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DOI:
10.1249/MSS.0000000000002034

Document Version
Peer reviewed version

Citation for published version (Harvard):
Shad, B, Thompson, J, Holwerda, A, Stocks, B, Elhassan, Y, Philp, A, van Loon, L & Wallis, G 2019, 'One week of step reduction lowers myofibrillar protein synthesis rates in young men', *Medicine and Science in Sports and Exercise*, vol. 51, no. 10, pp. 2125-2134. https://doi.org/10.1249/MSS.0000000000002034

One week of step reduction lowers myofibrillar protein synthesis rates in young men

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Running title: Step reduction and muscle protein synthesis

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ABSTRACT

PURPOSE: Across the lifespan, physical activity levels decrease and time spent sedentary typically increases. However, little is known about the impact that these behavioural changes have on skeletal muscle mass regulation. The primary aim of this study was to use a step reduction model to determine the impact of reduced physical activity and increased sedentary time on daily myofibrillar protein synthesis rates in healthy young men. METHODS: Eleven men (22±2 y) completed 7 days of habitual physical activity (HPA) followed by 7 days of step reduction (SR). Myofibrillar protein synthesis rates were determined during HPA and SR using the deuterated water (^2H_2O) method combined with the collection of skeletal muscle biopsies and daily saliva samples. Gene expression of selected proteins related to muscle mass regulation and oxidative metabolism were determined via real time RT-qPCR. RESULTS: Daily step count was reduced by approximately 91% during SR (from 13054±2763 to 1192±330 steps·d^{-1}; P<0.001) and this led to an increased contribution of sedentary time to daily activity (73±6 to 90±3%; P<0.001). Daily myofibrillar protein synthesis decreased by approximately 27% from 1.39±0.32 %·d^{-1} during HPA to 1.01±0.38 %·d^{-1} during SR (P<0.05). MAFbx and myostatin mRNA expression were up-regulated whereas mTOR, p53 and PDK4 mRNA expression were down-regulated following SR (P<0.05). CONCLUSION: One week of reduced physical activity and increased sedentary time substantially lowers daily myofibrillar protein synthesis rates in healthy young men.

KEY WORDS: Skeletal muscle, physical activity, inactivity, sedentary.

INTRODUCTION

Skeletal muscle mass, physical function and metabolic health progressively decline with advancing age. This could be attributed to the gradual reduction in levels of physical activity and/or the increase in sedentary behaviour that typically occurs across the lifespan (1). The importance of physical activity in maintaining skeletal muscle mass and function is well appreciated and recent evidence has implicated sedentariness, distinct from physical activity, as a risk factor for age-related loss of skeletal muscle mass and strength (2). However, the physiological processes which may contribute to the negative consequences of physical inactivity and sedentary behaviour on skeletal muscle mass and function are relatively unknown.

Skeletal muscle mass is governed by overall protein balance, which is determined by rates of muscle protein synthesis and breakdown. Any loss of muscle mass must be explained by an overall negative protein balance (i.e., muscle protein breakdown must exceed muscle protein synthesis). Extreme muscle disuse (i.e., bed rest or limb immobilization), where voluntary muscular contractile activity is essentially removed, results in substantial loss of skeletal muscle mass (3, 4). This is associated with reductions in postabsorptive muscle protein synthesis rates and the development of ‘anabolic resistance’; that is, a reduced stimulation of postprandial muscle protein synthesis (3). On the other hand, the impact of disuse on muscle protein breakdown rates in humans is poorly defined. Whilst disuse models provide invaluable information on the impact of severe muscle unloading, their extreme nature may not accurately reflect typical physically inactive and sedentary lifestyles (5).

Step reduction has been proposed as a model to more accurately examine the underlying physiology of physically inactive and sedentary individuals and also explore the physiological changes that occur when inactivity is enforced by injury, illness and/or other

significant life events (5-7). Step reduction has been shown to reduce insulin sensitivity (8, 9), but few studies have evaluated its impact on skeletal muscle mass regulation. Breen and colleagues reported reduced postprandial myofibrillar protein synthesis rates following 14 days of step reduction in older, overweight individuals (10). These findings are supported by more recent data showing reduced integrated myofibrillar protein synthesis rates throughout a two-week period of step reduction in older, overweight adults (11).

While these findings are important, there are currently no data available on muscle protein synthesis rates over shorter periods of time typically associated with recovery from injury and/or acute illness (i.e., ≤ one week). The above studies also describe responses in older, overweight individuals, but young and older individuals have distinct responses to muscle disuse (12, 13). The influence of step reduction on skeletal muscle mass regulation in young individuals has yet to be studied but is important to characterize to enhance our understanding of the impact of reduced physical activity and increased sedentary behaviour on skeletal muscle mass regulation at various stages across the lifespan.

Accordingly, the primary purpose of the present study was to use the step reduction model to determine the impact of short-term reduced physical activity and increased sedentary time on myofibrillar protein synthesis rates in healthy young men. The deuterated water (^2H_2O) approach was used as it allows myofibrillar protein synthesis rates to be measured under free-living conditions over time frames where quantifiable changes in muscle mass are unlikely to occur, providing important insight into longer-term muscle mass regulation (14-16). It was hypothesized that one week of reduced physical activity and increased sedentary time would reduce daily myofibrillar protein synthesis rates.

METHODS

Participants and ethical approval

Eleven healthy young men participated in the present study which took place between June 2016 and February 2018. All participants were recreationally active and self-reported engaging in structured physical activity ≥ 3 times/week for > 6 months prior to inclusion. Five of the participants reported undertaking only aerobic-based exercise, four of the participants reported undertaking only resistance-based exercise and two of the participants reported undertaking both aerobic and resistance-based exercise. None of the participants were competitive endurance and/or power athletes. The participants' baseline characteristics are presented in **Table 1**. Prior to providing informed written consent, each volunteer was informed of the experimental procedures and potential risks associated with the experimental intervention. Participants were screened prior to inclusion in the study and deemed healthy based on their responses to a general health questionnaire. Exclusion criteria included being a current or recent (last 6 months) smoker, hypertensive (≥ 140/90 mmHg), diagnosed with diabetes and/or suffering from a recent injury. Participants deemed eligible were subsequently fitted with an ActivPAL3^{TM} accelerometer (see *Accelerometry* section below) for 7 days to objectively assess daily step count. Any individual completing <7000 steps·d^{-1} was excluded from participating in the study. The study was approved by the National Research Ethics Service Committee West Midlands, Edgbaston, United Kingdom (Reference: 16/WM/0011) and conformed to standards for the use of human participants in research as outlined in the Declaration of Helsinki. The intervention was registered at clinicaltrials.gov prior to data collection (Identifier: NCT02624011).

Study overview

An overview of the study is presented in **Figure 1**. Following an initial $^{2}H_2O$ dosing day (day -2) and one maintenance day (see *$^{2}H_2O$ dosing protocol* section below), participants completed 7 days of habitual physical activity (HPA) followed by 7 days of step reduction (SR). For the first 7 days, participants were instructed to maintain their habitual physical

activity levels (i.e., regular ambulation and structured physical activity). During SR, participants were instructed to reduce their step count to ~1500 steps·d^{-1}, be as sedentary as possible and refrain from any form of structured physical activity for the remaining 7 days. A target daily step count of ~1500 steps·d^{-1} was set during SR as large-scale global data suggest that the average daily step count for adults is <5000 steps·d^{-1} (17). An ActivPAL3™ accelerometer (see *Accelerometry* section below) was worn throughout HPA and SR to objectively assess physical activity levels and sedentary time. As the ActivPAL3™ accelerometer does not provide visual feedback on daily step count, participants were also provided with a pedometer during the SR period to help prevent their daily step count exceeding the 1500 steps·d^{-1} threshold. A member of the investigative team was on call throughout the SR period to help participants with activities of daily living (e.g., food shopping) that were not practical within the step count parameters set out. Weighed 4-day food diaries were completed during HPA and SR (see *Dietary intake* section below). Muscle biopsies were collected on days 0, 7 and 14, saliva samples were collected daily and an oral glucose tolerance test (OGTT) was conducted on days 7 and 14.

Experimental visits

On the morning of day 0, participants arrived at the laboratory at 08:00 in a fasted state from 22:00 the evening before. After voiding, participants were weighed in light clothing to the nearest 0.1 kg (OHaus, Champ II scales, USA) and height measured to the nearest centimetre (Stadiometer, Seca, UK). Body composition (whole-body fat-free mass and body fat percentage) was subsequently determined by dual-energy X-ray absorptiometry (DXA, Discovery QDR W series; Hologic). Following the DXA scan, a saliva sample (see $^{2}H_2O$ *dosing protocol* section below) was obtained before collection of a muscle biopsy from the *vastus lateralis* muscle. Muscle biopsies were collected using the Bergström needle technique with manual suction under local anaesthesia (1% lidocaine). Muscle biopsy samples were

blotted and any visible fat, blood or connective tissue removed before snap freezing in liquid nitrogen and storing at -80°C for later analysis. Subsequent muscle biopsies (days 7 and 14) were taken from separate incisions in an alternating pattern between legs. Participants then consumed a single maintenance bolus of $^{2}H_2O$ (see *$^{2}H_2O$ dosing protocol* section below) before being fitted with an ActivPAL3^{TM} accelerometer prior to leaving the laboratory.

Following 7 days of HPA, participants returned to the laboratory at 08:00 on day 7, again in a fasted state from 22:00 the evening before. Participants were weighed prior to insertion of a 20G cannula into an antecubital vein to allow for repeated blood sampling during the OGTT. A saliva sample was subsequently obtained before collection of the second muscle biopsy. Following the muscle biopsy, a baseline blood sample was then drawn before participants completed an OGTT. Participants consumed 75 g dextrose as a 25% solution with subsequent blood samples drawn at 30, 60, 90 and 120 minutes to assess postprandial blood glucose, insulin and non-esterified fatty acid (NEFA) concentration responses. Blood samples were collected into EDTA-containing Vacutainers (BD, New Jersey, USA) prior to centrifugation at 1500 g for 15 minutes at 4°C. Aliquots containing plasma were stored at -80°C. Participants remained in a semi-supine position throughout the OGTT and once completed, consumed a single maintenance bolus of $^{2}H_2O$ before leaving the laboratory. Following 7 days of SR, participants arrived at 08:00 in a fasted state for the final laboratory visit (i.e., day 14) which was identical to the experimental protocol completed on day 7.

$^{2}H_2O$ dosing protocol

The $^{2}H_2O$ dosing protocol consisted of one dosing day and 16 maintenance days (14). On day -2, participants completed a $^{2}H_2O$ loading day. Following collection of a background saliva sample, participants were provided with 8 x 50 mL boluses of 70% $^{2}H_2O$ (Cambridge Isotope Laboratories, Massachusetts, USA) to increase deuterium (^{2}H) enrichment in body water to

0.5-1%. Approximately 60-90 minutes was allowed between each bolus to negate side effects (e.g., vertigo, nausea) previously reported upon consumption of large volumes of $^{2}H_2O$ over short periods of time. The $^{2}H_2O$ protocol was well tolerated with none of the participants reporting any adverse effects. For each subsequent day, participants were provided with a daily 50 mL maintenance bolus of $^{2}H_2O$ to consume. Participants were instructed to consume the daily bolus upon waking up to ensure consistency and minimize the risk of missed doses. The time at which each bolus was consumed was recorded and participants were instructed to bring the empty bottles back in on each laboratory visit to measure compliance. All boluses were returned void, suggesting full compliance with the $^{2}H_2O$ protocol.

To measure ^{2}H enrichment in body water, saliva samples were collected daily. Participants lightly chewed a cotton swab until completely saturated with saliva (~2-3 minutes). On days -2, 0, 7 and 14, swabs were collected in the laboratory, immediately placed in a 5 mL syringe and the saliva compressed into sample tubes and stored at -80°C for later analysis. On the remaining days when participants were not in the laboratory, daily saliva samples were collected at home and stored in pre-labelled falcon tubes in the fridge until the next laboratory visit where samples were stored as described above. Participants were instructed to provide their saliva sample at least 2 hours following their last $^{2}H_2O$ bolus and at least 30 minutes after their last meal or drink and to record the time at which the sample was collected.

Accelerometry

During the screening process, participants were fitted with an ActivPAL3^{TM} accelerometer (PAL Technologies Ltd., Glasgow, UK) to assess daily step count. Participants were also fitted with an ActivPAL3^{TM} accelerometer during HPA and SR to objectively assess physical activity levels and sedentary time. The ActivPAL3^{TM} accelerometer was attached to the

anterior of the upper thigh using waterproof dressing. Participants were required to wear the accelerometer at all times except when bathing. Complete 14-day accelerometry data were obtained from all 11 participants over the experimental intervention. During the 7-day period of SR, participants were also provided with a hip-worn pedometer (Yamax Digi-Walker SW-200) which provided visual feedback on their step count to aid compliance with the 1500 steps·d^{-1} requirement. Daily step count from the hip-worn pedometer was recorded by participants before bed. Accelerometry data were downloaded from devices using ActivPAL3^{TM} analysis software (PAL Technologies Ltd., Glasgow, UK, v7.2.32).

Dietary intake

The evening prior to each experimental visit on days 0, 7 and 14, participants received the same standardized meal (~689 kcal, providing ~55 energy% (En%) carbohydrate, ~20 En% protein, and ~25 En% fat). A weighed 4-day food diary was completed over the first 7-day period of HPA and over the second 7-day period of SR to evaluate energy and macronutrient intake. Participants were required to include two week-days and both weekend days in their recordings. Dietary records were analysed using Dietplan software (Forestfield Software Ltd., v6.70.67).

Plasma analyses

Plasma glucose (Glucose Oxidase kit, Instrumentation Laboratories, Cheshire, UK) and NEFA (NEFA kit, Randox, London, UK) concentrations were analysed in duplicate using enzymatic colorimetric assays using an ILAB 650 Clinical Chemistry Analyser (Instrumentation Laboratory, Warrington, UK). Plasma insulin concentrations were determined in duplicate using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen, California, United States, KAQ1251).

Body water ^{2}H enrichment

Body water ^{2}H enrichment was analysed from daily saliva samples collected throughout the study as previously described (14). Briefly, samples were centrifuged at 10000 g and then diluted 70-fold with ddH_2O. Subsequently, small plastic cups holding 4 mg of catalyst (5 % platinum on alumina, 325 mesh, Sigma-Aldrich, St. Louis, USA) were placed inside 3 mL glass vials (Labco Exetainer, Labco limited, Lampeter, UK) and 300 uL of diluted saliva was then added. Air in each vial was simultaneously evacuated and replaced by hydrogen gas. Once prepared, the vials were left at 21 °C for 24 hours for ^{2}H equilibration to occur between the hydrogen gas and the saliva samples. The ^{2}H enrichment of the hydrogen gas was then measured in duplicate on a GC-C-IRMS (Micromass 205 Optima IRMS fitted with a Multiprep and Gilson autoinjector, Micromass UK Limited, 206 Manchester, UK). Standard regression curves were applied to assess the linearity of the mass spectrometer and to account for ^{2}H loss during equilibration.

Myofibrillar bound ^{2}H-alanine enrichment

For measurement of ^{2}H-alanine enrichment in the myofibrillar fractions, ~50 mg wet muscle tissue was hand-homogenized on ice using a pestle in a standard extraction buffer (10 $\mu L \cdot mg^{-1}$). The samples were then spun at 2500 g for 5 minutes at 4ºC. The pellet was washed with 500 µL of ddH_2O and centrifuged at 250 g for 10 minutes at 4ºC. The myofibrillar protein was solubilized by adding 1 mL of 0.3 M NaOH and heating at 50ºC for 30 minutes with vortex mixing every 10 minutes. Samples were centrifuged at 9500 g for 5 minutes at 4ºC, the supernatant containing the myofibrillar proteins was collected and the collagen pellet was discarded. Myofibrillar proteins were precipitated by the addition of 1 mL of 1 M PCA and spinning at 700 g for 10 minutes at 4 ºC. The myofibrillar protein was washed twice with 70% ethanol and hydrolysed overnight in 2 mL of 6 M HCL at 110˚C. The free amino acids

from the hydrolysed myofibrillar protein pellet were dried under a nitrogen stream while being heated to 120 ºC. The free amino acids were then dissolved in 25% acetic acid solution, passed over cation exchange AG 50W-X8 resin columns (mesh size: 100-200, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA), and eluted with 2 M NH_4OH. Thereafter, the eluate was dried, and the purified amino acids were derivatized to their N(O,S)-ethoxycarbonyl ethyl esters. The derivatized samples were measured using a gas chromatography-isotope ratio mass spectrometer (GC-IRMS) (Thermo Fisher Scientific, MAT 253; Bremen, Germany) equipped with a pyrolysis oven and a 60 m DB-17MS column (no. 122-4762; Agilent, Wilmington, DE, USA) and 5 m precolumn. Ion masses 2 and 3 were monitored to determine the $^{2}H/^{1}H$ ratios of myofibrillar protein bound alanine. A series of known standards were applied to assess linearity of the mass spectrometer and to control for the loss of tracer.

Gene expression analysis

Total RNA was isolated from ~20 mg of frozen powdered muscle tissue by homogenising in 1 mL of TRI Reagent (Sigma Aldrich, Gillingham, UK) using an IKA T10 basic ULTRA-TURRAX homogenizer (IKA, Oxford, UK). To achieve phase separation, 200 µL of chloroform was added to each sample followed by vigorous shaking for 15 seconds, 15 minutes at ambient temperature and subsequent centrifugation at 12000 g for 15 minutes at 4°C. The RNA-containing supernatant was then removed and mixed with an equal volume of 2-propanol. RNA was purified on Reliaprep spin columns (Promega, Madison, Wisconsin, USA) using the manufacturer's instructions, which includes a DNase treatment step. A FLUOstar Omega microplate reader (LVis function) was used to determine the RNA concentration and purity of each sample. The ratio of absorbance at 260 nm and 280 nm was ≥ 2.0 for all samples. 900 ng of total RNA was reverse-transcribed to cDNA in 20 µL volumes using the nanoScript 2 RT kit and a combination of oligo(dT) and random primers

(Primerdesign, Southampton, UK) as per the manufacturer's instructions. The resultant cDNA was diluted to 10 ng/mL prior to RT-qPCR analysis. All analysis was performed in triplicate using Primerdesign custom designed primers (Supplementary Table 1) or commercially available 18S, GAPDH, TOP1, B2M and ACTB (Primerdesign Southampton, UK) and Precision plus qPCR Mastermix with low ROX and SYBR (Primerdesign Southampton, UK) on a QuantStudio3 Real-Time PCR System (Applied Biosystems, Thermo Fisher, UK). Dependent on the gene of interest, 10-50 ng of cDNA was added to each well in a 20 uL reaction volume. Thermal cycling conditions were 2 minutes at 95°C and 40 cycles of 10 seconds at 95°C and 60 seconds at 60°C. A post qPCR run melt curve (Applied Biosystems, Thermo Fisher, UK) was used to ascertain the specificity of each primer. qPCR results were analysed using Experiment Manager (Thermo Fisher). mRNA expression values are expressed as fold change relative to the average baseline (i.e., HPA) ΔCQ value using the $2^{-\Delta\Delta CQ}$ method (18). To control for RNA input, the geometric mean of the CQ values for TOP1, B2M and ACTB was used as an internal control as these were found to be the three most stable genes across all samples using RefFinder (RefFinder, RRID:SCR_000472) (19). All gene expression data are presented for n=10 as insufficient muscle tissue was available for RNA isolation for one participant. Statistical analysis was performed on the $2^{-\Delta\Delta CQ}$ transformed data.

Calculations

Total area under the curve (AUC) for plasma glucose and insulin concentrations was calculated using the trapezoidal method. The Matsuda index, an index of whole-body insulin sensitivity, was calculated as previously described (20). Myofibrillar protein fractional synthetic rate (FSR) was determined using the incorporation of ^{2}H-alanine into myofibrillar protein and the mean ^{2}H enrichment in body water between sequential biopsies, corrected by

a factor of 3.7, as the surrogate precursor based upon ^{2}H labelling during *de novo* alanine synthesis (14, 15). The standard precursor-product method was used to calculate FSR:

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

where E_{m1} and E_{m2} are the myofibrillar protein-bound ^{2}H-alanine enrichments between sequential muscle biopsies. $E_{precursor}$ represents the mean body water ^{2}H enrichment between sequential biopsies corrected by a factor of 3.7 based upon the ^{2}H labelling of alanine during *de novo* synthesis (14). *t* represents the time between sequential biopsies in days.

Statistics

Based on previous research (15), sample size calculations showed that n=9 would be sufficient to detect a difference in daily myofibrillar protein synthesis rates between HPA and SR conditions using a two-tailed paired samples t-test (95% power, α-level of 0.05, G*power version 3.1.9.2). Allowing for a 20% dropout rate, eleven participants were recruited. All statistical analyses were performed using SPSS 22.0 (SPSS, RRID:SCR_002865, Chicago, IL, USA). Differences between conditions (HPA vs. SR) for accelerometry, dietary intake, plasma insulin and glucose AUC, Matsuda index, myofibrillar protein FSR and gene expression were compared using paired sample t-tests. Body water ^{2}H enrichment was analysed using a one-factor repeated measures ANOVA with time as the within-subjects factor. A two-factor repeated measures ANOVA (condition x time) with condition (HPA vs. SR) and time (0, 30, 60, 90 and 120 minutes) as within-subjects factors was performed for analysis of plasma glucose, insulin and NEFA concentrations. Bonferroni post-hoc tests were conducted to correct for multiple comparisons when a significant condition x time interaction was identified. All data are presented as mean±SD.

RESULTS

Accelerometry

Daily step count was reduced by approximately 91% during SR (13054±2763 to 1192±330 steps·d^{-1}; P<0.001). Self-reported pedometer-derived daily step count during SR (1312±297 steps·d^{-1}) was highly correlated with accelerometer-derived daily step count (r = 0.851; P=0.001). The percentage of total time spent sedentary (73±6 to 90±3%; P<0.001) increased and percentage of total time spent standing (17±6 to 8±3%; P<0.001) and ambulatory (10.0±1.0 to 1.0±0.5%; P<0.001) decreased during SR. The number of daily transitions from a sitting to standing position was also significantly reduced during SR (46±8 to 31±10; P<0.001).

Body weight and dietary intake

Body weight was not different following HPA and SR (75.3±11.0 to 75.1±10.8 kg; P>0.05). Dietary intake during HPA and SR is presented in **Table 2**. Daily energy intake tended to decrease (P=0.07) whereas both daily protein intake (P<0.01) and protein intake relative to body weight (P<0.01) significantly decreased during SR. However, absolute carbohydrate and fat intake and the relative contribution of protein, carbohydrate and fat to overall energy intake were unchanged across the intervention (P>0.05).

Oral glucose tolerance

Following SR, fasting plasma glucose concentrations were unaltered (P>0.05; **Figure 2A**), whereas fasting plasma insulin concentrations increased (P<0.05; **Figure 2C**). In response to the OGTT, a significant main effect for time (P<0.001) was observed, with plasma glucose concentrations elevated at 30 minutes compared to all other time points (P<0.01), at 60 minutes compared to baseline and 120 minutes (P<0.05), and at 90 minutes compared to 120 minutes (P<0.05; **Figure 2A**). Plasma glucose AUC (703±118 to 788±79 mmol·120 min·L^{-1})

was not significantly altered by 7 days of SR (P>0.05; **Figure 2B**). In contrast, a significant condition x time interaction (P<0.01) was observed for plasma insulin, with greater plasma insulin concentrations at 60, 90 and 120 minutes of the OGTT following 7 days of SR (P<0.05; **Figure 2C**). In line with these findings, plasma insulin AUC (4590±1817 to 6287±1363 μIU·120 min·mL^{-1}) was significantly greater following SR (P<0.01; **Figure 2D**), corresponding with a decrease in the Matsuda index (6.5±1.8 to 4.5±0.7) (P<0.01; **Figure 2F)**. A significant main effect for time (P<.001) was also observed for plasma NEFA concentrations, with baseline values being significantly higher at baseline compared to all other time points (P<0.05), at 30 minutes compared to 60, 90 and 120 minutes (P<0.001) and at 60 minutes compared to 90 minutes (P<0.05; **Figure 2E**).

Body water ^{2}H enrichment

Figure 3A presents the mean body water ^{2}H enrichment on a day-by-day basis. Following the loading phase on day -2 and a single maintenance day on day -1, body water ^{2}H enrichment reached 0.54±0.09% (day 0). Body water ^{2}H enrichment did not change significantly over the duration of the study with an average body water ^{2}H enrichment of 0.59±0.12% during HPA and 0.64±0.17% during SR (P>0.05).

Myofibrillar protein synthesis

As shown in **Figure 3B**, daily myofibrillar protein synthesis rates decreased by approximately 27% from 1.39±0.32 %·d^{-1} during HPA to 1.01±0.38 %·d^{-1} during SR (P<0.05).

Gene expression

The skeletal muscle mRNA expression of genes implicated in muscle mass regulation and oxidative metabolism is presented in **Figure 4**. In relation to the regulation of muscle protein

synthesis, myostatin mRNA expression was increased following SR and this was paralleled by reduced mTOR mRNA expression (both $P<0.05$; **Figures 4C and 4D**). However, p70S6K mRNA expression was unchanged following SR ($P>0.05$; **Figure 4E**). In regards to muscle protein breakdown, MuRF1 mRNA expression was unchanged ($P>0.05$; **Figure 4A**), whereas MAFbx mRNA expression was up-regulated following SR ($P<0.05$; **Figure 4B**). p53 and PDK4 mRNA expression both decreased following SR (both $P<0.05$; **Figures 4F and 4G**) with no change in PGC-1α mRNA expression ($P>0.05$; **Figure 4H**).

DISCUSSION

The major novel finding of the present study was that one week of reduced physical activity and increased sedentary time led to a substantial (~27%) decline in daily myofibrillar protein synthesis rates. This decline in myofibrillar protein synthesis was associated with increased skeletal muscle mRNA expression of myostatin and MAFbx and decreased mRNA expression of mTOR. The present findings also show that one week of reduced physical activity and increased sedentary time led to a decline in whole-body insulin sensitivity, in addition to decreasing skeletal muscle mRNA expression of selected genes related to oxidative metabolism (i.e., PDK4 and p53). Together, these findings provide direct evidence that reduced physical activity and increased sedentary time alters the physiological processes which regulate skeletal muscle in healthy young individuals.

Across the lifespan, physical activity levels generally decrease and time spent sedentary typically increases (1). Likewise, injury, illness and/or other significant life events often necessitate short periods (typically 2-7 days) of reduced physical activity and increased sedentariness (6, 7). The findings of the present study demonstrate for the first time that just one week of reduced physical activity and increased sedentary time leads to significant (~27%) declines in daily myofibrillar protein synthesis rates in young healthy individuals

(**Figure 3B**). These findings extend previous observations of reduced postprandial and integrated myofibrillar protein synthesis rates following two weeks of step reduction in older, overweight adults (10, 11) and highlights the central role that day-to-day muscular contractile activity plays in regulating muscle protein synthesis rates. Promotion of regular physical activity and minimising sedentariness throughout the lifespan should be considered as integral to the maintenance of skeletal muscle health.

The findings of McGlory and colleagues are most comparable as they also applied $^{2}H_2O$ to measure daily myofibrillar protein synthesis rates (11). The ~27% decline in daily myofibrillar protein synthesis observed in the present study is substantially greater than the ~12% decline in integrated myofibrillar protein synthesis rates observed by McGlory and colleagues (11). This may be related to the greater relative change in daily step count induced by this step reduction intervention (~91%) compared to McGlory et al. (~70%) (11). Alternatively, this discrepancy could be explained by the duration of step reduction or differences in the populations studied (i.e., younger vs. older adults). For example, some, but not all, human muscle disuse studies have shown that 5-14 days of limb immobilization results in greater loss of muscle mass in younger individuals when compared to older individuals (12, 21). Thus, it could be hypothesized that a similar pattern of response is seen from the perspective of muscle protein synthesis, whereby younger individuals are more susceptible to changes in physical activity status than older individuals. In this regard, an older comparator group to directly assess age-related differences in the present study would have been informative and is an important avenue for future research.

A number of factors including habitual physical activity (10), diet composition (22), energy balance (23) and sleep (24) can influence day-to-day muscle protein synthesis rates. It is also well established that dietary protein/amino acid administration robustly stimulates muscle protein synthesis (25-27). Whilst dietary protein intake decreased from habitual levels during

SR, it is important to note that participants were still consuming 133±13 $g \cdot d^{-1}$ of dietary protein during the SR period **(Table 2)**. When expressed relative to body weight, this equates to a protein intake of 1.8±0.2 $g \cdot kg^{-1} \cdot d^{-1}$. This intake is well above the established recommended dietary allowance for protein of 0.8 $g \cdot kg^{-1}$ of body weight and is also greater than recently proposed changes to those recommendations (i.e., 1.2-1.6 $g \cdot kg^{-1}$ of body weight) (28). Energy balance can also influence muscle protein synthesis rates, with studies showing that energy restriction reduces myofibrillar protein synthesis rates in young and older individuals (23, 29, 30). In the present study, daily energy intake tended (P=0.07) to decrease during SR but body weight remained stable, suggesting that participants were not in negative energy balance. Whilst it is unlikely that modifications in dietary protein and/or energy intake contributed to the decline in daily myofibrillar protein synthesis rates during one week of SR, these factors and other dietary related variables (e.g., protein distribution across the day) cannot be completely ruled out and thus future studies should investigate the independent and combined impact of these variables on muscle mass regulation.

Previous studies that employed stable isotope infusion protocols within a laboratory setting provide some insight into what could explain the reduction in daily myofibrillar protein synthesis rates observed herein. For example, the reduction in regular muscular contractile activity undoubtedly contributed given that physical activity acts synergistically to enhance the muscle protein synthetic response to dietary protein/amino acids (24, 31, 32). In addition, two weeks of step reduction has previously been shown to induce the development of 'anabolic resistance' in older adults and thus it is possible that a similar phenomenon was captured in the long-term measurement of myofibrillar protein synthesis rates in the present study (10).

The precise acute metabolic mechanisms underpinning the step reduction-induced decline in myofibrillar protein synthesis rates remain to be confirmed in a younger population.

Nonetheless, in the present study, a coordinated up-regulation of myostatin expression and down-regulation of mTOR expression was observed in skeletal muscle following one week of SR **(Figures 4C and 4D)**. These findings are relatively consistent with previous studies that have observed increased myostatin expression following human muscle disuse (33, 34). Myostatin negatively regulates muscle mass in part via inhibition of the mechanistic target of rapamycin (mTOR), a key regulator of muscle protein synthesis (35). Heightened mRNA expression of myostatin in conjunction with lowered mRNA expression of mTOR is therefore entirely consistent with the observed reduction in myofibrillar protein synthesis rates.

To gain further insight into the impact of short-term reduced physical activity and increased sedentary time on muscle mass regulation, the gene expression of putative markers of muscle protein breakdown was also determined. Muscle-specific E3 ubiquitin ligases (e.g., muscle atrophy F-box (MAFbx) and muscle RING finger 1 (MuRF1)) selectively target muscle proteins for degradation via the 26S proteasome (36). In the present study, an increase in MAFbx expression was observed whereas MuRF1 expression remained unchanged **(Figures 4A and 4B)**. The disparity in the responsiveness of the E3 ubiquitin ligases to step reduction is intriguing but has been reported previously following bed rest and limb immobilization in humans (13, 34). It is possible that the observed decrease in myofibrillar protein synthesis rates was matched by a similar decrease in muscle protein breakdown, reflecting a reduced muscle protein turnover, although the increase in MAFbx expression following step reduction does not support this notion. However, this observation represents a single time point and may not necessarily reflect dynamic changes that occurred throughout the entire step reduction period. Clearly further research is required to provide greater insight into the relative importance of muscle protein breakdown in the context of reduced physical activity and increased sedentary time.

In line with previous findings, one week of reduced physical activity and increased sedentary time led to a decline in whole-body insulin sensitivity **(Figure 2)**. The increased plasma insulin response **(Figures 2C and 2D)**, without a significant change in the plasma glucose response to the OGTT **(Figure 2A and 2B)**, supports previous findings in young individuals (37) and likely represents a compensatory mechanism in order to maintain glycaemic control. This is in contrast to longer-term (2 weeks) step reduction, where both plasma glucose and insulin concentrations appear to be elevated in response to an OGTT (11).

The absence of muscle mass measures following step reduction may be considered a limitation of the present investigation. However, recent evidence has shown that myofibrillar protein synthesis rates measured using $^{2}H_2O$ are predictive of long-term changes in skeletal muscle mass (38). Thus, it is possible that the observed decline in daily myofibrillar protein synthesis would contribute to loss of muscle mass with chronic reduced physical activity and increased sedentary time. It should also be noted that structured physical activity was reduced *and* sedentary time was increased in the present study, precluding any conclusions being made on the independent impact of either of these distinct behaviours. However, given that a large proportion of the global population are both physically inactive and highly sedentary (1), the present findings are highly relevant. Finally, physical activity levels tend to be lower in women compared to men and thus future research utilising a similar study design in women is warranted (17).

In conclusion, one week of step reduction lowers daily myofibrillar protein synthesis rates and alters the expression of several genes within skeletal muscle related to muscle mass regulation and oxidative metabolism in healthy young men. Promotion of regular physical activity and minimising sedentariness throughout the lifespan should be considered as essential to the preservation of skeletal muscle health.

ACKNOWLEDGEMENTS

We would like to acknowledge Dr Helen Bradley and Dr Matthew Soden (both University of Birmingham, United Kingdom) for their assistance with blood sample analysis, Joy Goessens and Annemie Gijsen (both Maastricht University, The Netherlands) for their technical assistance with muscle sample analysis and Nina Salman for her assistance with body composition analysis. Finally, we thank the study volunteers for their willingness to take part in the study. B.J.S is funded by a University of Birmingham 'Exercise as Medicine' PhD studentship.

CONFLICTS OF INTEREST

None of the authors have any conflicts of interest or financial disclosures to declare. The results of the present study are presented clearly, honestly, and without fabrication, falsification, or inappropriate data manipulation and do not constitute endorsement by the American College of Sports Medicine.

AUTHOR CONTRIBUTIONS

B.J.S., L.J.C.v.L., J.L.T., and G.A.W. conception and design of research; B.J.S., Y.S.E., and G.A.W. performed experiments; B.J.S., A.M.H., and B.S. analysed samples; B.J.S. and G.A.W. prepared figures and drafted manuscript; B.J.S., A.M.H., B.S., Y.S.E., A.P., L.J.C.v.L., J.L.T., and G.A.W. edited and revised manuscript; B.J.S., A.M.H., B.S., Y.S.E., A.P., L.J.C.v.L., J.L.T., and G.A.W. approved final version of manuscript.

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664 **Table 1.** Participant characteristics at baseline

Variable	Value
Age (y)	22.2 ± 2.2
Height (m)	1.77 ± 0.08
Body mass (kg)	74.0 ± 11.0
BMI ($kg \cdot m^{-2}$)	23.4 ± 2.4
Body fat (%)	18.6 ± 3.2
Whole body FFM (kg)	60.0 ± 7.2
Leg FFM (kg)	20.2 ± 2.4

665 Values are mean±SD. n=11. BMI, body mass index; FFM, fat-free mass.

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669 **Table 2.** Dietary intake during habitual physical activity (HPA) and step reduction (SR)

Variable	HPA	SR
Energy intake (kcal·d^{-1})	2625 ± 732	2380 ± 864
Protein (g·kg^{-1}·d^{-1})	2.1 ± 0.7	1.8 ± 0.6*
Protein intake (g·d^{-1})	156 ± 51	133 ± 45*
Carbohydrate intake (g·d^{-1})	297 ± 142	279 ± 165
Fat intake (g·d^{-1})	83 ± 34	77 ± 33
Protein (En%)	26 ± 13	24 ± 12
Carbohydrate (En%)	46 ± 13	46 ± 12
Fat (En%)	28 ± 9	29 ± 10

670 Values are mean±SD. n=11. *(P<0.01) indicates a significant difference between HPA and SR
671 conditions.

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683 **FIGURE HEADINGS**

684 **Figure 1.** Study overview.

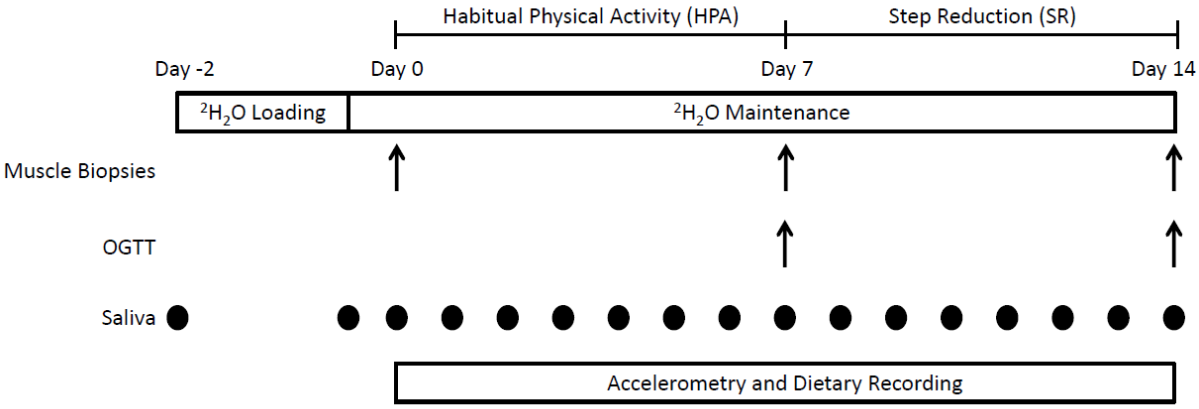
685 **Figure 2.** Plasma metabolite responses to an oral glucose tolerance test (OGTT) following 7
686 days of habitual physical activity (HPA) and 7 days of step reduction (SR) in young males
687 (n=11). Data are mean±SD. A two-factor repeated measures ANOVA was performed for
688 analysis of plasma glucose (A), insulin (C) and NEFA (E) responses to the OGTT. Plasma
689 glucose (B) and insulin (D) area under the curve (AUC) and Matsuda index (F) were
690 analysed using paired sample t-tests. A: significant main effect for time ($P<0.001$), *$P<0.01$
691 compared to all other time points, †$P<0.05$ compared to 0 and 120 minutes, ‡$P<0.05$
692 compared to 120 minutes. B: no significant effect. C: significant condition x time interaction
693 ($P<0.01$), *$P<0.05$ compared with corresponding HPA value. D: *$P<0.01$ compared with
694 HPA. E: significant main effect for time ($P<0.001$), *$P<0.05$ compared to all other time
695 points, †$P<0.001$ compared to 60, 90 and 120 minutes, ‡$P<0.05$ compared to 90 minutes. F:
696 *$P<0.01$ compared with corresponding HPA value.

697 **Figure 3.** Body water ^{2}H enrichment and daily myofibrillar protein fractional synthesis rates
698 (FSR) during 7 days of habitual physical activity (HPA) and 7 days of step reduction (SR) in
699 young males (n=11). Data are mean±SD. Body water ^{2}H enrichment (A) was analysed using
700 a one-factor repeated measures ANOVA. Myofibrillar protein FSR (B) was analysed using a
701 paired sample t-test. Body water ^{2}H enrichment remained in steady state for the duration of
702 the study ($P>0.05$). *($P<0.05$) indicates a significant difference between HPA and SR
703 conditions.

704 **Figure 4.** Skeletal muscle mRNA expression of muscle RING finger 1 (MuRF1; A) muscle
705 atrophy F-box (MAFbx; B), myostatin (C), the mechanistic target of rapamycin (mTOR; D),
706 ribosomal protein S6 kinase beta-1 (p70S6K; E), p53 (F), pyruvate dehydrogenase kinase 4

(PDK4; G) and peroxisome proliferator activated receptor gamma coactivator 1-alpha (PGC-1α; H) following 7 days of habitual physical activity (HPA) and 7 days of step reduction (SR) in young males (n=10). Data are mean±SD. Data were analysed using paired sample t-tests. *($P<0.05$) indicates a significant difference between HPA and SR conditions.

730 **Figure 1.**



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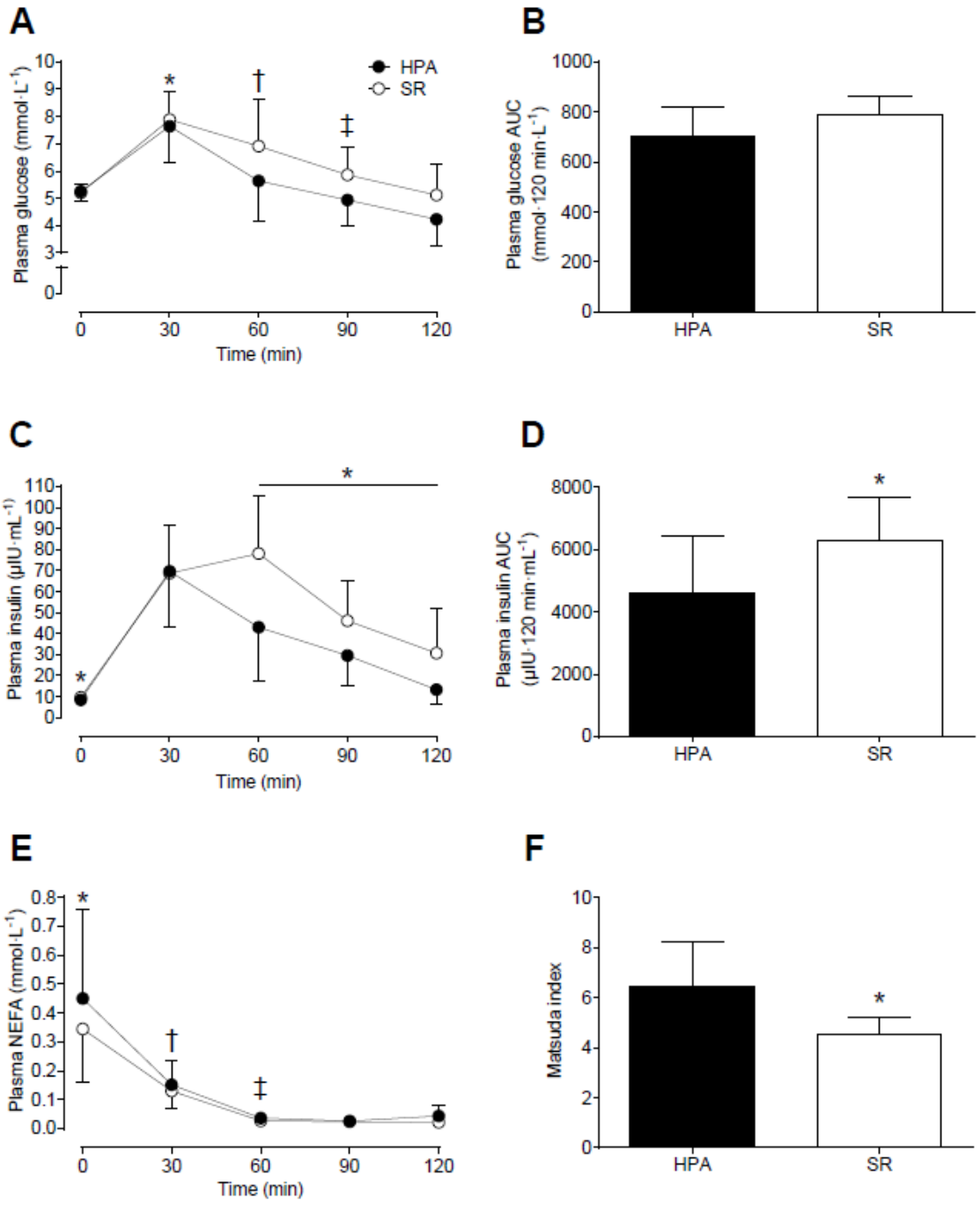
733 **Figure 2.**



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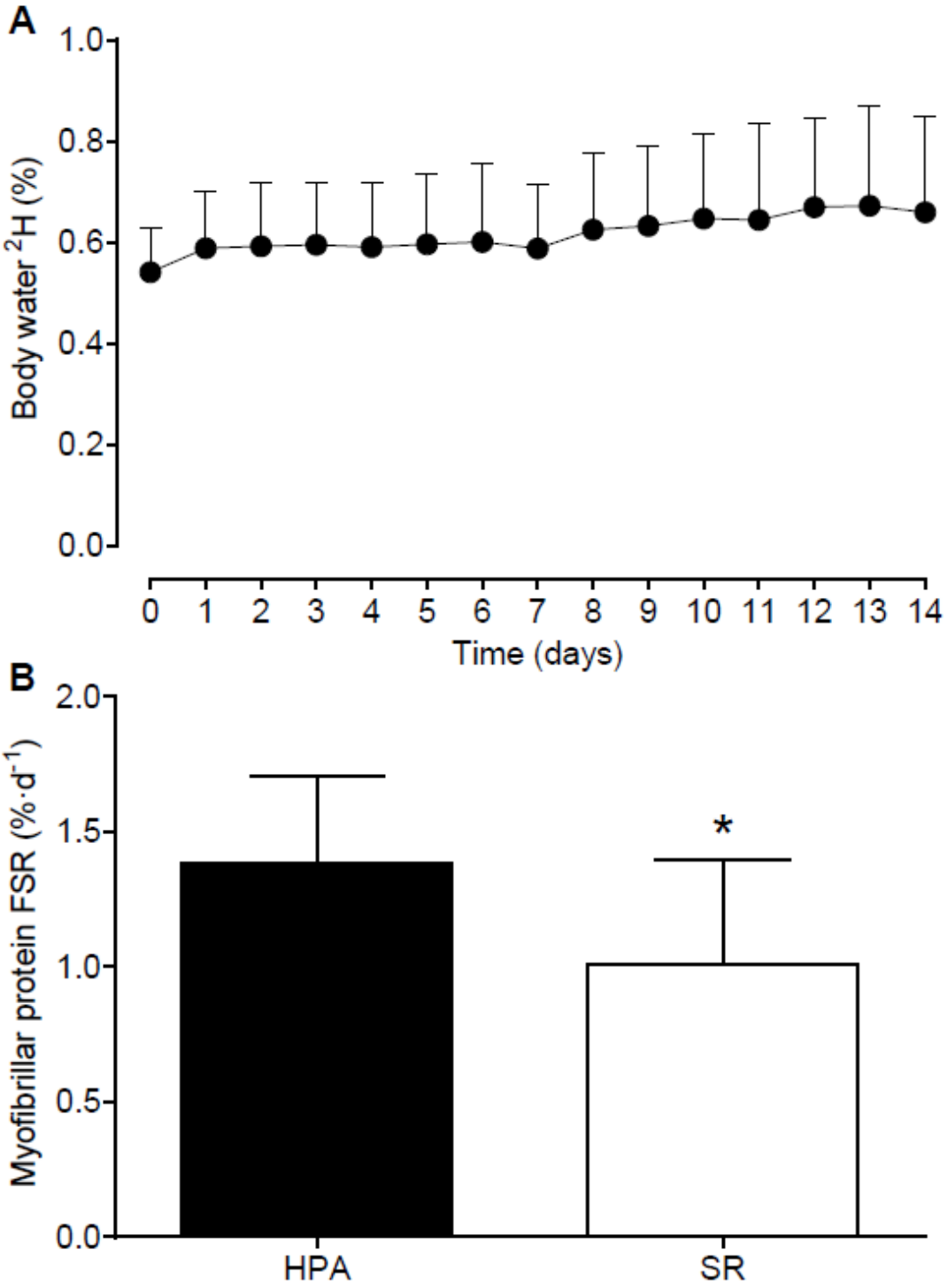
736 **Figure 3.**



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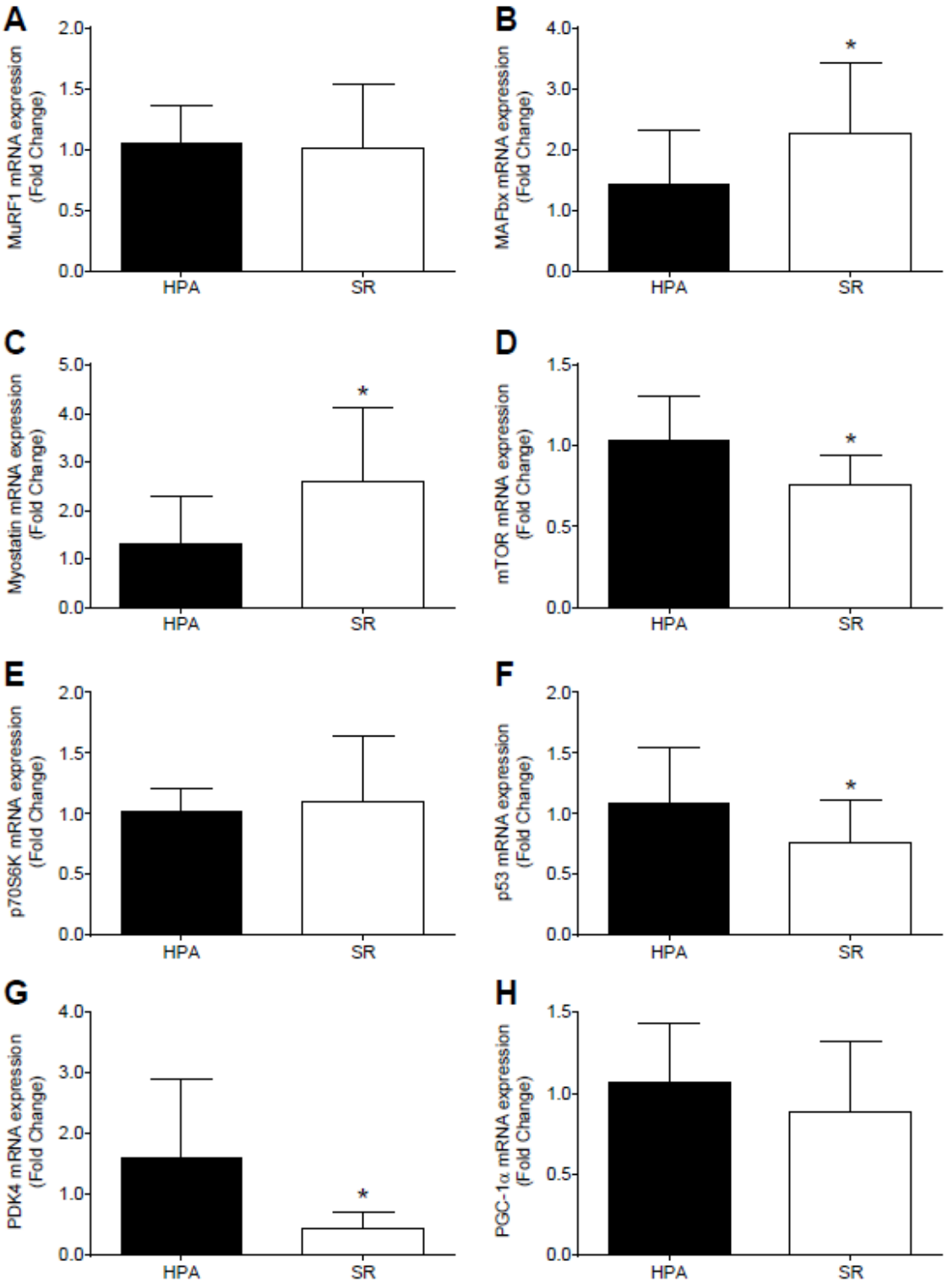
739 **Figure 4.**



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742 **Supplementary Table 1.** RT-qPCR primer sequences

Gene	Forward primer	Reverse primer
MuRF1	5’-GACGCCCTGAGAGCCATT-3’	5’-CCTCTTCCTGATCTTCTTCTTCAAT-3’
MAFbx	5’-AACTCAAATACAAAATAGGACGCTTT-3’	5’-CCTTCGCCTTCTCAAAACAAAC-3’
Myostatin	5’-GTCGAGACTCCTACAACAGTG-3’	5’-TCCAGTATACCTTGTACCGTCTT-3’
mTOR	5’-CTGATGCTGGACCGTCTGA-3’	5’-TCTTGTTAGTCTAAATGGAATCTTCTC-3’
p70S6K	5’-GCAAGCTGGACAAACTATCACA-3’	5’-CCACTGAGATAATACTTGTGCTATAATG-3’
p53	5’-GTGGAGTATTTGGATGACAGAAAC-3’	5’-GTAGTTGTAGTGGATGGTGGTAC-3’
PDK4	5’-GAGGGACACTCAGGACACTTTAC-3’	5’-TGGAGGAAACAAGGGTTCACAC-3’
PGC-1α	5’-TTGCTAAACGACTCCGAGAAC-3’	5’-GACCCAAACATCATACCCCAAT-3’

743 MuRF1, muscle RING finger 1; MAFbx, muscle atrophy F-box; mTOR, the mechanistic target of rapamycin;
744 p70S6K, ribosomal protein S6 kinase beta-1; PDK4, pyruvate dehydrogenase kinase 4; PGC-1α, peroxisome
745 proliferator activated receptor gamma coactivator 1-alpha.