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Protein dose requirements to maximize skeletal muscle protein synthesis after repeated bouts of resistance exercise in young trained women

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Abstract

Studies examining the effect of protein (PRO) feeding on post resistance exercise (RE) muscle protein synthesis (MPS) have primarily been performed in men, and little evidence is available regarding the quantity of PRO required to maximally stimulate MPS in trained women following repeated bouts of RE. We therefore quantified acute (4 h and 8 h) and extended (24 h) effects of two bouts of resistance exercise, alongside protein-feeding, in women, and the PRO requirement to maximize MPS. Twenty-four RE trained women (26.6 ± 0.7 years, mean ± SEM) performed two bouts of whole-body RE (3 × 8 repetitions/maneuver at 75% 1-repetition maximum) 4 h apart, with post-exercise ingestion of 15 g, 30 g, or 60 g whey PRO ($n = 8$/group). Saliva, venous blood, and a vastus lateralis muscle biopsy were taken at 0 h, 4 h, 8 h, and 24 h post-exercise. Plasma leucine and branched chain amino acids were quantified using gas chromatography mass spectrometry (GC–MS) after ingestion of D_2O. Fifteen grams PRO did not alter plasma leucine concentration or myofibrillar synthetic rate (MyoFSR). Thirty and sixty grams PRO increased plasma leucine concentration above baseline (105.5 ± 5.3 μM; 120.2 ± 7.4 μM, respectively) at 4 h (151.5 ± 8.2 μM, $p < 0.01$; 224.8 ± 16.0 μM, $p < 0.001$, respectively) and 8 h (176.0 ± 7.3 μM, $p < 0.001$; 281.7 ± 21.6 μM, $p < 0.001$, respectively). Ingestion of 30 g PRO increased MyoFSR above baseline (0.068 ± 0.005%/h) from 0 to 4 h (0.140 ± 0.021%/h, $p < 0.05$), 0 to 8 h (0.121 ± 0.012%/h, $p < 0.001$), and 0 to 24 h (0.099 ± 0.011%/h, $p < 0.01$). Ingestion of 60 g PRO increased MyoFSR above baseline (0.063 ± 0.003%/h) from 0 to 4 h (0.109 ± 0.011%/h, $p < 0.01$), 0 to 8 h (0.093 ± 0.008%/h, $p < 0.01$), and 0 to 24 h (0.086 ± 0.006%/h, $p < 0.01$). Post-exercise ingestion of 30 g or 60 g PRO, but not 15 g, acutely increased MyoFSR following two consecutive bouts of RE and extended the anabolic window over 24 h. There was no difference between the 30 g and 60 g responses.

KEYWORDS
muscle protein synthesis, protein dosing, resistance exercise

1 | INTRODUCTION

Skeletal muscle mass is dictated by a dynamic equilibrium; muscle proteins lost during postabsorptive periods are replenished in postprandial periods. Dietary protein (PRO) is vital for maintaining this dynamic equilibrium[1,2] since essential amino acid (EAA) constituents, and leucine in particular,[3] are the most crucial of the nutrients for muscle anabolism. PRO-mediated increases in muscle protein synthesis (MPS) are initiated after transport of EAAs into the muscle cell,[4] where leucine (although not exclusively) activates cell signaling cascades that lead to increased MPS.[5–7] Increasing plasma availability of EAAs by oral[8] or intravenous[9] supply, or by oral PRO intake[10] stimulates MPS. Resistance exercise in the fasted state can stimulate MPS alone[11] however, resistance exercise alongside hyperaminoacidaemia further stimulates MPS[12,13] and post resistance exercise PRO intake represents an important component of maximizing skeletal muscle adaptation to resistance exercise training.[14] The time course of this enhanced exercise-mediated sensitisation of PRO intake on MPS is poorly defined, but it is reported to be evident in trained participants 24 h after a single bout of resistance exercise[15] and up to 48 h[16] and 72 h[17] after resistance exercise in untrained subjects.

The type of PRO ingested, timing of PRO ingestion and amount of PRO ingested in any given serving influence the response of MPS following exercise.[18] The optimal dosing of PRO to maximize MPS has been the subject of much debate and in men is proposed to lie between 20 and 40 g of PRO[18–20] or 10 and 20 g EAAs.[8] The majority of studies examining the effect of PRO on MPS after exercise are primarily performed in men, and the optimal amount of PRO needed by women to show such a response is unknown. While some studies have shown that resting postabsorptive rates of MPS in young untrained individuals are similar in both men and women,[21–24] and the MPS response to amino acids and insulin are also similar in young men and women,[23] little evidence is available regarding the quantity of PRO required to maximally stimulate post resistance exercise MPS in trained women. Current PRO feeding guidelines for female athletes are based on studies in men, and further observations in women alone are needed to ensure recommendations are optimal for enhanced athletic performance. Furthermore, little is known about the response of MPS after repeated bouts of whole-body resistance exercise or how dietary PRO dosing impacts on any effects of repeated bouts of resistance exercise, particularly in trained women.

Thus, the requirements and design of this study were based on (i) the PRO requirement for maximizing MPS in trained women remains poorly understood, (ii) that the effect of multiple bouts of RE and PRO feeding on MPS in trained women is unknown, and (iii) that understanding these paradigms better should lead to improved recommendations and nutritional strategies for female athletes. Therefore, we aimed to understand the PRO dosing requirements to maximize post resistance exercise MPS, specifically myofibrillar fractional synthetic rate (MyoFSR) in trained women, and to understand both the acute (4 h and 8 h) and extended (24 h) effect of two bouts of resistance exercise on these MPS responses.

2 | MATERIALS AND METHODS

2.1 | Ethics

This study was approved by the Ministry of Defence (MOD) Research Ethics Committee (Ref. No. 892/MODREC/18) and the University of Nottingham Faculty of Medicine and Health Sciences Research Ethics Committee (Ref. No. 28–1086) and met the regulations outlined in the Declaration of Helsinki. The study was part of a MOD program of research aimed at supporting female soldiers in ground close combat, and where possible the study was designed to reflect the conditions of soldiers working in a field situation performing physical tasks. The study was performed at the David Greenfield Human Physiology Unit (DGHPU), University of Nottingham, and informed written consent was obtained from all individuals prior to enrolment in the study.

2.2 | Participants

The study recruited 24 healthy women (Figure 1; 26.6 ± 0.8 years, BMI 24.0 ± 0.5 kg m^2). All were non-smokers, non-vegetarian, and regularly (at least twice a week for the past 6 months) performed resistance-type exercise. Eligibility was assessed at a medical screening, which included anthropometric measurements, blood pressure assessment, a 12-lead electrocardiogram, and a venous blood sample for routine screening. Participants were excluded if they had cardiovascular disease, metabolic or endocrine abnormalities, or were taking regular medication to treat a medical condition. To ensure participants were resistance exercise trained, they were required

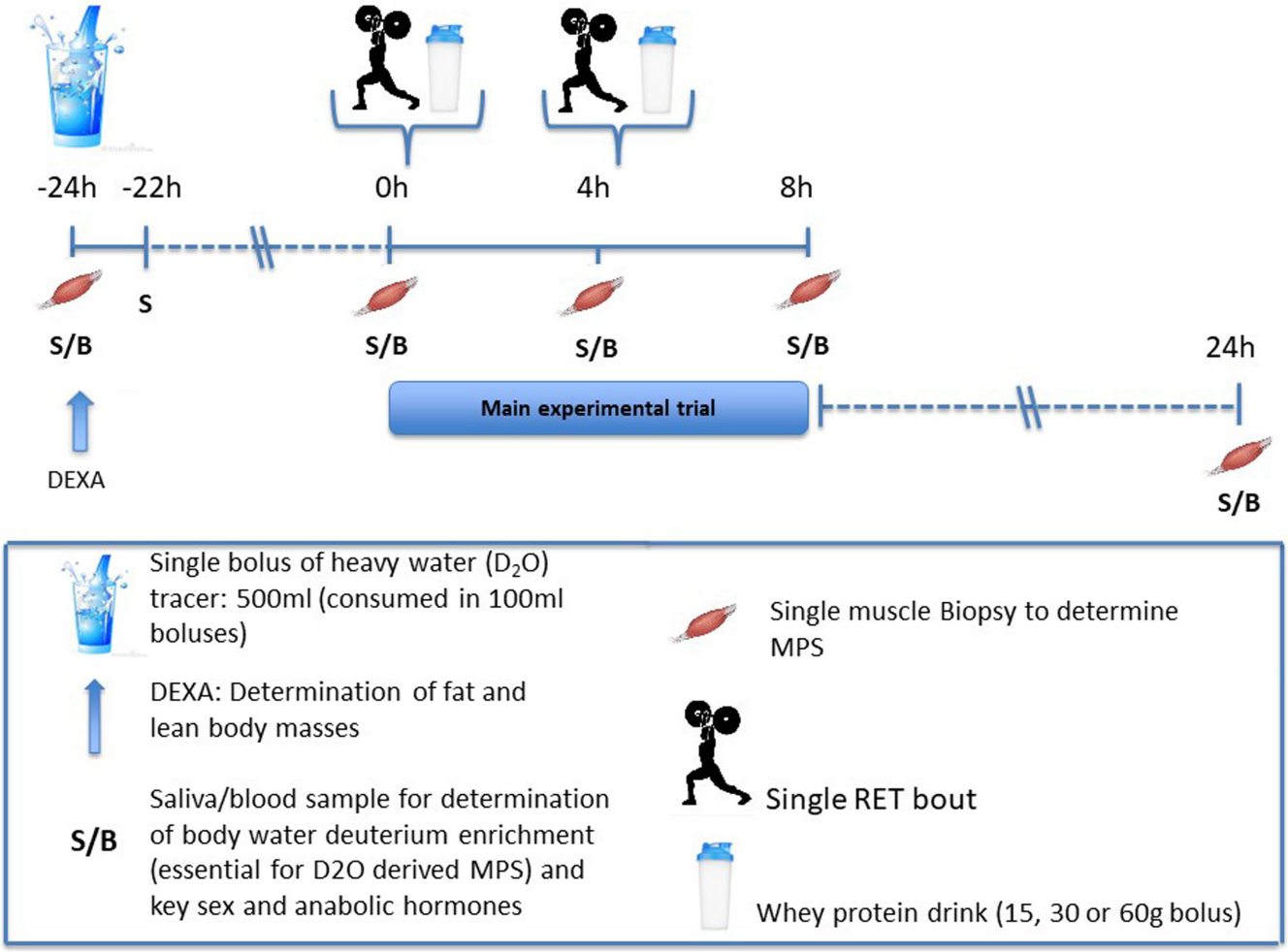

FIGURE 1 Schematic of the study protocol.

to perform a short exercise test that involved lifting a 30 kg Powerbag onto a platform at a height of 1.49 m to be included into the study. Women were tested in the early follicular phase of their menstrual cycle where known, and hormonal contraceptive users were included since female reproductive hormones do not affect rates of MPS at rest and after acute exercise.[25] The types of hormonal contraception used by participants included the combined oral contraceptive pill ($n = 7$), progesterone only contraceptive pill ($n = 2$), Mirena coil ($n = 4$), and the hormonal implant ($n = 2$). After screening, participants attended the laboratory on three further occasions (Figure 1).

2.3 | Initial resistance exercise test

Participants who passed their medical screening attended the laboratory for a whole-body resistance exercise test, which was performed at least 7 days before the main experimental trial. The purpose of this testing was to determine participant-specific exercise workloads to be employed in the main experimental trial. The exercises performed were latissimus dorsi pull down (lat pull down), single-leg press on both legs, and chest press using machine-based equipment (Paramount, True Fitness Technologies, MO, USA). One repetition-max (1RM) for each exercise was estimated using an eight-repetition submaximal protocol.[26] Participants were asked to refrain from strenuous exercise 48 h prior to the testing day to ensure they were fully rested. The testing protocol consisted of a 10 min warm-up on a stationary exercise bike, after which each participant performed the following regimen for each muscle group: a warm-up set of eight repetitions followed by eight repetitions at a weight where only eight repetitions could be achieved before failure, and then a set where failure was achieved before eight repetitions, these sets were interspersed with 2 min rest. One-RM was then calculated using the Brzycki equation[26]:

$$1\text{RM} = 100^{*}\ \text{load rep} / \left(102.78 - 2.78^{*}\ \text{rep}\right)$$

Load rep = workload value of repetitions performance, expressed in kg.

Rep: number of repetitions performed.

2.4 | Dual-energy X-ray absorptiometry (DXA) scan and heavy water (D_2O) ingestion

Twenty-four hours prior to the main experimental trial, participants attended the laboratory for a DXA scan (Lunar Prodigy DXA; GE Medical Systems, Bedford, UK) and D_2O ingestion after an overnight fast (see Figure 1). Prior to D_2O ingestion a baseline venous blood, saliva, and muscle biopsy sample were taken. The muscle biopsy was obtained from the vastus lateralis (VL) using the microbiopsy technique[27] with participants rested in a supine position. The muscle sample was snap frozen in liquid nitrogen and stored for analysis. Participants then consumed 500 mL of 70AP D_2O in 100 mL boluses over a period of 6 h to enable the assessment of baseline MyoFSR.[28]

2.5 | Main experimental trial

Twenty-four hours after the D_2O ingestion, participants attended the laboratory at 7.45 AM having fasted from 10 PM the night before and rested semi-supine on a bed. A saliva sample, venous blood sample, and a vastus lateralis muscle biopsy were taken before participants performed the resistance exercise protocol. Participants performed 3×8 reps at 75% 1RM for lat pull down, single-leg press (on both legs), and chest press with 2 min rest between sets, to stimulate MPS acutely.[29,30] After the exercise session, participants consumed a PRO drink consisting of either 15 g, 30 g, or 60 g unflavoured whey PRO (Iso:Pro Whey Protein Isolate, My Protein, Manchester, UK) with 7.5 g Bournville cocoa power (Cadburys, Mondelez UK, Birmingham, UK) and 1.5 g aspartame sweetener (Canderel, High Wycombe, UK) diluted with 200 mL water. Participants were assigned to drink either 15 g, 30 g, or 60 g PRO, and allocation was stratified to dominant leg 1RM leg strength, thereby ensuring each PRO group was matched for strength. We chose to use an absolute bolus protein supplement as this would mimic better the conditions in the field for soldiers, as opposed to ingestion of protein supplements relative to body mass. After consumption of the drink, participants rested semi-supine on a bed for 4 h. Participants then gave another saliva, venous blood, and muscle biopsy sample as described above and completed another bout of whole-body resistance exercise followed by consumption of another PRO drink as per the morning. Participants again rested semi-supine on a bed for a further 4 h before saliva, venous blood, and muscle biopsy samples were obtained (8 h after ingestion of the first PRO drink). While multiple RET bouts during a single day are uncommon, we aimed to approximate better the arduous training regimes performed by soldiers during field training and potential warfare situation. However, we acknowledge that this may not represent any additional aerobic-type activities performed by warfighters.

After the 8 h sample collection, participants were provided with food and beverages (55% carbohydrate, 30% fat, and 15% PRO) that contained their total energy requirements for the day calculated from the Harris Benedict equation (activity factor = 1.55). All food and drink were consumed after the 8 h saliva, venous blood, and muscle biopsy sample collection and before 10 PM that evening. The following day, participants arrived at the laboratory at 7.45 AM, fasted from 10 PM the night before and rested semi-supine on a bed. A final saliva, venous blood, and muscle biopsy sample was obtained 24 h post ingestion of the first PRO drink the day before.

2.6 | Blood analysis

Venous blood samples (~25 mL) collected during the study visits were aliquoted into sodium heparin and EDTA tubes and were centrifuged immediately after collection at 4400 × *g* for 10 min at 4°C to obtain plasma. Another aliquot was left to coagulate in spray-coated silica and polymer gel tubes for 20–30 min before centrifugation at 4400 × *g* for 10 min at 4°C to obtain serum. The supernatant was removed and stored at −80°C until further analysis.

2.6.1 | Testosterone, growth hormone, myostatin/GDF-8 and insulin-like growth factor 1 (IGF-1)

Serum concentration of testosterone was determined using a MILLIPLEX® MAP Human Magnetic Bead Panel kit (Merck Millipore, Darmstadt, Germany). Enzyme-linked immunosorbent assay (ELISA) kits were used to measure serum concentration of growth hormone (Abcam, Cambridge, UK), myostatin (R&D Systems, Abingdon, UK), and IGF-1 (R&D Systems, Abingdon, UK).

2.6.2 | Plasma amino acid analysis

Plasma amino acid (AA) concentration was determined using gas chromatography mass spectrometry (GC–MS) as described by Burd et al.[29] Plasma proteins were precipitated with ice-cold ethanol and the supernatant, containing free amino acids dried under N_2, was solubilized in 0.5 M HCl and the lipid fraction extracted into ethyl acetate. The remaining solution was dried down before being derivatized to their tert-Butyldimethylsilyl 2-[(3,5,6-trichloro-2-pyridyl)oxy]acetate (tBDMS) derivative. To determine AA concentrations,

an internal standard was added to each sample containing a stable isotopically labeled AA for all AA. AA were then quantified on a GC–MS (Agilent) alongside a standard curved of known AA concentrations that was run throughout each sequence. A pooled QC sample was run throughout to ensure repeatability.

2.7 | Saliva and skeletal muscle analysis

2.7.1 | Body water and determination of protein-bound alanine enrichment

Body water and muscle protein enrichment were measured as described elsewhere.[28] Saliva was heated for 4 h at 95°C to purify fractions of the body water. The vials were then cooled on ice, and the condensed body water was injected on a high-temperature conversion elemental analyzer (Thermo Finnigan, Thermo Scientific, Hemel Hempstead, UK) connected to an isotope ratio mass spectrometer (Delta V advantage, Thermo Scientific). To assess protein-bound alanine muscle fraction enrichment, ~30 mg of muscle was homogenized in ice-cold homogenization buffer to isolate myofibrillar proteins and centrifuged at 11000 × *g* for 15 min at 4°C. The pellet was resuspended in 500 μL mitochondrial extraction buffer (MEB) and homogenized by Dounce and centrifuged at 1000 × *g* for 5 min at 4°C. Insoluble collagen was separated following centrifugation from myofibrillar proteins that were solubilized in 750 μL NaOH and subsequently precipitated using 1 M perchloric acid (PCA) then pelleted by centrifugation. Following overnight hydrolysis at 110°C in a 0.1 M HCl and Dowex H+ resin slurry, the amino acids were eluted with 2 M NH_4OH and dried down.

Dried samples were resuspended in 60 μL distilled water, 32 μL methanol, 10 μL pyridine, and 8 μL methyl chloroformate. The n-methoxycarbonyl methyl esters of the AAs were then extracted after adding 100 μL chloroform. Incorporation of deuterium into the protein-bound alanine was determined by gas chromatography–pyrolysis–isotope ratio mass spectrometry (Delta V Advantage, Thermo, Hemel Hempstead, UK).

2.7.2 | Calculation of fractional and absolute synthetic rate

Myofibrillar fractional synthetic rate (MyoFSR) was calculated using the incorporation of deuterium in alanine in myofibrillar proteins ($APE_{Alanine}$) and deuterium enrichment of body water from the saliva samples, representing the precursor labeling between biopsies ($APE_{Precursor}$: corrected for the mean number of deuterium moieties incorporated per alanine, 3.7, and the dilution from the total number of hydrogens in the derivative, i.e., 11). The following calculation was used, where *t* represents the time between biopsies:

$$\text{MyoFSR}\,(\%/\text{h}) = -\ln\left(\frac{1-\left(\frac{APE_{Alanine}}{APE_{Precursor}}\right)}{t}\right).$$

3 | DATA HANDLING AND STATISTICAL ANALYSIS

The study is powered to the primary end point of feeding-induced increase in MyoFSR, which we have shown previously using the D_2O technique.[28] Using these data to calculate Cohen's d as an estimate of effect size, and at an alpha level of significance of 0.05, a sample size of eight per group is estimated to be sufficient to detect a difference within groups. Therefore, based upon group sizes of $n = 8$ determined from our primary power calculation, this provides us statistical power of 83%–93% to detect between group differences.

Data were analyzed using Graphpad Prism 9.4.1 (GraphPad Software Inc, San Diego, CA). Data were tested for normality using a Shapiro–Wilks test. Comparisons of baseline measurements were performed using one-way ANOVA with Tukey's post-hoc test to identify any differences between each group. A two-way repeated measures ANOVA with a Tukey's post-hoc test was performed to identify differences between PRO dose and time. Where data were not normally distributed (age, height, total body fat, leg press 1RM, baseline serum growth hormone, and energy content of dietary intake), comparisons were made between each PRO dose using a Kruskal–Wallis test with a Dunn's post-hoc test to identify any differences. All data are represented as mean ± SEM, unless otherwise stated, with statistical significance accepted at the $p < 0.05$ level.

4 | RESULTS

4.1 | Baseline characteristics

Table 1 shows subject physical characteristics and serum hormone concentrations for each study group at baseline. Subjects had been performing regular resistance exercise for 6.8 ± 0.9 years and trained on average 4.9 ± 0.4 times per week. There were no significant differences in age, BMI, total body fat, appendicular lean mass, dominant leg strength (leg press 1RM), serum testosterone, serum myostatin, serum growth hormone, and serum IGF-1 between groups.

4.2 | Dietary intake

There was no difference in the absolute intake of fat or carbohydrate between PRO dose groups on the experimental day (Table 2). The total energy intake was greater in those supplemented twice daily with 60 g PRO (11 445 ± 183.4 kJ) compared with supplementation with 15 g PRO twice daily (10 011 ± 367.4 kJ, $p < 0.05$) and 30 g PRO twice daily (9917 ± 457.8 kJ, $p < 0.05$). As expected, total daily PRO intake was also different between PRO dose groups, with the 30 g (148 ± 2.7 g, $p < 0.001$) and 60 g PRO (208 ± 3.2 g, $p < 0.001$) groups consuming more PRO than the 15 g group (121 ± 4.4 g), and the 60 g PRO group consuming more protein than the 30 g PRO group ($p < 0.001$). Similarly, relative daily PRO intake (g kg BW^{-1} d^{-1}) differed between groups, with the 30 g (2.3 ± 0.11 g, $p < 0.01$) and 60 g (3.2 ± 0.01 g, $p < 0.001$) PRO dose groups consuming more PRO than the 15 g group (1.8 ± 0.05 g), and the 60 g PRO group consuming more protein than the 30 g PRO group ($p < 0.001$).

4.3 | Amino acid enrichment

4.3.1 | Plasma branched-chain amino acid (BCAA) concentration

There was no difference in plasma BCAA concentration between groups at baseline (15 g, 391.9 ± 20.7 μM; 30 g, 351.0 ± 20.4 μM; 60 g, 395.7 ± 21.8 μM). There was a significant time × PRO dose interaction in plasma BCAA concentration ($p < 0.001$). Post-exercise PRO ingestion by the 15 g PRO group did not increase post-exercise plasma BCAA concentration from baseline at 4 h, 8 h, or 24 h (Figure 2A). Plasma BCAA concentration increased from baseline (351.0 ± 20.4 μM) at 8 h in the 30 g PRO group (467.7 ± 16.7 μM, $p < 0.05$) and from baseline (395.7 ± 21.8 μM) at 4 h (589.9 ± 37.7 μM, $p < 0.01$) and 8 h (732.2 ± 59.4 μM, $p < 0.01$) in the 60 g PRO group. Both groups returned to baseline concentrations at 24 h. Plasma BCAA concentration at 4 h was greater in the 60 g PRO group (589.9 ± 37.7 μM) when compared to the 15 g (367.8 ± 13.3 μM, $p < 0.01$) and 30 g (417.7 ± 22.7 μM, $p < 0.01$) PRO groups at the same time point. At 8 h, plasma BCAA concentration was greater in the 30 g PRO group (467.7 ± 16.7 μM) compared to 15 g PRO group (401.1 ± 18.9 μM, $p < 0.05$) and greater in the 60 g PRO group (732.2 ± 59.4 μM) compared to the 30 g PRO (467.7 ± 16.7 μM, $p < 0.01$) and 15 g PRO (401.1 ± 18.9 μM, $p < 0.01$) groups. No differences in plasma BCAA concentration were seen between groups at 24 h.

4.3.2 | Plasma leucine concentration

There was no difference in plasma leucine concentration between groups at baseline (15 g, 123.6 ± 6.2 μM; 30 g, 105.5 ± 5.3 μM; 60 g, 120.2 ± 7.4 μM). There was a significant time × PRO dose interaction in plasma leucine concentration ($p < 0.001$). Post-exercise PRO ingestion in the 15 g PRO group did not alter plasma leucine concentration from baseline at any time point (Figure 2B), but did increase concentrations from baseline in the 30 g and 60 g PRO groups (105.5 ± 5.3 μM; 120.2 ± 7.4 μM, respectively) at 4 h (151.5 ± 8.2 μM, $p < 0.01$; 224.8 ± 16.0 μM, $p < 0.001$ respectively) and 8 h (176.0 ± 7.3 μM, $p < 0.001$; 281.7 ± 21.6 μM, $p < 0.001$, respectively). Plasma leucine concentration was greater at 4 h in the 60 g PRO group

TABLE 1 Physical characteristics and serum hormone concentrations for each protein dose group at baseline.

Characteristic	Group		
Protein Dose (g)	15	30	60
Age (years)	26.3 ± 1.33	25.9 ± 2.57	27.6 ± 1.78
Height (m)	1.68 ± 0.02	1.65 ± 0.02	1.64 ± 0.02
Body mass (kg)	68.2 ± 3.89	64.6 ± 3.51	65.0 ± 2.44
BMI (kg m^2)	24.3 ± 1.10	23.6 ± 0.91	24.1 ± 0.84
Total body fat (%)	29.7 ± 2.98	29.5 ± 3.05	28.2 ± 2.34
Appendicular lean mass (kg)	20.8 ± 0.43	19.6 ± 0.79	20.1 ± 1.01
Dominant leg press 1RM (kg)	70.2 ± 5.19	71.5 ± 10.21	69.9 ± 4.04
Serum testosterone (ng mL^{-1})	0.6 ± 0.08	0.5 ± 0.10	0.5 ± 0.07
Serum myostatin (pg mL^{-1})	1694 ± 225	1984 ± 212	1958 ± 193
Serum growth hormone (pg mL^{-1})	2704 ± 731	1268 ± 818	1749 ± 619
Serum IGF-1 (ng mL^{-1})	118 ± 13.2	125 ± 13.6	119 ± 13.4

Note: Data expressed as mean ± SEM; $n = 8$ per group.

Abbreviations: BMI, body mass index; 1RM, one repetition maximum.

Macronutrient	Group		
Protein Dose (g)	15	30	60
Energy (kJ)	10011 ± 367.4	9917 ± 457.8	11445 ± 183.4*,$
Carbohydrate (g)	301 ± 9.2	274 ± 16.7	295 ± 7.6
Fat (g)	73 ± 5.1	70 ± 4.8	73 ± 2.6
Protein (g)	121 ± 4.4	148 ± 2.7***	208 ± 3.2***,$$$
Protein (g kg BW^{-1} d^{-1})	1.8 ± 0.05	2.3 ± 0.11**	3.2 ± 0.14***,$$$

Note: Data expressed as mean ± SEM; $n = 8$ per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ when compared to 15 g PRO and $^{\$}p < 0.05$, $^{\$\$\$}p < 0.001$ when compared to 30 g PRO.

Abbreviation: BW, body weight.

TABLE 2 Dietary macronutrient intake on the experimental day for each protein dose group (includes prescribed food intake and nutritional content of protein drinks).

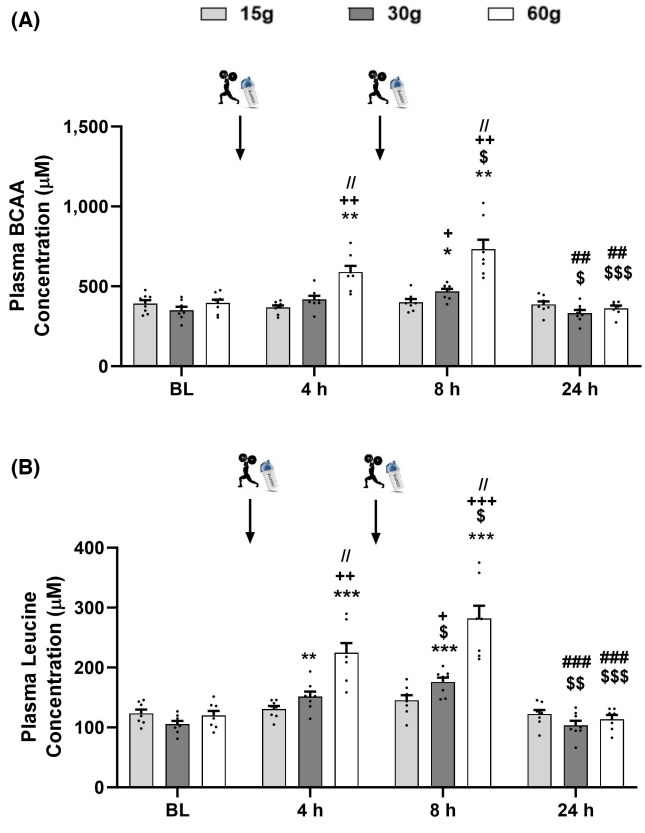

FIGURE 2 (A) Plasma branched-chain amino acid (BCAA) and (B) plasma leucine concentration between each PRO dose. Data expressed as mean ± SEM plus individual values; $n = 8$ per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to baseline (BL) in same PRO dose, $^{\$}p < 0.05$, $^{\$\$}p < 0.01$, $^{\$\$\$}p < 0.01$ compared to 4 h in same PRO dose. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$, $^{\#\#\#}p < 0.001$ compared to 8 h in same PRO dose. $^{+}p < 0.05$, $^{++}p < 0.01$, $^{+++}p < 0.001$ compared to 15 g PRO dose at the same timepoint. $^{//}p < 0.01$ compared to 30 g PRO at the same timepoint.

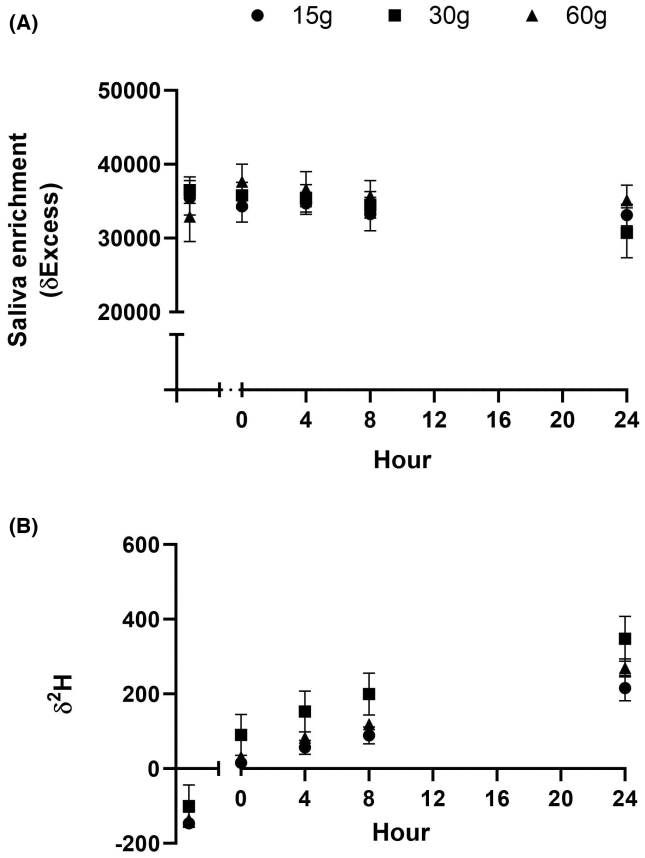

FIGURE 3 (A) Saliva body water enrichment and (B) incorporation of deuterium into protein-bound alanine for each PRO dose. Data expressed as mean ± SEM: $n = 8$ per group.

(224.8 ± 16.0 μM) compared to the 15 g (130.8 ± 5.1 μM, $p < 0.01$) and 30 g (151.5 ± 8.2 μM, $p < 0.01$) PRO groups. At 8 h, plasma leucine concentration was greater in the 30 g PRO group compared to 15 g PRO group (176.0 ± 7.3 μM vs. 145.5 ± 8.4 μM, respectively, $p < 0.05$), and greater in the 60 g PRO group (281.7 ± 21.6 μM) compared to the 30 g PRO (176.0 ± 7.3 μM, $p < 0.01$) and 15 g PRO (145.5 ± 8.4 μM, $p < 0.001$) groups. No differences in plasma leucine concentration were seen between groups at 24 h.

4.4 | Body water and protein-bound alanine

Body water enrichments were ~35000 δ, and due to the slow turnover rate of body water this level of enrichment

maintained a pseudo-steady state throughout the study period (Figure 3A). As expected, incorporation of deuterium into protein-bound alanine increased over time (Figure 3B).

4.5 | Cumulative myofibrillar fractional synthetic rate

There was no difference in cumulative MyoFSR between groups at baseline (15 g, 0.061 ± 0.009%/h; 30 g, 0.068 ± 0.005%/h; 60 g, 0.063 ± 0.003%/h). There was a significant time × PRO dose interaction in cumulative MyoFSR ($p < 0.05$). Post-exercise ingestion of 15 g PRO did not increase cumulative MyoFSR over time (Figure 4). In the 30 g PRO group MyoFSR was increased above baseline (0.068 ± 0.005%/h) from 0 to 4 h (0.140 ± 0.021%/h, $p < 0.05$), 0 to 8 h (0.121 ± 0.012%/h, $p < 0.01$), and 0 to 24 h (0.099 ± 0.011%/h, $p < 0.05$). In the 60 g PRO group cumulative MyoFSR was increased above baseline (0.063 ± 0.003%/h) from 0 to 4 h (0.109 ± 0.011%/h, $p < 0.01$), 0 to 8 h (0.093 ± 0.008%/h, $p < 0.01$), and 0 to 24 h (0.086 ± 0.006%/h, $p < 0.01$). There were no differences in cumulative MyoFSR between doses at any given time point.

4.6 | Blood hormones

There was no difference in the concentration of blood hormones between groups at baseline, and there was no time × dose interaction for any of the blood hormones (Table 3). Serum testosterone showed a time effect ($p < 0.001$) with levels decreasing from baseline to 8 h with the 15 g PRO group ($p < 0.01$). There was an effect of time in serum myostatin ($p < 0.01$), with concentrations at 4 h ($p < 0.05$) and 8 h ($p < 0.01$) being greater than baseline. There was an effect of time in serum IGF-1 ($p < 0.001$), with the concentration at 24 h in the 60 g PRO dose being greater than baseline ($p < 0.05$), 4 h ($p < 0.05$), and 8 h ($p < 0.05$). There were no differences in the concentration of serum growth hormone, testosterone, myostatin, or IGF-1 between the PRO doses at any given time point.

5 | DISCUSSION

To our knowledge, this is the first study to investigate the acute (0–4 h and 0–8 h) and extended (0–24 h) MyoFSR responses to two bouts of whole-body resistance exercise to understand the PRO dosing requirements to maximize post-exercise MPS responses in trained women. The

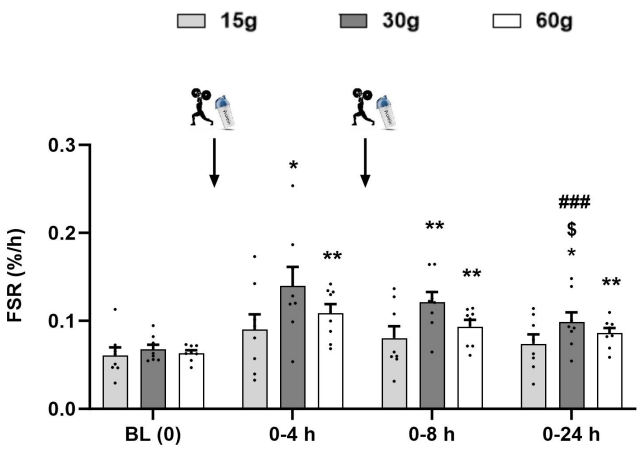

FIGURE 4 Baseline (BL) and cumulative muscle protein fractional synthetic rates (FSR, %/hour) for each PRO dose investigated. Data expressed as mean ± SEM plus individual values; $n = 8$ per group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to BL in same PRO dose group. $^{\$}p < 0.05$ compared to 4 h in the same PRO dose group. $^{\#\#\#}p < 0.001$ compared to 8 h in the same PRO dose group.

ingestion of a 15 g PRO bolus immediately after two bouts of resistance exercise, separated by 4 h, did not alter the MyoFSR response from baseline. Ingestion of 30 g and 60 g PRO immediately following each bout of resistance exercise increased MyoFSR above baseline from 0 to 4 h, 0 to 8 h, and 0 to 24 h. The magnitude of change in MyoFSR was not different between the 30 g and 60 g PRO feeding groups, suggesting that maximal increases in post-exercise MyoFSR was achieved with 30 g whey PRO in trained women performing whole-body resistance exercise.

The dose of high-quality PRO required to elicit a maximal increase in post-exercise MPS is reported to lie between 20 and 40 g.[18–20] This range was derived from studies of healthy young men, where MPS was measured over 0–5 h following a single bout of unilateral resistance exercise, or after one bout of whole-body resistance exercise.[20] In the present study, we quantified both the acute (0–4 h and 0–8 h) and extended (0–24 h) effects of two separate bouts of resistance exercise, alongside PRO feeding, on the cumulative MyoFSR response in trained women. Ingestion of 15 g PRO immediately post-exercise did not increase the rate of MyoFSR above baseline over 0–4 h or 0–8 h, which included two bouts of resistance exercise and PRO feeding. There was no increase above baseline in the rate of MyoFSR measured over 0–24 h (Figure 4), and no increase in plasma BCAA or leucine concentrations above baseline at 4 h, 8 h, and 24 h with this PRO dose. The failure of RE to increase MyoFSR above baseline at this dose may be explained by the large variation in response of MyoFSR between individuals when compared to the 60 g

TABLE 3 Serum hormone levels between each protein dose and over time.

	Timepoint			
	BL	4 h	8 h	24 h
Serum testosterone (ng ml^{-1})				
15 g	0.6 ± 0.08	0.5 ± 0.08	0.4 ± 0.05*	0.6 ± 0.09
30 g	0.5 ± 0.10	0.4 ± 0.06	0.3 ± 0.05	0.4 ± 0.07
60 g	0.5 ± 0.07	0.5 ± 0.11	0.4 ± 0.03	0.5 ± 0.05#
Serum growth hormone (pg mL^{-1})				
15 g	2704 ± 731	419 ± 267	1040 ± 247	1013 ± 353
30 g	1268 ± 818	568 ± 289	759 ± 199	804 ± 331
60 g	1749 ± 619	1046 ± 504	1996 ± 513	1094 ± 342
Serum myostatin (pg mL^{-1})				
15 g	1694 ± 225	1676 ± 203	1555 ± 223	1582 ± 215
30 g	1984 ± 212	1752 ± 153	1701 ± 156	1868 ± 246
60 g	1958 ± 193	1835 ± 203	1720 ± 178	1881 ± 172
Serum IGF-1 (ng mL^{-1})				
15 g	118 ± 13.2	113 ± 14.2	111 ± 14.9	119 ± 16.9
30 g	125 ± 13.6	129 ± 14.3	129 ± 12.4	146 ± 17.5
60 g	119 ± 13.4	120 ± 16.3	125 ± 16.8	143 ± 18.6*,$,#

Note: Values expressed as mean ± SEM: $n = 8$ per group.

*$p < 0.05$ when compared to baseline.

$$p < 0.05$ compared to 4 h.

#$p < 0.05$ compared to 8 h within the same PRO dose.

dose. Our findings contrast to Moore et al.,[31] where both 5 g and 10 g whey PRO ingested after bilateral lower limb resistance exercise in men increased mixed muscle FSR above baseline over 0–4 h (0.5-fold and 1.0-fold, respectively), with 10 g PRO elevating plasma BCAA and leucine over baseline, peaking 1 h post-exercise. It is difficult to ascertain in our study whether 15 g PRO would increase BCAA and leucine immediately post-exercise due to the sampling timepoint at 4 h; however, as no increases were seen in MyoFSR with this PRO dose our results suggest that after whole-body resistance exercise, greater amounts of PRO are required to elicit a stimulus to increase aminoacidemia and MyoFSR above resting values, in trained women. Higher PRO doses of 30 g and 60 g increased MyoFSR above baseline after the first bout of exercise (0–4 h; 1.1-fold and 0.7-fold, respectively), concomitant with increased plasma leucine concentration at both PRO doses and increased plasma BCAA concentration at 60 g PRO (Figure 2). Many studies in men have reported increased MPS post acute resistance exercise with PRO feeding,[8,19,31,32] and our data are consistent with evidence in resistance-trained men where 20 g PRO post acute unilateral lower limb resistance exercise maximally increases MyoFSR after 4 h (1-fold above baseline).[18] There is less evidence in resistance-trained women, although West et al. have shown 25 g whey PRO consumed after a bout of lower body resistance exercise increases MyoFSR above baseline over 1–5 h post-exercise in physically active women.[32] Macnaughton and colleagues[20] detected 19% greater stimulation of MyoFSR following whole-body resistance exercise in young men consuming 40 g of PRO compared to those consuming 20 g. We demonstrate that increasing PRO feeding to 60 g after whole-body resistance exercise in women does not increase MyoFSR over 0–4 h above that seen with 30 g, despite greater plasma BCAA and leucine concentrations; an excess of dietary PRO intake does not cause an increase in MPS,[33] termed the "muscle full" phenomenon. This concept is based on the notion that an upper limit of amino acid delivery must be achieved before muscle cells no longer use amino acids as substrate for MPS and, instead, divert amino acids toward catabolic processes.[18] Similar to our study, Moore et al.[31] and Witard et al.[18] have shown increased AA availability with 40 g PRO ingested after acute lower limb resistance exercise does not lead to increased MPS above that seen with 20 g PRO. Witard et al.[18] also showed increased AA oxidation, urea production rates, and plasma urea concentrations with 40 g PRO ingestion, suggesting the metabolic fate of excess amino acids is predominantly oxidation or excretion. Our study demonstrated that the "muscle full" phenomenon is present in trained women after resistance exercise with 60 g PRO ingestion, and that 30 g is an appropriate PRO dose for maximal MyoFSR post acute resistance exercise for resistance-trained women.

Few studies have examined the effect of within-day multiple bouts of whole-body resistance exercise and PRO feeding on the cumulative MyoFSR response. We showed both 30 g and 60 g PRO after a second bout of resistance exercise increased MyoFSR above baseline over 0–8 h (0.8-fold and 0.5-fold, respectively) and extended the anabolic window over 0–24 h (0.5-fold and 0.4-fold, respectively). These changes were linked with increases in plasma leucine concentrations above baseline at 8 h with the 30 g and 60 g PRO doses and plasma BCAA at 8 h with the 60 g PRO dose (Figure 2). There were no differences in cumulative MyoFSR between the 0–4 h, 0–8 h, and 0–24 h timepoints with either PRO dose, despite higher concentrations of both plasma BCAA and leucine at 8 h compared to 4 h. This observation suggests that an additional bout of resistance exercise with PRO feeding during the day leads to increased aminoacidemia but does not stimulate the MyoFSR response above the initial bout of resistance exercise in trained women, most likely due to the "muscle full" effect. We did show that multiple bouts of resistance exercise and PRO feeding (30 g and 60 g PRO) extends the anabolic window over 24 h in trained women, with cumulative MyoFSR elevated above baseline. It is well known that a single bout of resistance exercise in untrained volunteers increases muscle FSR above baseline up to 48 h post-exercise[16] and in trained men, alongside PRO feeding, muscle FSR is elevated up to 24 h post-exercise.[8,15,34] In addition, in trained individuals it has been shown that the magnitude of increase in MyoPS to resistance exercise and PRO feeding is similar to untrained individuals.[35] However, there are limited studies in women looking at the MPS response longer than 12 h to exercise and feeding. West et al. have shown an intake of 25–30 g PRO post whole-body resistance exercise in trained women does not increase MPS above baseline over 24 h[32], and our data suggest a greater stimulus of two bouts of exercise with PRO feeding, plus total daily PRO intakes greater than 1.8 g/kg BW, may be needed to extend the anabolic window to 24 h in trained women.

While many studies we acknowledge have utilized stable isotope-labeled AA infusions to measure MPS,[18,20] we have adopted the use of an oral bolus of D_2O tracer to measure MyoFSR over 24 h, and it may be argued that any differences seen in the MPS response to exercise and feeding could have been influenced by the measurement technique we employed. However, Wilkinson et al. reported changes in MPS in response to EAA feeding using a D_2O oral tracer and L-[*ring*-$^{13}C_6$] phenylalanine tracer in the same individuals produced comparable findings, and that D_2O can accurately quantify MPS over a period as short as 3 h.[28] Importantly, the use of an oral D_2O tracer negates the use of cannulation and intravenous infusions, limits the number of muscle biopsies required, and allows measurement of MPS in the free-living state and over a longer period (>12 h), which is an advantage over traditional tracer methods.

In the present study, we saw little change in serum testosterone, growth hormone, or myostatin after whole-body resistance exercise and PRO feeding (Table 3). Similarly, West et al.[32] showed little change in serum testosterone levels up to 1 h after acute lower body resistance exercise and 25 g PRO feeding despite increased MPS. We did show increased serum IGF-1 concentration from baseline after 24 h with 60 g PRO postresistance exercise; however, this was not linked with greater increases in MyoFSR over and above that seen with 30 g PRO. Overall, it seems likely that exercise and protein feeding induced changes in circulating testosterone, growth hormone, myostatin, and IGF-1 concentrations play a limited role in the acute regulation of post-exercise MyoFSR in trained women. However, it should be noted that the protocol employed may not have allowed for key hormonal changes to be captured, as many studies have shown hormone levels can return to normal after 60 min[36] and earlier sampling time points would be recommended in future studies.

Leucine rich whey PRO is known to be a superior protein source to maximize post resistance exercise MyoPS; however, the use of non-animal derived dietary protein sources to stimulate MPS is receiving attention, for example, soy PRO[37,38] and vegan sources such as mycoprotein.[39] While some studies suggest these PRO sources do not increase MPS to the same extent as whey PRO,[40] recent work indicates that when PRO sources are matched for AA content then comparable post-exercise protein synthetic responses are seen.[41] Clearly, more work would be needed to identify if the MPS response to PRO feeding and RE in trained women seen in the present study could be replicated with other PRO sources.

In conclusion, 30 g and 60 g PRO dosing regimens increased MyoFSR above baseline after one bout (4 h) and two bouts (8 h) of resistance exercise and over 24 h; however, no changes were seen with 15 g PRO. While both 30 g and 60 g PRO extended the post-exercise anabolic window above baseline, the magnitude of increased MyoFSR with PRO feeding was no greater with the 60 g dose suggesting that maximal MyoFSR post whole-body resistance exercise was achieved with 30 g or greater of whey PRO. Trained women should supplement with 30 g high-quality protein immediately following whole-body resistance exercise to support increased post-exercise MyoFSR.

6 | PERSPECTIVE

Current research evaluating the impact of nutritional strategies on muscle adaptations to resistance exercise are

predominantly performed in men, with that of women being under-represented. Protein provision after resistance exercise is known to enhance muscle PRO synthesis;[12,13] however, the amount of PRO required in trained women is currently unknown, particularly when performing repeated bouts of whole-body resistance exercise. Our dose response study showed that the provision of 30 g PRO, given immediately after two bouts of whole-body resistance exercise separated by 4 h, increased MyoFSR above baseline in resistance-trained women, but 15 g PRO did not. Increasing PRO provision to 60 g post-exercise did not increase the magnitude over the 30 g PRO dose response. Therefore, we recommend trained women who perform resistance exercise should consume 30 g of high-quality PRO immediately after each bout of exercise. The results of this study will improve current PRO feeding guidelines for female athletes to maximize their training adaptations and exercise performance.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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