Short-term muscle disuse induces a rapid and sustained decline in daily myofibrillar protein synthesis rates

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Abstract

Introduction: Short-term muscle disuse has been reported to lower both post-absorptive and post-prandial myofibrillar protein synthesis rates. This study assessed the impact of disuse on daily myofibrillar protein synthesis rates following short-term (2 and 7 days) muscle disuse under free living conditions. **Methods**: Thirteen healthy young men (age, 20±1 y; BMI, 23±1 $kg \cdot m^{-2}$) underwent 7 days of unilateral leg immobilization via a knee brace with the non-immobilized leg acting as a control. Four days prior to immobilization participants ingested 400 mL 70% deuterated water, with 50 mL doses consumed daily thereafter. Upper leg bilateral MRI scans and muscle biopsies were collected before, and after 2 and 7 days of immobilization to determine quadriceps volume and daily myofibrillar protein synthesis rates. **Results**: Immobilization reduced quadriceps volume in the immobilized leg by 1.7±0.3 and 6.7±0.6 % after 2 and 7 days, respectively, with no changes in the control leg. Over the one week immobilization period myofibrillar protein synthesis rates were 36±4% lower in the immobilized (0.81±0.04%·d^{-1}) compared with the control (1.26±0.04%·d^{-1}) leg (P<0.001). Myofibrillar protein synthesis rates in the control leg did not change over time (P=0.775), but in the immobilized leg were numerically lower during the 0-2 day period (16±6%, 1.11±0.09%·d^{-1}, P=0.153) and were significantly lower during the 2-7 day period (44±5%, 0.70±0.06%·d^{-1}, P<0.001) when compared with the control leg. **Conclusion**: One week of muscle disuse induces a rapid and sustained decline in daily myofibrillar protein synthesis rates in healthy young men.

Introduction

The recovery from injury or illness requires otherwise healthy individuals to undergo a period of short term (≤ one week) muscle disuse and/or physical inactivity, during which rapid skeletal muscle atrophy and declines in functional and metabolic capacity occur (12, 43). It has also been proposed that the accumulation of such short periods of disuse may contribute to the development of age related sarcopenia (16, 42). Despite the clinical relevance, the physiological mechanisms responsible for muscle disuse atrophy are yet to be fully elucidated. A loss of skeletal muscle mass must ultimately be explained by a chronic alteration in muscle protein synthesis and/or breakdown rates. We (47) and others (8, 17, 18, 20, 21) have shown that experimental muscle disuse results in a decline in post-absorptive muscle protein synthesis rates, without any apparent change in muscle protein breakdown rates (22). More recently, we (47) and others (15) have also reported that disuse brings about a resistance to the anabolic properties of protein ingestion. Consequently, it has been suggested that impairments in post-absorptive and post-prandial muscle protein synthesis rates largely explain (uncomplicated) muscle disuse atrophy in humans (34, 35, 45).

To date, data concerning disuse induced alterations in muscle protein synthesis rates have relied on the continuous infusion of stable isotope labelled amino acids, under laboratory conditions, and their subsequent incorporation into serial muscle biopsies to capture hour-by-hour muscle protein synthesis rates at limited time points before, during or after a disuse intervention. While this allows the controlled assessment of muscle protein synthesis rates, these measurements can only be made over a few hours before potential tracer recycling (i.e. protein bound tracers being released back into the precursor pools) becomes a confounding variable. As a result, these measurements capture only a small snapshot of time (e.g. 1-2%) that a volunteer spends undergoing muscle disuse, and do not account for the combined effects of all lifestyle factors that may contribute to chronically altered muscle protein synthesis rates (e.g. repeated and

varied mixed meal ingestion, whole body and altered physical activity patterns, hormonal and diurnal metabolic fluctuations, etc.). Consequently, it is of importance to establish whether measurements of how disuse affects acute muscle protein synthesis rates translate to daily and free living muscle protein synthesis rates and, therefore, can be quantitatively predictive of muscle disuse atrophy. Moreover, the difficulties in obtaining multiple acute measures of muscle protein synthesis rates within the same individual explains the lack of data fully detailing how muscle protein turnover is temporally regulated during short-term disuse. This is of interest since it has been suggested that both the rate of atrophy and the contribution of alterations in muscle protein synthesis rates may differ in the first two/three days of disuse compared with subsequent time points (2).

Recently the regular ingestion of deuterated water as a means to measure muscle protein synthesis rates has regained popularity in the field due to its capacity to assess free living muscle protein synthesis rates during an intervention lasting days (5, 23, 31, 46) or weeks (9, 33). This methodology has recently been applied to assess cumulative (25) and temporal (9) changes in free living muscle protein synthesis rates throughout resistance training programs of varying durations but, to date, has only minimally been directed at muscle disuse atrophy research (29, 30). In the present work we applied oral deuterated water dosing methods to assess the temporal impact of disuse on free living and daily myofibrillar protein synthesis rates during one week of limb immobilization in healthy young men. We hypothesized that disuse would reduce myofibrillar protein synthesis rates within 2 days, and to a greater extent than after one week, and this would primarily explain the observed rate of muscle disuse atrophy.

Methods

Participants

Thirteen healthy young men (age=20±1 y, BMI=23.4±0.9 kg·m^{-2}) were included in the present study. Only young males were included in the present study since both age (43) and sex (10) can influence the rate of muscle disuse atrophy, and our goal was to maintain a homogeneous population for the current study. 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 kg·m^{-2} 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, consumption of any nutritional supplement prior to and during 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). All subjects were informed of the nature and possible risks of the experimental procedures before providing written informed consent. The study was part of a wider study assessing muscle disuse atrophy that was approved by The Sport and Health Science Ethics committee of the University of Exeter (151021/B/02), in accordance with the guidelines set out in the Declaration of Helsinki. This study was registered as a clinical trial with clinicaltrials.gov (NCT02984332).

Experimental design

A graphical representation of the experimental study design can be seen in **Figure 1**. Following successful completion of the screening visit eligible participants attended the laboratory for 5 experimental visits across 11 days and this included a 7 day period of unilateral leg immobilization using a leg brace and ambulation via the use of crutches. To measure daily myofibrillar protein synthesis rates throughout the immobilization period participants underwent a deuterium oxide dosing protocol (described below). 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 (25). Participants arrived at the laboratory on day 5 of the experimental protocol at ~0800 h for the first of three experimental test days. During this visit, bilateral muscle biopsies were collected from the *m. vastus lateralis* and an MRI scan was conducted of both thigh muscles (at ~0830 h; participants were transported to and from the MRI scanner via a wheelchair to ensure no contraction or weight bearing occurred before/after the MRI scans and biopsies). Thereafter, a 7 day immobilization period was started at ~0900 h. After 2 and 7 days of immobilization subjects returned to the laboratory at ~0800 h for an identical test day. Muscle biopsies were obtained under local anesthesia, using the percutaneous Bergstrom needle biopsy technique (6), from the *m. vastus lateralis* of both legs ~15 cm above the patella and ~3 cm below the fascia. Immediately following a muscle biopsy the muscle tissue was quickly assessed and any blood or non-muscle tissue was dissected and discarded. The muscle samples were immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

Immobilization protocol

The model of immobilization used was a unilateral leg brace (X-ACT Donjoy brace, DJO global, Vista, CA, USA) with the participant ambulating on crutches (after receiving

instruction) throughout the immobilization period. The immobilized leg was randomized and was counterbalanced for leg dominance with the non-immobilized leg acting as a within-subject control. 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. Subjects 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 (24), no participants were excluded. 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.

Deuterated water dosing protocol

The deuterated water dosing protocol was based on our previous work (25). Day 1 of the experimental protocol acted as a D_2O loading day where participants consumed 400 mL 70% D_2O (i.e. 280 mL pure 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. 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). One participant reported some mild feelings of vertigo and dizziness during the loading day which passed after approx. 2 h. Three hours (~1100 h) after the daily D_2O maintenance dose a daily saliva sample was collected using a cotton mouth

swab (Celluron, Hartmann, Germany) which the participant lightly chewed for ~1 min until saturated with saliva. The saturated sponge was placed into an empty syringe where the swab was squeezed to release the saliva into a collection tube. The saliva samples were used to assess the body water ^{2}H enrichment and were stored in the participant's freezer until they were returned at the next study visit. Additional blood samples were collected in the fasted state at the start (i.e. ~0800 h) of each test day (i.e. day 5 [pre], 7 [after 2 days of immobilization] and 12 [post]). Venous blood samples were collected from the antecubital vein via venipuncture technique and collected into EDTA-containing vacutainers which were centrifuged at 4,000 rpm 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.

Diet and physical activity

For 3 days prior to immobilization subjects' physical activity was measured using an accelerometer (GENEactiv, Activinsights, Cambridgeshire, UK) that was attached to the non-dominant wrist. Subjects were instructed to wear the accelerometer continuously with data being collected at a 60 Hz sampling frequency. Subjects' physical activity was measured again throughout the 7 day immobilization period. Subjects' were instructed to refrain from vigorous physical activity during immobilization but to attempt 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 total physical activity (all intensities) using standard cut-off points (11). Subjects' diets were recorded for 3 days (two

week days and one weekend day) prior to immobilization by a self-reported written diet diary following detailed instructions and advice from a member of the research team. Subjects were asked to refrain from alcohol intake and maintain a similar diet during and throughout the immobilization period and this was assessed by two further 2 day diet diaries in the first and last two days of immobilization, these were averaged to create an 'immobilization diet'. Dietary analyses for the calculation of energy and macronutrient intakes were completed using specialized nutrition software (Nutritics Professional Nutritional Analysis Software; Swords, Co. Dublin).

Magnetic resonance imaging and quadriceps volume calculation

MRI scan methodology of the upper legs for the determination of quadriceps volume has been described in detail previously (4). 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, a 4-element sense body radiofrequency coil was wrapped around both thighs. Philips on-line MRI software was used to analyze the images obtained in the axial plane (the same experimenter performed all manual segmentation of the images). Starting at the most distal image (approximately mid patella) where each muscle group could be fully delineated, the muscle of interest was manually segmented to calculate slice cross sectional area. Moving proximally every third image was analyzed until complete delineation of the muscle groups of interest could no longer be identified (the greater trochanter). Muscle volume was calculated using a previously published method (30) where the total CSA for all images was calculated and multiplied by the slice gap plus the distance between slices (linear interpolation) (in this

200 case a total 3 cm, 5 mm slice thickness, 25 mm slice gap), summarized by the following
201 equation:

202 $$muscle\ volume = \sum_{aCSA} \cdot\ (slice\ thickness + slice\ gap)$$

203

204 *Body water deuterium enrichment*

205 Body water deuterium enrichment was measured using the saliva samples collected daily
206 throughout the course of the study. All saliva samples were centrifuged at 10,000 g and were
207 subsequently diluted 70-fold with ddH_2O to lower deuterium enrichments so that they were in
208 the detection range of the isotope ratio mass spectrometer (IRMS). Following the dilution step,
209 samples were prepared for analysis on the IRMS using the protocol described by Scrimgeour
210 et al., (39). In summary, small plastic cups holding 4 mg of 5% platinum on alumina, 325 mesh
211 (Sigma-Aldrich, St. Louis, MO) were placed inside 3 ml glass vials (Labco Exetainer; Labco,
212 Lampeter, UK). Subsequently 300 µL of the diluted saliva samples was placed into the vials,
213 vials were then sealed using rubber septums and a screw cap. The air within each vial was
214 simultaneously evacuated and replaced by hydrogen gas. Vials were then left for 24 h at 21°C
215 for deuterium equilibration between the hydrogen gas and saliva samples to occur. The
216 deuterium enrichment of the hydrogen gas was then measured in duplicate on a IRMS
217 (Micromass Optima IRMS fitted with a multiprep and Gilson auto-injector; Micromass,
218 Manchester, UK). Standard regression curves were applied from a series of known standard
219 enrichment values against the measured values to assess the linearity of the mass spectrometer
220 and to account for deuterium loss during equilibration.

221

222 *Plasma free [^{2}H]alanine enrichments*

223 Plasma amino acid enrichments were determined by gas chromatography-mass spectrometry
224 analysis (GC-MS; Agilent 5975C MSD & 7890A GC, Wilmington, USA). First the plasma

225 samples were deproteinized using dry 5-sulfosalicylic acid. Subsequently free amino acids
226 were purified using cation exchange chromatography (AG 50W-X8 resin, mesh size 100-200
227 μm, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA). The purified amino acids
228 were converted to their *tert*-butyldimethylsilyl (*tert*-BDMS) derivatives with MTBSTFA
229 before analysis via GC-MS. The plasma free alanine mass isotopomers (M and M+1) were
230 measured using selective ion monitoring at *m/z* 232 and 233. Standard regression curves were
231 applied from a series of known standard enrichment values against the measured values to
232 assess the linearity of the mass spectrometer and to account for any isotope fractionation.
233
234 *Myofibrillar bound ^{2}H alanine enrichments*
235 Myofibrillar protein-enriched fraction was extracted from ~50 mg of wet weight muscle tissue
236 by hand-homogenization on ice using a pestle in a standard extraction buffer (10 μl/mg). The
237 samples were centrifuged at 2,500 *g* for 5 min at 4°C and the pellet was then washed with 500
238 μl of ddH2O and again centrifuged at 2,500 *g* for 10 min at 4°C. The myofibrillar protein was
239 solubilized by adding 1 ml of 0.3 M NaOH and heating for 30 min at 50°C with samples being
240 vortexed every 10 min. Samples were then centrifuged for 10 min at 9,500 *g* and 4°C, the
241 supernatant containing the myofibrillar protein was kept and the collagen protein pellet was
242 discarded. The myofibrillar proteins were precipitated by the addition of 1 ml of 1 M PCA and
243 spun at 700 *g* and 4°C for 10 min. Myofibrillar proteins were then washed with 70% ethanol
244 twice and hydrolyzed overnight in 2 mL of 6 M HCL at 110°C. The free amino acids from the
245 hydrolyzed myofibrillar protein pellet were dried under a nitrogen stream while being heated
246 at 120°C. The free amino acids were subsequently dissolved in 25% acetic acid solution and
247 passed over cation exchange AG 50W-X8 resin columns (mesh size: 100-200, ionic form:
248 hydrogen; Bio-Rad Laboratories, Hercules, CA) and eluted with 2 M NH_4OH. Following this
249 the eluted amino acids were dried and the purified amino acids were derivatized to their

N(O,S)-ethoxycarbonyl ethyl esters (28). The derivatized were measured using a gas-chromatograph-isotope ratio mass spectrometer (GC-IRMS; Thermo Fisher Scientific, MAT 253; Bremen, Germany) equipped with a pyrolysis oven and a 60m DB-17MS column (no. 122-4762; Agilent, Wilmington, DE) and a 5 m precolumn. Ion masses 2 and 3 were analyzed to determine the $^{2}H/^{1}H$ ratios of muscle protein-bound alanine. A series of known standards was used to assess the linearity of the mass spectrometer and to control for the loss of tracer.

Skeletal muscle mRNA analyses

Skeletal muscle mRNA expression of 48 genes involved in the regulation of skeletal muscle mass and metabolism were analyzed (gene names and symbols are listed in Table 2) as described previously (11). In brief, total RNA was isolated from ~20 mg of frozen muscle tissue using TRIzol Reagent (Life Technologies, Invitrogen) according to the supplier's instructions. Total RNA was quantified spectrophotometrically at 260 nm and RNA purity was assessed as the ratio of readings at 260/280 nm (Nanodrop ND-1000 Spectrophotometer, Thermo Fisher Scientific). Subsequently first strand cDNA was synthesized from 150 ng of RNA using a SuperScript III cDNA synthesis kit (cat. no. 11752-050; Invitrogen, Life Technologies, CA, USA). Taqman low-density custom designed array cards (ABI Applied Biosystems, Foster City, CA, USA) were used for the relative quantification of expression of the 48 selected genes. Each card ran eight samples simultaneously against 48 Taqman gene expression assay probes that were pre-fixed into each well on the card. In summary, 50 µL of Taqman universal master mix (ABI, Applied Biosystems) was added to 150 ng of RNA equivalent cDNA in an RNase free Eppendorf with RNase free water being added to a make a final reaction volume of 100 µL. Samples were vortexed and centrifuged (briefly) then pipetted into sample reservoirs on the micro fluidic array cards, following this the array cards were centrifuged (1000 rpm for 1 min) (Hereaus 3 S-R Microfuge, Thermo Fisher Scientific,

Waltham, MA, USA) and then run on a QuantStudio 12K Flex Real-Time PCR system (ABI, Applied Biosystems). Relative quantification of the genes was performed using the delta delta Ct method ($2^{-\Delta\Delta Ct}$), data were normalized to the geometric mean of *GAPDH* and *18s* genes (47).

Calculations

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

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

where E_{m1} and E_{m2} are the myofibrillar muscle protein-bound enrichments on either day 0 and 2, 0 and 7, and 2 and 7 respectively. $E_{precursor}$ represents either mean plasma free [^{2}H] alanine or mean body water deuterium enrichment corrected by a factor of 3.7 based on deuterium labelling of alanine during *de novo* synthesis (15, 24) (mean enrichment between day 0-2, 0-7 or 2-7). *t* represents the time between biopsies (day 0-2, 0-7 or 2-7).

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). A paired samples *t*-test was used to compare myofibrillar protein synthesis rates in the control vs immobilized legs between days 0 and 7, and physical activity and dietary intake between pre and during immobilization. Two-way repeated measures ANOVAs with leg (immobilized and control) and time (day 0-2 and day 2-7 [for myofibrillar protein synthesis rates] and days 0, 2 and 7 [for MRI]) as within subjects factors were used to compare differences in myofibrillar protein synthesis rates over time, gene expression and quadriceps volume. Pearson's correlation coefficient was used to

assess the relationship between tracer precursor pools. For all ANOVAs, 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

Diet and physical activity analyses

Table 1 shows participants' habitual dietary intake and physical activity data averaged for 3 days preceding, and for 4 (for dietary intake) and 7 days (for physical activity) during the immobilization period. No differences in energy or macronutrient intake (fat, carbohydrate and protein) were observed between pre and during immobilization (all $P>0.05$). Light and vigorous physical activity were not different between pre and during immobilization ($P>0.05$). Moderate physical activity reduced from pre to during immobilization ($P=0.032$).

Quadriceps muscle volume

Quadriceps muscle volumes of the control and immobilized legs were determined by MRI (previously reported in detail in Kilroe et al, 2019). There were no difference in quadriceps volume between legs pre-immobilization (control leg = 2315±120 cm^3, immobilized leg = 2342±129 cm^3 [$P=0.993$]) and the control leg remained unchanged throughout the study ($P=0.981$). However, a significant time *x* leg interaction was detected ($P<0.001$) such that quadriceps volume of the immobilized leg had decreased by 1.7±0.3% after 2 days (to 2301±127 cm^3; $P=0.007$) and by a further 5.0±0.6% between days 2 and 7 days (to 2186±117 cm^3; $P<0.001$), resulting in a total decrease in quadriceps muscle volume of 6.7±0.6% ($P<0.001$) over the full week of immobilization.

Precursor pool enrichments

Saliva deuterium enrichments (**Figure 2A**) reached 0.69±0.02% on day 0 (start of immobilization), and averaged 0.71±0.02%, 0.78±0.03% and 0.76±0.02% over 0-2, 2-7 and 0-7 time points, respectively. Saliva deuterium enrichments showed a modest increase over the

immobilization period (main effect of time, $P<0.001$) such that enrichments at day 6 (0.80±0.02%, $P=0.024$) and 7 (0.83±0.03%, $P<0.001$) were elevated above day 0, and day 7 enrichments were higher than day 1 (0.70±0.02%, $P=0.003$). Plasma free [^{2}H] alanine enrichments (Figure 2A) reached 2.8±0.1 mole percent excess (MPE) on day 0 and averaged 3.1±0.2 MPE over the week of immobilization. Plasma free [^{2}H] alanine enrichments also showed a slight increase over the immobilization period (main effect of time, $P<0.001$) such that day 2 (3.1±0.1 MPE, $P=0.017$) and day 7 (3.5±0.1 MPE, $P<0.001$) enrichments were higher than day 0 (2.8±0.1 MPE). A Pearson's correlation coefficient showed that the saliva deuterium and the plasma [^{2}H] alanine precursor pools were strongly correlated (data from all time points, 0-2, 2-7 and 0-7 collapsed into one test) (r=0.959, $P<0.001$) (**Figure 2B**). Plasma free [^{2}H] alanine enrichments were, on average, 3.93-fold greater than saliva deuterium enrichments (**Figure 2C**). It should be acknowledged that we have not determined muscle intracellular free [^{2}H] alanine enrichments in the present work. It is conceivable that our intervention may have influenced global muscle alanine metabolism divergently across legs which could affect local precursor enrichments. However, our (27) and others' (9) previous observations that muscle intracellular free [^{2}H] alanine does not differ substantially across legs even when unilateral leg interventions are applied (e.g. daily resistance-type exercise) and correlate tightly with plasma free [^{2}H] alanine enrichments, suggest the latter to be a valid precursor pool.

Daily myofibrillar protein synthesis rates

Myofibrillar protein bound [^{2}H] alanine enrichments showed significant time ($P<0.001$), leg ($P<0.001$) and time *x* leg interaction ($P<0.001$) effects. After 2 days of immobilization, myofibrillar protein bound [^{2}H] alanine enrichments increased by 20±10 % more in the control (0.0695±0.0045 MPE) compared with the immobilized (0.0579±0.0042 MPE) leg ($P=0.511$).

After 7 days, myofibrillar bound protein [^{2}H] alanine enrichments increased over time in both legs but by 53±11 % more in the control (0.2448±0.0096 MPE) compared with the immobilized (0.1596±0.0079 MPE) leg (P<0.001). Daily myofibrillar FSRs (%·d^{-1}) were calculated using mean (of each individual's data during that time period) saliva deuterium enrichments ((corrected by a factor of 3.7 to account for the labeling coefficient between saliva and plasma; (15, 25)) and then separately using mean plasma free [^{2}H] alanine enrichments (again, an individual's own data averaged over the period of interest) as precursor pools over the 0-2, 2-7 and 0-7 time-points of immobilization (**Figure 3**). Over the entire week of immobilization daily myofibrillar FSR, calculated using either the saliva deuterium enrichment (control leg = 1.26±0.05%·d^{-1} and immobilized leg = 0.81±0.04%·d^{-1}) or plasma free [^{2}H] alanine enrichment (control leg = 1.14±0.05%·d^{-1}; immobilized leg = 0.71±0.04%·d^{-1}) as the precursor pools were 36±4% (P<0.001) and 37±4% (P<0.001) lower, respectively, in the immobilized compared with the control leg. A Pearson's r product moment correlation showed a strong correlation between FSR calculated from the two precursor pools with data collapsed for both legs over the full week of immobilization (r = 0.982, P<0.001 [Figure 3C]).

When assessing the temporal changes in myofibrillar FSR over the early (i.e. 0-2 days) and late (i.e. 2-7 days) immobilization periods and using the saliva deuterium enrichment precursor, significant main effects of time (P=0.020), condition (P<0.001) and a time x condition interaction (P=0.046) were detected. Specifically, myofibrillar FSR was 16±6% (P=0.153) and 44±5% (P<0.001) lower at 0-2 (control leg = 1.32±0.08%·d^{-1}; immobilized leg = 1.11±0.09%·d^{-1}) and 2-7 days (control leg = 1.24±0.07%·d^{-1}; immobilized leg = 0.70±0.06%·d^{-1}) in the immobilized compared with the control leg. Myofibrillar FSR remained unchanged over time in the control leg but declined by 37±11% from 0-2 to 2-7 days (P=0.005) in the immobilized leg. When using the plasma free [^{2}H] alanine enrichment to assess temporal changes in myofibrillar FSR over the early (i.e. 0-2 days) and late (i.e. 2-7 days) immobilization

period, significant main effects of time (P=0.013), condition (P<0.001) and a time x condition interaction *(P*=0.048) were detected. Specifically, myofibrillar FSR was 16±6% (P=0.159) and 44±5% (P<0.001) lower at 0-2 (control leg = 1.19±0.07%·d^{-1}; immobilized leg = 1.01±0.08%·d^{-1}) and 2-7 days (control leg = 1.11±0.07%·d^{-1}; immobilized leg = 0.62±0.05%·d^{-1}) in the immobilized compared with the control leg. Myofibrillar FSR remained unchanged over time in the control leg but declined by 38±11 % from 0-2 to 2-7 days (P=0.003) in the immobilized leg. A Pearson's r product moment correlation showed a strong correlation between the two precursor pools for both legs over both the 0-2 and 2-7 day time points (both legs and time periods collapsed into one test) (r=0.995, P<0.001 [Figure 3F]).

Skeletal muscle gene expression

The skeletal muscle expression of genes involved in insulin signaling and muscle protein synthesis/growth factors, muscle protein breakdown, carbohydrate and lipid metabolism, and mitochondrial metabolism/angiogenesis/force transduction are shown in **Figures 5, 6, 7 and 8,** respectively. The remaining genes of the 46 analyzed are shown in **Figure 9**. Thirteen of the analyzed genes (*P70S6K*, *EIF-4EBP1*, *REDD1*, *MTSN*, *GS1*, *HK2*, *FAT*, *CPT1B*, *PPARa*, *DNML1*, *TFAM*, *SCGA*, *ANGPT1*) showed no changes throughout the experiment (time, condition and interaction effects all P>0.05). Twenty genes exhibited a time effect (all P<0.05) only, such that they changed to an equivalent extent (either an increase [*AKT2*, *LAT1*, *PAT1*, *CLPN1*, *GDF11*, *FASN*, *MFN1*, *ITGB1*, *HIF1a*, *DES*, *mTOR*, *SHREBP1*, *BECN1*, *PDK4*, *FOXO1* and *SNAT2*] or a decrease [*PYGM*]) in both the control and immobilized legs. Some of these genes (*ACABa*, *DMD* and *VEGFa*) also exhibited a treatment effect (all P<0.05) without any interaction (all P>0.05). Thirteen genes (*MAFbx*, *PGC1a*, *PSMB1*, *NFKb*, *IRS1*, *ACTN3*, *PI3K*, *MuRF1*, *FOXO3*, *CASP3*, *CD36*, *CLPN3*, *GLUT4*) displayed an interaction effect such that divergent responses occurred between legs over time (all P<0.05). With one

exception (*PI3K*) these responses were restricted to 7, but not 2, days of immobilization. For example, after 7 days of immobilization the relative expression of *PSMB1* (Figure 6F) and *MAFBx* (Figure 6I) increased by 83±15% and 123±26 % (both $P<0.001$), respectively, in the immobilized leg only. *NFKb* (Figure 6G) increased in both legs at 7 days but to a greater extent in the immobilized compared with the control leg (115±24 vs 59±23 %; $P=0.027$). Muscle mRNA expression of *IRS1* (Figure 5A), *ACNT3* (Figure 8I) and *PGC1a* (Figure 8B) remained unchanged in the control leg ($P>0.05$), but reduced by 54±9, 77±7 and 52±6 %, respectively, in the immobilized leg after 7 days (all, $P<0.001$). Only *PI3K* (Figure 5B) expression increased after both 2 (87±24%, $P<0.001$) and 7 (181±17%, $P<0.001$) days in the immobilized leg with no change in the control leg ($P=0.774$). *MuRF1* (Figure 6J), *FOXO3* (Figure 6B), *CASP3* (Figure 6D), *CD36* (Figure 7E) and *CLPN3* (Figure 6E) all displayed trends for a divergent response in relative expression between legs over the immobilization period (interaction effects all $P<0.10$). The relative expression of *MuRF1* and *FOXO3* did not change after 2 days but displayed a trend to increase by 175±51 and 112±22 %, respectively, and *CLPN3* a trend to decrease by 37±5%, after 7 days in the immobilized leg (all, $P<0.05$]), with no changes in the control leg (all, $P>0.05$). *CASP3* increased in both legs at 7 days but to a greater extent in the immobilized compared with control leg (179±34 vs 92±40 %; $P=0.313$). Muscle *CD36* expression displayed a trend to increase in the control leg after 2 (73±51%) and 7 (65±49%) days, with no change in the immobilized leg ($P=0.994$).

Discussion

In the current study we applied a deuterated water approach and performed MRI scans to assess the temporal impact of one week of unilateral leg immobilization on daily free living cumulative myofibrillar protein synthesis rates and quadriceps muscle volume, respectively. We report several novel observations. First, immobilization lowered daily free living myofibrillar protein synthesis rates by 36% (compared with the control leg) over one week of immobilization, and the magnitude of this decline was positively correlated with the observed decline in quadriceps volume. Second, this decline in myofibrillar protein synthesis rates occurred rapidly within 2 days, but to a greater extent in the latter part (2-7 days) of the week of immobilization. Finally, one week, but not two days, of muscle disuse was associated with a coordinated upregulation in the muscle expression of genes involved in the ubiquitin proteasome mediated muscle protein breakdown pathway.

Skeletal muscle loss during a period of disuse has been attributed to a decline in both basal (post-absorptive) (8, 20, 43) and post-prandial muscle protein synthesis rates (13, 43). Currently there are fewer data available on the impact of disuse on muscle protein synthesis rates throughout the day, which includes both basal, post-prandial and overnight muscle protein synthesis rates. To gain more insight into the impact of disuse on daily muscle protein synthesis rates over 2, and up to 7 days of immobilization we applied the use of deuterated water. The ingestion of deuterated water has been applied to assess muscle protein synthesis rates in response to acute (27) and longer term (9, 33) resistance training, as well as in response to various nutritional interventions (34). Recently, other groups have used the provision of deuterated water to measure the effect of disuse on muscle protein synthesis rates (29, 30). In line with previous work from our group(s) (24, 38) we show that our oral deuterated water regimen resulted in a sustained elevation in body water deuterium (~0.76±0.02%; Figure 2A) and plasma deuterated alanine (~3.1±0.2 MPE; Figure 2A) enrichments throughout the

experimental period. The data from both precursor pools also correlated tightly (Figure 2B), and quantitatively related to one another in agreement with previous work (Figure 2C; (27). Combined with the unilateral immobilization approach (a within subject comparison of muscle contraction status with identical precursor pool supply) we were able to calculate that disuse reduced free living daily myofibrillar protein synthesis rates by ~36% over a week of immobilization (Figure 3B). This reduction is quantitatively in line with what would be predicted from previous studies that have assessed the effects of 5 to 14 days of leg immobilization on myofibrillar protein synthesis rates using both stable isotope labelled amino acid tracers (8, 22, 43) and deuterium oxide approaches (31). Moreover, we also report that the individual decline in myofibrillar protein synthesis rates was strongly positively correlated with the amount of muscle tissue lost during the week of disuse (Figure 4A). Thus, we provide robust evidence to show that a chronic (i.e. presumably for 24 h per day) decline in daily myofibrillar protein synthesis rates plays a major role in driving muscle disuse atrophy *in vivo* in humans. Worthy of note, this decline occurred despite our volunteers maintaining a relatively high dietary protein intake of 1.6 $g \cdot kg \cdot d^{-1}$ (see Table 1), suggesting that the impairments were exclusively a result of the disuse *per se*, and not further augmented by any compensatory decrease in protein (or energy) intake. In addition, the decline in synthetic rate was observed within the myofibrillar fraction of the muscle protein pool, underlining that targeting the maintenance (or stimulation) of myofibrillar protein synthesis rates during short-term disuse should clearly represent a primary strategy to combat the loss of muscle mass and associated declines in function (11, 29, 44).

A clear picture of how muscle protein synthesis rates change over time during a period of disuse is not yet available, primarily due to the technical difficulties associated with making multiple acute measurements of myofibrillar protein synthesis rates within the same person (21). As a result, it is not clear how quickly myofibrillar protein synthesis rates decline consequent with

disuse, or whether the magnitude of decline is dependent on the duration of disuse. Experiments utilizing static molecular markers within muscle tissue have suggested that muscle disuse atrophy may be differentially regulated within the first few days compared with one or more weeks (1, 40). In the present work we show that although the decline in MPS rates did not reach statistical significance over the first 2 days, the *daily* decline in myofibrillar protein synthesis rates during this period (i.e. 8% per day [or 16% in total]; Figures 3D and E) was of a similar magnitude as the *daily* decline observed over days 2-7 (i.e. 9% per day [or 45% in total]; Figures 3D and E). Clearly, the decline in muscle protein synthesis rates as a major factor in driving muscle loss (Figure 3D and E) is a process that occurs rapidly and seems to be a key process in explaining the fast decline in quadriceps volume that we observed during the first 2 days. However, though the daily rate of decline was similar across the two time periods, the absolute decline in myofibrillar protein synthesis rates consequently occurred to a much greater extent during the latter phase of the disuse period (i.e. 2-7 days). This could suggest that the effect of disuse is cumulative with time, at least for a few days. Alternatively, these data may indicate that an immediate drop in myofibrillar protein synthesis rates occurs, but is variable in the time it takes to manifest across individuals (11/13 subjects decline after 2 days and 13/13 decline after 7 days). Of note, the tight correlation of declining myofibrillar protein synthesis rates with rate of muscle atrophy was less clear when examining the early phase of disuse (Figure 4). It is therefore interesting to speculate to what extent declines in myofibrillar protein synthesis rates may *quantitatively* explain the observed muscle atrophy.

Previous work, using data obtained from acute measurements of hourly muscle protein synthesis rates have estimated that reductions in muscle protein synthesis rates can fully (36) (or even over) explain (22) observed muscle atrophy after 3-6 weeks of leg immobilization. In contrast, we have previously estimated from our work that reductions in basal and/or post-prandial muscle protein synthesis rates could explain ~80% of the muscle atrophy seen during

525 5-14 days of disuse (49). However, these estimations rely on a number of assumptions; most
526 importantly that measuring myofibrillar protein synthesis rates after a period of disuse reflect
527 the changes in chronic myofibrillar protein synthesis rates that occur throughout the entire
528 period of disuse, and usually inferring muscle mass from single slice cross sectional
529 measurements. In the present study, we have captured a cumulative myofibrillar protein
530 synthetic response throughout the entire period of disuse, alongside the calculation of
531 quadriceps mass. If, as has previously been done (36), we assume that muscle protein
532 breakdown rates remained unchanged with disuse, and assume in the control leg that daily
533 MPB rates were equivalent to daily MPS rates, we can calculate the expected loss of muscle
534 mass as a result of decreased daily MPS rates as follows; net muscle protein loss = daily MPB
535 – daily MPS. Using this approach, we calculate a net muscle protein loss of $0.21\%\cdot d^{-1}$ and
536 $0.45\%\cdot d^{-1}$ over the first 2 days and entire week, respectively. Given we know the muscle mass
537 of the participants' quadriceps at baseline, this equates to an expected muscle protein loss from
538 the immobilized quadriceps of 11 and 76 g over 2 and 7 days, respectively, directly attributable
539 to reduced MPS rates. Surprisingly this only accounts for 25 and 47% of the 42 and 162 g
540 estimated (measured) muscle loss. Despite improving the resolution of such calculations, our
541 methods still involve a number of assumptions to arrive at these figures. Most notably, that
542 muscle density does not differ substantially across volunteers or in response to disuse, that
543 vastus lateralis muscle protein turnover rates are analogous to the entire quadriceps mass,
544 alanine synthesis rates are representative of other (and all) amino acids, that myofibrillar
545 protein atrophy is equivalent to total protein loss and that the absence of a 'true precursor pool'
546 (i.e. labelled tRNA) does not considerably alter the absolute rates arrived at. Since it is likely
547 such limitations induce some error in the calculated contribution of muscle protein synthesis to
548 muscle atrophy, indirect conclusions on the reciprocal role of muscle protein breakdown are
549 difficult to make with confidence. However, the fact we can only account for (less than) half

of the observed muscle atrophy due to impaired MPS rates is surprising and not in line with previous work (21, 35). Consequently, it is intriguing for future work to endeavor to make accurate and direct measures of muscle protein breakdown in humans to establish any (temporal) causative role in human muscle disuse atrophy. This is an elusive, but highly consequential, physiological question that was beyond the scope of the present work.

With the difficulty of measuring *in vivo* MPB rates within the constraints of the current design, we applied a custom-designed low-density RT-qPCR microarray gene card to measure the relative expression of 46 targeted genes that encode proteins associated with the regulation of muscle mass, muscle deconditioning/reconditioning and muscle metabolism, with a specific sub-focus on genes involved in MPB. Thirteen genes were unaffected with a further 20 showing an equivalent change in both legs ((likely due to a repeated biopsy effect (44)). Thirteen of our selected genes changed differentially in the immobilized compared with control leg, and a striking observation is that 7 of those genes are involved in muscle protein breakdown, 4 of which encode proteins involved in the ubiquitin proteasome system (UPS) (*PSMB1* [Figure 5F], *MAFbx* [Figure 5I], *MuRF1* [Figure 5J]) (7) and associated transcription factors (e.g. *FOXO3* [Figure 5B]). This coordinated change in gene expression of components of the UPS typically manifested as an increase after 7 but not 2 days of immobilization. In contrast, genes involved in other muscle protein breakdown pathways, for example the calcium dependent calpain system (3), were either unaffected (*CLPN1* [Figure 5]) or tended to be reduced after 7 but not 2 days of immobilization (*CLPN3* [Figure 5]). These findings are line with and extend on our (47) and other research groups' (29) previous findings that genes specifically involved in the ubiquitination and degradation of myofibrillar proteins show a transcriptional rise consequent with disuse. Previous work has suggested that such a rise in gene expression is not immediate (11) but rapid (between 2 and 7 days) and transient (typically subsiding at time points beyond 1-2 weeks), and possibly indicative of an increased rate of muscle protein

breakdown and/or metabolic deconditioning during this early phase (42, 45, 46). With respect to the latter, the expression of various genes involved in insulin mediated glucose uptake (e.g. *IRS1* and *GLUT4*) also declined after 7 but not 2 days of immobilization, interestingly at time points known to succeed the physiological manifestation of insulin resistance (13, 36, 39, 47). Collectively, therefore, our gene array data point to widespread muscle deconditioning stretching beyond solely a loss of muscle mass. A limitation of the present study is that only males were included. While this allowed for a more homogenous population to allow us to precisely measure and describe temporal, muscle specific disuse atrophy, it prevents the data being generalizable to females. Given that sex-based differences in the rate of muscle disuse atrophy likely exist (10), it is of importance that future work assesses whether similar results are demonstrable in females.

In conclusion, muscle disuse induces a rapid decline in muscle volume (within 2 days) that continues at a similar rate for one week. The decline in muscle volume is accompanied by a ~36% decline in daily myofibrillar protein synthesis rates in healthy young men over one week of disuse. These data highlight the key responsible role that declining myofibrillar protein synthesis rates play in the development of muscle disuse atrophy *in vivo* in humans.

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604 **Author contributions**

605 S. P. K., J. F., S. R. J., and B. T. W. performed the experiments; A. M. H., A. P. G., B. P. L.,
606 B. T. W., and S. P. K. performed the biological analyses. S. P. K., and B. T. W. analyzed the
607 data; S. P. K., L. J.C v. L., and B. T. W. interpreted results of experiments; S. P. K., prepared
608 figures; S. P. K., and B. T. W drafted manuscript; S. P. K., L. J.C v. L., and B. T. W. edited
609 and revised manuscript; S. P. K., J. F., S. R. J., A. M. H., A. P. G., L. J.C v. L., and B. T. W.
610 approved the final version of the manuscript.
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Figure Legends

Figure 1. Study Schematic. Thirteen healthy young males underwent 7 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, habitual dietary intake recorded by self-reported written diet diary. Blood, venous blood sample. Arrows represent bilateral *m. vastus lateralis* muscle biopsies, (i.e. taken from the control and immobilized legs).

Figure 2. Stable isotope precursor pool data. Plasma free ^{2}H-alanine (MPE) (left y-axis) and saliva ^{2}H (%) (right y-axis) enrichments displayed over the week of immobilization (A), with data analyzed using one-way repeated measures ANOVAs, and the correlation (B) between saliva ^{2}H (%) and plasma free ^{2}H-alanine (MPE) analyzed by a Pearsons' correlation analysis. C, displays comparison of individual participant saliva enrichment (%) and plasma free ^{2}H-alanine (MPE) enrichments at day 0, 2 and 7 timepoints. a = significant difference from day 0 in corresponding precursor pool $P<0.05$, b = significant difference from day 1 in saliva precursor pool $P<0.05$. Data are means±SEM, $n=13$.

Figure 3. Free living myofibrillar fractional synthesis rates (%·d^{-1}). Graphs A and B show free living myofibrillar FSR (%·d^{-1}) over the full week calculated from plasma and saliva precursor pools, respectively, as individually assessed by paired samples t-test. Graphs D and E show free living myofibrillar FSR between days 0 – 2 and 2 - 7 of immobilization calculated from plasma and saliva precursor pools, respectively, as assessed by two-way repeated measures ANOVA (leg

x time), with time (T), condition (C) and interaction (I) effects displayed above each graph. Where a significant time x treatment interaction was detected Bonferroni post hoc tests were conducted to locate individual differences; *** denotes $P<0.001$ significant difference between legs at corresponding timepoint, ## denotes $P<0.01$ significant difference within leg between 0-2 and 2-7 timepoints. Graphs C and F show correlations between myofibrillar FSRs calculated from the two different precursors between day 0-7 (C) and 0-2 and 2-7 days combined (F), analyzed by Pearson's correlation analyses. Data are means±SEM for the non-correlation graphs, $n=13$.

Figure 4. Correlations between the change in quadriceps volume in the immobilized leg and the difference between control and immobilized leg myofibrillar FSR across the following timepoints; A) days 0 – 7, B) days 0 – 2 and C) days 2 – 7. Data analyzed by Pearson's correlation analyses, R^2 and P values displayed on each graph, $n=13$.

Figure 5. Skeletal muscle mRNA expression of genes involved in muscle protein synthesis and/or identified as growth factors, in healthy young males before and following 2 and 7 days of unilateral leg immobilization in the control and immobilized legs. Data from each gene were analyzed using a two way repeated measures ANOVA (leg *x* time) and time (T), condition (C) (i.e. leg) and interaction (I) effects are displayed above each graph. Where a significant interaction was found, Bonferroni post hoc tests were applied to locate individual differences, *, ** and *** denotes $P<0.05$, $P<0.01$ and $P<0.001$ significant differences from day 0 within the same leg, #, ## and ### denotes $P<0.05$, $P<0.01$ and $P<0.001$ significant differences from day 2 within the same leg. Relative quantification of the genes was performed using the delta delta Ct method ($2^{-\Delta\Delta Ct}$), with data normalized to the geometric mean of two housekeeping genes (*GAPDH* and *18s*) and pre-

immobilization (i.e. day 0; normalized to a value of 1) in both the control and immobilized legs. Data are means±SEM, *n*=13.

Figure 6. Skeletal muscle mRNA expression of genes involved in muscle protein breakdown in healthy young males before and following 2 and 7 days of unilateral leg immobilization in the control and immobilized legs. Data from each gene were analyzed using a two way repeated measures ANOVA (leg *x* time) and time (T), condition (C) (i.e. leg) and interaction (I) effects are displayed above each graph. Where a significant interaction was found, Bonferroni post hoc tests were applied to locate individual differences, * and *** denotes $P<0.05$ and $P<0.001$ significant differences from day 0 within the same leg, #, ## and ### denotes $P<0.05$, $P<0.01$ and $P<0.001$ significant differences from day 2 within the same leg. Relative quantification of the genes was performed using the delta delta Ct method ($2^{-\Delta\Delta Ct}$), with data normalized to the geometric mean of two housekeeping genes (*GAPDH* and *18s*) and pre-immobilization (i.e. day 0; normalized to a value of 1) in both the control and immobilized legs. Data are means±SEM, *n*=13.

Figure 7. Skeletal muscle mRNA expression of genes involved in carbohydrate and/or lipid metabolism in healthy young males before and following 2 and 7 days of unilateral leg immobilization in the control and immobilized legs. Data from each gene were analyzed using a two way repeated measures ANOVA (leg *x* time) and time (T), condition (C) (i.e. leg) and interaction (I) effects are displayed above each graph. Where a significant interaction was found, Bonferroni post hoc tests were applied to locate individual differences, *, ** and *** denotes $P<0.05$, $P<0.01$ and $P<0.001$ significant differences from day 0 within the same leg, ## and ### denotes $P<0.01$ and $P<0.001$ significant differences from day 2 within the same leg. Relative

quantification of the genes was performed using the delta delta Ct method ($2^{-\Delta\Delta Ct}$), with data normalized to the geometric mean of two housekeeping genes (*GAPDH* and *18s*) and pre-immobilization (i.e. day 0; normalized to a value of 1) in both the control and immobilized legs. Data are means±SEM, *n*=13.

Figure 8. Skeletal muscle mRNA expression of genes involved in carbohydrate and/or lipid metabolism in healthy young males before and following 2 and 7 days of unilateral leg immobilization in the control and immobilized legs. Data from each gene were analyzed using a two way repeated measures ANOVA (leg *x* time) and time (T), condition (C) (i.e. leg) and interaction (I) effects are displayed above each graph. Where a significant interaction was found, Bonferroni post hoc tests were applied to locate individual differences, *, ** and *** denotes $P<0.05$, $P<0.01$ and $P<0.001$ significant differences from day 0 within the same leg, ## and ### denotes $P<0.01$ and $P<0.001$ significant differences from day 2 within the same leg. Relative quantification of the genes was performed using the delta delta Ct method ($2^{-\Delta\Delta Ct}$), with data normalized to the geometric mean of two housekeeping genes (*GAPDH* and *18s*) and pre-immobilization (i.e. day 0; normalized to a value of 1) in both the control and immobilized legs. Data are means±SEM, *n*=13.

Figure 9. Skeletal muscle mRNA expression of A) Dynamin like protein 1, B) Desmin, C) Pyruvate dehydrogenase kinase 4 and d) hexokinase 2 in healthy young males before and following 2 and 7 days of unilateral leg immobilization in the control and immobilized legs. Data from each gene were analyzed using a two way repeated measures ANOVA (leg *x* time) and time (T), condition (C) (i.e. leg) and interaction (I) effects are displayed above each graph. Relative

quantification of the genes was performed using the delta delta Ct method ($2^{-\Delta\Delta Ct}$), with data normalized to the geometric mean of two housekeeping genes (*GAPDH* and *18s*) and pre-immobilization (i.e. day 0; normalized to a value of 1) in both the control and immobilized legs. Data are means±SEM, n=13.

Table 1. Dietary intake and physical activity levels during a habitual period and during one week of single leg immobilization.

	Pre-immobilization	During immobilization
Energy intake ($MJ \cdot d^{-1}$)	11.1±0.7	11.3±1.0
($Kcal \cdot d^{-1}$)	(2647±167)	(2689±288)
Protein intake ($g \cdot d^{-1}$)	119±7	115±12
Protein intake ($g \cdot kg^{-1} \cdot d^{-1}$)	1.6±0.1	1.6±0.1
Protein intake (En%)	18±1	17.3±1
Carbohydrate intake ($g \cdot d^{-1}$)	298±25	308±42
Carbohydrate intake (En%)	45±2	46±2
Fat intake ($g \cdot d^{-1}$)	102±10	108±11
Fat intake (En%)	35±2	36±2
Light physical activity ($h \cdot d^{-1}$)	1.4±0.2	1.0±0.1
Moderate physical activity ($h \cdot d^{-1}$)	2.6±0.4	1.7±0.2*
Vigorous physical activity ($h \cdot d^{-1}$)	0.3±0.1	0.1±0.1
Total physical activity ($h \cdot d^{-1}$)	4.3±0.5	2.8±0.2*

Values represent means±SEM, n=13. *=significant difference from pre-immobilization value, $P<0.05$.

Table 2. Names and symbols of the gene expression assay targets preloaded upon PCR microfluidic cards.

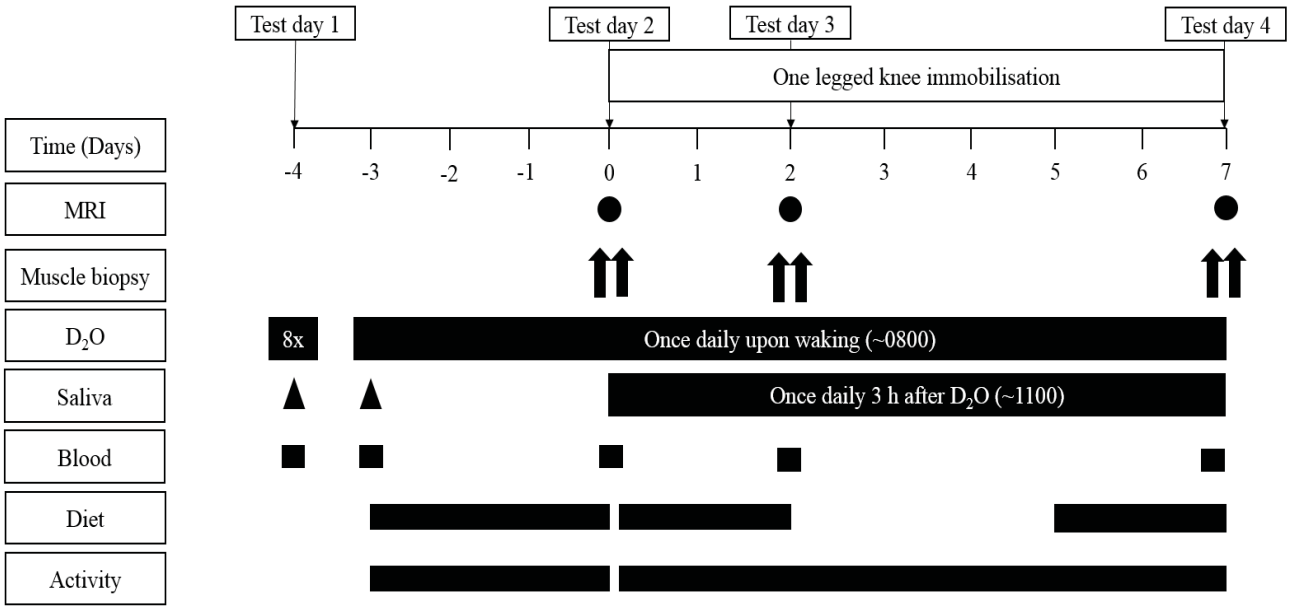
Gene Name (human skeletal muscle)	Symbol
Insulin receptor substrate 1	*IRS1*
Phosphoinositide-3-kinase regulatory subunit 1	*PI3K*
AKT serine/threonine kinase 2	*AKT2*
Mechanistic target of rapamycin	*mTOR*
Ribosomal protein S6 kinase B2	*RPS6KB1 (P70s6k)*
Eukaryotic translation initiation factor 4E binding protein 1	*EIF4EBP1 (4E-BP1)*
Solute carrier family 7 member 5	*SLC7A5 (LAT1)*
Solute carrier family 38 member 2	*SLC38A2 (SNAT2)*
Solute carrier family 36 member 1	*SLC36A1 (PAT1)*
DNA damage inducible transcript 4	*DDIT4 (REDD1)*
Eukaryotic 18S rRNA	*18s rRNA*
Forkhead box O1	*FOXO1*
Forkhead box O3	*FOXO3*
Calpain 1	*CLPN1*
Calpain 3	*CLPN3*
Caspase 3	*CASP3*
Proteasome subunit beta 1	*PSMB1*
Nuclear factor kappa B subunit 1	*NFKB*
F-box protein 32	*MAFbx*
Tripartite motif containing 63	*TRIM63 (MuRF1)*
Beclin 1	*BECN1*

Myostatin	*MTSN*
Growth differentiation factor 11	*GDF11*
Solute carrier family 2 member 4	*GLUT4*
Glycogen Phosphorylase (muscle)	*PYGM*
Glycogen synthase 1	*GS1*
Hexokinase 2	*HK2*
Pyruvate dehydrogenase kinase 4	*PDK4*
CD36 molecule	*CD36*
Fatty acid synthase	*FASN*
Sterol regulatory element binding transcription factor 1	*SHREBP1*
Carnitine palmitoyltransferase 1B,	*CPT1B*
Acetyl-CoA carboxylase beta	*ACACA*
Peroxisome proliferator activated receptor alpha	*PPARa*
PPARG coactivator 1 alpha	*PGC1a*
Mitofusin 1	*MFN1*
Dynamin 1 like protein	*DNM1L*
Transcription factor A, mitochondrial	*TFAM*
Dystrophin	*DMD*
Sarcoglycan alpha	*SGCA*
Laminin subunit alpha 2	*LAMA2*
Integrin subunit beta 1	*ITGB1*
Actinin alpha 3	*ACTN1*
Desmin	*DES*
Vascular endothelial growth factor A	*VEGF*

Hypoxia inducible factor 1 alpha subunit	*HIF-1*
Angiopoietin 1	*ANGPT1*
Glyceraldehyde-3-phosphate dehydrogenase	*GAPDH*

Symbols in brackets represent gene synonyms.

Figure 1



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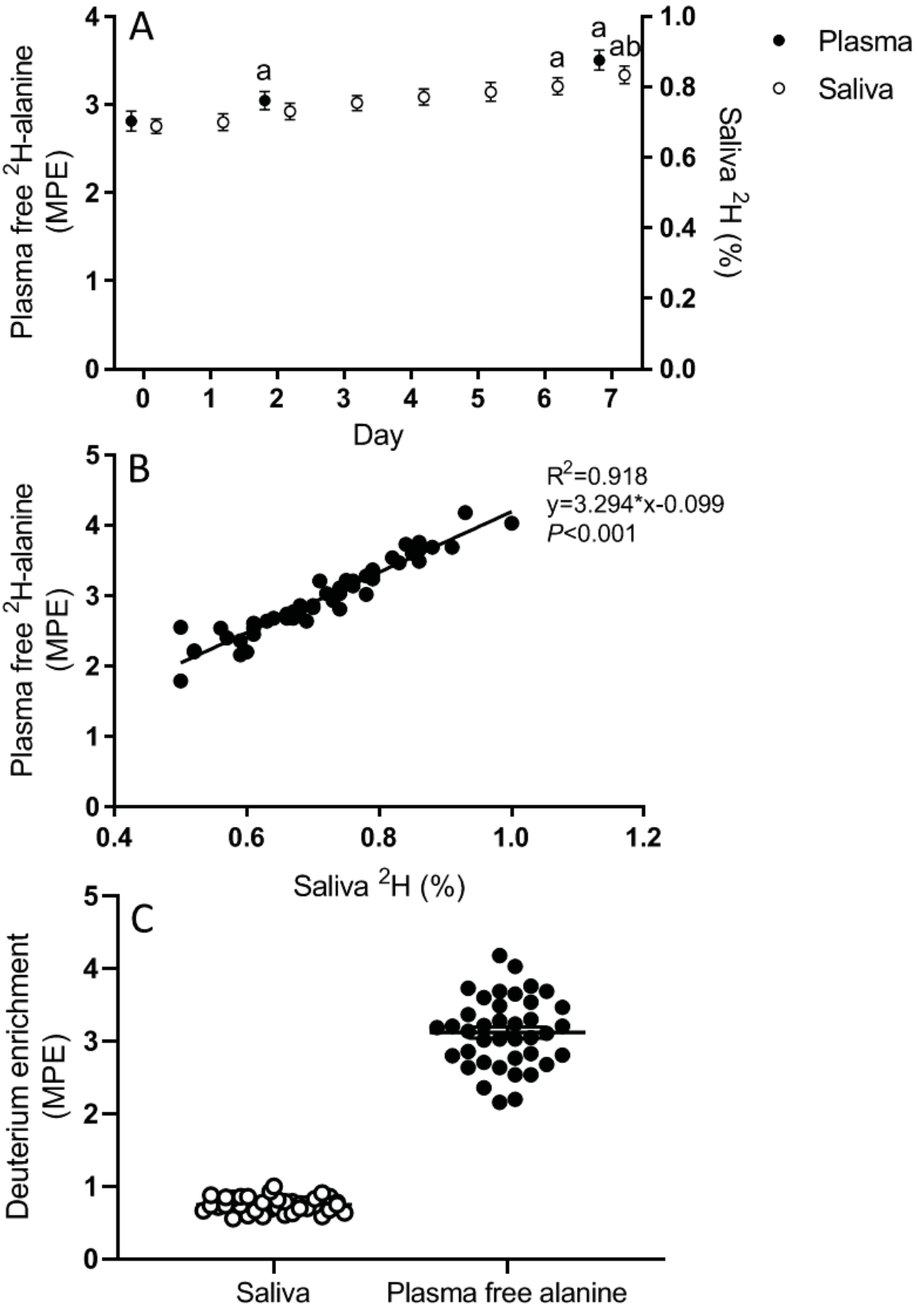

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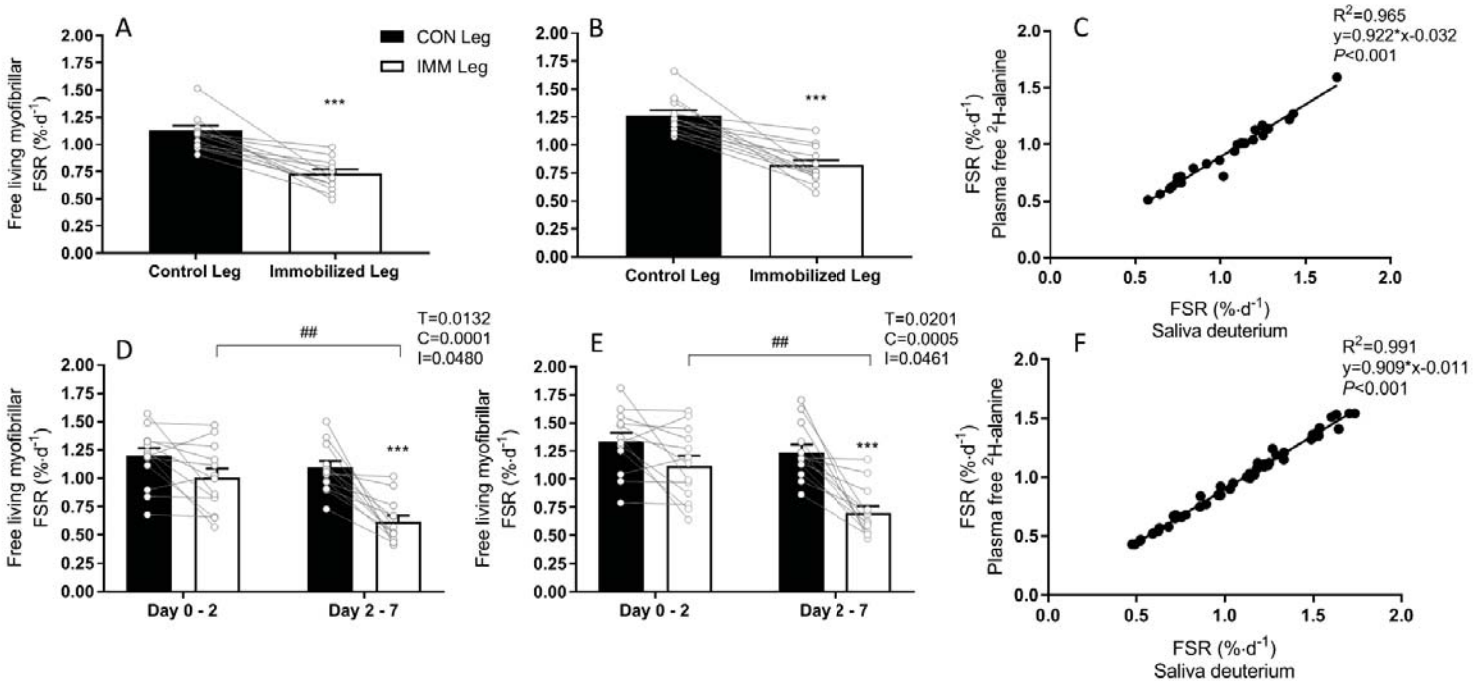

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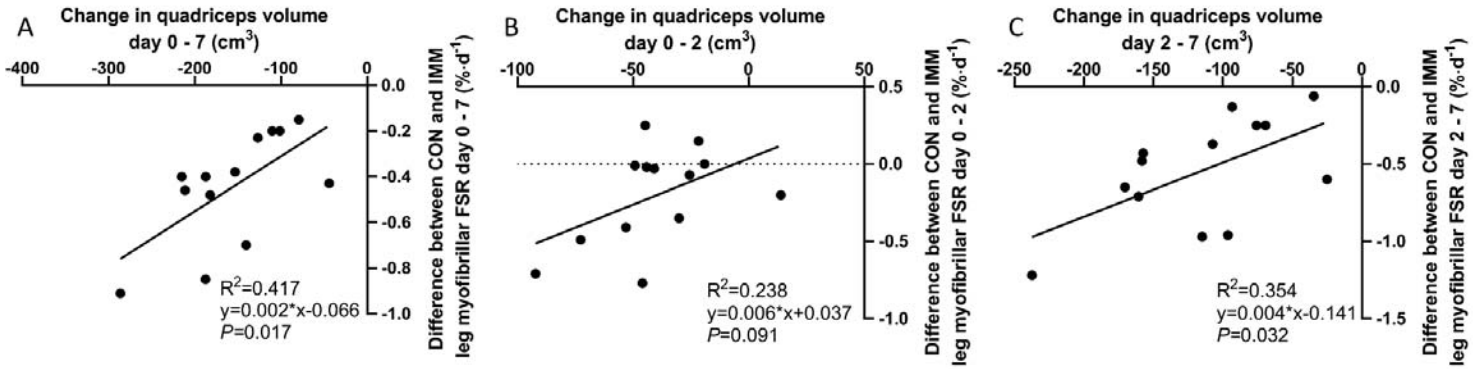

Figure 5

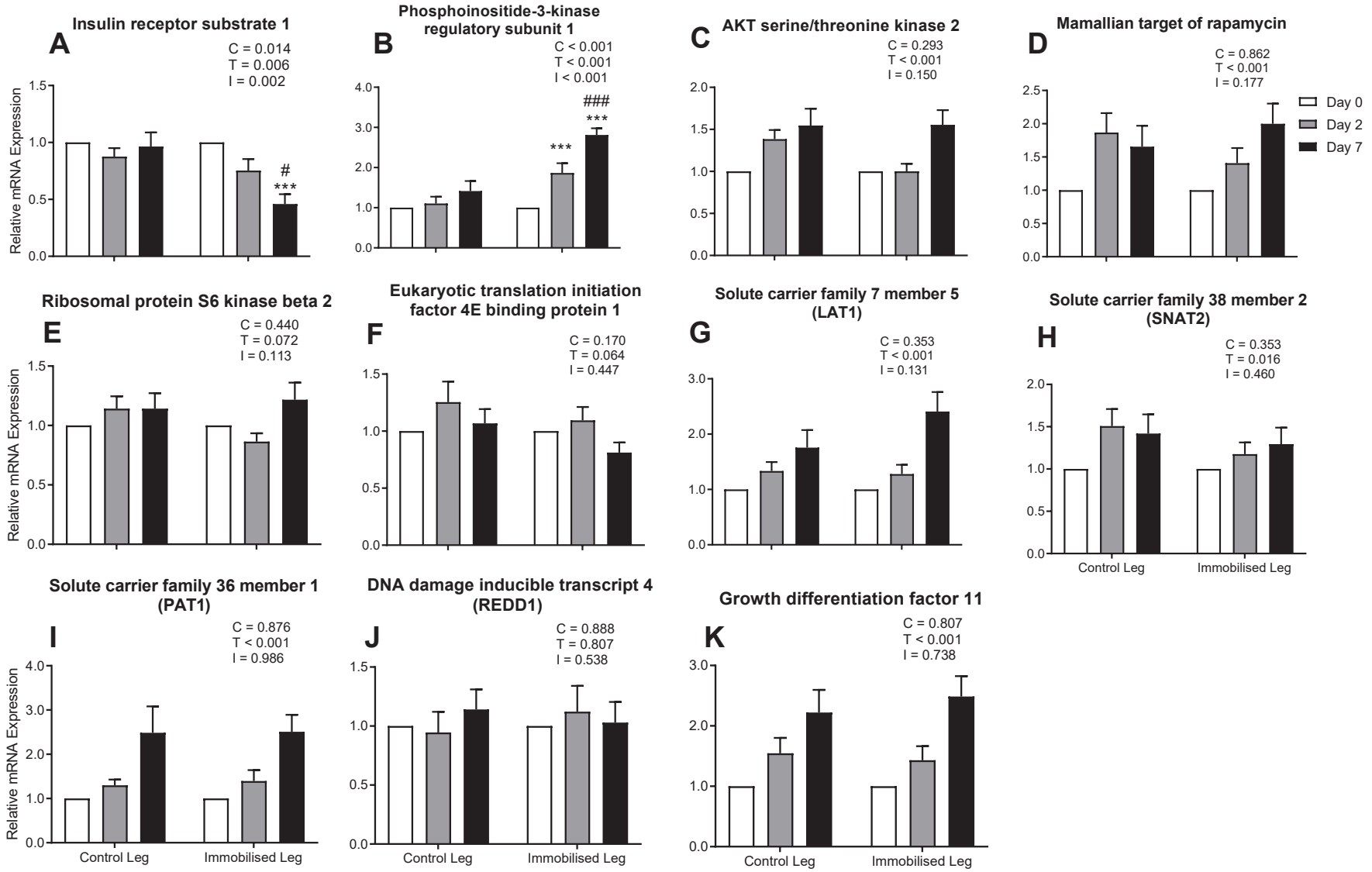

Figure 6

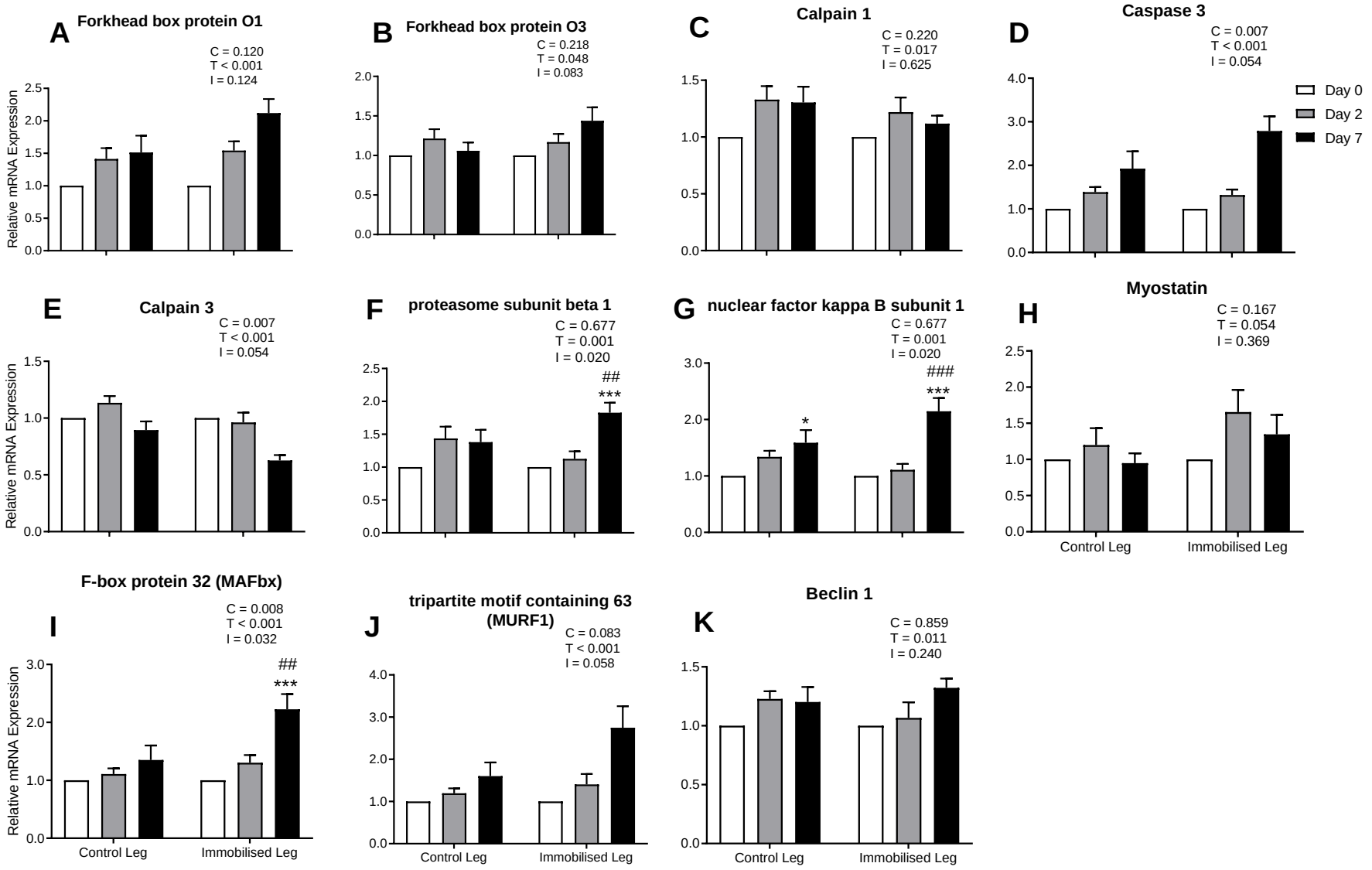

Figure 7

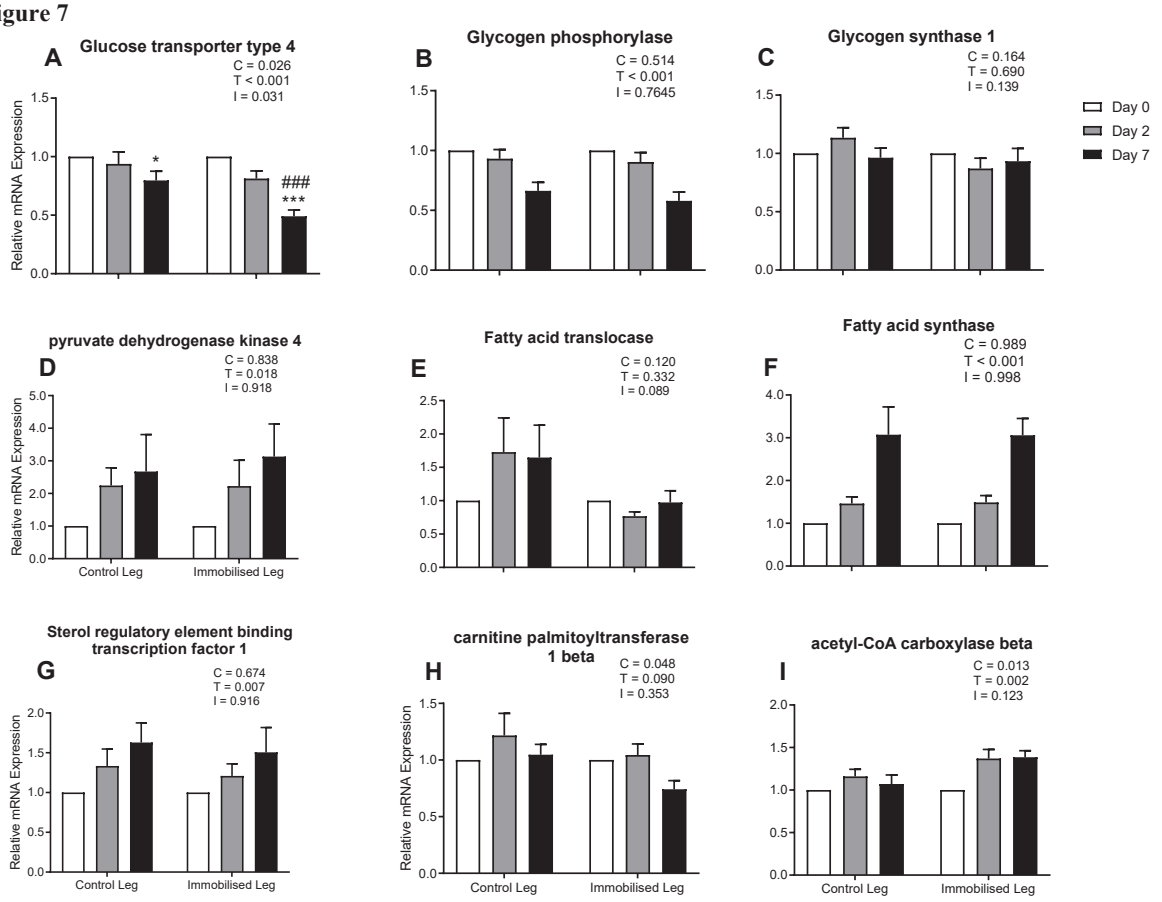

Figure 8

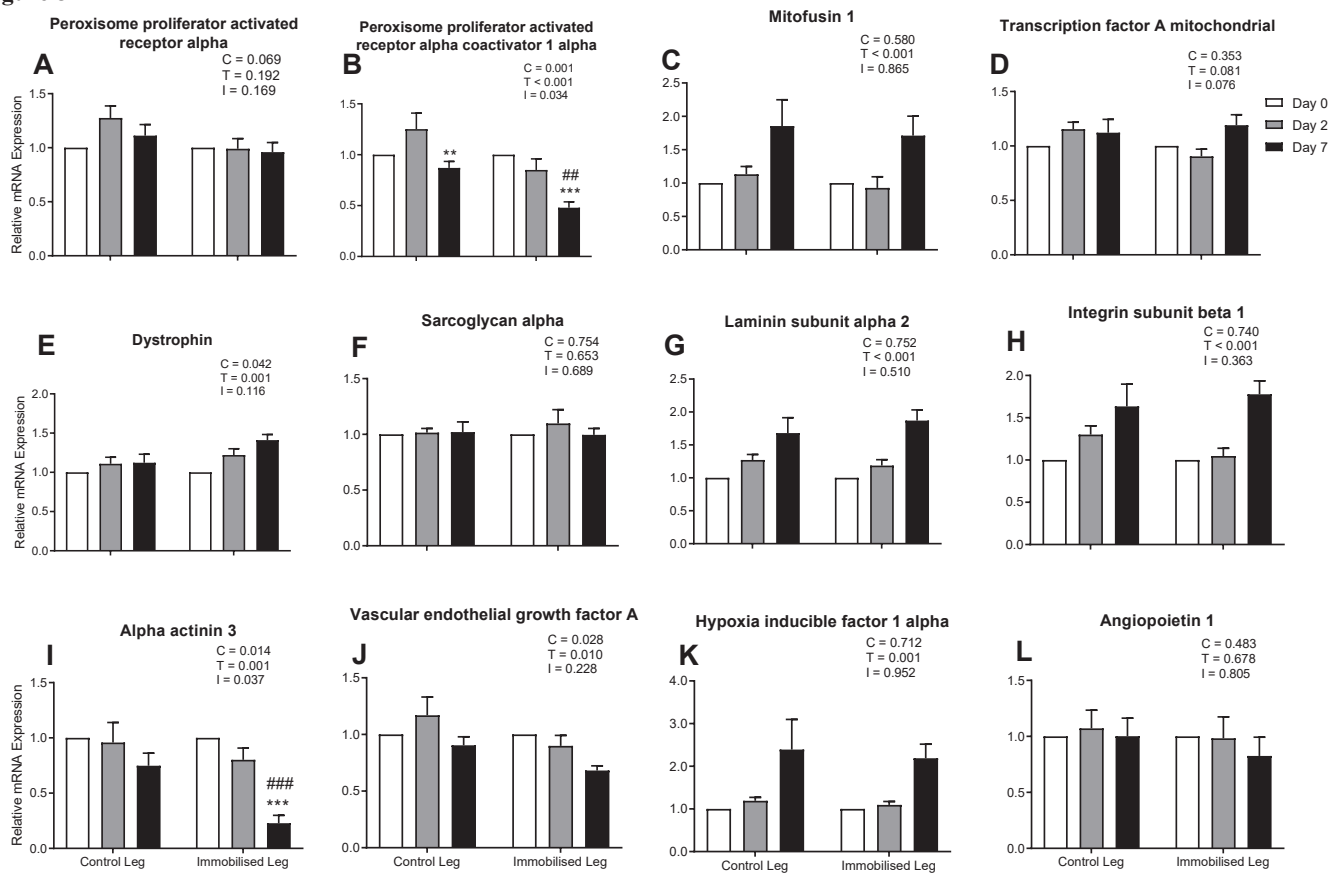

Figure 9