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Obesity and Eating Disorders

Dose-Response of Myofibrillar Protein Synthesis To Ingested Whey Protein During Energy Restriction in Overweight Postmenopausal Women: A Randomized, Controlled Trial

Mads S. Larsen [1,2], Oliver C. Witard [3], Lars Holm [4], Paula Scaife [5], Rikke Hansen [6], Kenneth Smith [5], Kevin D. Tipton [7], Maike Mose [8], Mads B. Bengtsen [8], Katrine M. Lauritsen [8], Ulla R. Mikkelsen [2], Mette Hansen [1,*]

[1] *Department of Public Health, Aarhus University, Denmark;* [2] *Arla Foods Ingredients Group P/S, Denmark;* [3] *Centre for Human and Applied Physiological Sciences, School of Basic and Medical Biosciences, Faculty of Life Sciences and Medicine, King's College London, London, UK;* [4] *School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham, UK;* [5] *Metabolic Physiology, Medical Research Council and Arthritis Research United Kingdom Centre for Excellence in Musculoskeletal Ageing, School of Graduate Entry Medicine and Health, University of Nottingham, Derby, UK;* [6] *Aalborg University, Denmark;* [7] *Department of Sport and Exercise Sciences, Durham University, UK;* [8] *Medical Research Laboratory, Institute for Clinical Medicine, Aarhus University, Denmark*

A B S T R A C T

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 characterized 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 postexercise.

Design: Forty middle-aged (58.6±0.4 y), overweight (BMI: 28.6±0.4), postmenopausal women were randomly assigned to 1 of 4 groups: Three groups underwent 5 d 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 d of an energy balanced diet (EBW35, $n = 10$). Myofibrillar fractional synthetic rate (FSR) was calculated under basal, fed (FED) and postexercise (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.

Trial registry: clinicaltrials.gov (ID: NCT03326284).

Keywords: females, middle-aged, obesity, weight loss, muscle protein synthesis

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.

* Corresponding author. *E-mail address:* mhan@ph.au.dk (M. Hansen).

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Introduction

The worldwide prevalence of overweight and obese middle-aged (40–65 y) adults represents an increasingly important public health challenge within the discipline of human and clinical nutrition [1, 2]. Accordingly, considerable attention has focused on optimizing 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 focused 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 with 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 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 to 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 d of diet-induced energy restriction. The whey protein doses (15 g, 35 g, 60 g) were selected to characterize 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 after 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 (ie, 15 g $<$ 35 g $<$ 60 g) after short-term energy restriction (primary outcome). Secondly, we hypothesized that rates of MPS would be augmented with resistance exercise compared with rest, regardless of protein dose. Finally, we hypothesized the MPS response to ingestion of 35 g whey protein would be attenuated after a period of energy restriction vs. energy balance in middle-aged, postmenopausal, women.

Methods

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 to 65 y, postmenopausal (defined as no menstrual bleeding for 6 mo, follicle-stimulating hormone (FSH) concentration $>$ 30 IU/L, estrogen concentration $<$ 50 pmol/L), nonsmokers and recorded a 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 following the standards of the local ethics committee of Central Denmark Region (1-10-72-56-17) and the Declaration of Helsinki.

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 postexercise (FED-EX) after a 5-d 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 after a controlled 5-d period in energy balance to determine the influence of energy status on MPS rates. In total, 40 women were randomly assigned to 1 of 4 groups (Figure 1). Three groups underwent a 5-d 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) before 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

TABLE 1
Participant characteristics

	ERW15 ($n = 10$)		ERW35 ($n = 10$)		ERW60 ($n = 10$)		EBW35 ($n = 10$)	
	Mean (SD)							
Age (y)	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)
Estrogen 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.
1-RM, one-repetition maximum; FSH, follicle-stimulating hormone; TG, triglycerides; BMI, body mass index.

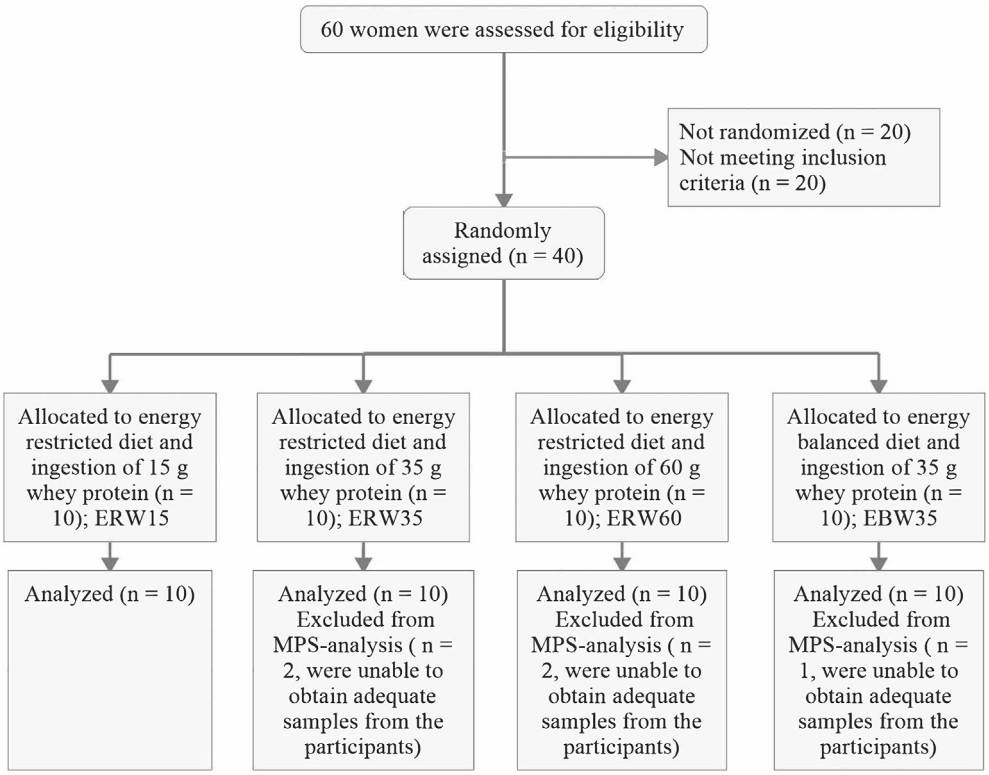


FIGURE 1. Flowchart of enrolment process.

procedure, we were unsuccessful in obtaining sufficient tissue from 5 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).

Screening visit

Eligible participants attended the laboratory after an overnight fast >1 wk before conducting the experimental trial. A blood sample was analyzed for routine biomarkers of general metabolic health and sex hormone concentrations. Women with concentrations of estrogen < 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 4 treatments. The participants were blinded to the protein-dose allocation.

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

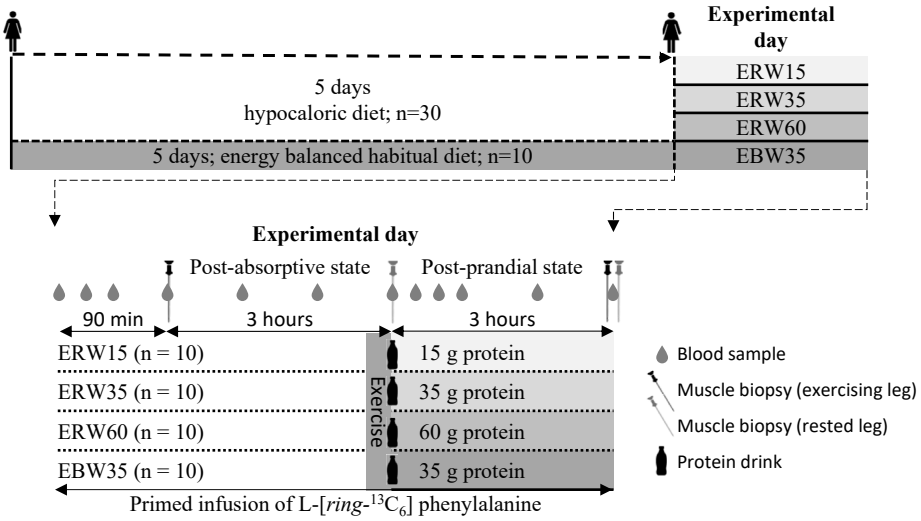


FIGURE 2. Overview of study design and experimental trial. Blood samples were collected prior to initiation of L-(*ring*-$^{13}C_6$)phenylalanine infusion (–270 min; Baseline) and periodically thereafter during the experimental day. A single bout of unilateral leg resistance exercise was initiated 20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

conducted after a self-administered 10 min warm-up on an ergometer bike. Leg assigned to exercise was randomly selected, ie, independent of dominance.

Diet and physical activity control

Participants commenced their assigned diets 5 d before the experimental visit. Energy-restricted groups (ERW15, ERW35 and ERW60) 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 $\pm$ 51 kcal/d (ERW15); 2024 $\pm$ 29 kcal/d (ERW35); 2098 $\pm$ 43 kcal/d (ERW60); 2026 $\pm$ 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 to 10,000 steps as quantified by a Yamax pedometer (Yamax PZ270 Power Walker Lite, Yamasa Tokei Keike Co., Ltd, Japan). Noncaloric drinks (eg, black coffee and tea) were permitted ad libitum until 24 h before 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.

Infusion protocol

Participants reported to the laboratory at 7:30 a.m. after an overnight fast. Body weight was measured and 2 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 $\times$ 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 to 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 2 further muscle biopsies were obtained from the exercised (FED-EX) and nonexercised (FED) leg.

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 flavor 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 after bolus ingestion of 40 g of whey protein [31], we adjusted the beverage enrichment of L-[*ring*-$^{13}C_6$]-phenylalanine as follows depending

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
Σ Essential 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
Σ Nonessential amino acids	52.7

on the whey protein dose: 15 g protein dose: 10%; the 35 g dose: 8.5%, and the 60 g dose: 6.25%.

Muscle biopsy and blood sampling

All blood samples were dispensed into prechilled 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 4 muscle biopsies (2 from each leg; ~250 mg) were obtained from the vastus lateralis (~12–15 cm proximal to patella) under local anesthesia (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.

Analytical procedures

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).

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 2 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 the 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 ($P > 0.05$).

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 2 biopsy samples, $E_{precursor}$ is the arterialized blood precursor defined as the area under the curve (AUC) for plasma enrichments of labeled phenylalanine over the 3-h incorporation periods. $\Delta time$ is the time interval between muscle biopsies.

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 assumed 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 analyzed for main effects and any interaction between the 2 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 analyzed 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 AUC (*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.

Results

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-d 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, $P = 0.221$)

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 analyzed 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.

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

Plasma glucose, serum insulin and urea concentrations

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

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

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

Plasma phenylalanine enrichments

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

Myofibrillar fractional synthetic rate

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

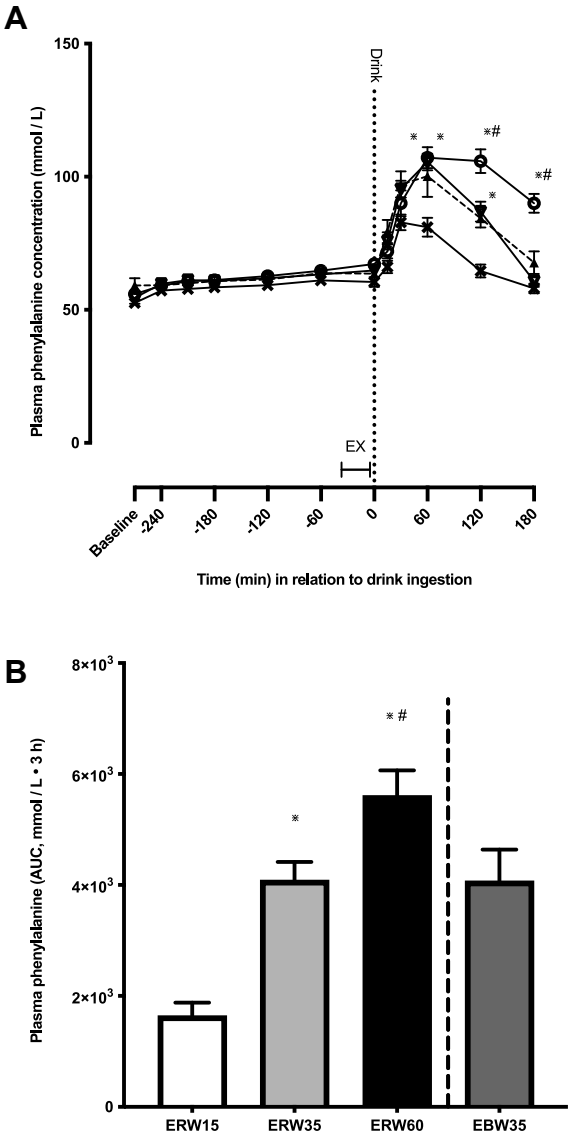


FIGURE 3. Plasma phenylalanine concentration expressed over time (A) and as *i*AUC (B) in energy-restricted and energy balanced groups. ERW15 (✕), energy-restricted diet with ingestion of 15 g whey protein; ERW35 (▽), energy-restricted diet with ingestion of 35 g whey protein; EBW35 (▲), energy balanced 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.

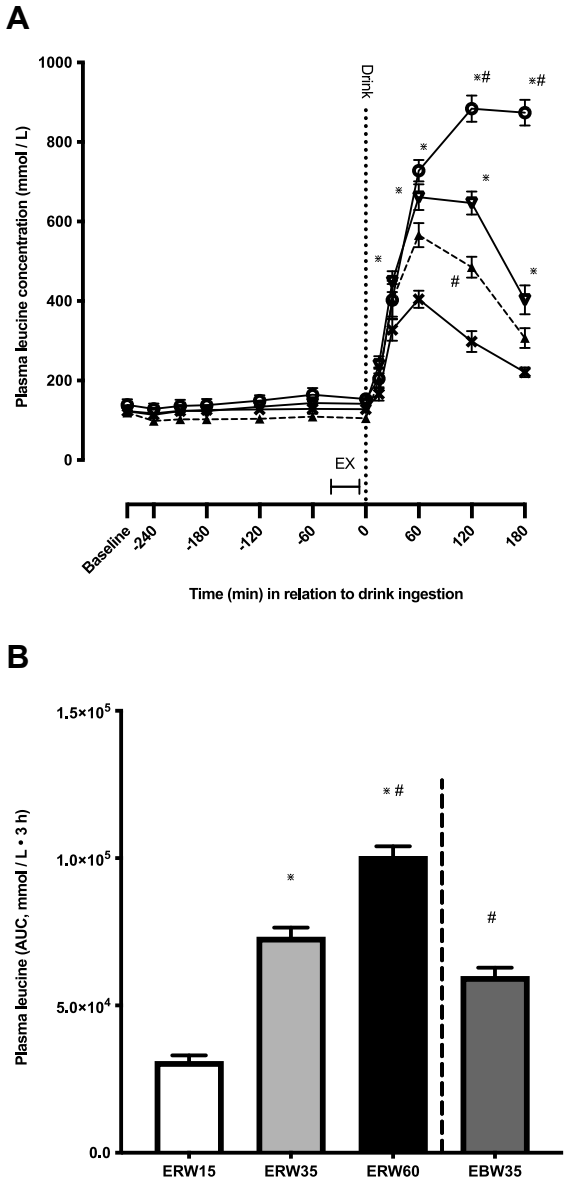
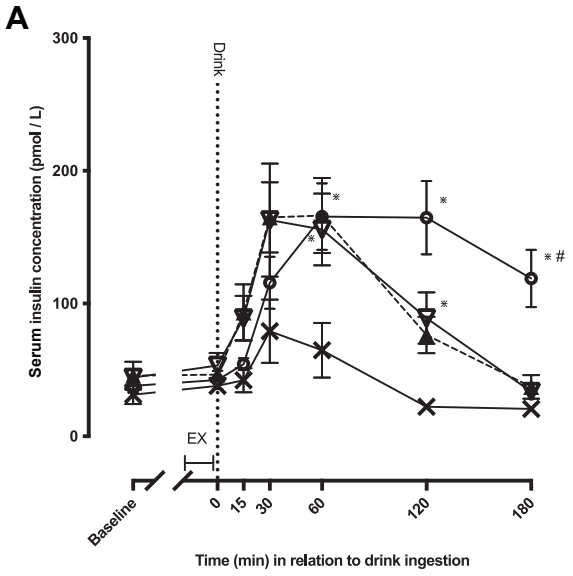


FIGURE 4. Plasma leucine concentrations expressed over time (A) and as *i*AUC (B) in energy-restricted and energy balanced groups. ERW15 (✕), energy-restricted diet with ingestion of 15 g whey protein; ERW35 (▽), energy-restricted diet with ingestion of 35 g whey protein; EBW35 (▲), energy balanced diet 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.



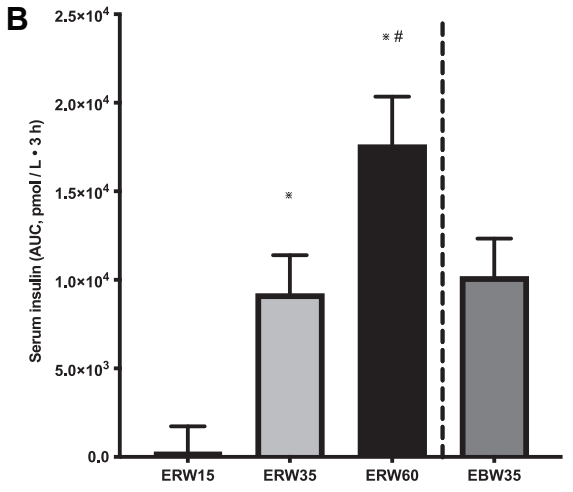


FIGURE 5. Serum insulin concentrations expressed over time (A) and as *i*AUC (B) in energy-restricted and energy balanced groups. ERW15 (✕), energy-restricted diet with ingestion of 15 g whey protein; ERW35 (▽), energy-restricted diet with ingestion of 35 g whey protein; EBW35 (▲), energy balanced diet 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.

(+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

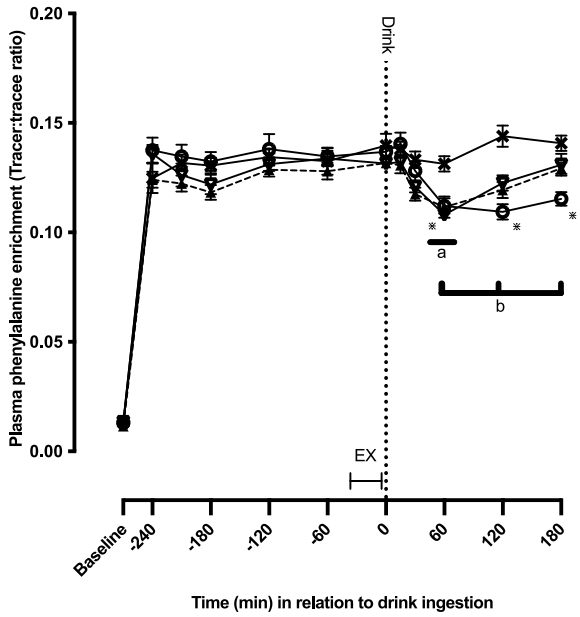


FIGURE 6. Arterialized plasma phenylalanine enrichment expressed over time in energy-restricted and energy-balanced groups. ERW15 (✕), energy-restricted diet with ingestion of 15 g whey protein; ERW35 (▽), energy-restricted diet with ingestion of 35 g whey protein; EBW35 (▲), energy-balanced diet with 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.

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).

Discussion

This clinical randomized 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 postexercise (FED-EX) after 5 d of energy restriction. In addition, we examined the influence of energy status (ie, 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

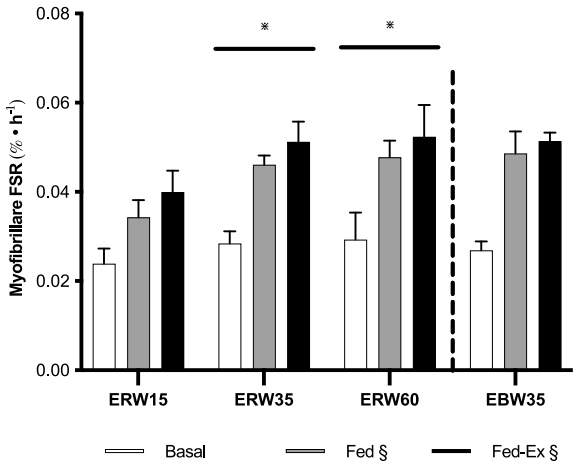


FIGURE 7. Myofibrillar fractional synthesis rate (FSR) in response to graded doses of ingested whey protein in exercised and rested muscles in energy-restricted and energy-balanced groups. A mixed effect model was used for statistical analysis with *protein* dose (ERW15, ERW35, EBW35, ERW60) and *condition* (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, $n = 8$).

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) after 5 d 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 subpopulation is to ingest 35 g of high-quality protein per meal during a weight loss program.

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 nonanabolic (ie, oxidation) rather than incorporated 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, ie, aggregate protein synthesis rates across all body tissues combined, rather than tissue-specific (ie, 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. Although 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 LBM in middle-aged women that undertook a 4-mo weight loss trial. In addition, the impairment in basal myofibrillar FSR following 5 d of energy restriction in resistance-trained young adults was restored after 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 d 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 postmenopausal 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 in muscle protein breakdown [16]. Moreover, an extended period of energy restriction (21 d) was shown to elicit a suppressed postprandial response of MPS to 20 g of ingested milk protein [54] when daily protein intake was restricted to the RDA (0.8 g/kg BM/d). Hence, based on acute metabolic studies in healthy young adults, the primary metabolic driver of LBM loss during energy deficit appears to be phase dependent, with basal rates of MPS impaired during the early phase of energy restriction, and the postprandial response of MPS attenuated during later periods of energy restriction. Refuting our original hypothesis, we report no differences in basal or postprandial (FED or FED-EX conditions) myofibrillar FSR between EBW35 and ERW35 groups, despite the 2 kg decline in body mass in ERW35 after the diet period. This counter-intuitive finding was likely attributed to differences in experimental design between past [14, 15] and present studies. We utilized a parallel, between-subjects, design to determine the influence of energy status on myofibrillar FSR, whereas previous studies employed a more sensitive within-subject crossover design with participants serving as their own control [14–16]. Interestingly, previous studies in physically-active young adults have demonstrated a high protein diet (1.6–2.4 g/kg/d) to be effective in preserving basal and postprandial rates of MPS and reducing loss of LBM during short-term energy restriction [54]. Hence, a follow up study that manipulates dietary protein intake during a longer-term (weeks to months) period of energy restriction is warranted in a clinical population of overweight, postmenopausal women.

A strength of the present study relates to the novelty in terms of investigating the protein-dose MPS response relationship under conditions of energy deficit in a clinically relevant, homogenous sample of middle-aged, overweight, postmenopausal women. Moreover, fraction-specific measurements of myofibrillar FSR were conducted under basal, fed and exercised-fed conditions, and thus provided comprehensive insight into post-absorptive, postprandial and exercise-stimulated responses of MPS to energy restriction. However, we acknowledge several limitations. First, for practical reasons, the trial was conducted as a single-blinded study. In this regard, the investigators that performed the experimental trial and statistical analysis were not blinded to group allocation. However, all sample analyses for the measurement of MPS (primary endpoint) were performed by blinded investigators, and thus the single-blinded nature of the trial was unlikely to bias study findings. Second, although measurements of MPS were conducted under multiple conditions, i.e., resting and postexercise, energy balance and energy restriction, the study was powered based on previous dose-response studies conducted in energy balance. Third, the energy restriction period was severe (~800 kcal/d) and short-term (5 d) and thus direct translation of our findings to clinically relevant (20% energy deficit for weeks to months) periods of weight loss must be considered with caution. Fourth, due to limited available muscle tissue, it was not possible to use intracellular $^{13}C_6$ phenylalanine enrichments as the true precursor in the calculation of myofibrillar FSR and instead plasma $^{13}C_6$ phenylalanine enrichments were used for the calculation of MPS. Finally, we did not conduct measurements of muscle protein breakdown alongside MPS. Hence, it was not possible to calculate the response of net muscle protein balance to protein feeding during energy deficit. Interestingly, previous studies have reported an increased stimulation of muscle protein breakdown following 10 d of moderate (20%) energy deficit [17], suggesting a mechanistic action of muscle proteolysis in muscle mass loss during diet-induced energy restriction, at least over prolonged periods of weight loss. Moreover, future studies are warranted to establish the dose-response of MPS to ingested protein during weight loss in other clinical populations that experience muscle loss, i.e., sarcopenic obese older adults, over chronic periods of diet-induced weight loss. Deuterium oxide tracer methodology is ideally suited to the measurement of free-living, integrated, rates of MPS over prolonged periods of weight loss [55], and thus once fully reestablished in the field of muscle protein metabolism, may be utilized in future studies to inform protein recommendations for muscle mass retention during weight loss in clinical populations.

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 program.

Author Contribution

The authors' responsibilities were as follows—MSL, MH, OCW, KDT, LH and URM designed research; MSL, RH, MM, MBB, KML, LH, KS, and PS conducted research; URM provided the protein supplements for the study; MSL and MH analyzed data; MSL, OCW and MH wrote the paper; MSL and MH had primary responsibility for final content. All authors read and approved the final manuscript.

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Norway. Nutrilett, The Danish Dairy Research Foundation, Toyota Foundation, and Innovation Fund Denmark had no influence on study design, implementation, analysis or interpretation of the data.

Conflict of Interest

Mette Hansen reports financial support to research and supplies of products for research from Arla Foods Ingredients Group P/S and financial support from The Danish Dairy Research Foundation and Toyota Foundation, Denmark. In addition, Mette Hansen reports supplies of products from Orkla Health, Norway, to the present project. Mads S. Larsen was in the project period employed as an industrial PhD student at Arla Food Ingredients P/S funded by the public fund, Innovation Fund Denmark, and Arla Food Ingredients P/S, but enrolled as a PhD student at Faculty of Health, Aarhus University. Mette Hansen from Aarhus University was the main PhD supervisor. Ulla R. Mikkelsen employed at Arla Food Ingredients Group P/S was affiliated with the project as an industrial PhD-supervisor. Lars Holm and Maike Mose report a relationship with Arla Foods that includes funding grants. Oliver C. Witard, Kevin D. Tipton, Katrine Meyer Lauritsen, Mads Bisgaard Bengtsen, Rikke Hansen, Kenneth Smith and Paula Scaife declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

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

Acknowledgments

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.tjnut.2023.08.011.

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