main effect off time, $P < 0.001$; main effect of group (*protein dose*), $P = 0.129$; time × group interaction: $P < 0.001$. Analysis of *energy status:* Main effect of time *(ERW35 vs EBW35), P* < 0.001; main effect of group (*ERW35 vs EBW35*), $P = 0.277$; time × group interaction: $P < 0.664$. ⁎significant difference from ERW15 at corresponding timepoint; [a]significant difference from time 0 for ERB35; [b]significant difference from time 0 for ERW60. Data are expressed as means ± SEM (ERW15, n = 10; ERW35, n = 8; EBW35, n = 9; ERW60, n = 8). EX, exercise.

Figure 7. Myofibrillar fractional synthesis rate (FSR) in response to graded doses of ingested whey protein in exercised and rested muscles in energy restricted and energy balanced groups. A mixed

effect model was used for statistical analysis with *protein dose* (ERW15, ERW35, EBW35, ERW60) and *condition* (BASAL, FED, FED-EX) serving as independent variables in the fixed part of the model. Analysis of *protein dose* response: Main effect of group (*protein dose*; ERW15, ERW35, ERW60): $P = 0.006$; main effect of condition (BASAL, FED, FED-EX): $P < 0.001$; protein dose × condition interaction: $P = 0.7442$. Analysis of *energy status*: Main effect of group (ERW35 vs EBW35), $P < 0.744$; main effect of condition (BASAL, FED, FED-EX), $P < 0.001$; time × group interaction, $P = 0.996$.⁎ significant difference compared to ERW15. § significant difference compared to BASAL across protein dose groups. Data are expressed as means ± SEM (ERW15, n = 10; ERW35, n = 8; EBW35, n = 9; ERW60, n = 8).

Reporting checklist for randomised trial.

Based on the CONSORT guidelines.

Instructions to authors

Complete this checklist by entering the page numbers from your manuscript where readers will find each of the items listed below.

Your article may not currently address all the items on the checklist. Please modify your text to include the missing information. If you are certain that an item does not apply, please write "n/a" and provide a short explanation.

Upload your completed checklist as an extra file when you submit to a journal.

In your methods section, say that you used the CONSORTreporting guidelines, and cite them as:

Schulz KF, Altman DG, Moher D, for the CONSORT Group. CONSORT 2010 Statement: updated guidelines for reporting parallel group randomised trials

		Reporting Item	Page Number
Title and Abstract			
Title	#1a	Identification as a randomized trial in the title.	1
Abstract	#1b	Structured summary of trial design, methods, results, and conclusions	3
Introduction			
Background and objectives	#2a	Scientific background and explanation of rationale	4
Background and objectives	#2b	Specific objectives or hypothesis	6
Methods			
Trial design	#3a	Description of trial design (such as parallel, factorial) including allocation ratio.	7

Trial design	#3b	Important changes to methods after trial commencement (such as eligibility criteria), with reasons	N/A
Participants	#4a	Eligibility criteria for participants	7
Participants	#4b	Settings and locations where the data were collected	6
Interventions	#5	The experimental and control interventions for each group with sufficient details to allow replication, including how and when they were actually administered	8
Outcomes	#6a	Completely defined prespecified primary and secondary outcome measures, including how and when they were assessed	12
Sample size	#7a	How sample size was determined.	12
Sample size	#7b	When applicable, explanation of any interim analyses and stopping guidelines	N/A
Randomization - Sequence generation	#8a	Method used to generate the random allocation sequence.	8
Randomization - Sequence generation	#8b	Type of randomization; details of any restriction (such as blocking and block size)	8
Randomization - Allocation concealment mechanism	#9	Mechanism used to implement the random allocation sequence (such as sequentially numbered containers), describing any steps taken to conceal the sequence until interventions were assigned	8
Randomization - Implementation	#10	Who generated the allocation sequence, who enrolled participants, and who assigned participants to interventions	8
Blinding	#11a	If done, who was blinded after assignment to interventions (for example, participants, care providers, those assessing outcomes) and how.	6

Blinding	#11b	If relevant, description of the similarity of interventions	9
Statistical methods	#12a	Statistical methods used to compare groups for primary and secondary outcomes	12
Statistical methods	#12b	Methods for additional analyses, such as subgroup analyses and adjusted analyses	12
Outcomes	#6b	Any changes to trial outcomes after the trial commenced, with reasons	11
Results			
Participant flow diagram (strongly recommended)	#13a	For each group, the numbers of participants who were randomly assigned, received intended treatment, and were analysed for the primary outcome	7
Participant flow	#13b	For each group, losses and exclusions after randomization, together with reason	7
Recruitment	#14a	Dates defining the periods of recruitment and follow-up	6
Recruitment	#14b	Why the trial ended or was stopped	N/A
Baseline data	#15	A table showing baseline demographic and clinical characteristics for each group	28
Numbers analysed	#16	For each group, number of participants (denominator) included in each analysis and whether the analysis was by original assigned groups	12
Outcomes and estimation	#17a	For each primary and secondary outcome, results for each group, and the estimated effect size and its precision (such as 95% confidence interval)	13
Outcomes and estimation	#17b	For binary outcomes, presentation of both absolute and relative effect sizes is recommended	N/A
Ancillary analyses	#18	Results of any other analyses performed, including subgroup analyses and adjusted analyses, distinguishing pre-specified from exploratory	13
Harms	#19	All important harms or unintended effects in each group (For specific guidance see CONSORT for harms)	7

Discussion			
Limitations	#20	Trial limitations, addressing sources of potential bias, imprecision, and, if relevant, multiplicity of analyses	20
Interpretation	#22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	15
Registration	#23	Registration number and name of trial registry	2
Generalisability	#21	Generalisability (external validity, applicability) of the trial findings	20
Other information			
Interpretation	#22	Interpretation consistent with results, balancing benefits and harms, and considering other relevant evidence	15
Registration	#23	Registration number and name of trial registry	2
Protocol	#24	Where the full trial protocol can be accessed, if available	7
Funding	#25	Sources of funding and other support (such as supply of drugs), role of funders	21

60 women were assessed for eligibility

Not randomized (n = 20)
Not meeting inclusion criteria (n = 20)

Randomly assigned (n = 40)

Allocated to energy restricted diet and ingestion of 15 g whey protein (n = 10); ERW15

Allocated to energy restricted diet and ingestion of 35 g whey protein (n = 10); ERW35

Allocated to energy restricted diet and ingestion of 60 g whey protein (n = 10); ERW60

Allocated to energy balanced diet and ingestion of 35 g whey protein (n = 10); EBW35

Analyzed (n = 10)

Analyzed (n = 10)
Excluded from MPS-analysis (n = 2, were unable to obtain adequate samples from the participants)

Analyzed (n = 10)
Excluded from MPS-analysis (n = 2, were unable to obtain adequate samples from the participants)

Analyzed (n = 10)
Excluded from MPS-analysis (n = 1, were unable to obtain adequate samples from the participants)

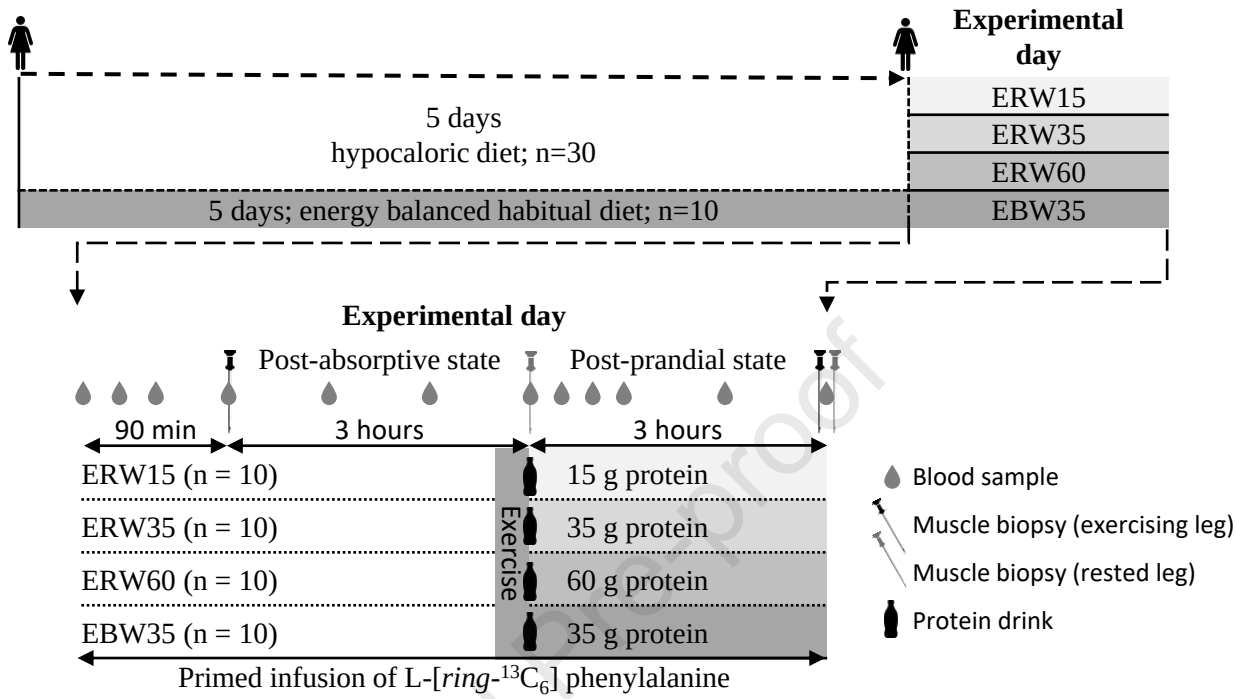
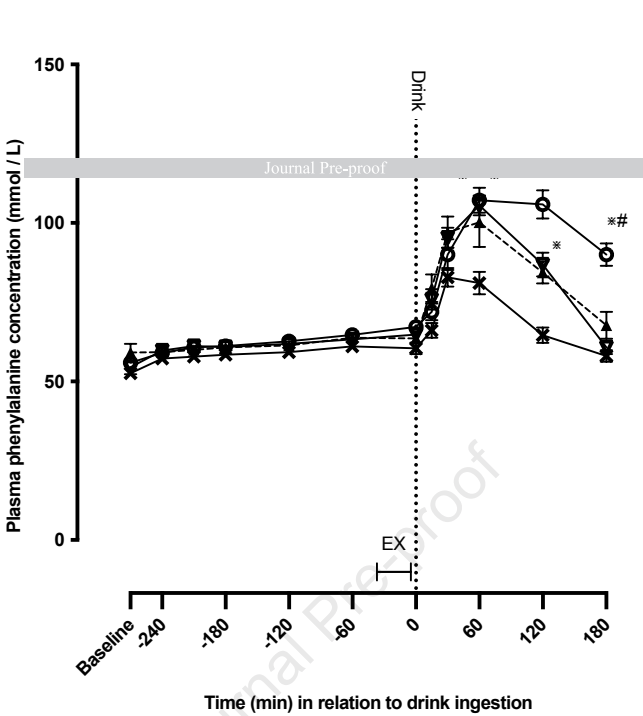


Figure 3. Plasma phenylalanine concentration



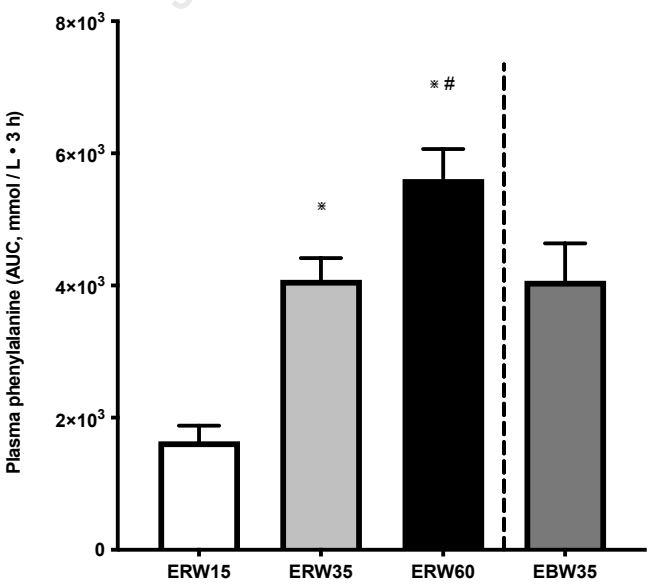
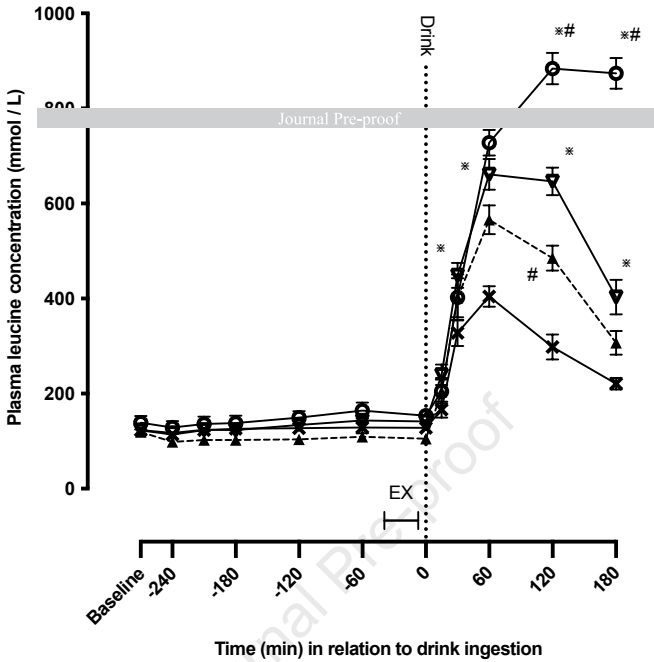


Figure 4. Plasma leucine concentration

a



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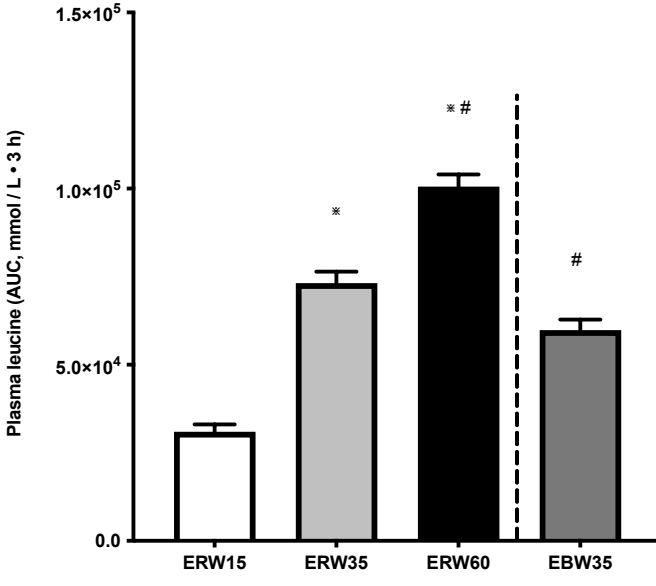
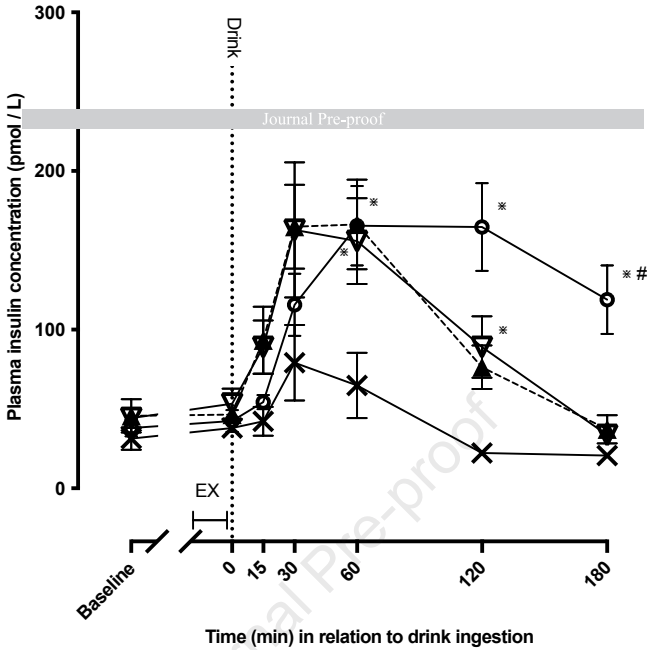


Figure 5. Serum insulin

a



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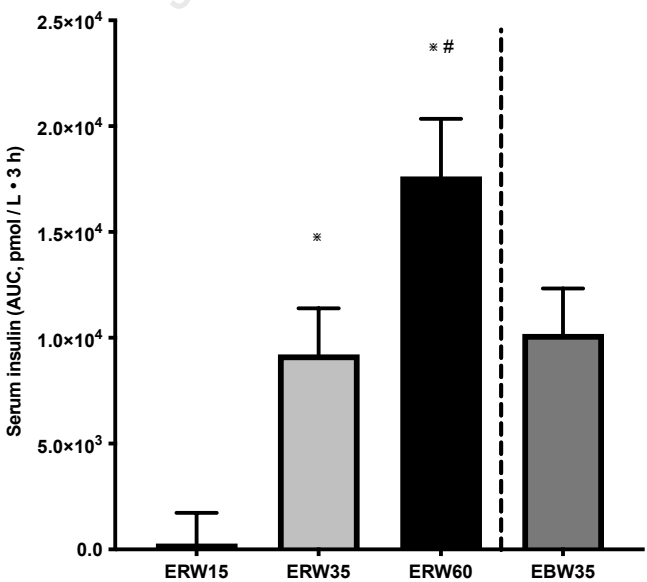

Figure 6. Plasma phenylalanine enrichment

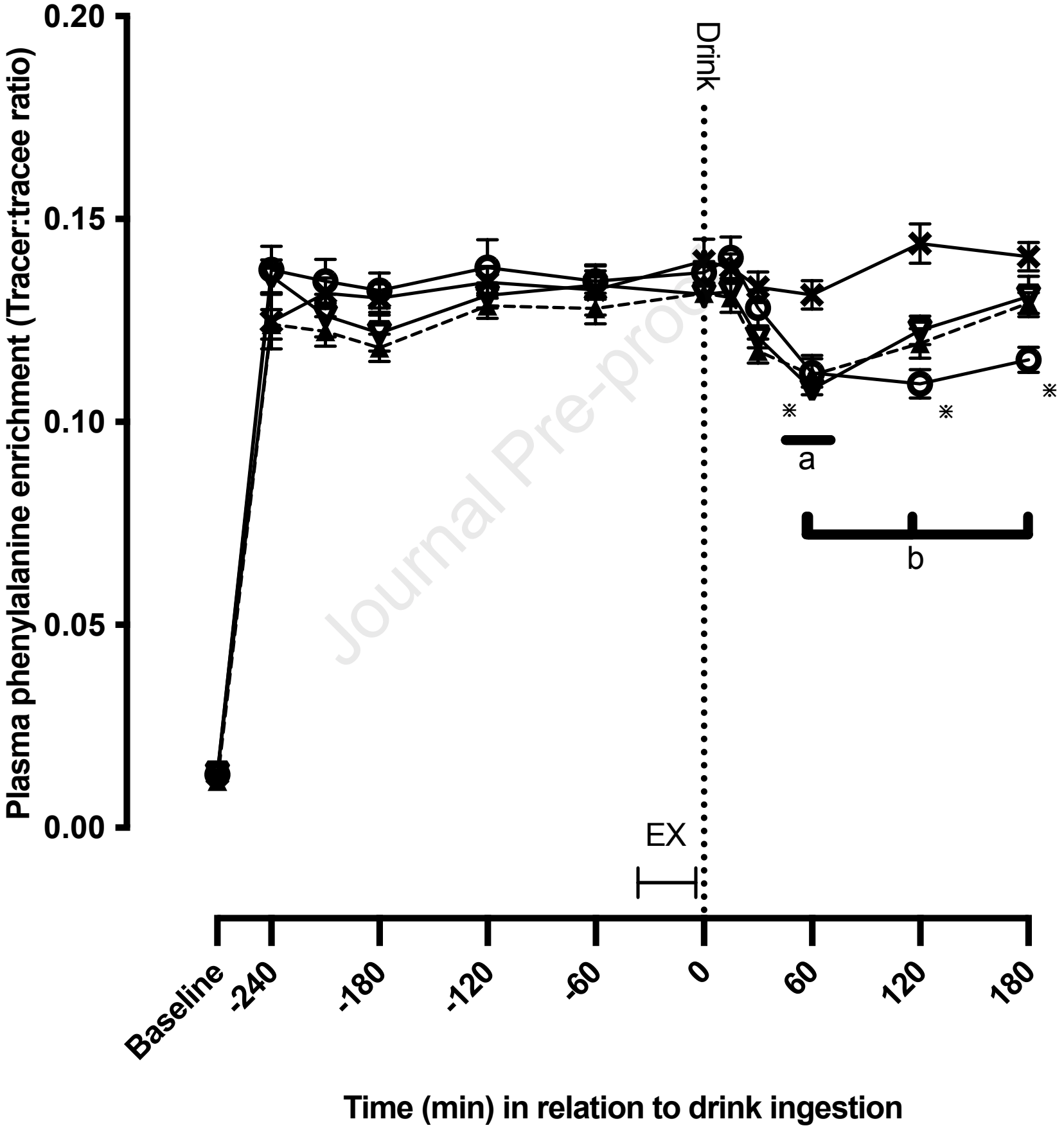

Figure 7. Myofibrillar protein synthesis