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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Abbreviations

BMI, body mass index, BCAA, branch chain amino acids, BMR, basal metabolic rate, CON, control leg, CSA, cross sectional area, D_2O, deuterium oxide, ddH2O, doubly distilled water, EAA, essential amino acids, FSR, fractional synthesis rate, GC-IRMS, Gas chromatography – isotope ratio mass spectrometry, GCMS, Gas chromatography mass spectrometry, ^2H, deuterium, ^1H, hydrogen, HIGH, high protein diet of 1.6 g·kg bm·d^{-1}, IMM, immobilized leg, IPAQ, International physical activity questionnaire, LOW, low protein diet of 0.5 g·kg bm·d^{-1}, MET, Metabolic equivalent, MRI, magnetic resonance imaging, MPB, muscle protein breakdown, MPE, mole percent excess, MPS, muscle protein synthesis, MyoPS, myofibrillar protein synthesis, NAOH, sodium hydroxide, NO, negligible protein diet of 0.15 g·kg bm·d^{-1}, PAL, physical activity level, PCA, perchloric acid, RDA, recommended daily allowance, T, tesla, t, time, UK, United Kingdom, USA, Unites States of America, $\dot{V}O_2$peak, peak maximal oxygen uptake, 1RM, one repetition maximum.

Abstract

Background: Short-term (<1 week) 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. **Objective:** To assess the influence of graded dietary protein intakes on daily MyoPS rates and the loss of muscle mass during 3 days of disuse. **Design:** 33 healthy young men (age, 22±1 y; BMI, 23±1 $kg \cdot m^{-2}$) first consumed the same standardized diet for 5 days providing 1.6 g protein·kg $bm \cdot d^{-1}$. Thereafter, participants underwent a 3 day period of unilateral leg immobilization during which they were randomized into one of three eucaloric diets containing a relatively high, low, or no protein at all (HIGH: 1.6, LOW: 0.5, NO: 0.1 $g \cdot kg\ bm \cdot d^{-1}$; 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 *M. vastus lateralis* biopsies were performed for the measurements of *M. quadriceps* volume and daily MyoPS rates, respectively. **Results:** *M. quadriceps* 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 *M. quadriceps* 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 ^2H-alanine enrichments during immobilization (~2.5 MPE) 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 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.

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 focussed on short term periods of muscle disuse (≤ one week) which are common in clinical settings (1). We and others have shown that merely 2-5 days of disuse already results in substantial loss of muscle mass (2, 3, 4, 5), with associated declines in strength (3, 4, 5). As a result, there is an eagerness of researchers to develop effective (nutritional) countermeasures (e.g. 6, 3, 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 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 two days 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 may 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 bm·d^{-1}) (11). Though 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 days of bed-rest or immobilization has been reported to attenuate losses of muscle mass,

strength and/or whole body nitrogen (17, 18, 19). However, studies where dietary protein supplementation has been applied during 5-60 days 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·kg bm·d^{-1}), low (0.5 g·kg bm·d^{-1}) and negligible (0.15 g·kg bm·d^{-1}) daily dietary protein intakes influence daily MyoPS rates determined using the deuterated water approach, and muscle mass loss determined via MRI during a three day period of unilateral leg immobilization in healthy males. We hypothesised 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, UK (for the consort flow chart please see **Supplemental Figure 1**). Participants were allocated sequential numbers at the time of screening which were then used as the only identifiable characteristic for all documents containing participant information, and were randomised into groups using an online randomiser (http://www.randomization.com/), recruitment and testing was ended once 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; a (family) history of deep vein thrombosis/cardiovascular disease, metabolic disorders (e.g. type 2 diabetes), musculoskeletal/orthopedic disorders, a body mass index of above 28.5 or below 18.5 kg·m^{-2}, participation in a structured resistance training program within 6 months prior to the study, any musculoskeletal injury of the legs within 12 months before the study, use of anticoagulants, any contraindications to MRI scanning (e.g. metallic implants), and consumption of any nutritional supplement prior to and during the study. Participants who consumed a habitual dietary protein intake of above 1.8 or below 0.6 g·kg·d^{-1} were also excluded from the study. During the screening participants' height, body mass and blood pressure were measured, body composition was also assessed by air displacement plethysmography (BODPOD; Life Measurement, Inc. CA, USA). 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 (METs) (21). Participants' habitual diets were recorded for 3 days (two week days and one 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 to 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 (170712/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 onto the study participants attended the laboratory in the fasted state for 5 experimental visits across 13 days. This included an 8 day fully controlled dietary intervention period with the final 3 days of the dietary controlled period involving unilateral leg immobilization using a leg brace and ambulation with crutches. Pre-immobilization (visit 1) comprehensive unilateral muscle strength and maximal aerobic capacity ($\dot{V}O_2peak$) testing were conducted (protocols described below). Thereafter, five days prior to immobilization (visit 2) all participants attended the laboratory to collect their first 5 days of food to commence a fully controlled, pre-immobilization standardized diet with protein intake fixed at 1.6 $g \cdot kg\ bm \cdot d^{-1}$. To measure daily myofibrillar protein synthesis (MyoPS) rates throughout the immobilization period participants underwent a deuterium oxide dosing protocol (described below) beginning on visit 3 (i.e. one

day 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 ~0800 h and a single *M. 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 pre-immobilization MRI scan of both thigh muscles. Thereafter, participants were randomised, in a single (participant) blind manner into one of three 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·kg bm·d^{-1}. Participants were fitted with a leg brace to induce immobilization of one leg and were given crutches for ambulation, and provided with 3 days of food supply in line with their allotted diet, and this signified the commencement of the 3 day immobilization period. Following the immobilization period participants returned to the laboratory for the final visit (visit 5) where *M. 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 anaesthesia, using the percutaneous Bergstrom needle biopsy technique (23) from the *M. vastus lateralis* approx. 15 cm above the patella and approx. ~2 cm below the fascia. Immediately following muscle biopsies, the tissue was quickly assessed and any blood or non-muscle tissue was dissected and discarded. The muscle samples were immediately frozen in liquid nitrogen within one min and stored at -80°C until further analysis. Thereafter, participants were transported via wheel chair to undergo further MRI scans of the thighs of both legs, and finally further unilateral 1RM 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 days prior to immobilization (days 2-5 of the pre-immobilization diet) and for the entirety of the immobilization period participants' physical activity was measured using an accelerometer (GENEactiv, Activinsights, Cambridgeshire, UK) worn on the non-dominant 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 one week before and throughout the 8 day 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; Swords, Co. Dublin).

Magnetic resonance imaging for determination of M. quadriceps volume

Prior to and post immobilization muscle volume of the *M. quadriceps* of both legs was determined via MRI. We described the MRI methodology for the determination of *M. quadriceps* volume in detail previously (11). In brief, a 1.5 tesla (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 x 500 mm, reconstructed matrix 512 x 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 *M. quadriceps* were analysed via manual segmentation using Philips on-line 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 *M. quadriceps* muscle undergoes rapid atrophy during disuse and accounts for the vast majority of total *M. quadriceps* volume loss. *M. quadriceps* volume was calculated using a previously published method (25) where the total CSA for all images was calculated and multiplied by the slice gap plus the distance between slices (linear interpolation) (in this case a total 2 cm, comprised of a 5 mm slice thickness and a 15 mm slice gap), summarized by the following equation:

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

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 x 50 mL boluses (CK Isotopes Ltd, Leicestershire, UK). Upon arrival at the laboratory (0730 h) 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 ~0800 h with the remaining doses being consumed every 1.5 h thereafter. Participants stayed at the university until 4 out 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 (~0800 h). Blood samples were collected during the test days (i.e. day 5 [pre], 7 [after 2 days of immobilization] and 12 [post]). Venous blood samples were collected from the antecubital vein via venepuncture and collected into EDTA-containing vacutainers which were

centrifuged at 2,500 *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, Shirley, NY, USA). Isometric, isokinetic concentric and isokinetic eccentric strength for both knee extension (i.e. *M. quadriceps* strength) and flexion (i.e. *M. hamstrings* strength) were all determined in the stated order. After warm-up repetitions at 50, 75 and 85 % of self-determined one repetition maximum (1-RM) participants performed 3 x 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 minute rest and the 2 exercise modalities by a 5 minute 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 minute break between the two contraction types, contraction speed was $60°\cdot s^{-1}$ over the central 80° range of motion (verified by goniometry) out of each participant's full range of motion (e.g. from full extension to full flexion). Then following a 5 minute break the same isokinetic concentric and eccentric contractions were repeated for knee flexion.

Unilateral leg peak maximal oxygen uptake ($\dot{V}O_2$peak) was assessed using a previously validated single leg ramp exercise test to exhaustion (27, 28). In brief, a custom designed counterweight pedal (11.4 kg) was fitted to the crank of an electronically braked cycle ergometer (Lode Corival, Groningen, The Netherlands). Participants cycled with one leg, with

the non-exercising 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 analyser (Cortex Metalyzer 3B gas analyser, Cortex, Germany). For all exercises the (to be) immobilized leg was always performed first followed by the control leg.

Dietary control

Nutritional information for the pre-immobilization (5 days immediately before immobilization) and immobilization (3 day immobilization period) diets is provided in Table 2. Basal metabolic rate (BMR) was estimated using the Henry equations based on age, gender, and weight (29). Individual energy requirements were then calculated by multiplying the participants' BMR and PAL (calculated from the IPAQ as described above). Thereafter, an individual 8 day 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 preparation were provided to the participants which they prepared at home, and a log was provided to record the times of consumption of each meal. For the first 5 days of the 8 day dietary control (i.e. pre-immobilization period) all participants consumed a diet containing 1.6 g·kg bm·d^{-1}, 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 other 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 four meals (~27±1, 28±1, 28±1 and 28±1 g at breakfast, lunch, dinner and a pre-sleep whey protein beverage) and participants were instructed to consume their meals approximately 4-5 h apart, throughout the day to optimise 24 h muscle protein synthesis rates (13, 30, 31). At each experimental visit

participants' body mass was measured (seca 703 column scale, seca GmbH & Co. KG, Hamburg, Germany) wearing light clothing and the researchers discussed with the participants any questions or issues that may have arisen with the diet, and in the event of any substantial weight change (>0.5 kg, with the same upward or downward trend on two consecutive visits) energy content of the next two days was adjusted (via the reduction/increase in carbohydrate). Following the 5 day pre immobilization period, volunteers commenced with the 3 day immobilization period during which volunteers were randomised into either the HIGH (1.6 g·kg bm·d^{-1}; *n*=11) LOW (0.5 g·kg bm·d^{-1}; *n*=11) or NO (0.15 g·kg bm·d^{-1}; *n*=11) protein groups. The HIGH group therefore maintained the pre-immobilization 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. 1.6 g·kg bm·d^{-1} was selected as 'high' based on being double the UK RDA (32), being in line with current recommendations for restricting muscle loss during disuse (10, 14, 33) 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 LOW group was selected as 0.5 g·kg bm ·d^{-1} since 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 (34). 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·kg bm·d^{-1}). All food items in the 1.6 and 0.5 g·kg bm·d^{-1} protein groups were purchased from commercial retailers. To reduce protein intake to 0.15 g·kg bm·d^{-1} certain food products given to this group were purchased from a company that produces specialized low/zero protein food (Promin Metabolics, Stockport, UK). 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 days (11). Briefly, the brace (X-ACT Donjoy brace, DJO global, Vista, CA, USA) was applied and the participant can then ambulate on crutches (after receiving instructions) throughout the immobilization period. The immobilized leg was randomized and counterbalanced for leg dominance with the non-immobilized 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 per day 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 resulted in exclusion from the study (11, 35), though 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 gas chromatography-mass spectrometry analysis (GC-MS; Agilent 5975C MSD & 7890A GC, Wilmington, USA). 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, Hercules, CA). The purified amino acids were converted to their *tert*-butyldimethylsilyl (*tert*-BDMS) derivatives with MTBSTFA 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 ddH_2O was added until a total volume of 10 ml was achieved [10 µL/mg]). The samples were centrifuged at 2,500 *g* and 4°C for 5 min and the pellet was then washed with 500 µL of ddH2O and again centrifuged at 2,500 *g* and 4°C for 10 min. The myofibrillar protein was solubilized by adding 1 mL of 0.3 M NaOH and heating for 30 min at 50°C with samples being vortexed every 10 min. Samples were then centrifuged for 10 min at 9,500 *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 of 1 M PCA and spun at 700 *g* and 4°C for 10 min. Myofibrillar proteins were then washed with 70% ethanol twice and hydrolyzed overnight in 2

326 mL of 6 M HCL at 110°C. The free amino acids from the hydrolyzed myofibrillar protein pellet
327 were dried under a nitrogen stream while being heated at 120°C. The free amino acids were
328 subsequently dissolved in 25% acetic acid solution and passed over cation exchange AG 50W-
329 X8 resin columns (mesh size: 100-200, ionic form: hydrogen; Bio-Rad Laboratories, Hercules,
330 CA) and eluted with 2 M NH_4OH. Following this the eluted amino acids were dried and the
331 purified amino acids were derivatized to their N(O,S)-ethoxycarbonyl ethyl esters (36). The
332 derivatized amino acids were measured using a gas-chromatograph-isotope ratio mass
333 spectrometer (GC-IRMS; Thermo Fisher Scientific, MAT 253; Bremen, Germany) equipped
334 with a pyrolysis oven and a 60m DB-17MS column (no. 122-4762; Agilent, Wilmington, USA)
335 and a 5 m precolumn. Ion masses 2 and 3 were analyzed to determine the $^2H/^1H$ ratios of muscle
336 protein-bound alanine. A series of known standards was used to assess the linearity of the mass
337 spectrometer and to control for the loss of tracer.
338
339 *Calculations*
340 Myofibrillar protein fractional synthesis rates (FSR) were calculated based on the incorporation
341 of [^{2}H] alanine into myofibrillar protein and the mean free plasma [^{2}H] alanine enrichment
342 throughout the immobilization period as a precursor. FSR was calculated using the standard
343 precursor-product method expressed as daily rates as follows:

344 $$FSR\ (\% \cdot\ \text{day}^{-1}) = \left(\frac{E_{m2} - E_{m1}}{E_{precursor}\ x\ t}\right) x\ 100$$

345 where E_{m1} and E_{m2} are the myofibrillar muscle protein-bound enrichments on day 0 and 3.
346 $E_{precursor}$ represents mean plasma free [^{2}H] alanine enrichment (mean enrichment between day
347 0-3). *t* represents the time between biopsies (day 0-3). FSRs were calculated in both legs
348 separately using the biopsy collected from the immobilized leg as baseline for both legs.
349
350

Statistics

All data are presented as means±SEM and all statistical analyses were conducted in GraphPad Prism version 7.0 (GraphPad Software, San Diego, CA, USA). The study sample size was based on a previously reported one week muscle disuse dietary controlled intervention in healthy young males (37). 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 *M. quadriceps* muscle mass (SD=0.3, 80% power, α=0.05). A two-way repeated measures ANOVA with leg (control vs immobilized; treated as the repeated factor) and group (HIGH, LOW and NO) as factors were used to compare MyoPS rates. Three-way repeated measures ANOVAs (time [pre vs post], leg [control vs immobilized] and group [HIGH, LOW and NO] as factors, with time and leg considered as repeated factors) were used to compare *M. quadriceps* volume and isometric, concentric and eccentric leg strength and unilateral leg $\dot{V}O_2$peak data. A two-way repeated measures ANOVA with group (HIGH, LOW and NO) and time (habitual vs pre vs during immobilization) as factors was used to assess for differences in dietary intake parameters. Two-way 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 days preceding (pre) and during the 3 day 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 x group interaction effects; all $P>0.05$) across groups. Moderate physical activity significantly declined in the HIGH and NO protein group only ($P<0.05$). As expected, all dietary parameters were identical across groups during the pre-immobilization dietary controlled period. By design, energy intake was equivalent across groups during immobilization, but dietary protein intake differed (group, time and group x time effects; all $P<0.001$) such that HIGH was greater than LOW and NO (both; $P<0.001$) and LOW was greater that NO ($P<0.001$). This resulted in differences in CHO 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 CHO intake than LOW ($P<0.001$). There were no significant differences between groups for fat intake during immobilization (group and the group x time effects; both $P>0.05$, time effect; $P<0.001$). (Fat intake [En%] was significantly reduced from pre to during immobilization in the NO group [$P<0.05$]).

M. quadriceps muscle volume

M. quadriceps muscle volumes calculated from MRI are displayed in **Figure 2**. There were no significant differences in *M. quadriceps* muscle volume between legs or between groups pre

immobilization (group x 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 *M. quadriceps* muscle volume of the control leg was unaffected by immobilization ($P>0.05$). *M. quadriceps* volume of the immobilized leg reduced significantly during immobilization (leg x 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 post immobilization = 1396±81 cm^3, $P<0.001$), LOW (pre = 1439±105 to post immobilization = 1400±101 cm^3, $P<0.001$) and NO (pre = 1499±83 to post immobilization = 1469±81 cm^3, $P<0.05$) groups, respectively (Figure 2B); however, these changes did not differ across groups (Figure 2B) (group x time and group x leg x time interactions; $P>0.05$).

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

M. quadriceps and *M. hamstrings* muscle strength data are displayed in **Figures 3 and 4**, respectively. There were no significant differences in any contraction type for the *M. quadriceps* or *M. hamstrings* muscle strength between legs or between groups pre immobilization (group x leg interaction effect, $P>0.05$). No parameter of strength was altered throughout the experiment in the control leg for either the *M. quadriceps* or *M. hamstrings* muscles ($P>0.05$) and there were no significant differences between groups ($P>0.05$). Immobilization decreased *M. quadriceps* maximal isometric (HIGH = by 24±8%, LOW = by 24±6%, NO = by 26±5%; time x leg effect; $P<0.001$), concentric (HIGH = by 23±8%, LOW = by 22±6%, NO = by 25±4%; time x leg effect; $P<0.001$) and eccentric (HIGH = by 16±7 %, LOW = by 18±8 %, NO = by 23±6 %; time x leg effect; $P<0.001$) strength to a similar extent across groups (group x leg interaction; $P>0.05$ for all 3 contraction types). In contrast, immobilization only reduced *M. hamstrings* maximal concentric strength (HIGH = by 7±2 %, LOW = by 8±6 %, NO = by 5±5 %) (time x leg; $P<0.05$), again with no significant differences

between groups (group x leg interaction; $P>0.05$), whereas *M. hamstrings* 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 ($P>0.05$), group ($P>0.05$) in either leg (interactions all; $P>0.05$).

Plasma free ^2H 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 percent excess (MPE) at the start of immobilization in the HIGH, LOW and NO groups, respectively, and tended (time effect; $P=0.06$) to increase post immobilization. However, there were no significant differences in plasma free [^{2}H] alanine enrichments between groups ($P>0.05$).

Daily myofibrillar protein synthesis rates

Myofibrillar protein bound [^{2}H] alanine enrichments increased pre- to post- immobilization (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 x time interaction; $P<0.001$). After the 3 day 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 ($P>0.05$), or any group interactions (all $P>0.05$) such that dietary protein intake had no effect.

Daily myofibrillar protein FSRs (%·d^{-1}) 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 vs 1.08±0.04 %·d^{-1}), LOW (1.57±0.10 vs 1.16±0.06 %·d^{-1}) and NO (1.40±0.08 vs 1.03±0.07 %·d^{-1}) groups 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 day) period of muscle disuse (knee immobilization) in healthy young men on daily myofibrillar protein synthesis (MyoPS) rates, muscle mass and function. We report that 3 days of immobilization resulted in a considerable decline in daily MyoPS rates and loss of *M. quadriceps* 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 (38). 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 (32) (i.e. 1.6 g·kg bm·d^{-1}) and in line with current recommendations to limit muscle disuse atrophy (10, 14, 33). 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 role 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 five days 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, one group consumed a diet virtually devoid of protein, and another group a sub-optimal amount indicative of consumption levels during hospitalization (34). In line with our previous work

(4), immobilization lowered MyoPS rates by ~28% (or ~9%·d^{-1}) 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 their daily protein intake equally across four meals (breakfast, lunch, dinner, 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, 31) and appropriately timed (13, 39, 38) to maximise daily MyoPS rates. It would be remiss of us not to mention that significant differences in daily MyoPS rate across groups also did not occur in the *non*-immobilized 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 *M. quadriceps* within two days (11). In line with those data, and previous reports (4, 5, 15, 40), we observed a ~2.4% (i.e. 0.8%·d^{-1}) decline in *M. quadriceps* volume following 3 days of immobilization. However, atrophy was comparable across the dietary intervention groups with the high, low and zero protein intake groups' *M. quadriceps* volume declining by 2.3 (~0.8%·d^{-1}), 2.7 (~0.9%·d^{-1}) and 2.0% (~0.7%·d^{-1}), 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^{-1} decline), *M. hamstrings* strength was remarkably resistant to disuse induced declines, whereas *M. quadriceps* concentric (~8%·d^{-1}), eccentric (~6%·d^{-1}) and isometric (~8%·d^{-1}) muscle strength

all declined at rates in line with the literature (40, 41). Though the numerical decrease in single leg V̇O2peak seen with immobilization was not significant (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 which have increased protein intakes to; 1.6 (vs 1.1) g·kg bm·d^{-1} during 5 days of immobilization in older men (3); 1.3 (vs 1.0) g·kg bm·d^{-1} during 2 weeks of immobilization in young men (42); and to 1.6 (vs 1.0) g·kg bm·d^{-1} during 29 days 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 bm·d^{-1}) during disuse does not modulate the rate of muscle loss. We have reasoned previously that such findings may 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 may be explained by the reduced MPS response to each dietary protein meal that occurs consequent with disuse (i.e. 'anabolic resistance'; (5, 35, 43)). Our data extend on 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 essential amino acid (EAA) or leucine supplementation has been applied during a period of disuse has generally (17, 18, 37, 44), 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 the equivalent of ~87-158 (1.2-1.9 g·kg bm·d^{-1}) g total protein was consumed in the treatment conditions, and thus generally higher than the present and previous data concerning protein manipulation. Alternatively, the specific provision of high dose EAA and/or leucine may 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 and ~15-19 g per day, respectively (17, 18, 37, 44), compared with ~51 and ~8 g in the present work, or ~19-50 and ~3-10 g in previous protein manipulation studies 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 a dramatic increase in the threshold required for EAA/leucine to stimulate intracellular anabolic signalling pathways rates occurs. The inference therefore would be that maximising 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 not all leucine supplementation studies have been successful at attenuating muscle disuse atrophy (6). Additionally, the model of disuse (i.e. single limb immobilization vs whole body bedrest) is a further important consideration. The amount of inactive tissue will undoubtedly affect amino acid availability during disuse, and could therefore conceivably contribute towards discrepancies across studies or nutritional strategies depending on the nature of disuse. It may be that future nutritional strategies may be more effective by focussing on sensitising the intracellular anabolic signalling 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 ageing (45, 46) and disuse (7).

In conclusion, graded dietary protein intakes of 0.15, 0.5 or 1.6 g·kg bm·d^{-1} did not influence the rapid decline in MyoPS, muscle mass or function during 3 days of unilateral leg

571 immobilization. This study is the first to evaluate the role of dietary protein intake *per se* under
572 controlled dietary conditions on the rate of skeletal muscle deconditioning during short-term
573 muscle disuse.

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Conflict of interest statement

No conflicts of interest, financial or otherwise, are declared by the authors.

Author contributions

S. P. K., L. J.C v. L., and B. T. W. designed the research; S. P. K., J. F., S. R. J., A. M. H., A. P. G., and B. T. W. conducted the research. S. P. K and B. T. W. Analyzed the data and performed statistical analysis. S. P. K., L. J. C. v. L., and B. T. W., wrote the paper. S. P. K. has final responsibility for the final content.

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Tables

Table 1. Participants' characteristics and habitual diet.

	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 ($kg \cdot m^{-2}$)	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 ($MJ \cdot d^{-1}$)	11.7±0.6	10.7±0.7	11.2±0.8
($Kcal \cdot d^{-1}$)	(2800±64)	(2548±163)	(2680±202)
Protein intake ($g \cdot d^{-1}$)	107±6	122±14	127±14
Protein intake ($g \cdot kg\ bm \cdot d^{-1}$)	1.5±0.1	1.7±0.1	1.7±0.1
Protein intake (En%)	15±1	19±2	19±1
CHO intake ($g \cdot d^{-1}$)	373±28	277±22	281±25
Carbohydrate intake (En%)	51±4	44±3	43±3
Fat intake ($g \cdot d^{-1}$)	116±8	100±11	109±11
Fat intake (En%)	39±3	35±2	37±2

Values represent means±SEM, n=11 per group. Data were statistically analyzed with a one way ANOVA. No statistically significant differences were found between groups for any parameter.

Table 2. Dietary intake and physical activity levels during dietary controlled periods pre and during a 3 day period of unilateral knee immobilization.

	HIGH		LOW		NO	
	Pre	During	Pre	During	Pre	During
Energy intake ($MJ \cdot d^{-1}$)	11.6±0.3	11.7±0.3	11.5±0.4	11.5±0.5	11.7±0.3	11.8±0.3
($Kcal \cdot d^{-1}$)	(2777±64)	(2788±62)	(2741±104)	(2747±114)	(2791±74)	(2801±80)
Protein intake ($g \cdot d^{-1}$)	116±4	116±4	113±7	36±2*[a]	116±4	10±0.4*[a, b]
Protein intake ($g \cdot kg\ bm \cdot d^{-1}$)	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]
Protein intake (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 ($g \cdot d^{-1}$)	362±12	368±13	341±11	426±19*[a]	361±11	525±13*[a, b]
Carbohydrate intake (En%)	52±1	53±1	50±1	62±1*[a]	52±1	71±2*[a, b]
Fat intake ($g \cdot d^{-1}$)	88±4	85±4	98±4	91±5	90±4	78±4
Fat intake (En%)	29±1	28±2	32±1	30±1	29±1	23±1*[a, b]
Light physical activity ($h \cdot d^{-1}$)	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 physical activity ($h \cdot d^{-1}$)	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 physical activity ($h \cdot d^{-1}$)	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 physical activity ($h \cdot d^{-1}$)	3.4±0.3	2.3±0.2*	3.7±0.4	2.6±0.2*	4.6±0.6	2.4±0.4*

Values represent means±SEM, n=11 per group. Data were analyzed by using a two-way repeated measures ANOVA (with time and group as factors). *=significant difference from pre immobilization value, $P<0.001$, [a] = significant difference from HIGH group during immobilization $P<0.05$, [b] = significant difference from LOW group during immobilization $P<0.001$. En% = % of total energy intake. Pre denotes the 5 day period of controlled diet before immobilization. During denotes the 3 day immobilization period.

Figure Legends

Figure 1. Study protocol. Thirty-three healthy young males underwent 3 days of unilateral leg immobilization via knee brace. MRI, Magnetic resonance imaging. D_2O, deuterated water ingestion. Activity, physical activity measured continuously by GENEactiv wrist watch accelerometry. Diet, all participants underwent 5 days of fully controlled pre-immobilization diet relatively high in dietary protein (1.6 g·kg bm·d^{-1}) before being randomized into three groups of varying protein intake HIGH (1.6 g·kg bm·d^{-1}), LOW (0.5 g·kg bm·d^{-1}) and NO (0.15 g·kg bm·d^{-1}) for the 3 day immobilization period. Blood, venous blood sample collection. Arrows represent *M. vastus lateralis* muscle biopsies, (i.e. taken from the immobilized leg only at pre-immobilization and both control and immobilized legs post-immobilization). Strength, unilateral maximal isometric, concentric and eccentric contractions of both the *M. quadriceps* and *M. hamstrings* measured by isokinetic dynamometry. $\dot{V}O_2$peak, single leg peak oxygen uptake was measured using a ramp cycling test to exhaustion. Control and immobilized legs completed all strength and aerobic capacity tests separately.

Figure 2. *M. quadriceps* muscle volume of the control and immobilized legs pre and post 3 days of unilateral leg immobilization where participants consumed a fully controlled energy balanced diet containing a HIGH (n=11; 1.6 g·kg bm·d^{-1}), LOW (n=11; 0.5 g·kg bm·d^{-1}) or NO (n=11; 0.15 g·kg bm·d^{-1}) dietary protein content (A). A three-way repeated measures ANOVA (leg [control vs immobilized] x time [pre vs post] x 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 x group interaction P>0.05, leg x time interaction P<0.001, group x time interaction P>0.05, leg x group x time interaction P>0.05. Bonferroni post tests were conducted to locate individual

differences; *** and * denotes a significant difference from pre immobilization within the same group at $P<0.001$ and $P<0.05$, respectively. B expresses the relative difference in *M. quadriceps* volume between the control and immobilized legs for HIGH, LOW and NO groups. Data were analyzed by a two-way repeated measures ANOVA with leg (control vs immobilized; treated as the repeated factor) and group (HIGH vs LOW vs NO) as factors. Main effect of leg $P<0.001$, group $P>0.05$, leg x group interaction was $P<0.05$. Bonferroni post tests were conducted to locate individual differences; * and *** denote a significant difference from pre immobilization within the same group at $P<0.05$ and $P<0.001$, respectively. Data presented are means±SEM.

Figure 3. *M. quadriceps* muscle strength assessed by isokinetic dynamometry. Maximal isometric (3-repetition average) (A), isokinetic concentric (C) and isokinetic eccentric (E) (both 5-repetition average) *M. quadriceps* strength for the control and immobilized legs pre- and post- immobilization for the three groups HIGH (n=11; 1.6 g·kg bm·d^{-1}), LOW (n=11; 0.5 g·kg bm·d^{-1}) and NO (n=11; 0.15 g·kg bm·d^{-1}). Data were analyzed by three-way repeated measures ANOVA (with leg [control vs immobilized] x time [pre vs post] x 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 group ($P>0.05$), but for isometric and concentric *M. quadriceps* strength a main effect of leg was detected $P<0.05$, but for eccentric the main effect of leg was $P>0.05$. For all three exercises (graphs A, C and E) the main effect of time was $P<0.001$. The group x leg and group x time interactions were $P>0.05$ for 3 graphs (A, C and E), the leg x time interactions were $P<0.001$ for all 3 graphs (A, C and E). The group x leg x time interactions were $P>0.05$ for all three graphs. Graphs B, D and F show the relative change in maximal isometric, concentric and eccentric *M. quadriceps* strength respectively, for the control and immobilized leg and for the HIGH, LOW and NO groups. Data were analyzed by two-way repeated measures ANOVA

with leg (control vs immobilized; treated as the repeated factor) and group (HIGH, LOW and NO) as factors. The main effect of group was $P>0.05$ for all 3 graphs (B, D, F), the main effect of leg was $P<0.05$ for isometric (B) and eccentric (E) exercises and $P<0.01$ for concentric (D). * and ** denote the main effect of leg at $P<0.05$ and $P<0.01$ level respectively. The group x leg interaction effects were $P>0.05$ for all 3 exercises (graph B, D, F). Data are means±SEM.

Figure 4. *M. hamstrings* muscle strength assessed by isokinetic dynamometry. Maximal isometric (3-repetition average) (A), isokinetic concentric (C) and isokinetic eccentric (E) (both 5-repetition average) *M. hamstrings* strength, for the control and immobilized legs pre- and post- immobilization for three groups HIGH (n=11; 1.6 g·kg bm·d^{-1}), LOW (n=11; 0.5 g·kg bm·d^{-1}) and NO (n=11; 0.15 g·kg bm·d^{-1}). Data were analyzed by three-way repeated measures ANOVA (with leg [control vs immobilized] x time [pre vs post] x 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 x leg and group x time interactions were $P>0.05$ for 3 graphs (A, C and E), the leg x time interactions were $P>0.05$ for isometric (A) and eccentric (E), but $P<0.05$ for concentric (C). The group x leg x time interactions were $P>0.05$ for all three graphs. Graphs B, D and F show the relative change in maximal isometric, concentric and eccentric *M. hamstrings* strength respectively, for the control and immobilized leg and for the HIGH, LOW and NO groups. Data were analyzed by two-way repeated measures ANOVA with leg (control vs immobilized; treated as the repeated factor) and group (HIGH, LOW and NO) as factors. The main effect of group was $P>0.05$ for all 3 graphs (B, D, F), the main effect of leg was $P<0.05$ for concentric (C) and $P>0.05$ for eccentric (E) exercises and concentric (D). * denotes the main effect of leg $P<0.05$. The group x leg interaction effects were $P>0.05$ for all 3 exercises (graph B, D, F). Data are means±SEM.

Figure 5. $\dot{V}O_2$peak achieved during a unilateral cycling ramp test. The graph expresses data for both the control and immobilized leg and for both pre- and post- immobilization. Data were analysed by three-way repeated measures ANOVA (with leg [control vs immobilized] x time [pre vs post] x group [HIGH, LOW and NO] as factors, with time and leg considered as repeated factors). Main effect of both group and leg were $P>0.05$, but time was $P<0.05$. The group x time, group x leg and leg x time interactions were all $P>0.05$, the group x time x leg interaction was $P>0.05$. Data were analyzed by two-way ANOVA with leg and group as factors. Main effects of group, leg were both $P>0.05$, the group x time interaction was $P>0.05$. Data are means±SEM; $n=11$ per group.

Figure 6. Plasma free ^{2}H-alanine (MPE) enrichments pre and post a 3 day period of unilateral knee immobilization where participants consumed a fully controlled energy balance diet containing a HIGH ($n=11$; 1.6 g·kg bm·d^{-1}), LOW ($n=11$; 0.5 g·kg bm·d^{-1}) and NO ($n=11$; 0.15 g·kg bm·d^{-1}) dietary protein content. Data were analyzed using a two-way repeated measures ANOVA with group (HIGH vs LOW vs NO) and time (pre vs post immobilization) as factors. Main effect of group $P>0.05$, time $P>0.05$ and group x time interaction $P>0.05$. Data are means±SEM.

Figure 7. Daily myofibrillar fractional synthesis rates (FSR; %·d^{-1}) over a 3 day immobilization period via unilateral knee immobilization calculated from the plasma ^{2}H-alanine precursor pool, where participants consumed a fully controlled energy balance diet containing a HIGH ($n=11$; 1.6 g·kg bm·d^{-1}), LOW ($n=11$; 0.5 g·kg bm·d^{-1}) and NO ($n=11$; 0.15 g·kg bm·d^{-1}) dietary protein content. Data were assessed by two-way repeated measures repeated measures ANOVA with leg (control vs immobilized; treated as the repeated factors)

and group (HIGH vs LOW vs NO) as factors. Main effect of leg $P<0.001$ (***), group $P>0.05$, leg x group interaction $P>0.05$. B expresses the difference in daily myofibrillar fractional synthesis rates between the control and immobilized leg for the three groups. Data were analyzed by one way ANOVA, $P>0.05$. Data are means±SEM.

Figure 1

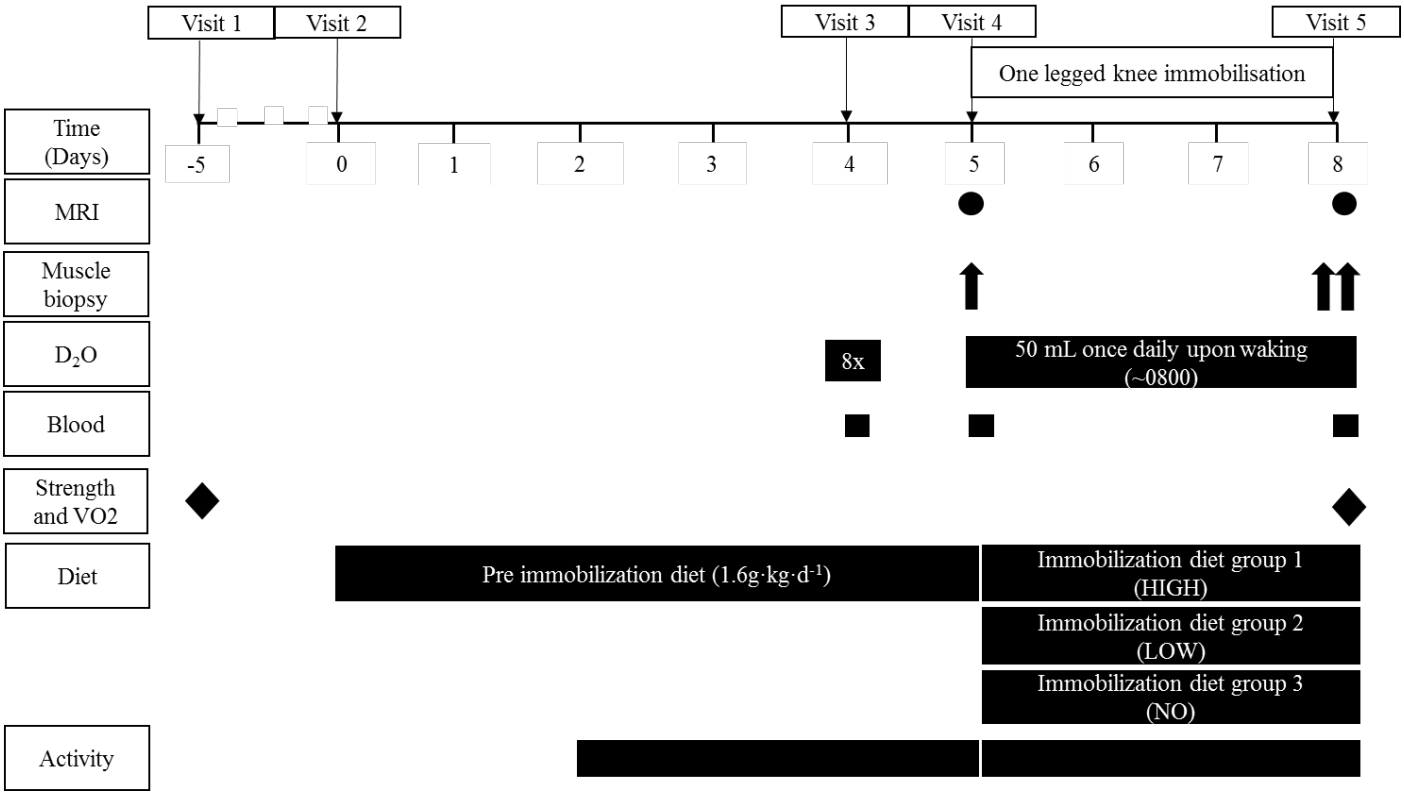

Visit 1
Visit 2
Visit 3
Visit 4
Visit 5
One legged knee immobilisation
Time (Days)
-5
0
1
2
3
4
5
6
7
8
MRI
Muscle biopsy
D2O
8x
50 mL once daily upon waking (~0800)
Blood
Strength and VO2
Diet
Pre immobilization diet (1.6g·kg·d-1)
Immobilization diet group 1 (HIGH)
Immobilization diet group 2 (LOW)
Immobilization diet group 3 (NO)
Activity

Figure 2

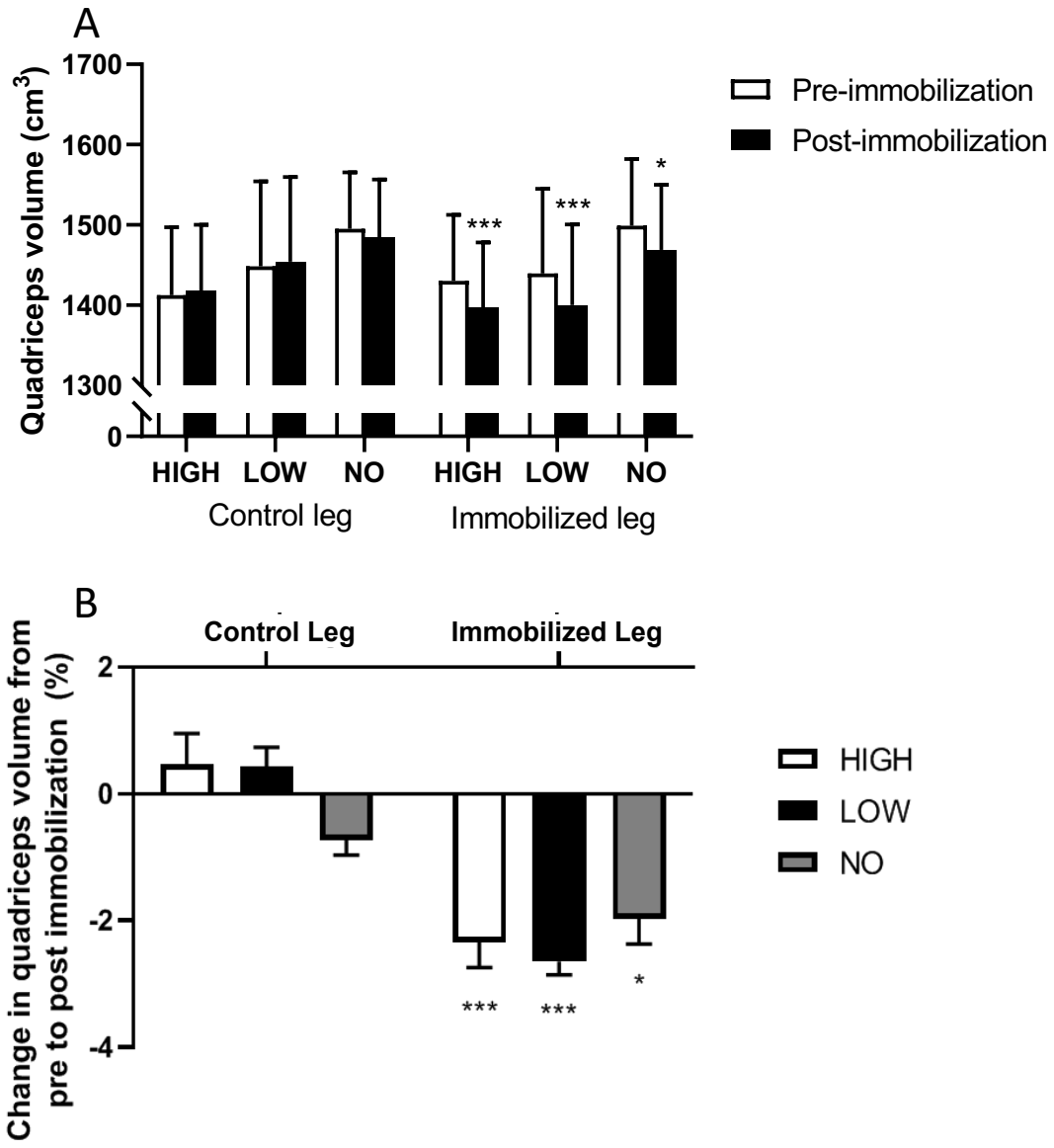

A
Quadriceps volume (cm3)
1700
1600
1500
1400
1300
0
HIGH
LOW
NO
HIGH
LOW
NO
Control leg
Immobilized leg
***
***
*
Pre-immobilization
Post-immobilization
B
Control Leg
Immobilized Leg
Change in quadriceps volume from pre to post immobilization (%)
2
0
-2
-4
***
***
*
HIGH
LOW
NO

Figure 3

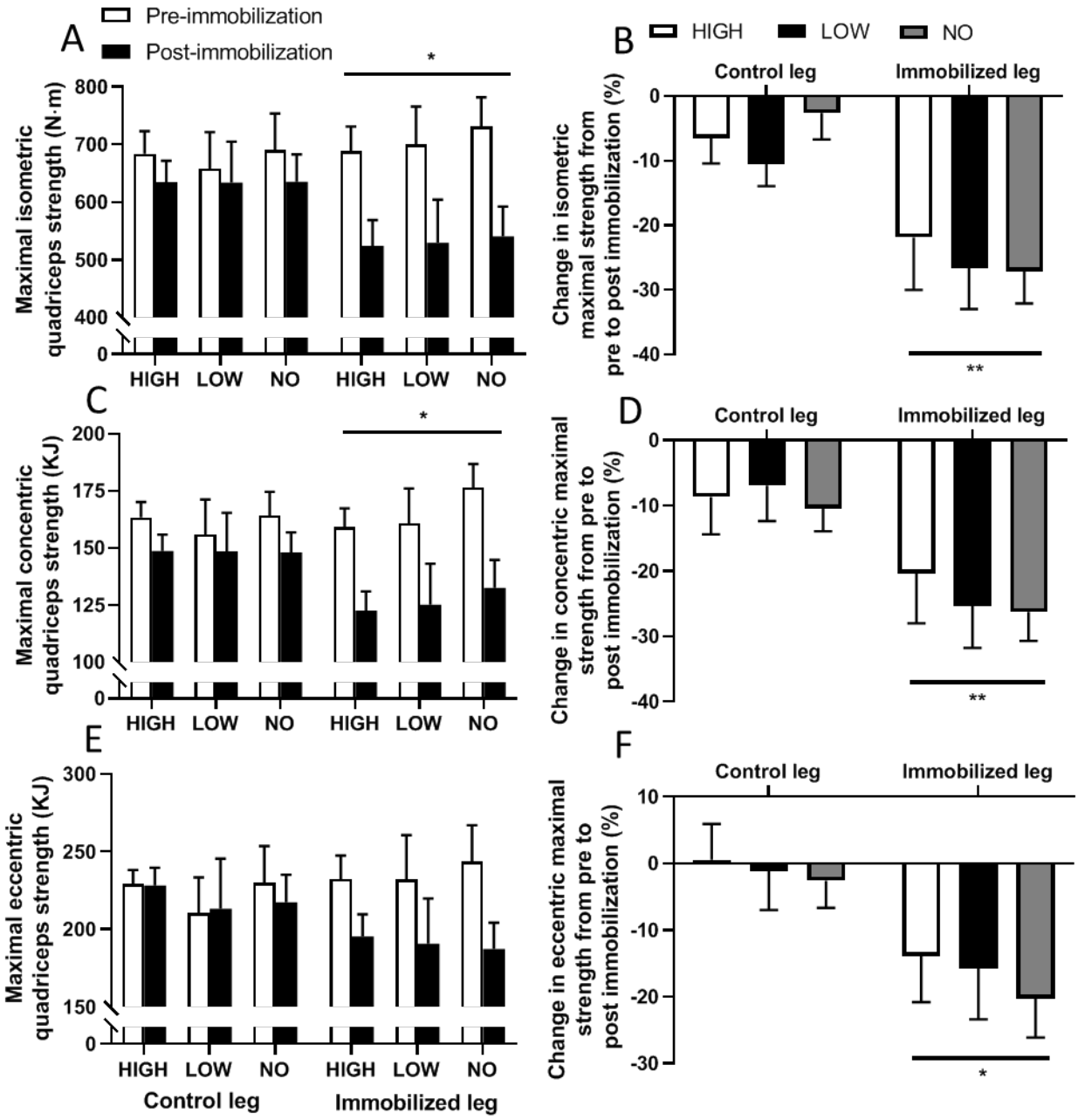

A
Pre-immobilization
Post-immobilization
Maximal isometric quadriceps strength (N·m)
800
700
600
500
400
0
*
HIGH LOW NO HIGH LOW NO
B
HIGH LOW NO
Control leg
Immobilized leg
Change in isometric maximal strength from pre to post immobilization (%)
0
-10
-20
-30
-40
**
C
Maximal concentric quadriceps strength (KJ)
200
175
150
125
100
0
*
HIGH LOW NO HIGH LOW NO
D
Control leg
Immobilized leg
Change in concentric maximal strength from pre to post immobilization (%)
0
-10
-20
-30
-40
**
E
Maximal eccentric quadriceps strength (KJ)
300
250
200
150
0
HIGH LOW NO HIGH LOW NO
Control leg
Immobilized leg
F
Control leg
Immobilized leg
Change in eccentric maximal strength from pre to post immobilization (%)
10
0
-10
-20
-30
*

Figure 4

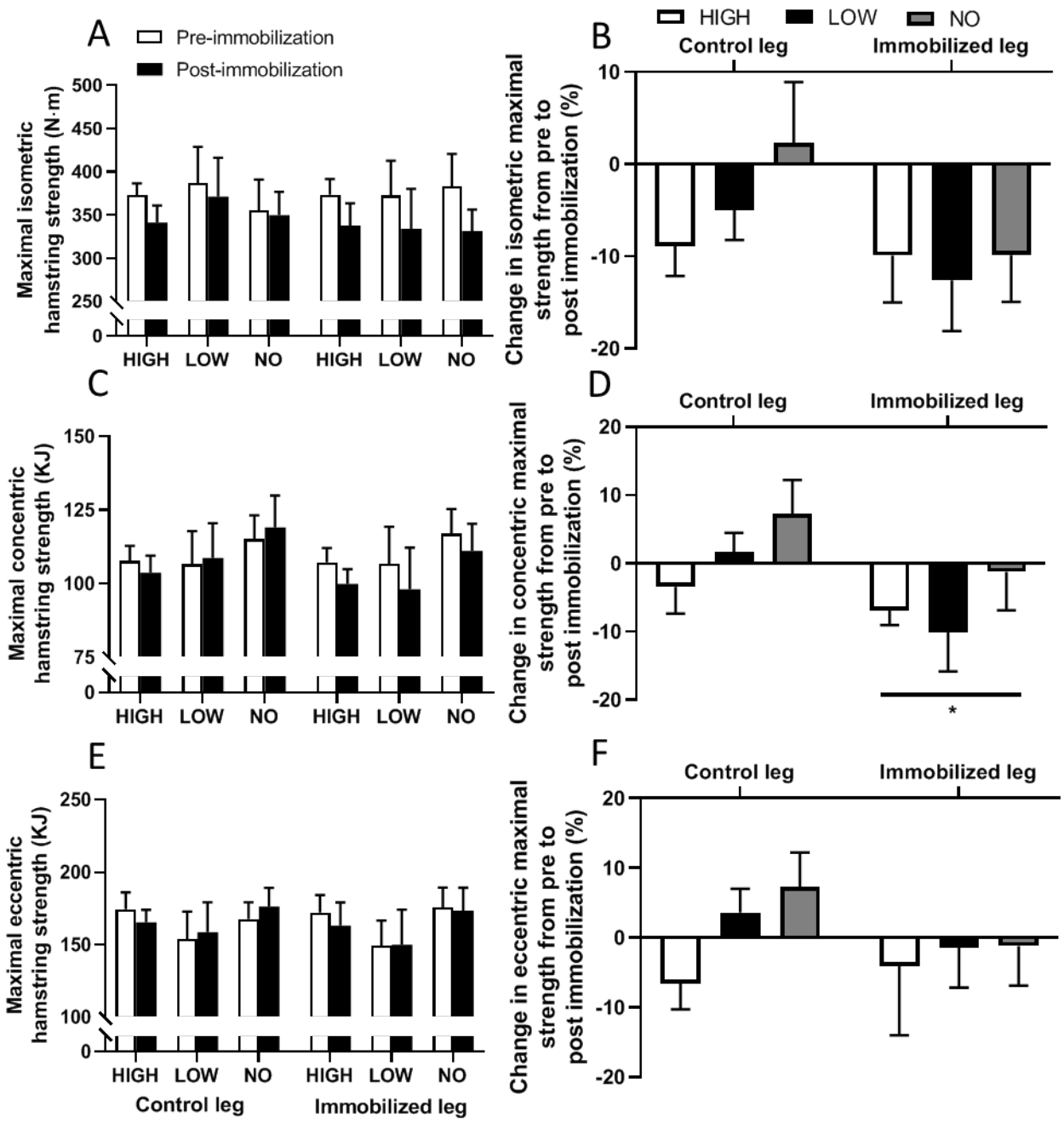

A
Pre-immobilization
Post-immobilization
Maximal isometric hamstring strength (N·m)
500
450
400
350
300
250
0
HIGH LOW NO HIGH LOW NO
B
HIGH LOW NO
Control leg
Immobilized leg
Change in isometric maximal strength from pre to post immobilization (%)
10
0
-10
-20
C
Maximal concentric hamstring strength (KJ)
150
125
100
75
0
HIGH LOW NO HIGH LOW NO
D
Control leg
Immobilized leg
Change in concentric maximal strength from pre to post immobilization (%)
20
10
0
-10
-20
*
E
Maximal eccentric hamstring strength (KJ)
250
200
150
100
0
HIGH LOW NO HIGH LOW NO
Control leg
Immobilized leg
F
Control leg
Immobilized leg
Change in eccentric maximal strength from pre to post immobilization (%)
20
10
0
-10
-20

Figure 5

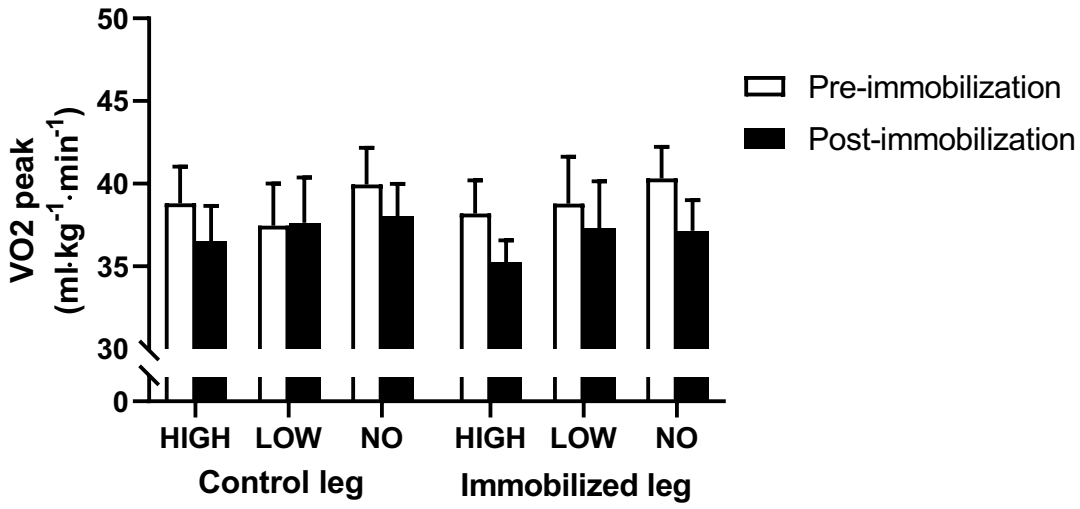

50
45
40
35
30
0
VO2 peak
(ml·kg-1·min-1)
HIGH
LOW
NO
HIGH
LOW
NO
Control leg
Immobilized leg
Pre-immobilization
Post-immobilization

Figure 6

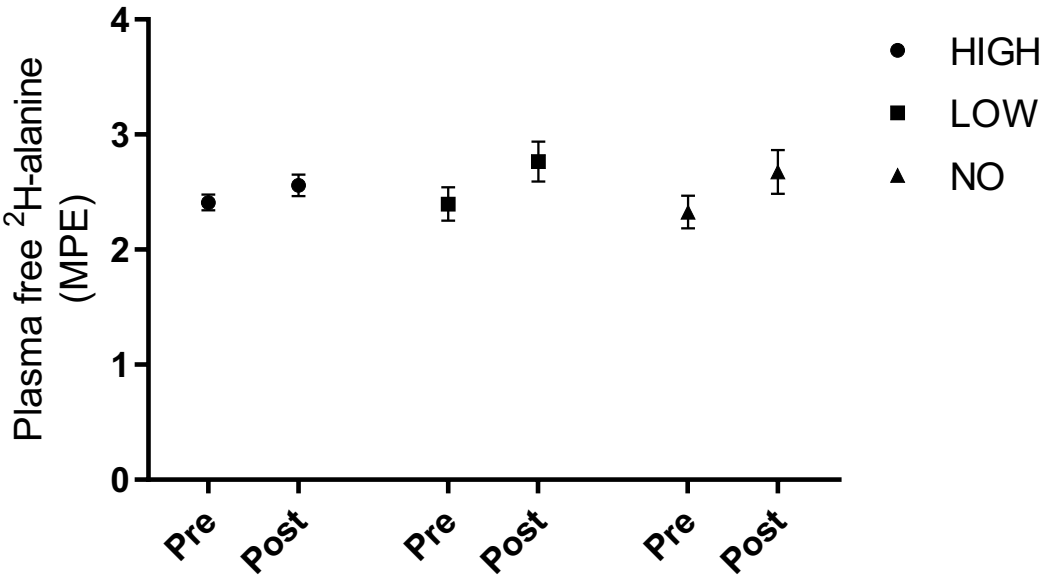
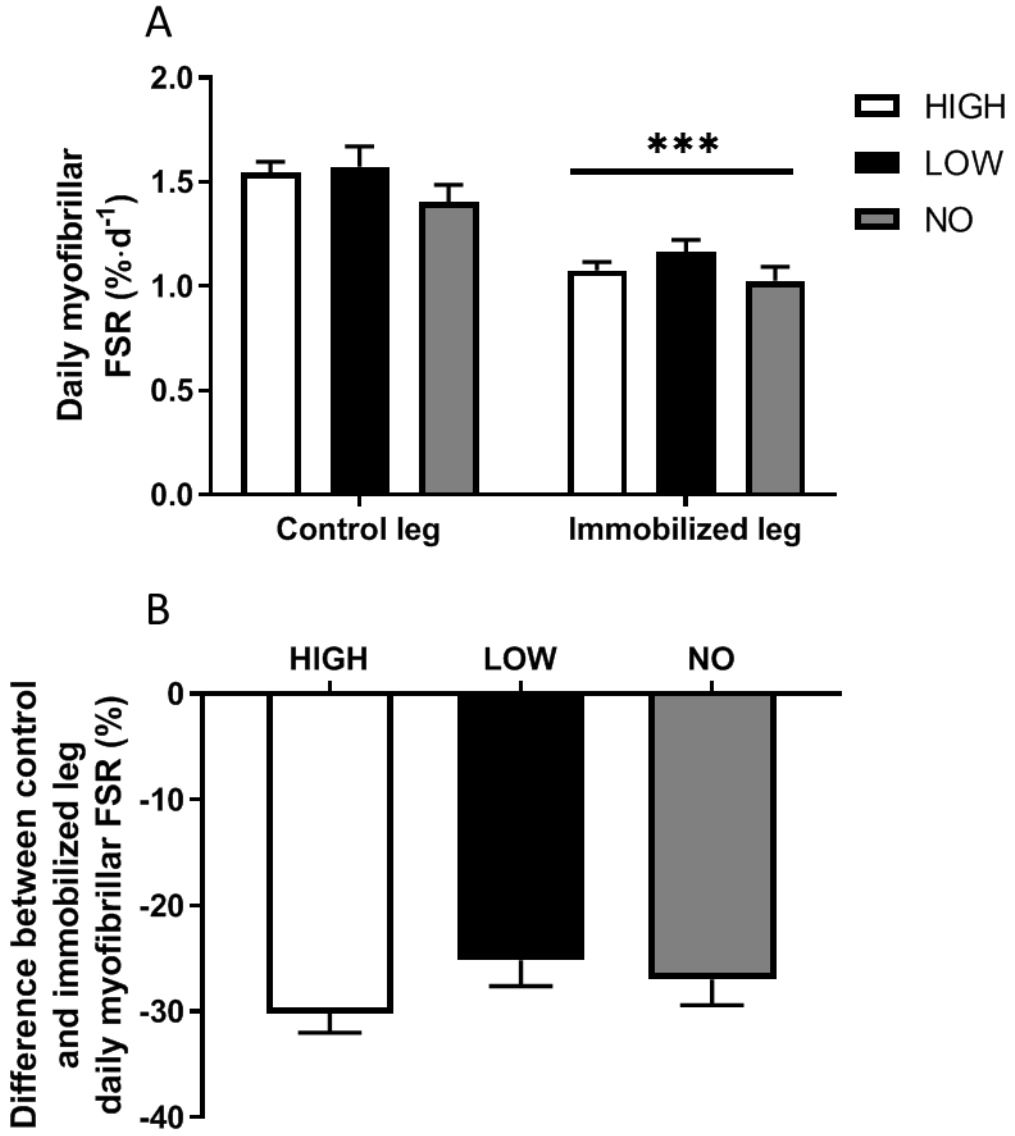

Plasma free ^{2}H-alanine (MPE)
4
3
2
1
0
Pre
Post
Pre
Post
Pre
Post
HIGH
LOW
NO

Figure 7


A
2.0
1.5
1.0
0.5
0.0
Daily myofibrillar FSR (%·d-1)
***
HIGH
LOW
NO
Control leg
Immobilized leg
B
HIGH
LOW
NO
0
-10
-20
-30
-40
Difference between control and immobilized leg daily myofibrillar FSR (%)