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Dietary protein intake does not modulate daily myofibrillar protein synthesis rates or loss of muscle mass and function during short-term immobilization in young men: a randomized controlled trial

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ABSTRACT

Background: Short-term (<1 wk) muscle disuse lowers daily myofibrillar protein synthesis (MyoPS) rates resulting in muscle mass loss. The understanding of how daily dietary protein intake influences such muscle deconditioning requires further investigation.

Objectives: To assess the influence of graded dietary protein intakes on daily MyoPS rates and the loss of muscle mass during 3 d of disuse.

Methods: Thirty-three healthy young men (aged 22 ± 1 y; BMI = 23 ± 1 kg/m^2) initially consumed the same standardized diet for 5 d, providing 1.6 g protein/kg body mass/d. Thereafter, participants underwent a 3-d period of unilateral leg immobilization during which they were randomly assigned to 1 of 3 eucaloric diets containing relatively high, low, or no protein (HIGH: 1.6, LOW: 0.5, NO: 0.15 g protein/kg/d; $n = 11$ per group). One day prior to immobilization participants ingested 400 mL deuterated water (D_2O) with 50-mL doses consumed daily thereafter. Prior to and immediately after immobilization upper leg bilateral MRI scans and vastus lateralis muscle biopsies were performed to measure quadriceps muscle volume and daily MyoPS rates, respectively.

Results: Quadriceps muscle volume of the control legs remained unchanged throughout the experiment ($P > 0.05$). Immobilization led to 2.3 ± 0.4%, 2.7 ± 0.2%, and 2.0 ± 0.4% decreases in quadriceps muscle volume ($P < 0.05$) of the immobilized leg in the HIGH, LOW, and NO groups ($P < 0.05$), respectively, with no significant differences between groups ($P > 0.05$). D_2O ingestion resulted in comparable plasma free [^{2}H]-alanine enrichments during immobilization (~2.5 mole percentage excess) across groups ($P > 0.05$). Daily MyoPS rates during immobilization were 30 ± 2% (HIGH), 26 ± 3% (LOW), and 27 ± 2% (NO) lower in the immobilized compared with the control leg, with no significant differences between groups ($P > 0.05$).

Conclusions: Three days of muscle disuse induces considerable declines in muscle mass and daily MyoPS rates. However, daily protein intake does not modulate any of these muscle deconditioning responses. Clinical trial registry number: NCT03797781 *Am J Clin Nutr* 2021;113:548–561.

Keywords: skeletal muscle, atrophy, dietary protein, immobilization, muscle protein synthesis

Introduction

Recovery from illness or injury often requires a period of muscle disuse, which typically occurs in the form of bed rest or limb immobilization. Recent research has focused on short-term periods of muscle disuse (≤1 wk), which are common in clinical settings (1). We and others have shown that merely 2–5 d of disuse already results in substantial loss of muscle mass (2–5), with associated declines in strength (3–5). As a result, there is an eagerness of researchers to develop effective (nutritional) countermeasures (e.g., 3, 6, 7).

Skeletal muscle mass loss must ultimately be underpinned by a chronic imbalance between muscle protein synthesis (MPS) and breakdown (MPB) rates. We have previously shown that

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Data described in the manuscript, code book, and analytic code will be made available upon request pending approval by the corresponding author.

Supplemental Figure 1 is available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: BCAA, branched-chain amino acid; BMR, basal metabolic rate; CSA, cross-sectional area; ddH_2O, doubly distilled water; D_2O, deuterium oxide; EAA, essential amino acid; FSR, fractional synthesis rate; IPAQ, International Physical Activity Questionnaire; MPB, muscle protein breakdown; MPE, mole percentage excess; MPS, muscle protein synthesis; MyoPS, myofibrillar protein synthesis; $\dot{V}O_2$peak, peak maximal oxygen uptake; 1-RM, 1 repetition maximum.

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TABLE 1 Participants' characteristics and habitual diet[1]

	HIGH	LOW	NO
Age, y	22 ± 1	22 ± 1	20 ± 1
Height, m	1.78 ± 0.08	1.74 ± 0.09	1.79 ± 0.09
Body mass, kg	72 ± 1	70 ± 1	72 ± 1
BMI, kg/m^2	23 ± 1	23 ± 1	23 ± 1
Body fat, %	15 ± 1	15 ± 1	17 ± 1
Lean mass, kg	61 ± 1	60 ± 1	60 ± 1
Systolic blood pressure, mmHg	126 ± 1	124 ± 1	124 ± 1
Diastolic blood pressure, mmHg	76 ± 1	71 ± 1	76 ± 1
Mean arterial blood pressure, mmHg	94 ± 1	90 ± 1	93 ± 1
Energy intake			
MJ/d	11.7 ± 0.6	10.7 ± 0.7	11.2 ± 0.8
kcal/d	2800 ± 64	2548 ± 163	2680 ± 202
Protein intake			
g/d	107 ± 6	122 ± 14	127 ± 14
g/kg body mass/d	1.5 ± 0.1	1.7 ± 0.1	1.7 ± 0.1
En%	15 ± 1	19 ± 2	19 ± 1
CHO intake			
g/d	373 ± 28	277 ± 22	281 ± 25
En%	51 ± 4	44 ± 3	43 ± 3
Fat intake			
g/d	116 ± 8	100 ± 11	109 ± 11
En%	39 ± 3	35 ± 2	37 ± 2

[1] Values represent means ± SEM, $n = 11$ per group. Data were statistically analyzed with a 1-factor ANOVA. No statistically significant differences were found between groups for any parameter. Participants consumed a fully controlled energy-balanced diet containing a HIGH ($n = 11$; 1.6 g/kg body mass/d), LOW ($n = 11$; 0.5 g/kg/d), or NO ($n = 11$; 0.15 g/kg/d) dietary protein content. CHO, carbohydrate; En%, percentage of total energy intake.

postabsorptive and postprandial MPS rates decline within a few days of disuse (5). This translates to chronically lower free-living daily myofibrillar protein synthesis (MyoPS) rates during disuse, an effect that manifests within just 2 d, and can explain a large part of muscle atrophy (4). Dietary protein ingestion stimulates MPS rates and inhibits MPB rates which, under normal conditions, allows for postprandial net protein accretion within muscle tissue (8). As a consequence it has been speculated that increasing dietary protein consumption during a period of disuse might alleviate the loss of muscle mass (9, 10). However, we recently showed substantial declines in daily MyoPS rates and muscle disuse atrophy despite participants reporting relatively high habitual dietary protein intakes (1.6 g/kg body mass/d) (11). Although this would theoretically have provided sufficient dietary protein to stimulate MyoPS rates throughout the day (12, 13) and limit muscle atrophy (10, 14), observed rates of muscle loss were in line with the literature (15, 16).

Studies where essential/branched chain amino acid (EAA/BCAA) or protein supplementation has been applied during more prolonged disuse report inconsistent findings concerning loss of muscle mass and function. For example, high-dose EAA/BCAA supplementation during 6–28 d of bed rest or immobilization has been reported to attenuate losses of muscle mass, strength, and/or whole body nitrogen (17–19). However, studies where dietary protein supplementation has been applied during 5–60 d of immobilization or bed rest have typically shown no effect on losses of muscle mass or function (3, 20). For the development of effective nutritional countermeasures, it is important to develop a clear picture of how daily dietary protein intake per se (rather than supplementation) influences muscle protein metabolism and mass during disuse. To date, no studies have manipulated total habitual dietary protein consumption under controlled dietary conditions during (short-term) disuse to establish the link between dietary protein intake, daily MyoPS rates, and muscle atrophy.

In the present work we conducted a dose–response study comparing how high (1.6 g protein/kg body mass/d), low (0.5 g/kg/d), and negligible (0.15 g/kg/d) daily dietary protein intakes influence daily MyoPS rates determined using the deuterated water approach, and muscle mass loss determined via MRI, during a 3-d period of unilateral leg immobilization in healthy males. We hypothesized that declining dietary protein intakes would lead to a greater decline in daily MyoPS rates and a consequent increase in the rate of loss of muscle mass and function.

Methods

Participants and general screening

Thirty-three healthy young men (age = 21 ± 1 y; BMI = 23 ± 1 kg/m^2) were included in the present study (see **Table 1** for participants' characteristics) and participated in this parallel-group, randomized controlled trial. The trial was conducted between November 2018 and December 2019 within the Nutritional Physiology Unit at the Department of Sport and Health Sciences at the University of Exeter, Exeter, United Kingdom (for the CONSORT flow chart see **Supplemental Figure 1**). Participants were allocated sequential numbers at the time of screening that were then used as the only identifiable characteristic for all documents containing participant information. They were then randomly assigned to groups using

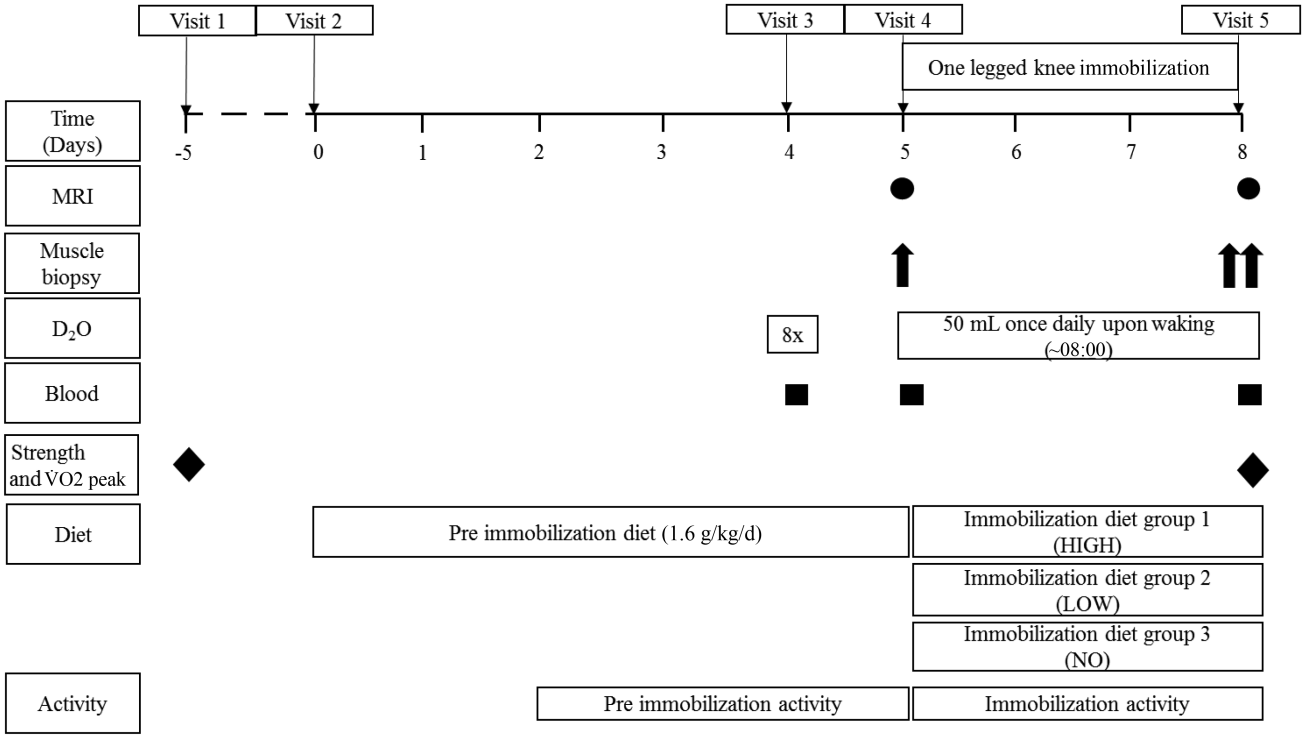

FIGURE 1 Study protocol. Thirty-three healthy young males underwent 3 d of unilateral leg immobilization via knee brace. Activity: physical activity measured continuously by GENEactiv wrist watch (Activinsights) accelerometry. Diet: all participants underwent 5 d of fully controlled preimmobilization diet relatively high in dietary protein (1.6 g/kg body mass/d) before being randomly assigned to 1 of 3 groups of varying protein intake: HIGH (1.6 g/kg/d), LOW (0.5 g/kg/d), and NO (0.15 g/kg/d) for the 3-d immobilization period. Blood: venous blood sample collection. D_2O: deuterated water ingestion. $\dot{V}O_2$peak: measurement of single leg peak oxygen uptake using a ramp cycling test to exhaustion. Arrows represent vastus lateralis muscle biopsies (i.e., taken from the immobilized leg only at preimmobilization and from both control and immobilized legs postimmobilization). Strength: unilateral maximal isometric, concentric, and eccentric contractions of both the quadriceps and hamstring muscles measured by isokinetic dynamometry. Control and immobilized legs completed all strength and aerobic capacity tests separately.

an online randomizer (http://www.randomizer.org). Recruitment and testing were ended when the trial was fully recruited according to the a priori calculation. Participants attended the laboratory for a routine medical screening and completed a general medical questionnaire to assess their eligibility for participation, and to ensure no adverse health conditions were present. Exclusion criteria included: *1*) a (family) history of deep vein thrombosis/cardiovascular disease, metabolic disorders (e.g., type 2 diabetes), or musculoskeletal/orthopedic disorders; *2*) a BMI >28.5 or <18.5; *3*) participation in a structured resistance training program within 6 mo prior to the study; *4*) any musculoskeletal injury of the legs within 12 mo before the study; *5*) use of anticoagulants; *6*) any contraindications to MRI scanning (e.g., metallic implants); and *7*) consumption of any nutritional supplement prior to and during the study. Participants whose habitual dietary protein intake was >1.8 g/kg/d or <0.6 g/kg/d were also excluded from the study. During the screening participants' height, body mass, and blood pressure were measured, and body composition was also assessed by air displacement plethysmography (BODPOD; Life Measurement, Inc). The participants also completed the International Physical Activity Questionnaire (IPAQ). This was used to estimate the participants' physical activity level by reporting time spent sedentary, or undertaking light, moderate, and vigorous activities, and multiplying this by the metabolic equivalents for these activities (21). Participants' habitual diets were recorded for 3 d (2 weekdays and 1 weekend day) prior to the dietary controlled period by a self-reported written diet diary following detailed instructions and advice from a member of the research team. Furthermore, participants visited the laboratory shortly after the screening, where they were familiarized with the exercise tests (described below). All participants were informed of the nature and possible risks of the experimental procedures before providing written informed consent. The study was approved by the Sport and Health Science Ethics Committee of the University of Exeter (170,712/B/01), in accordance with the guidelines set out in the Declaration of Helsinki, and registered as a clinical trial with clinicaltrials.gov (NCT03797781).

Experimental protocol

A graphical representation of the study design is shown in **Figure 1**. Following acceptance into the study participants attended the laboratory in the fasted state for 5 experimental visits across 13 d. This included an 8-d fully controlled dietary intervention period, with the final 3 d of the dietary controlled period involving unilateral leg immobilization using a leg brace and ambulation with crutches. Preimmobilization (visit 1), comprehensive unilateral muscle strength and maximal aerobic capacity (peak maximal oxygen uptake; $\dot{V}O_2$peak) testing were

conducted (protocols described below). Thereafter, 5 d prior to immobilization (visit 2), all participants attended the laboratory to collect their first 5 d of food to commence a fully controlled preimmobilization standardized diet with protein intake fixed at 1.6 g/kg body mass/d. To measure daily MyoPS rates throughout the immobilization period participants underwent a deuterium oxide (D_2O) dosing protocol (described below) beginning on visit 3 (i.e., 1 d prior to commencing immobilization). This protocol was designed to achieve and maintain 0.8–1.0% body water deuterium enrichment during the measurement periods in line with our previous work (11, 22). The following day (visit 4) participants arrived at the laboratory at ~08:00 and a single vastus lateralis muscle biopsy was obtained from the (to be) immobilized leg. Following this, participants were transported to an MRI scanner by wheelchair, avoiding any weight-bearing activity and underwent a preimmobilization MRI scan of both thigh muscles. Thereafter, participants were randomly allocated, in a single (participant) blind manner, into 1 of 3 isoenergetic dietary controlled experimental groups ($n = 11$ per group) where protein intakes differed between groups: 1.6 (HIGH), 0.5 (LOW), or 0.15 (NO) g protein/kg body mass/d. Participants were fitted with a leg brace to induce immobilization of 1 leg and were given crutches for ambulation, and provided with 3 d of food supply in line with their allotted diet; this signified the commencement of the 3-d immobilization period. Following the immobilization period participants returned to the laboratory for the final visit (visit 5) where vastus lateralis muscle biopsies were collected from both the immobilized and control legs (the brace was only removed for the biopsy, MRI, and exercise testing procedures). Muscle biopsies were all obtained under local anesthesia, using the percutaneous Bergstrom needle biopsy technique (23) from the vastus lateralis muscle ~15 cm above the patella and ~2 cm below the fascia. Immediately following muscle biopsies, the tissue was quickly assessed and any blood or nonmuscle tissue was dissected and discarded. The muscle samples were immediately frozen in liquid nitrogen within 1 min and stored at −80°C until further analysis. Thereafter, participants were transported via wheelchair to undergo further MRI scans of the thighs of both legs, and finally further unilateral 1 repetition maximum (1-RM) strength and $\dot{V}O_2$peak testing of both legs separately was performed. This signified the end of the experiment, when weight-bearing activity of both legs was then permitted.

Physical activity and habitual dietary intake

For 3 d prior to immobilization (days 2–5 of the preimmobilization diet) and for the entirety of the immobilization period participants' physical activity was measured using an accelerometer (GENEactiv; Activinsights) worn on the nondominant wrist. Participants were instructed to wear the accelerometer continuously with data being collected at a 60-Hz sampling frequency. Participants were instructed to refrain from vigorous physical activity during immobilization but to attempt to maintain their habitual activity levels despite using crutches for ambulation (to avoid whole body sedentariness during immobilization). Physical activity data from the GENEActiv accelerometers were converted into 60-s epochs and used to estimate time spent performing light, moderate, and vigorous physical activity using standard cut-off points (11). Participants were asked to refrain from alcohol intake for 1 wk before and throughout the 8-d dietary control period. Dietary analyses for the calculation of habitual energy and macronutrient intakes were completed using specialized nutrition software (Nutritics Professional Nutritional Analysis Software).

MRI for determination of quadriceps muscle volume

Prior to and following immobilization, the volume of the quadriceps muscle of both legs was determined via MRI. We have previously described the MRI methodology for the determination of quadriceps muscle volume in detail (11). In brief, a 1.5-T MRI scanner was used to make axial plane images over the full length of the femur. A T1-weighted 3D turbo spin echo sequence was used (field of view 500 × 500 mm, reconstructed matrix 512 × 512 mm, echo time 15 ms, repetition time 645 ms, slice thickness 5 mm, slice gap 5 mm) with the subject lying still in the supine position, and a 4-element sense body radiofrequency coil was wrapped around both thighs. On average ~45 images were acquired along the length of the femur, with the bottom 25% (from the lateral femoral condyle working proximally) and top 25% (from the greater trochanter working distally) excluded (7, 18). All other images in the axial plane in the middle 50% area of the quadriceps muscle were analyzed via manual segmentation using Philips online MRI software. The same experimenter (SPK) performed all manual segmentation of the images. We (11) and others (24) have shown that this region of the quadriceps muscle undergoes rapid atrophy during disuse and accounts for the vast majority of total quadriceps muscle volume loss. Quadriceps muscle volume was calculated using a previously published method (25) where the total cross-sectional area (CSA) for all images was calculated and multiplied by the slice gap plus the distance between slices (linear interpolation) (in this case a total of 2 cm, comprising a 5-mm slice thickness and a 15-mm slice gap), summarized by the following equation:

$$muscle\ volume = \sum_{aCSA} \cdot (slice\ thickness + slice\ gap) \quad (1)$$

Deuterated water protocol

The deuterated water dosing protocol was based on our previous work (4, 22). Day 1 of the experimental protocol acted as a D_2O loading day where participants consumed 400 mL 70% D_2O separated over the day as 8 × 50-mL boluses (CK Isotopes Ltd). Upon arrival at the laboratory (07:30) background blood and saliva samples were collected before the first bolus of D_2O was ingested. The first dose of D_2O was consumed at ~08:00, with the remaining doses being consumed every 1.5 h thereafter. Participants stayed at the university until 4 of the 8 loading-day D_2O doses had been consumed, with the remaining D_2O doses being consumed at home under instruction of timings (i.e., leaving 1.5 h between each). Every day following the loading day participants consumed a maintenance dose of D_2O (50 mL) upon waking (~08:00). Blood samples were collected during the test days [i.e., day 5 (pre), day 7 (after 2 d of immobilization), and day 12 (post)]. Venous blood samples were collected from the antecubital vein via venepuncture and collected into EDTA-containing Vacutainers (BD), which were centrifuged at

TABLE 2 Dietary intake and physical activity levels of HIGH, LOW, and NO groups during dietary controlled periods preimmobilization and during a 3-d period of unilateral knee immobilization[1]

	HIGH		LOW		NO	
	Pre	During	Pre	During	Pre	During
Energy intake						
MJ/d	11.6 ± 0.3	11.7 ± 0.3	11.5 ± 0.4	11.5 ± 0.5	11.7 ± 0.3	11.8 ± 0.3
kcal·d^{-1}	(2777 ± 64)	(2788 ± 62)	(2741 ± 104)	(2747 ± 114)	(2791 ± 74)	(2801 ± 80)
Protein intake						
g/d	116 ± 4	116 ± 4	113 ± 7	36 ± 2*,a	116 ± 4	10 ± 0.4*,a,b
g/kg body mass/d	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	0.51 ± 0.1*,a	1.6 ± 0.1	0.14 ± 0.1*,a,b
En%	18 ± 1	18 ± 1	16 ± 0.4	5 ± 0.1*,a	17 ± 0.2	1.4 ± 0.03*,a,b
Protein per meal, g	28 ± 1	28 ± 1	27 ± 2	10 ± 1*,a	28 ± 1	3 ± 0.1*,a,b
CHO intake						
g/d	362 ± 12	368 ± 13	341 ± 11	426 ± 19*,a	361 ± 11	525 ± 13*,a,b
En%	52 ± 1	53 ± 1	50 ± 1	62 ± 1*,a	52 ± 1	71 ± 2*,a,b
Fat intake						
g/d	88 ± 4	85 ± 4	98 ± 4	91 ± 5	90 ± 4	78 ± 4
En%	29 ± 1	28 ± 2	32 ± 1	30 ± 1	29 ± 1	23 ± 1*,a,b
Physical activity						
Light, h/d	1.0 ± 0.1	0.7 ± 0.1*	1.2 ± 0.1	0.8 ± 0.1*	1.3 ± 0.1	0.8 ± 0.1*
Moderate, h/d	2.1 ± 0.2	1.6 ± 0.2*	2.2 ± 0.3	1.7 ± 0.2	3.0 ± 0.5	1.5 ± 0.2*
Vigorous, h/d	0.3 ± 0.1	0.1 ± 0.1*	0.3 ± 0.1	0.1 ± 0.02*	0.3 ± 0.1	0.1 ± 0.1*
Total, h/d	3.4 ± 0.3	2.3 ± 0.2*	3.7 ± 0.4	2.6 ± 0.2*	4.6 ± 0.6	2.4 ± 0.4*

[1] Values represent means ± SEM, $n = 11$ per group. Data were analyzed by using a 2-factor repeated measures ANOVA (with time and group as factors). *Significantly different from preimmobilization value ($P < 0.001$); [a]significantly different from HIGH group during immobilization ($P < 0.05$); [b]significantly different from LOW group during immobilization ($P < 0.001$). "Pre" denotes the 5-d period of controlled diet before immobilization; "During" denotes the 3-d immobilization period. CHO, carbohydrate; En%, percentage of total energy intake.

2500 *g* for 10 min at 4°C. Aliquots of plasma were frozen in liquid nitrogen and stored at −80°C until further analysis took place. To ensure uniformity and compliance with the D_2O protocol participants were provided with a log to record the times they consumed the D_2O and were provided with enough doses to last until their next study visit, at which point containers were returned, counted, and subsequent doses were provided.

Muscle strength and single leg cycling $\dot{V}O_2$peak testing

Unilateral knee extension and flexion contractions were performed using isokinetic dynamometry (Biodex System 3). Isometric, isokinetic concentric, and isokinetic eccentric strength for both knee extension (i.e., quadriceps muscle strength) and flexion (i.e., hamstring muscles' strength) were all determined in the stated order. After warm-up repetitions at 50%, 75%, and 85% of self-determined 1-RM, participants performed 3 × 3-s maximal isometric repetitions of knee extension followed by knee flexion. Knee angle was fixed at 60° of flexion (0° being full extension) and repetitions were separated by a 2-min rest, and the 2 exercise modalities by a 5-min rest. Subsequently participants performed 5 repetitions of maximal knee extension isokinetic concentric exercises, and this was repeated for knee extension isokinetic eccentric exercises. Repetitions were sequential with a 2-min break between the 2 contraction types; contraction speed was 60°/s over the central 80° range of motion (verified by goniometry) of each participant's full range of motion (e.g., from full extension to full flexion). Then, following a 5-min break, the same isokinetic concentric and eccentric contractions were repeated for knee flexion.

Unilateral leg $\dot{V}O_2$peak was assessed using a previously validated single-leg ramp exercise test to exhaustion (26, 27). In brief, a custom-designed counterweight pedal (11.4 kg) was fitted to the crank of an electronically braked cycle ergometer (Lode Corival). Participants cycled with 1 leg, with the nonexercising leg resting on a stationary stool. The counterweight assisted with the upstroke of the cycling phase and eliminated the need to pull up on the pedal. Whole-body expired gases were collected via a facemask and oxygen consumption was measured using an online gas analyzer (Cortex Metalyzer 3B gas analyzer). For all exercises the (to be) immobilized leg was always performed first followed by the control leg.

Dietary control

Nutritional information for the preimmobilization (5 d immediately before immobilization) and immobilization (3-d immobilization period) diets is provided in **Table 2**. Basal metabolic rate (BMR) was estimated using the Henry equations based on age, gender, and weight (28). Individual energy requirements were then calculated by multiplying the participants' BMR and physical activity level (calculated from the IPAQ as described above). Thereafter, an individual 8-d meal plan was designed for each participant with all food prepared, weighed, and packaged in house in the Nutritional Physiology Unit's research kitchen facility. Throughout the study all ingredients and instructions/information for meal preparation were provided to the participants, who prepared the meals at home, and a log was provided to record the times of consumption of each meal. For the first 5 d of the 8-d dietary control (i.e., preimmobilization period) all participants

consumed a diet containing 1.6 g protein/kg body mass/d, with ~30% of their energy being provided by fat and the remainder from carbohydrates (~50–55%; variation due to different energy requirements in parallel with clamped protein intake). Alcohol consumption and any food or drinks (except water, but including tea and coffee) other than that provided were prohibited during the study. Dietary protein intake was equally distributed across 4 meals (~27 ± 1 g, 28 ± 1 g, 28 ± 1 g, and 28 ± 1 g at breakfast, lunch, dinner, and a presleep whey protein beverage, respectively) and participants were instructed to consume their meals ~4–5 h apart, throughout the day to optimize 24-h muscle protein synthesis rates (13, 29, 30). At each experimental visit participants' body mass was measured (seca 703 column scale; seca GmbH & Co KG) wearing light clothing, and the researchers discussed with the participants any questions or issues that might have arisen with the diet. In the event of any substantial weight change (>0.5 kg, with the same upward or downward trend on 2 consecutive visits) energy content of the next 2 d was adjusted (via the reduction/increase in carbohydrate). Following the 5-d preimmobilization period, volunteers commenced with the 3-d immobilization period during which they were randomly assigned to either the HIGH (1.6 g/kg/d; $n = 11$), LOW (0.5 g/kg/d; $n = 11$), or NO (0.15 g/kg/d; $n = 11$) protein groups. The HIGH group therefore maintained the preimmobilization diet precisely, whereas the LOW group had ~68% (~77 g) of their protein [and ~7% (~7 g) of fat] replaced by ~25% (~85 g) more carbohydrate, and the NO protein group had ~91% (~106 g) of their protein [and ~13% (~12 g) of fat] replaced by ~45% (~164 g) of carbohydrate. The amounts of dietary protein were selected to represent a wide spectrum to allow a true dose–response to be investigated. The protein intake level of 1.6 g/kg/d was selected as "high" based on being double the UK RDA (31), in line with current recommendations for restricting muscle loss during disuse (10, 14, 32) and also consistent with habitual protein intakes reported in our previous work investigating daily MPS rates and muscle disuse atrophy in young healthy men (4). The protein intake level of the LOW group was selected as 0.5 g/kg/d because this is considerably below (38%) the current RDA and also representative of dietary protein intakes that might be expected in patients undergoing a period of disuse in a hospital setting (33). The NO group was designed to remove dietary protein as a stimulus for MPS rates as far as possible while being practically achievable during a diet maintaining energy balance (i.e., 0.15 g protein/kg/d). All food items in the 1.6 and 0.5 g/kg/d protein groups were purchased from commercial retailers. To reduce protein intake to 0.15 g/kg/d, certain food products given to this group were purchased from a company that produces specialized low/zero-protein food (Promin Metabolics). Example meals on each diet consisted of the following: breakfast: scrambled eggs, and beans on toast (HIGH); jam on toast (LOW); low-protein oatmeal (NO); lunches: a chicken sandwich with snacks (e.g., biscuits, fruit) (HIGH); ham sandwich with fruit (LOW); and vegetable soup with low-protein bread rolls (NO); dinner: chicken tikka masala curry with rice and vegetables (onion, green beans, tomatoes) (HIGH); vegetarian stir fry (vegetables, stir fry sauce, and rice noodles) (LOW); vegetarian pizza [low-protein pizza base, vegetables (sweetcorn, mushrooms, tomatoes, onion, pepper), tomato puree) (NO).

Immobilization protocol

We have previously used the knee brace approach to achieve unilateral leg immobilization and consequent declines in daily MPS rates and muscle mass over 2 and 7 d (11). Briefly, the brace (X-ACT Donjoy brace; DJO global) was applied and the participant could then ambulate on crutches (after receiving instructions) throughout the immobilization period. The immobilized leg was randomly selected and counterbalanced for leg dominance, with the nonimmobilized leg acting as a within-subject control (for both MPS rates and muscle mass measurements). Using the hinge of the brace the knee was fixed at an angle of 40° flexion (full knee extension = 0°) to ensure no weight-bearing occurred. Participants were instructed that all ground contact, and muscle contraction (except for ankle rotation exercises twice daily to activate the venous muscle pump), in the immobilized leg were forbidden. Adhesive tape with the experimenter's signature inscribed was placed around the straps of the brace. Breaking of the tape would indicate that the brace had been altered and result in exclusion from the study (11, 34), although it was not necessary to exclude any participants based on this in the present study. A plastic shower cover was provided to the participants to wear over the brace when showering. Daily contact was maintained with the subject throughout the study to ensure proper compliance.

Plasma free [^{2}H]-alanine enrichments

Plasma amino acid enrichments were determined by GC-MS analysis (Agilent 5975C MSD and 7890A GC). First the plasma samples were deproteinized using dry 5-sulfosalicylic acid. Subsequently free amino acids were purified using cation exchange chromatography (AG 50W-X8 resin; mesh size 100–200 μm; ionic form: hydrogen; Bio-Rad Laboratories). The purified amino acids were converted to their *tert*-butyldimethylsilyl derivatives with MTBSTFA (N-tert-Butyldimethylsilyl-N-methyltrifluoroacetamide) before analysis via GC-MS. The plasma free alanine mass isotopomers (M and M + 1) were measured using selective ion monitoring at *m/z* 232 and 233. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation.

Myofibrillar bound [^{2}H]-alanine enrichments

Myofibrillar protein–enriched fraction was extracted from ~50 mg of wet weight muscle tissue by hand homogenization on ice using a pestle in a standard extraction buffer [670 μL 1M sucrose, 500 μL 1M Tris/HCl, 500 μL 1M KCl, 100 μL 1M EDTA, and doubly distilled water (ddH_2O) was added until a total volume of 10 mL was achieved (10 μL/mg)]. The samples were centrifuged at 2500 *g* and 4°C for 5 min and the pellet was then washed with 500 μL of ddH_2O and again centrifuged at 2500 *g* and 4°C for 10 min. The myofibrillar protein was solubilized by adding 1 mL 0.3M NaOH and heating for 30 min at 50°C with samples being vortexed every 10 min. Samples were then centrifuged for 10 min at 9500 *g* and 4°C, the supernatant containing the myofibrillar protein was kept, and the collagen protein pellet was discarded. The

myofibrillar proteins were precipitated by the addition of 1 mL 1M perchloric acid and centrifuged at 700 *g* and 4°C for 10 min. Myofibrillar proteins were then washed with 70% ethanol twice and hydrolyzed overnight in 2 mL 6M HCL at 110°C. The free amino acids from the hydrolyzed myofibrillar protein pellet were dried under a nitrogen stream while being heated at 120°C. The free amino acids were subsequently dissolved in 25% acetic acid solution and passed over cation-exchange AG 50W-X8 resin columns (mesh size: 100–200 μm; ionic form: hydrogen; Bio-Rad Laboratories) and eluted with 2M NH_4OH. Following this the eluted amino acids were dried and the purified amino acids were derivatized to their N(O,S)-ethoxycarbonyl ethyl esters (35). The derivatized amino acids were measured using a GC-isotope ratio mass spectrometer (MAT 253; Thermo Fisher Scientific) equipped with a pyrolysis oven and a 60-m DB-17MS column (no. 122–4762; Agilent) and a 5-m precolumn. Ion masses 2 and 3 were analyzed to determine the $^2H/^1H$ ratios of muscle protein–bound alanine. A series of known standards was used to assess the linearity of the MS and to control for the loss of tracer.

Calculations

Myofibrillar protein fractional synthesis rates (FSRs) were calculated based on the incorporation of [^{2}H]-alanine into myofibrillar protein and the mean free plasma [^{2}H]-alanine enrichment throughout the immobilization period as a precursor. FSR was calculated using the standard precursor-product method expressed as daily rates as follows:

$$FSR\ \left(\% \cdot \text{day}^{-1}\right) = \left(\frac{E_{m2} - E_{m1}}{E_{precursor} \times t}\right) \times 100 \qquad (2)$$

where E_{m1} and E_{m2} are the myofibrillar muscle protein–bound enrichments on days 0 and 3; $E_{precursor}$ represents mean plasma free [^{2}H]-alanine enrichment (mean enrichment between days 0 and 3); and t represents the time between biopsies (days 0 to 3). FSRs were calculated in both legs separately using the biopsy collected from the immobilized leg as baseline for both legs.

Statistics

All data are presented as means ± SEM, and all statistical analyses were conducted in GraphPad Prism version 7.0 (GraphPad Software). The study sample size was based on a previously reported 1-wk muscle disuse dietary controlled intervention in healthy young males (36). A sample size of 33 (11 per group) was anticipated to detect a 0.3% difference between HIGH, LOW, and NO protein intakes groups on quadriceps muscle mass (SD = 0.3, 80% power, $\alpha = 0.05$). A 2-factor repeated measures ANOVA, with leg (control compared with immobilized; treated as the repeated factor) and group (HIGH, LOW, and NO) as factors, was used to compare MyoPS rates. Three-factor repeated measures ANOVAs [time (pre compared with post), leg (control compared with immobilized), and group (HIGH, LOW, and NO) as factors, with time and leg considered as repeated factors] were used to compare quadriceps muscle volume and isometric, concentric, and eccentric leg strength and unilateral leg $\dot{V}O_2$peak data. A 2-factor repeated measures ANOVA, with group (HIGH, LOW, and NO) and time (habitual compared with pre compared with during immobilization) as factors, was used to assess for differences in dietary intake parameters. Two-factor repeated measures (time) ANOVAs were used to assess how physical activity and plasma [^{2}H]-alanine enrichments differed between groups from pre to during immobilization. For all ANOVAs, data were checked and no ANOVA model assumptions were violated; when a significant interaction was found Bonferroni post hoc tests were applied to locate individual differences. Statistical significance was set at $P < 0.05$.

Results

Physical activity and diet

Table 1 displays participants' characteristics and habitual dietary intakes, and Table 2 depicts dietary intake parameters and physical activity during the dietary controlled period for 5 d preceding (pre) and during the 3-d immobilization period. There were no significant differences in habitual energy or macronutrient intake or habitual physical activity levels between the groups ($P > 0.05$). Light, vigorous, and total physical activity significantly reduced from pre to during immobilization (time effects; all $P < 0.05$) and to a similar extent (time × group interaction effects; all $P > 0.05$) across groups. Moderate physical activity significantly declined in the HIGH and NO protein groups only ($P < 0.05$). As expected, all dietary parameters were identical across groups during the preimmobilization dietary controlled period. By design, energy intake was equivalent across groups during immobilization, but dietary protein intake differed (group, time, and group × time effects; all $P < 0.001$) such that HIGH was greater than LOW and NO (both $P < 0.001$) and LOW was greater than NO ($P < 0.001$). This resulted in differences in carbohydrate intake across groups (group, time, and group x time effects; all $P < 0.001$) where HIGH was lower than NO ($P < 0.001$) and LOW ($P < 0.05$), with the NO group also having higher carbohydrate intake than LOW ($P < 0.001$). There were no significant differences between groups for fat intake during immobilization (group and the group × time effects, both $P > 0.05$; time effect, $P < 0.001$). [Fat intake (percentage of total energy intake) was significantly reduced from pre to during immobilization in the NO group ($P < 0.05$)].

Quadriceps muscle volume

Quadriceps muscle volumes calculated from MRI are displayed in **Figure 2**. There were no significant differences in quadriceps muscle volume between legs or between groups preimmobilization (group × leg interaction effect, $P > 0.05$) (control leg: HIGH = 1412 ± 85 cm^3, LOW = 1448 ± 106 cm^3, NO = 1495 ± 71 cm^3; immobilized leg: HIGH = 1430 ± 82 cm^3, LOW = 1439 ± 105 cm^3, NO = 1499 ± 83 cm^3). The quadriceps muscle volume of the control leg was unaffected by immobilization ($P > 0.05$). Quadriceps volume of the immobilized leg reduced significantly during immobilization (leg × time; $P < 0.001$) by 2.3 ± 0.4%, 2.7 ± 0.2%, and 2.0 ± 0.4% in the HIGH (pre- = 1430 ± 82 to postimmobilization = 1396 ± 81 cm^3; $P < 0.001$), LOW (pre- = 1439 ± 105 to postimmobilization = 1400 ± 101 cm^3; $P < 0.001$), and NO (pre- = 1499 ± 83 to postimmobilization = 1469 ± 81 cm^3; $P < 0.05$) groups, respectively (Figure 2B); however, these changes did not differ across groups (Figure 2B) (group × time and group × leg × time interactions; $P > 0.05$).

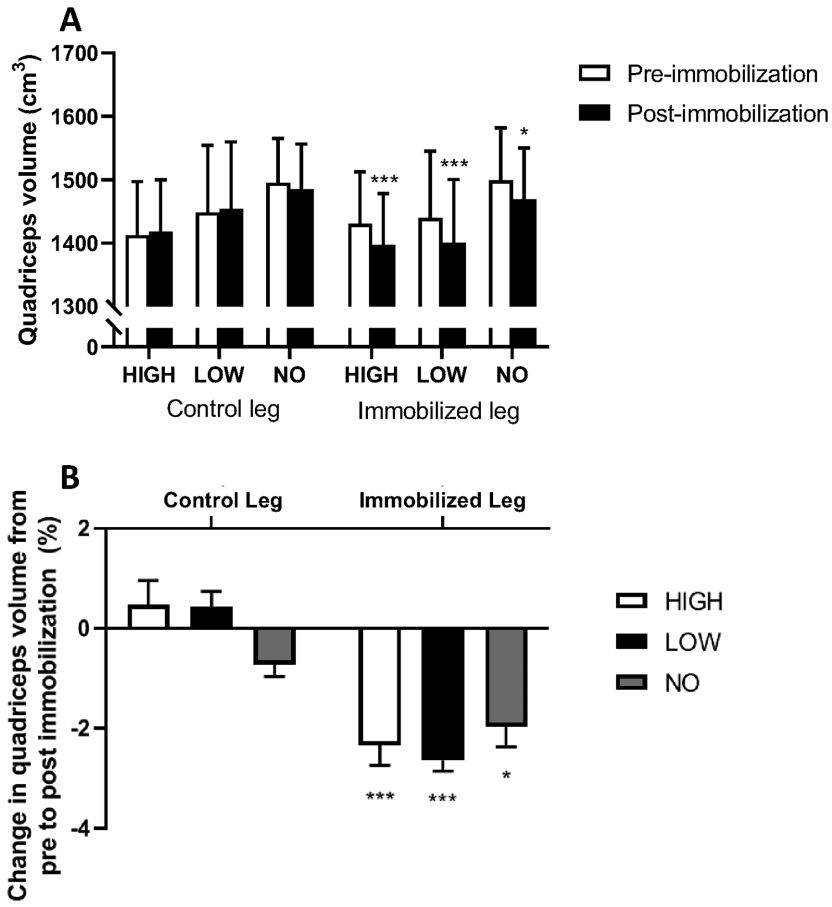

FIGURE 2 (A) Quadriceps muscle volume of the control and immobilized legs pre and post 3 d of unilateral leg immobilization where participants consumed a fully controlled energy-balanced diet containing a HIGH ($n = 11$; 1.6 g/kg body mass/d), LOW ($n = 11$; 0.5 g/kg/d), or NO ($n = 11$; 0.15 g/kg/d) dietary protein content. A 3-factor repeated measures ANOVA [leg (control compared with immobilized) × time (pre compared with post) × group (HIGH, LOW, and NO) as factors, with time and leg considered as repeated factors] was conducted to assess for statistical differences. Main effect of leg $P > 0.05$, group $P > 0.05$, and time $P < 0.001$. Leg × group interaction $P > 0.05$; leg × time interaction $P < 0.001$; group × time interaction $P > 0.05$; leg × group × time interaction $P > 0.05$. Bonferroni post tests were conducted to locate individual differences; *,***significantly different from preimmobilization within the same group: $^{*}P < 0.05$, $^{***}P < 0.001$. (B) Relative difference in quadriceps muscle volume between the control and immobilized legs for HIGH, LOW, and NO groups. Data were analyzed by a 2-factor repeated measures ANOVA with leg (control compared with immobilized; treated as the repeated factor) and group (HIGH compared with LOW compared with NO) as factors. Main effect of leg $P < 0.001$, group $P > 0.05$, leg × group interaction $P < 0.05$. Bonferroni post tests were conducted to locate individual differences; *,*** significantly different from preimmobilization within the same group: $^{*}P < 0.05$, $^{***}P < 0.001$. Data presented are means ± SEM.

Muscle strength and single leg cycling $\dot{V}O_2$peak

Quadriceps and hamstring muscles' strength data are displayed in **Figures 3** and **4**, respectively. There were no significant differences in any contraction type for the quadriceps or hamstring muscles' strength between legs or between groups preimmobilization (group × leg interaction effect, $P > 0.05$). No parameter of strength was altered throughout the experiment in the control leg for either the quadriceps or hamstring muscles ($P > 0.05$) and there were no significant differences between groups ($P > 0.05$). Immobilization decreased quadriceps maximal isometric (HIGH = by 24 ± 8%, LOW = by 24 ± 6%, NO = by 26 ± 5%; time × leg effect; $P < 0.001$), concentric (HIGH = by 23 ± 8%, LOW = by 22 ± 6%, NO = by 25 ± 4%; time × leg effect; $P < 0.001$), and eccentric (HIGH = by 16 ± 7%, LOW = by 18 ± 8%, NO = by 23 ± 6%; time × leg effect; $P < 0.001$) strength to a similar extent across groups (group × leg interaction; $P > 0.05$ for all 3 contraction types). In contrast, immobilization only reduced hamstrings' maximal concentric strength (HIGH = by 7 ± 2%, LOW = by 8 ± 6%, NO = by 5 ± 5%; time × leg; $P < 0.05$), again with no significant differences between groups (group × leg interaction; $P > 0.05$), whereas hamstring muscles' maximal isometric and eccentric strength were unaffected by immobilization (all main and interaction effects; $P > 0.05$, except hamstring isometric time effect; $P < 0.01$). Unilateral $\dot{V}O_2$peak (**Figure 5**) was not affected by immobilization ($P > 0.05$) or group ($P > 0.05$) in either leg (interactions all $P > 0.05$).

Plasma free [^{2}H]-alanine precursor pool enrichment

Plasma free [^{2}H]-alanine enrichments (**Figure 6**) were 2.4 ± 0.1, 2.4 ± 0.1, and 2.3 ± 0.1 mole percentage excess (MPE) at the start of immobilization in the HIGH, LOW, and NO groups, respectively, and tended (time effect; $P = 0.06$) to

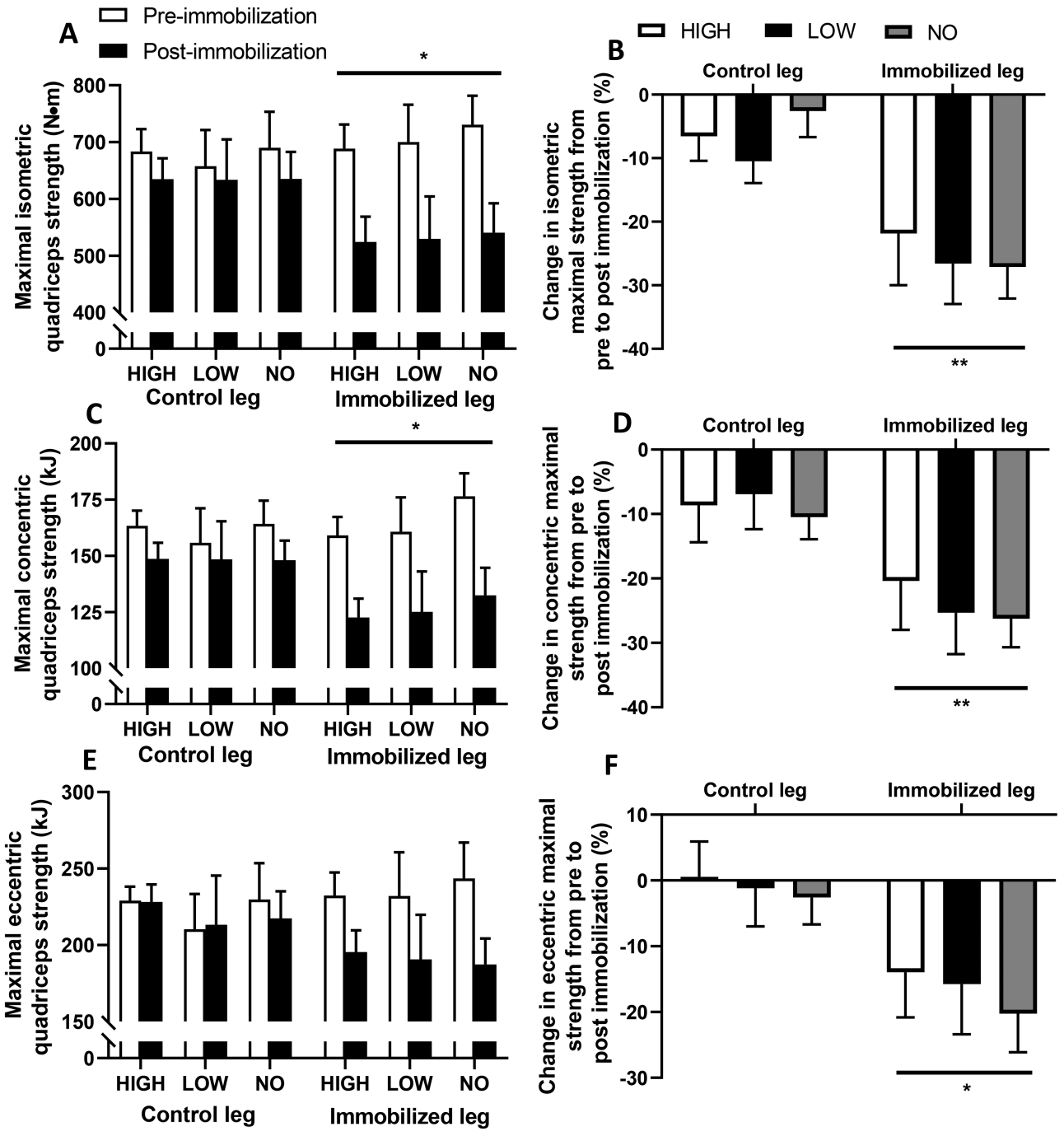

FIGURE 3 Quadriceps muscle strength assessed by isokinetic dynamometry. Maximal isometric (3-repetition average) (A), isokinetic concentric (C), and isokinetic eccentric (E) (both 5-repetition average) quadriceps muscle strength for the control and immobilized legs pre- and postimmobilization for the 3 groups HIGH ($n = 11$; 1.6 g protein/kg body mass/d), LOW ($n = 11$; 0.5 g/kg/d), and NO ($n = 11$; 0.15 g/kg/d). Data were analyzed by 3-factor repeated measures ANOVA [with leg (control compared with immobilized) × time (pre compared with post) × group (HIGH, LOW, and NO) as factors, with time and leg considered as repeated factors]. A, C, and E all show no main effect of group ($P > 0.05$), but for isometric and concentric quadriceps strength a main effect of leg was detected ($P < 0.05$), and for eccentric the main effect of leg was $P > 0.05$. For all 3 exercises (A, C, and E) the main effect of time was $P < 0.001$. The group × leg and group × time interactions were $P > 0.05$ for A, C, and E, and the leg × time interactions were $P < 0.001$ for A, C, and E. The group × leg × time interactions were $P > 0.05$ for A, C, and E. (B, D, and F) Relative change in maximal isometric, concentric, and eccentric quadriceps muscle strength, respectively, for the control and immobilized leg and for the HIGH, LOW, and NO groups. Data were analyzed by 2-factor repeated measures ANOVA with leg (control compared with immobilized; treated as the repeated factor) and group (HIGH, LOW, and NO) as factors. The main effect of group was $P > 0.05$ for B, D, and F; the main effect of leg was $P < 0.05$ for isometric (B) and eccentric (E) exercises, and $P < 0.01$ for concentric (D). *,**Denotes the main effect of leg: $^{*}P < 0.05$, $^{**}P < 0.01$. The group × leg interaction effects were $P > 0.05$ for all 3 exercises (B, D, F). Data are means ± SEM.

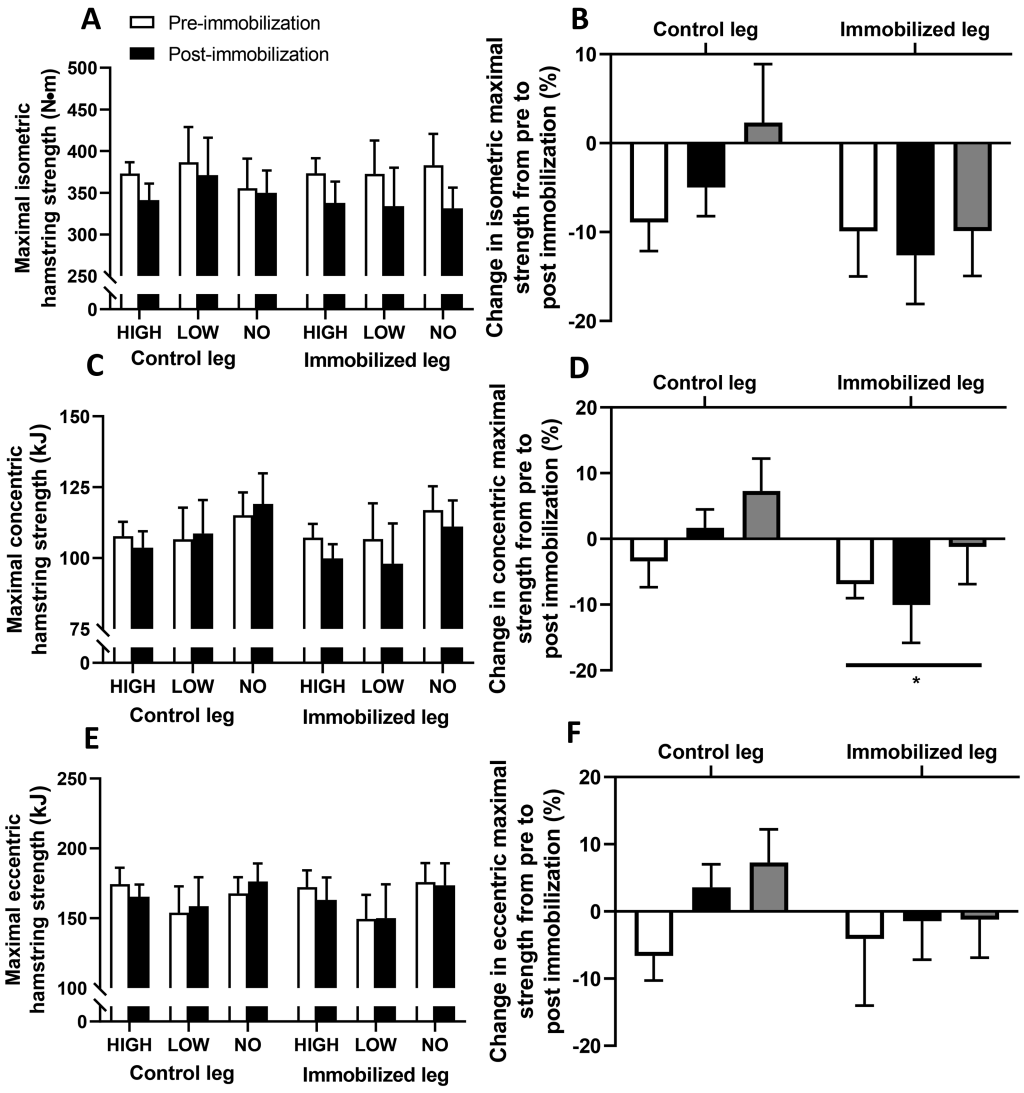

FIGURE 4 Hamstring muscles' strength assessed by isokinetic dynamometry. Maximal isometric (3-repetition average) (A), isokinetic concentric (C), and isokinetic eccentric (E) (both 5-repetition average) hamstring strength, for the control and immobilized legs pre- and postimmobilization for 3 groups: HIGH ($n = 11$; 1.6 g protein/kg body mass/d), LOW ($n = 11$; 0.5 g/kg/d), and NO ($n = 11$; 0.15 g/kg/d). Data were analyzed by 3-factor repeated measures ANOVA [with leg (control compared with immobilized) × time (pre compared with post) × group (HIGH, LOW, and NO) as factors, with time and leg considered as repeated factors]. A, C, and E all showed no main effect of leg ($P > 0.05$); main effect of time was $P > 0.05$ for concentric (C) and eccentric (E), but $P < 0.01$ for isometric (A). Group × leg and group × time interactions were $P > 0.05$ for A, C, and E; the leg × time interactions were $P > 0.05$ for isometric (A) and eccentric (E), but $P < 0.05$ for concentric (C). The group × leg × time interactions were $P > 0.05$ for A, C, and E. (B, D, and F) Relative change in maximal isometric, concentric, and eccentric hamstring muscles' strength, respectively, for the control and immobilized leg and for the HIGH, LOW, and NO groups. Data were analyzed by 2-factor repeated measures ANOVA with leg (control compared with immobilized; treated as the repeated factor) and group (HIGH, LOW, and NO) as factors. The main effect of group was $P > 0.05$ for B, D, and F; the main effect of leg was $P < 0.05$ for concentric (C) and $P > 0.05$ for eccentric (E) and concentric (D) exercises. *Denotes the main effect of leg: $^*P < 0.05$. The group × leg interaction effects were $P > 0.05$ for all 3 exercises (B, D, and F). Data are means ± SEM.

increase postimmobilization. However, there were no significant differences in plasma free [^{2}H]-alanine enrichments between groups ($P > 0.05$).

Daily MyoPS rates

Myofibrillar protein–bound [^{2}H]-alanine enrichments increased pre- to postimmobilization (main effect of time; $P < 0.001$), and showed differences between legs (main effect of group; $P < 0.001$) with the control leg increasing more than the immobilized leg (group × time interaction; $P < 0.001$). After the 3-d immobilization period myofibrillar protein–bound [^{2}H]-alanine enrichments increased by 44 ± 4%, 35 ± 4%, and 39 ± 5% more in the control compared with the immobilized leg in the HIGH (control leg to 0.1149 ± 0.0045 MPE; immobilized leg to 0.0797 ± 0.0024 MPE), LOW (control leg to 0.1191 ± 0.0057 MPE; immobilized leg to 0.0885 ± 0.0036 MPE), and NO (control leg to 0.1041 ± 0.0041 MPE; immobilized leg to 0.0755 ± 0.0031 MPE) groups, respectively (data not shown). There were no significant differences between groups

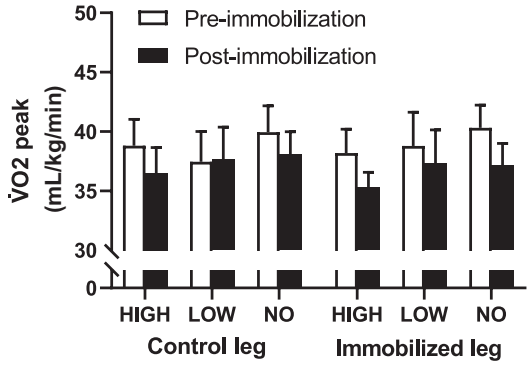

FIGURE 5 Peak maximal oxygen uptake ($\dot{V}O_2$peak) achieved during a unilateral cycling ramp test showing data for both the control and immobilized leg and for both pre- and postimmobilization. Data were analyzed by 3-factor repeated measures ANOVA [with leg (control compared with immobilized) × time (pre compared with post) × group (HIGH, LOW, and NO) as factors, with time and leg considered as repeated factors]. Main effects of both group and leg were $P > 0.05$, but time was $P < 0.05$. The group × time, group × leg, and leg × time interactions were all $P > 0.05$; the group × time × leg interaction was $P > 0.05$. Data were analyzed by 2-factor ANOVA with leg and group as factors. Main effects of group and leg were both $P > 0.05$; the group × time interaction was $P > 0.05$. Data are means ± SEM; $n = 11$ per group.

($P > 0.05$), or any group interactions (all $P > 0.05$) such that dietary protein intake had no effect.

Daily myofibrillar protein FSRs (percentage per day) during immobilization, calculated separately in the control and immobilized legs using mean plasma free [^{2}H]-alanine enrichments as the precursor pool, are displayed in **Figure 7**. Daily myofibrillar protein FSRs were 30 ± 2%, 26 ± 3%, and 27 ± 2% lower in the immobilized compared with the control leg in the HIGH (1.55 ± 0.05%/d compared with 1.08 ± 0.04%/d), LOW (1.57 ± 0.10%/d compared with 1.16 ± 0.06%/d), and NO (1.40 ± 0.08%/d compared with 1.03 ± 0.07%/d) groups,

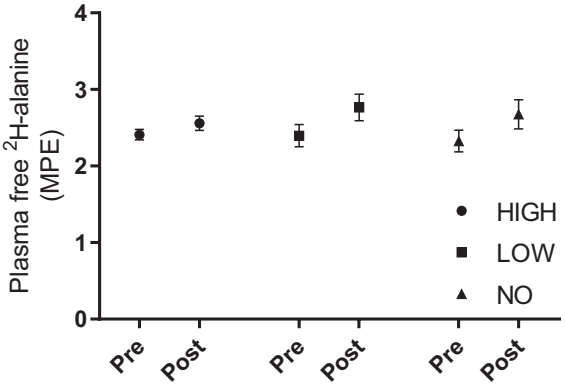

FIGURE 6 Plasma free [^{2}H]-alanine enrichments pre and post a 3-d period of unilateral knee immobilization where participants consumed a fully controlled energy-balanced diet containing a HIGH ($n = 11$; 1.6 g/kg body mass/d), LOW ($n = 11$; 0.5 g/kg/d), and NO ($n = 11$; 0.15 g/kg/d) dietary protein content. Data were analyzed using a 2-factor repeated measures ANOVA with group (HIGH compared with LOW compared with NO) and time (pre- compared with postimmobilization) as factors. Main effect of group ($P > 0.05$), time ($P > 0.05$), and group × time interaction ($P > 0.05$). Data are means ± SEM. MPE, mole percentage excess.

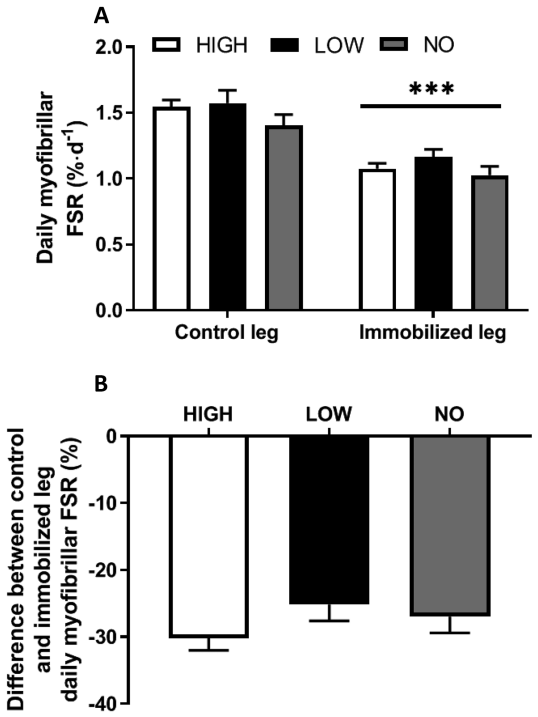

FIGURE 7 (A)Daily myofibrillar fractional synthesis rates (FSRs) over a 3-d immobilization period via unilateral knee immobilization calculated from the plasma [^{2}H]-alanine precursor pool, where participants consumed a fully controlled energy-balanced diet containing a HIGH ($n = 11$; 1.6 g/kg body mass/d), LOW ($n = 11$; 0.5 g/kg/d), and NO ($n = 11$; 0.15 g/kg/d) dietary protein content. Data were assessed by 2-factor repeated measures ANOVA with leg (control compared with immobilized; treated as the repeated factors) and group (HIGH compared with LOW compared with NO) as factors. Main effect of leg (***$P < 0.001$), group ($P > 0.05$), and leg × group interaction ($P > 0.05$). (B) Difference in daily myofibrillar FSRs between the control and immobilized leg for the 3 groups. Data were analyzed by 1-factor ANOVA ($P > 0.05$). Data are means ± SEM.

respectively, with no significant differences between groups ($P > 0.05$).

Discussion

We assessed the impact of graded intakes of daily dietary protein during a short-term (3-d) period of muscle disuse (knee immobilization) in healthy young men on daily MyoPS rates, muscle mass, and function. We report that 3 d of immobilization resulted in a considerable decline in daily MyoPS rates and loss of quadriceps muscle volume and leg muscle strength. However, none of these muscle deconditioning responses to immobilization were modulated by daily dietary protein intake, despite our design spanning a virtual absence of protein through to relatively high intakes.

Dietary protein ingestion transiently stimulates MyoPS rates for 2–5 h (37). As such, the repeated postprandial stimulation at each meal contributes considerably to daily, 24-h MyoPS rates, and thus muscle mass maintenance. We recently demonstrated that a major physiological driver of muscle loss during short-term disuse is a considerable decline in daily, free-living MyoPS rates (4). Importantly, in that work we reported that disuse lowered daily MyoPS rates and consequently induced muscle atrophy despite participants (self-) reporting habitual protein

intakes double that of the daily UK RDA (31) (i.e., 1.6 g/kg body mass/d) and in line with current recommendations to limit muscle disuse atrophy (10, 14, 32). However, this study was not conducted under dietary controlled conditions, nor did we compare groups according to protein intakes, and thus in the present work we aimed to establish the impact of daily dietary protein intake on daily MyoPS rates and muscle loss during disuse. We recreated the relatively high habitual protein intake previously observed (which was also in line with the habitual protein intakes of the current participants; see Table 2) and habituated all participants to this diet for 5 d prior to immobilization. This allowed the application of a dose–response approach during the immobilization period only, where a control group maintained the same intake, 1 group consumed a diet virtually devoid of protein, and another group a suboptimal amount indicative of consumption levels during hospitalization (33). In line with our previous work (4), immobilization lowered MyoPS rates by ∼28% (or ∼9%/d) compared with the control legs. However, contrary to our hypothesis, this decline was comparable across the groups, with the higher, low, and negligible protein intakes resulting in 30%, 26%, and 27% lower MyoPS rates, respectively, in the immobilized compared with control legs. As well as the considerable difference in total protein intakes across groups, these data also occurred in the face of the control group being provided with their daily protein intake equally across 4 meals (breakfast, lunch, dinner, and pre-bed, resulting in ∼28 g protein per meal) each separated by ∼4 h. We had reasoned such an approach would result in sufficient protein per meal (12, 30) and appropriately timed (13, 38, 37) to maximize daily MyoPS rates. It would be remiss of us not to mention that there were also no significant differences in daily MyoPS rate across groups in the *nonimmobilized* leg. It is likely that this represents a type 2 error given that the NO protein group displayed (numerically) lower daily MyoPS rates coupled with a numerical loss (compared with a numerical gain in the other groups) of muscle mass.

In line with the lack of effect of dietary protein intake on daily MyoPS rates, we also observed no impact on muscle mass loss during disuse (Figure 2). We have recently shown that leg immobilization results in substantial atrophy of the quadriceps muscle within 2 d (11). In line with those data, and previous reports (4, 5, 15, 39), we observed a ∼2.4% (i.e., 0.8%/d) decline in quadriceps muscle volume following 3 d of immobilization. However, atrophy was comparable across the dietary intervention groups, with the high, low, and zero protein intake groups' quadriceps muscle volume declining by 2.3% (∼0.8%/d), 2.7 (∼0.9%/d), and 2.0% (∼0.7%/d), respectively. These data were also consistent with the lack of effect of dietary protein intake on the decline in a wide array of muscle function tests following immobilization (Figures 5 and 6). Indeed, with the exception of concentric contractions (2%/d decline), hamstring muscle strength was remarkably resistant to disuse-induced declines, whereas quadriceps muscle concentric (∼8%/d), eccentric (∼6%/d), and isometric (∼8%/d) strength all declined at rates in line with the literature (39, 40). Though the numerical decrease in single leg $\dot{V}O2peak$ seen with immobilization was nonsignificant (Figure 7), comparable effects were seen across groups. Accordingly, our data conclusively show that the decline in muscle function during short-term disuse is rapid, but not modulated by dietary protein intake.

Previous studies have also found that manipulating protein intake during disuse does not modulate the rate of muscle disuse atrophy. For example, protein supplementation studies that have increased protein intakes to 1.6 (compared with 1.1) g/kg/d during 5 d of immobilization in older men (3); to 1.3 (compared with 1.0) g/kg/d during 2 wk of immobilization in young men (41); and to 1.6 (compared with 1.0) g/kg/d during 29 d of bed rest in young men and women (20), have all shown no protective effect on muscle mass. Taken together, therefore, it would seem that dietary protein consumption within "normal ranges" (i.e., ≤1.6 g/kg/d) during disuse does not modulate the rate of muscle loss. We have reasoned previously that such findings could be due to control groups also consuming adequate protein (3). However, our data refute this notion, with the novel observation that daily MyoPS rates and muscle loss are still not modulated even when relatively high, evenly spaced protein intakes are compared with low or negligible protein diets. Collectively, these studies' findings can be explained by the reduced MPS response to each dietary protein meal that occurs consequent with disuse—that is, "anabolic resistance" (5, 34, 42). Our data extend the concept of disuse-induced anabolic resistance by implying that this phenomenon manifests virtually immediately (given the short time frame of disuse), is not overcome by modest increases in protein intake, and is not exacerbated by dramatic reductions in protein intake.

Research where specific EAA or leucine supplementation has been applied during a period of disuse has generally (17, 18, 36, 43), but not always (6), attenuated muscle loss. It is possible that this apparent discrepancy with protein supplementation studies can be attributed to such approaches translating to a greater amount of total protein provided. That is, extrapolating total daily protein intakes from these supplementation studies suggests that the equivalent of ∼87–158 g (1.2–1.9 g/kg/d) total protein was consumed in the treatment conditions, thus generally higher than the present and previous data concerning protein manipulation. Alternatively, the specific provision of high-dose EAA and/or leucine could have had the capacity to overcome/compensate for muscle anabolic resistance more effectively than the present work. In support, such supplementation studies have provided daily EAA and leucine intakes of ∼43–88 g/d and ∼15–19 g/d, respectively (17, 18, 36, 43), compared with ∼51 g and ∼8 g in the present work, or ∼19–50 g and ∼3–10 g in previous protein manipulation studies that were ineffective at attenuating muscle disuse atrophy (3, 19, 20). This would imply that the availability of amino acids per se is not limiting to MPS during disuse, but rather there occurs a dramatic increase in the threshold required for EAA/leucine to stimulate intracellular anabolic signaling pathways. The inference therefore would be that maximizing selective intracellular transport of these key amino acids (rather than raising global amino acid availability) is the prudent goal during disuse. However, such a notion clearly warrants further research, especially given that not all leucine supplementation studies have been successful at attenuating muscle disuse atrophy (6). Additionally, the model of disuse (i.e., single limb immobilization compared with whole-body bed rest) is a further important consideration. The amount of inactive tissue will undoubtedly affect amino acid availability during disuse, and could therefore conceivably contribute toward discrepancies across studies or nutritional strategies depending on the nature of disuse. It could be that future nutritional strategies might

be more effective by focusing on sensitizing the intracellular anabolic signaling pathways (rather than increasing the stimulus), which appears to explain the proposed beneficial effects of prolonged fish oil supplementation on MPS rates and muscle mass maintenance in aging (44, 45) and disuse (7).

In conclusion, graded dietary protein intakes of 0.15, 0.5, or 1.6 g/kg body mass/d did not influence the rapid decline in MyoPS, muscle mass, or function during 3 d of unilateral leg immobilization. To our knowledge, this study is the first to evaluate the impact of dietary protein intake per se under controlled dietary conditions on the rate of skeletal muscle deconditioning during short-term muscle disuse.

The authors' contributions were as follows—SPK, LvL, BTW: designed the research; SPK, JF, SJ, AH, AG, BTW: conducted the research; SPK, BTW: analyzed the data and performed statistical analysis; SPK, LvL, BTW: wrote the paper; SPK: had final responsibility for the final content; and all authors: read and approved the final manuscript.

The authors report no conflicts of interest.

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