

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

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## 1 Abstract

2 **Introduction:** Short-term muscle disuse has been reported to lower both post-absorptive and  
3 post-prandial myofibrillar protein synthesis rates. This study assessed the impact of disuse on  
4 daily myofibrillar protein synthesis rates following short-term (2 and 7 days) muscle disuse  
5 under free living conditions. **Methods:** Thirteen healthy young men (age,  $20\pm 1$  y; BMI,  $23\pm 1$   
6  $\text{kg}\cdot\text{m}^{-2}$ ) underwent 7 days of unilateral leg immobilization via a knee brace with the non-  
7 immobilized leg acting as a control. Four days prior to immobilization participants ingested  
8 400 mL 70% deuterated water, with 50 mL doses consumed daily thereafter. Upper leg bilateral  
9 MRI scans and muscle biopsies were collected before, and after 2 and 7 days of immobilization  
10 to determine quadriceps volume and daily myofibrillar protein synthesis rates. **Results:**  
11 Immobilization reduced quadriceps volume in the immobilized leg by  $1.7\pm 0.3$  and  $6.7\pm 0.6$  %  
12 after 2 and 7 days, respectively, with no changes in the control leg. Over the one week  
13 immobilization period myofibrillar protein synthesis rates were  $36\pm 4\%$  lower in the  
14 immobilized ( $0.81\pm 0.04\%\cdot\text{d}^{-1}$ ) compared with the control ( $1.26\pm 0.04\%\cdot\text{d}^{-1}$ ) leg ( $P<0.001$ ).  
15 Myofibrillar protein synthesis rates in the control leg did not change over time ( $P=0.775$ ), but  
16 in the immobilized leg were numerically lower during the 0-2 day period ( $16\pm 6\%$ ,  
17  $1.11\pm 0.09\%\cdot\text{d}^{-1}$ ,  $P=0.153$ ) and were significantly lower during the 2-7 day period ( $44\pm 5\%$ ,  
18  $0.70\pm 0.06\%\cdot\text{d}^{-1}$ ,  $P<0.001$ ) when compared with the control leg. **Conclusion:** One week of  
19 muscle disuse induces a rapid and sustained decline in daily myofibrillar protein synthesis rates  
20 in healthy young men.

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## 26 **Introduction**

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

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

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

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

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76 **Methods**

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78 *Participants*

79 Thirteen healthy young men (age=20±1 y, BMI=23.4±0.9 kg·m<sup>-2</sup>) were included in the present  
80 study. Only young males were included in the present study since both age (43) and sex (10)  
81 can influence the rate of muscle disuse atrophy, and our goal was to maintain a homogeneous  
82 population for the current study. Participants attended the laboratory for a routine medical  
83 screening and completed a general medical questionnaire to assess their eligibility for  
84 participation, and to ensure no adverse health conditions were present. Exclusion criteria  
85 included; a (family) history of deep vein thrombosis/cardiovascular disease, metabolic  
86 disorders (e.g. type 2 diabetes), musculoskeletal/orthopedic disorders, a body mass index of  
87 above 28.5 kg·m<sup>-2</sup> or below 18.5 kg·m<sup>-2</sup>, participation in a structured resistance training  
88 program within 6 months prior to the study, any musculoskeletal injury of the legs within 12  
89 months before the study, use of anticoagulants, consumption of any nutritional supplement  
90 prior to and during the study. During the screening participants height, body mass and blood  
91 pressure were measured, body composition was also assessed by air displacement  
92 plethysmography (BODPOD; Life Measurement, Inc. CA, USA). All subjects were informed  
93 of the nature and possible risks of the experimental procedures before providing written  
94 informed consent. The study was part of a wider study assessing muscle disuse atrophy that  
95 was approved by The Sport and Health Science Ethics committee of the University of Exeter  
96 (151021/B/02), in accordance with the guidelines set out in the Declaration of Helsinki. This  
97 study was registered as a clinical trial with clinicaltrials.gov (NCT02984332).

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### 101 *Experimental design*

102 A graphical representation of the experimental study design can be seen in **Figure 1**. Following  
103 successful completion of the screening visit eligible participants attended the laboratory for 5  
104 experimental visits across 11 days and this included a 7 day period of unilateral leg  
105 immobilization using a leg brace and ambulation via the use of crutches. To measure daily  
106 myofibrillar protein synthesis rates throughout the immobilization period participants  
107 underwent a deuterium oxide dosing protocol (described below). This protocol was designed  
108 to achieve and maintain 0.8-1.0% body water deuterium enrichment during the measurement  
109 periods in line with our previous work (25). Participants arrived at the laboratory on day 5 of  
110 the experimental protocol at ~0800 h for the first of three experimental test days. During this  
111 visit, bilateral muscle biopsies were collected from the *m. vastus lateralis* and an MRI scan  
112 was conducted of both thigh muscles (at ~0830 h; participants were transported to and from  
113 the MRI scanner via a wheelchair to ensure no contraction or weight bearing occurred  
114 before/after the MRI scans and biopsies). Thereafter, a 7 day immobilization period was started  
115 at ~0900 h. After 2 and 7 days of immobilization subjects returned to the laboratory at ~0800  
116 h for an identical test day. Muscle biopsies were obtained under local anesthesia, using the  
117 percutaneous Bergstrom needle biopsy technique (6), from the *m. vastus lateralis* of both legs  
118 ~15 cm above the patella and ~3 cm below the fascia. Immediately following a muscle biopsy  
119 the muscle tissue was quickly assessed and any blood or non-muscle tissue was dissected and  
120 discarded. The muscle samples were immediately frozen in liquid nitrogen and stored at -80°C  
121 until further analysis.

122

### 123 *Immobilization protocol*

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

126 instruction) throughout the immobilization period. The immobilized leg was randomized and  
127 was counterbalanced for leg dominance with the non-immobilized leg acting as a within-  
128 subject control. Using the hinge of the brace the knee was fixed at an angle of 40° flexion (full  
129 knee extension = 0°) to ensure no weight bearing occurred. Subjects were instructed that all  
130 ground contact, and muscle contraction (except for ankle rotation exercises twice per day to  
131 activate the venous muscle pump), in the immobilized leg were forbidden. Adhesive tape with  
132 the experimenter's signature inscribed was placed around the straps of the brace. Breaking of  
133 the tape would indicate that the brace had been altered and resulted in exclusion from the study  
134 (24), no participants were excluded. A plastic shower cover was provided to the participants to  
135 wear over the brace when showering. Daily contact was maintained with the subject throughout  
136 the study to ensure proper compliance.

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### 138 *Deuterated water dosing protocol*

139 The deuterated water dosing protocol was based on our previous work (25). Day 1 of the  
140 experimental protocol acted as a D<sub>2</sub>O loading day where participants consumed 400 mL 70%  
141 D<sub>2</sub>O (i.e. 280 mL pure D<sub>2</sub>O) separated over the day as 8 x 50 mL boluses (CK Isotopes Ltd,  
142 Leicestershire, UK). Upon arrival at the laboratory (0730 h) background blood and saliva  
143 samples were collected before the first bolus of D<sub>2</sub>O was ingested. The first dose of D<sub>2</sub>O was  
144 consumed at ~0800 h with the remaining doses being consumed every 1.5 h. Participants stayed  
145 at the university until 4 out of the 8 loading day D<sub>2</sub>O doses had been consumed, with the  
146 remaining D<sub>2</sub>O doses being consumed at home under instruction of timings (i.e. leaving 1.5 h  
147 between each). Every day following the loading day participants consumed a maintenance dose  
148 of D<sub>2</sub>O (50 mL) upon waking (~0800 h). One participant reported some mild feelings of vertigo  
149 and dizziness during the loading day which passed after approx. 2 h. Three hours (~1100 h)  
150 after the daily D<sub>2</sub>O maintenance dose a daily saliva sample was collected using a cotton mouth

151 swab (Celluron, Hartmann, Germany) which the participant lightly chewed for ~1 min until  
152 saturated with saliva. The saturated sponge was placed into an empty syringe where the swab  
153 was squeezed to release the saliva into a collection tube. The saliva samples were used to assess  
154 the body water  $^2\text{H}$  enrichment and were stored in the participant's freezer until they were  
155 returned at the next study visit. Additional blood samples were collected in the fasted state at  
156 the start (i.e. ~0800 h) of each test day (i.e. day 5 [pre], 7 [after 2 days of immobilization] and  
157 12 [post]). Venous blood samples were collected from the antecubital vein via venipuncture  
158 technique and collected into EDTA-containing vacutainers which were centrifuged at 4,000  
159 rpm for 10 min at 4°C. Aliquots of plasma were frozen in liquid nitrogen and stored at -80°C  
160 until further analysis took place. To ensure uniformity and compliance with the D<sub>2</sub>O protocol  
161 participants were provided with a log to record the times they consumed the D<sub>2</sub>O and were  
162 provided with enough doses to last until their next study visit, at which point containers were  
163 returned, counted and subsequent doses were provided.

164

### 165 *Diet and physical activity*

166 For 3 days prior to immobilization subjects' physical activity was measured using an  
167 accelerometer (GENEActiv, Activinsights, Cambridgeshire, UK) that was attached to the non-  
168 dominant wrist. Subjects were instructed to wear the accelerometer continuously with data  
169 being collected at a 60 Hz sampling frequency. Subjects' physical activity was measured again  
170 throughout the 7 day immobilization period. Subjects' were instructed to refrain from vigorous  
171 physical activity during immobilization but to attempt maintain their habitual activity levels  
172 despite using crutches for ambulation (to avoid whole body sedentariness during  
173 immobilization). Physical activity data from the GENEActiv accelerometers were converted  
174 into 60 s epochs and used to estimate time spent performing total physical activity (all  
175 intensities) using standard cut-off points (11). Subjects' diets were recorded for 3 days (two

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

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#### 185 *Magnetic resonance imaging and quadriceps volume calculation*

186 MRI scan methodology of the upper legs for the determination of quadriceps volume has been  
187 described in detail previously (4). In brief, a 1.5 tesla (T) MRI scanner was used to make axial  
188 plane images over the full length of the femur. A T1-weighted 3D turbo spin echo sequence  
189 was used (field of view 500 x 500 mm, reconstructed matrix 512 x 512 mm, echo time 15 ms,  
190 repetition time 645 ms, slice thickness 5 mm, slice gap 5 mm) with the subject lying still in the  
191 supine position, a 4-element sense body radiofrequency coil was wrapped around both thighs.  
192 Philips on-line MRI software was used to analyze the images obtained in the axial plane (the  
193 same experimenter performed all manual segmentation of the images). Starting at the most  
194 distal image (approximately mid patella) where each muscle group could be fully delineated,  
195 the muscle of interest was manually segmented to calculate slice cross sectional area. Moving  
196 proximally every third image was analyzed until complete delineation of the muscle groups of  
197 interest could no longer be identified (the greater trochanter). Muscle volume was calculated  
198 using a previously published method (30) where the total CSA for all images was calculated  
199 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:

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

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<sub>2</sub>O 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 [<sup>2</sup>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  $\mu\text{m}$ , 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 $^2\text{H}$ 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  $\mu\text{l}/\text{mg}$ ). The  
237 samples were centrifuged at 2,500  $g$  for 5 min at 4°C and the pellet was then washed with 500  
238  $\mu\text{l}$  of ddH<sub>2</sub>O 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<sub>4</sub>OH. Following this  
249 the eluted amino acids were dried and the purified amino acids were derivatized to their

250 N(O,S)-ethoxycarbonyl ethyl esters (28). The derivatized were measured using a gas-  
251 chromatograph-isotope ratio mass spectrometer (GC-IRMS; Thermo Fisher Scientific, MAT  
252 253; Bremen, Germany) equipped with a pyrolysis oven and a 60m DB-17MS column (no.  
253 122-4762; Agilent, Wilmington, DE) and a 5 m precolumn. Ion masses 2 and 3 were analyzed  
254 to determine the  $^2\text{H}/^1\text{H}$  ratios of muscle protein-bound alanine. A series of known standards  
255 was used to assess the linearity of the mass spectrometer and to control for the loss of tracer.

256

### 257 *Skeletal muscle mRNA analyses*

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

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

278

### 279 *Calculations*

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

$$284 \quad \text{FSR (\%} \cdot \text{day}^{-1}) = \left( \frac{E_{m2} - E_{m1}}{E_{precursor} \times t} \right) \times 100$$

285 where  $E_{m1}$  and  $E_{m2}$  are the myofibrillar muscle protein-bound enrichments on either day 0 and  
286 2, 0 and 7, and 2 and 7 respectively.  $E_{precursor}$  represents either mean plasma free [ $^2\text{H}$ ] alanine  
287 or mean body water deuterium enrichment corrected by a factor of 3.7 based on deuterium  
288 labelling of alanine during *de novo* synthesis (15, 24) (mean enrichment between day 0-2, 0-7  
289 or 2-7).  $t$  represents the time between biopsies (day 0-2, 0-7 or 2-7).

290

### 291 *Statistics*

292 All data are presented as means $\pm$ SEM and all statistical analyses were conducted in GraphPad  
293 Prism version 7.0 (GraphPad Software, San Diego, CA, USA). A paired samples  $t$ -test was  
294 used to compare myofibrillar protein synthesis rates in the control vs immobilized legs between  
295 days 0 and 7, and physical activity and dietary intake between pre and during immobilization.  
296 Two-way repeated measures ANOVAs with leg (immobilized and control) and time (day 0-2  
297 and day 2-7 [for myofibrillar protein synthesis rates] and days 0, 2 and 7 [for MRI]) as within  
298 subjects factors were used to compare differences in myofibrillar protein synthesis rates over  
299 time, gene expression and quadriceps volume. Pearson's correlation coefficient was used to

300 assess the relationship between tracer precursor pools. For all ANOVAs, when a significant  
301 interaction was found Bonferroni post-hoc tests were applied to locate individual differences.

302 Statistical significance was set at  $P < 0.05$ .

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## 325 **Results**

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### 327 *Diet and physical activity analyses*

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

334

### 335 *Quadriceps muscle volume*

336 Quadriceps muscle volumes of the control and immobilized legs were determined by MRI  
337 (previously reported in detail in Kilroe et al, 2019). There were no difference in quadriceps  
338 volume between legs pre-immobilization (control leg =  $2315\pm120$  cm<sup>3</sup>, immobilized leg =  
339  $2342\pm129$  cm<sup>3</sup> [ $P=0.993$ ]) and the control leg remained unchanged throughout the study  
340 ( $P=0.981$ ). However, a significant time  $\times$  leg interaction was detected ( $P<0.001$ ) such that  
341 quadriceps volume of the immobilized leg had decreased by  $1.7\pm0.3\%$  after 2 days (to  
342  $2301\pm127$  cm<sup>3</sup>;  $P=0.007$ ) and by a further  $5.0\pm0.6\%$  between days 2 and 7 days (to  $2186\pm117$   
343 cm<sup>3</sup>;  $P<0.001$ ), resulting in a total decrease in quadriceps muscle volume of  $6.7\pm0.6\%$   
344 ( $P<0.001$ ) over the full week of immobilization.

345

### 346 *Precursor pool enrichments*

347 Saliva deuterium enrichments (**Figure 2A**) reached  $0.69\pm0.02\%$  on day 0 (start of  
348 immobilization), and averaged  $0.71\pm0.02\%$ ,  $0.78\pm0.03\%$  and  $0.76\pm0.02\%$  over 0-2, 2-7 and 0-  
349 7 time points, respectively. Saliva deuterium enrichments showed a modest increase over the

350 immobilization period (main effect of time,  $P<0.001$ ) such that enrichments at day 6  
351 ( $0.80\pm 0.02\%$ ,  $P=0.024$ ) and 7 ( $0.83\pm 0.03\%$ ,  $P<0.001$ ) were elevated above day 0, and day 7  
352 enrichments were higher than day 1 ( $0.70\pm 0.02\%$ ,  $P=0.003$ ). Plasma free [ $^2\text{H}$ ] alanine  
353 enrichments (Figure 2A) reached  $2.8\pm 0.1$  mole percent excess (MPE) on day 0 and averaged  
354  $3.1\pm 0.2$  MPE over the week of immobilization. Plasma free [ $^2\text{H}$ ] alanine enrichments also  
355 showed a slight increase over the immobilization period (main effect of time,  $P<0.001$ ) such  
356 that day 2 ( $3.1\pm 0.1$  MPE,  $P=0.017$ ) and day 7 ( $3.5\pm 0.1$  MPE,  $P<0.001$ ) enrichments were  
357 higher than day 0 ( $2.8\pm 0.1$  MPE). A Pearson's correlation coefficient showed that the saliva  
358 deuterium and the plasma [ $^2\text{H}$ ] alanine precursor pools were strongly correlated (data from all  
359 time points, 0-2, 2-7 and 0-7 collapsed into one test) ( $r=0.959$ ,  $P<0.001$ ) (**Figure 2B**). Plasma  
360 free [ $^2\text{H}$ ] alanine enrichments were, on average, 3.93-fold greater than saliva deuterium  
361 enrichments (**Figure 2C**). It should be acknowledged that we have not determined muscle  
362 intracellular free [ $^2\text{H}$ ] alanine enrichments in the present work. It is conceivable that our  
363 intervention may have influenced global muscle alanine metabolism divergently across legs  
364 which could affect local precursor enrichments. However, our (27) and others' (9) previous  
365 observations that muscle intracellular free [ $^2\text{H}$ ] alanine does not differ substantially across legs  
366 even when unilateral leg interventions are applied (e.g. daily resistance-type exercise) and  
367 correlate tightly with plasma free [ $^2\text{H}$ ] alanine enrichments, suggest the latter to be a valid  
368 precursor pool.

369

#### 370 *Daily myofibrillar protein synthesis rates*

371 Myofibrillar protein bound [ $^2\text{H}$ ] alanine enrichments showed significant time ( $P<0.001$ ), leg  
372 ( $P<0.001$ ) and time  $\times$  leg interaction ( $P<0.001$ ) effects. After 2 days of immobilization,  
373 myofibrillar protein bound [ $^2\text{H}$ ] alanine enrichments increased by  $20\pm 10\%$  more in the control  
374 ( $0.0695\pm 0.0045$  MPE) compared with the immobilized ( $0.0579\pm 0.0042$  MPE) leg ( $P=0.511$ ).

375 After 7 days, myofibrillar bound protein [ $^2\text{H}$ ] alanine enrichments increased over time in both  
376 legs but by  $53\pm 11\%$  more in the control ( $0.2448\pm 0.0096$  MPE) compared with the immobilized  
377 ( $0.1596\pm 0.0079$  MPE) leg ( $P<0.001$ ). Daily myofibrillar FSRs ( $\% \cdot \text{d}^{-1}$ ) were calculated using  
378 mean (of each individual's data during that time period) saliva deuterium enrichments  
379 ((corrected by a factor of 3.7 to account for the labeling coefficient between saliva and plasma;  
380 (15, 25)) and then separately using mean plasma free [ $^2\text{H}$ ] alanine enrichments (again, an  
381 individual's own data averaged over the period of interest) as precursor pools over the 0-2, 2-  
382 7 and 0-7 time-points of immobilization (**Figure 3**). Over the entire week of immobilization  
383 daily myofibrillar FSR, calculated using either the saliva deuterium enrichment (control leg =  
384  $1.26\pm 0.05\% \cdot \text{d}^{-1}$  and immobilized leg =  $0.81\pm 0.04\% \cdot \text{d}^{-1}$ ) or plasma free [ $^2\text{H}$ ] alanine enrichment  
385 (control leg =  $1.14\pm 0.05\% \cdot \text{d}^{-1}$ ; immobilized leg =  $0.71\pm 0.04\% \cdot \text{d}^{-1}$ ) as the precursor pools were  
386  $36\pm 4\%$  ( $P<0.001$ ) and  $37\pm 4\%$  ( $P<0.001$ ) lower, respectively, in the immobilized compared  
387 with the control leg. A Pearson's  $r$  product moment correlation showed a strong correlation  
388 between FSR calculated from the two precursor pools with data collapsed for both legs over  
389 the full week of immobilization ( $r = 0.982$ ,  $P<0.001$  [Figure 3C]).

390 When assessing the temporal changes in myofibrillar FSR over the early (i.e. 0-2 days) and late  
391 (i.e. 2-7 days) immobilization periods and using the saliva deuterium enrichment precursor,  
392 significant main effects of time ( $P=0.020$ ), condition ( $P<0.001$ ) and a time x condition  
393 interaction ( $P=0.046$ ) were detected. Specifically, myofibrillar FSR was  $16\pm 6\%$  ( $P=0.153$ ) and  
394  $44\pm 5\%$  ( $P<0.001$ ) lower at 0-2 (control leg =  $1.32\pm 0.08\% \cdot \text{d}^{-1}$ ; immobilized leg =  
395  $1.11\pm 0.09\% \cdot \text{d}^{-1}$ ) and 2-7 days (control leg =  $1.24\pm 0.07\% \cdot \text{d}^{-1}$ ; immobilized leg =  $0.70\pm 0.06\% \cdot \text{d}^{-1}$ )  
396 in the immobilized compared with the control leg. Myofibrillar FSR remained unchanged  
397 over time in the control leg but declined by  $37\pm 11\%$  from 0-2 to 2-7 days ( $P=0.005$ ) in the  
398 immobilized leg. When using the plasma free [ $^2\text{H}$ ] alanine enrichment to assess temporal  
399 changes in myofibrillar FSR over the early (i.e. 0-2 days) and late (i.e. 2-7 days) immobilization

400 period, significant main effects of time ( $P=0.013$ ), condition ( $P<0.001$ ) and a time x condition  
401 interaction ( $P=0.048$ ) were detected. Specifically, myofibrillar FSR was  $16\pm 6\%$  ( $P=0.159$ )  
402 and  $44\pm 5\%$  ( $P<0.001$ ) lower at 0-2 (control leg =  $1.19\pm 0.07\% \cdot d^{-1}$ ; immobilized leg =  
403  $1.01\pm 0.08\% \cdot d^{-1}$ ) and 2-7 days (control leg =  $1.11\pm 0.07\% \cdot d^{-1}$ ; immobilized leg =  $0.62\pm 0.05\% \cdot d^{-1}$ )  
404 in the immobilized compared with the control leg. Myofibrillar FSR remained unchanged  
405 over time in the control leg but declined by  $38\pm 11\%$  from 0-2 to 2-7 days ( $P=0.003$ ) in the  
406 immobilized leg. A Pearson's r product moment correlation showed a strong correlation  
407 between the two precursor pools for both legs over both the 0-2 and 2-7 day time points (both  
408 legs and time periods collapsed into one test) ( $r=0.995$ ,  $P<0.001$  [Figure 3F]).

409

#### 410 *Skeletal muscle gene expression*

411 The skeletal muscle expression of genes involved in insulin signaling and muscle protein  
412 synthesis/growth factors, muscle protein breakdown, carbohydrate and lipid metabolism, and  
413 mitochondrial metabolism/angiogenesis/force transduction are shown in **Figures 5, 6, 7 and 8**,  
414 respectively. The remaining genes of the 46 analyzed are shown in **Figure 9**. Thirteen of the  
415 analyzed genes (*P70S6K*, *EIF-4EBP1*, *REDD1*, *MTSN*, *GSI*, *HK2*, *FAT*, *CPT1B*, *PPAR $\alpha$* ,  
416 *DNML1*, *TFAM*, *SCGA*, *ANGPT1*) showed no changes throughout the experiment (time,  
417 condition and interaction effects all  $P>0.05$ ). Twenty genes exhibited a time effect (all  $P<0.05$ )  
418 only, such that they changed to an equivalent extent (either an increase [*AKT2*, *LAT1*, *PAT1*,  
419 *CLPN1*, *GDF11*, *FASN*, *MFN1*, *ITGB1*, *HIF1 $\alpha$* , *DES*, *mTOR*, *SHREBP1*, *BECN1*, *PDK4*,  
420 *FOXO1* and *SNAT2*] or a decrease [*PYGM*]) in both the control and immobilized legs. Some  
421 of these genes (*ACAB $\alpha$* , *DMD* and *VEGF $\alpha$* ) also exhibited a treatment effect (all  $P<0.05$ )  
422 without any interaction (all  $P>0.05$ ). Thirteen genes (*MAFbx*, *PGC1 $\alpha$* , *PSMB1*, *NFKb*, *IRS1*,  
423 *ACTN3*, *PI3K*, *MuRF1*, *FOXO3*, *CASP3*, *CD36*, *CLPN3*, *GLUT4*) displayed an interaction  
424 effect such that divergent responses occurred between legs over time (all  $P<0.05$ ). With one

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

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**450 Discussion**

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

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

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

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

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

520 Previous work, using data obtained from acute measurements of hourly muscle protein  
521 synthesis rates have estimated that reductions in muscle protein synthesis rates can fully (36)  
522 (or even over) explain (22) observed muscle atrophy after 3-6 weeks of leg immobilization. In  
523 contrast, we have previously estimated from our work that reductions in basal and/or post-  
524 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

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

555 With the difficulty of measuring *in vivo* MPB rates within the constraints of the current design,  
556 we applied a custom-designed low-density RT-qPCR microarray gene card to measure the  
557 relative expression of 46 targeted genes that encode proteins associated with the regulation of  
558 muscle mass, muscle deconditioning/reconditioning and muscle metabolism, with a specific  
559 sub-focus on genes involved in MPB. Thirteen genes were unaffected with a further 20 showing  
560 an equivalent change in both legs ((likely due to a repeated biopsy effect (44)). Thirteen of our  
561 selected genes changed differentially in the immobilized compared with control leg, and a  
562 striking observation is that 7 of those genes are involved in muscle protein breakdown, 4 of  
563 which encode proteins involved in the ubiquitin proteasome system (UPS) (*PSMB1* [Figure  
564 5F], *MAFbx* [Figure 5I], *MuRF1* [Figure 5J]) (7) and associated transcription factors (e.g.  
565 *FOXO3* [Figure 5B]). This coordinated change in gene expression of components of the UPS  
566 typically manifested as an increase after 7 but not 2 days of immobilization. In contrast, genes  
567 involved in other muscle protein breakdown pathways, for example the calcium dependent  
568 calpain system (3), were either unaffected (*CLPN1* [Figure 5]) or tended to be reduced after 7  
569 but not 2 days of immobilization (*CLPN3* [Figure 5]). These findings are line with and extend  
570 on our (47) and other research groups' (29) previous findings that genes specifically involved  
571 in the ubiquitination and degradation of myofibrillar proteins show a transcriptional rise  
572 consequent with disuse. Previous work has suggested that such a rise in gene expression is not  
573 immediate (11) but rapid (between 2 and 7 days) and transient (typically subsiding at time  
574 points beyond 1-2 weeks), and possibly indicative of an increased rate of muscle protein

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

586 In conclusion, muscle disuse induces a rapid decline in muscle volume (within 2 days) that  
587 continues at a similar rate for one week. The decline in muscle volume is accompanied by a  
588 ~36% decline in daily myofibrillar protein synthesis rates in healthy young men over one week  
589 of disuse. These data highlight the key responsible role that declining myofibrillar protein  
590 synthesis rates play in the development of muscle disuse atrophy *in vivo* in humans.

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

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<sub>2</sub>O, 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 <sup>2</sup>H-alanine (MPE) (left y-axis) and saliva <sup>2</sup>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 <sup>2</sup>H (%) and plasma free <sup>2</sup>H-alanine (MPE) analyzed by a Pearsons' correlation analysis. C, displays comparison of individual participant saliva enrichment (%) and plasma free <sup>2</sup>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  $\pm$  SEM,  $n = 13$ .

**Figure 3.** Free living myofibrillar fractional synthesis rates ( $\% \cdot d^{-1}$ ). Graphs A and B show free living myofibrillar FSR ( $\% \cdot d^{-1}$ ) 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 $\pm$ 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  $\times$  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 ( $2^{-\Delta\Delta C_t}$ ), 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  $\times$  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 ( $2^{-\Delta\Delta C_t}$ ), 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 $\pm$ 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  $\times$  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 ( $2^{-\Delta\Delta C_t}$ ), 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 $\pm$ 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  $\times$  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 ( $2^{-\Delta\Delta C_t}$ ), 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 $\pm$ 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·d <sup>-1</sup> )	11.1±0.7	11.3±1.0
(Kcal·d <sup>-1</sup> )	(2647±167)	(2689±288)
Protein intake (g·d <sup>-1</sup> )	119±7	115±12
Protein intake (g·kg <sup>-1</sup> ·d <sup>-1</sup> )	1.6±0.1	1.6±0.1
Protein intake (En%)	18±1	17.3±1
Carbohydrate intake (g·d <sup>-1</sup> )	298±25	308±42
Carbohydrate intake (En%)	45±2	46±2
Fat intake (g·d <sup>-1</sup> )	102±10	108±11
Fat intake (En%)	35±2	36±2
Light physical activity (h·d <sup>-1</sup> )	1.4±0.2	1.0±0.1
Moderate physical activity (h·d <sup>-1</sup> )	2.6±0.4	1.7±0.2*
Vigorous physical activity (h·d <sup>-1</sup> )	0.3±0.1	0.1±0.1
Total physical activity (h·d <sup>-1</sup> )	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	<i>IRS1</i>
Phosphoinositide-3-kinase regulatory subunit 1	<i>PI3K</i>
AKT serine/threonine kinase 2	<i>AKT2</i>
Mechanistic target of rapamycin	<i>mTOR</i>
Ribosomal protein S6 kinase B2	<i>RPS6KB1 (P70s6k)</i>
Eukaryotic translation initiation factor 4E binding protein 1	<i>EIF4EBP1 (4E-BP1)</i>
Solute carrier family 7 member 5	<i>SLC7A5 (LAT1)</i>
Solute carrier family 38 member 2	<i>SLC38A2 (SNAT2)</i>
Solute carrier family 36 member 1	<i>SLC36A1 (PAT1)</i>
DNA damage inducible transcript 4	<i>DDIT4 (REDD1)</i>
Eukaryotic 18S rRNA	<i>18s rRNA</i>
Forkhead box O1	<i>FOXO1</i>
Forkhead box O3	<i>FOXO3</i>
Calpain 1	<i>CLPN1</i>
Calpain 3	<i>CLPN3</i>
Caspase 3	<i>CASP3</i>
Proteasome subunit beta 1	<i>PSMB1</i>
Nuclear factor kappa B subunit 1	<i>NFKB</i>
F-box protein 32	<i>MAFbx</i>
Tripartite motif containing 63	<i>TRIM63 (MuRF1)</i>
Beclin 1	<i>BECN1</i>

Myostatin	<i>MTSN</i>
Growth differentiation factor 11	<i>GDF11</i>
Solute carrier family 2 member 4	<i>GLUT4</i>
Glycogen Phosphorylase (muscle)	<i>PYGM</i>
Glycogen synthase 1	<i>GSI</i>
Hexokinase 2	<i>HK2</i>
Pyruvate dehydrogenase kinase 4	<i>PDK4</i>
CD36 molecule	<i>CD36</i>
Fatty acid synthase	<i>FASN</i>
Sterol regulatory element binding transcription factor 1	<i>SHREBP1</i>
Carnitine palmitoyltransferase 1B, Acetyl-CoA carboxylase beta	<i>CPT1B</i> <i>ACACA</i>
Peroxisome proliferator activated receptor alpha	<i>PPARα</i>
PPARG coactivator 1 alpha	<i>PGC1α</i>
Mitofusin 1	<i>MFN1</i>
Dynamin 1 like protein	<i>DNM1L</i>
Transcription factor A, mitochondrial	<i>TFAM</i>
Dystrophin	<i>DMD</i>
Sarcoglycan alpha	<i>SGCA</i>
Laminin subunit alpha 2	<i>LAMA2</i>
Integrin subunit beta 1	<i>ITGB1</i>
Actinin alpha 3	<i>ACTN1</i>
Desmin	<i>DES</i>
Vascular endothelial growth factor A	<i>VEGF</i>

Hypoxia inducible factor 1 alpha subunit

*HIF-1*

Angiopoietin 1

*ANGPT1*

Glyceraldehyde-3-phosphate dehydrogenase

*GAPDH*

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Symbols in brackets represent gene synonyms.

**Figure 1**

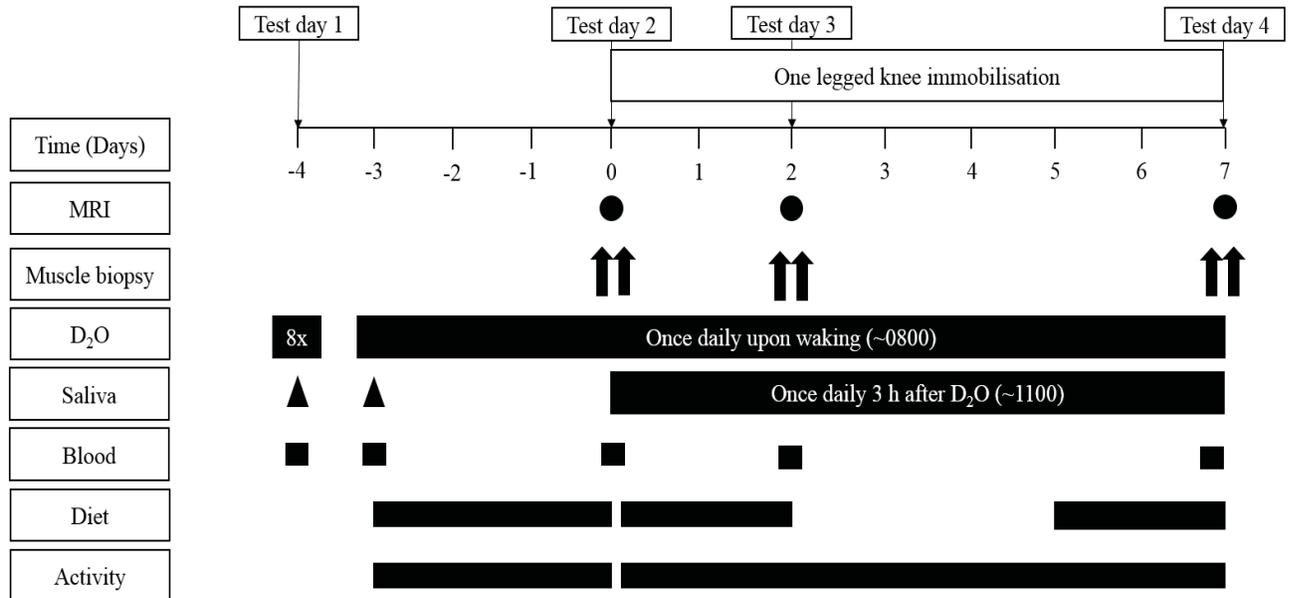


Figure 2

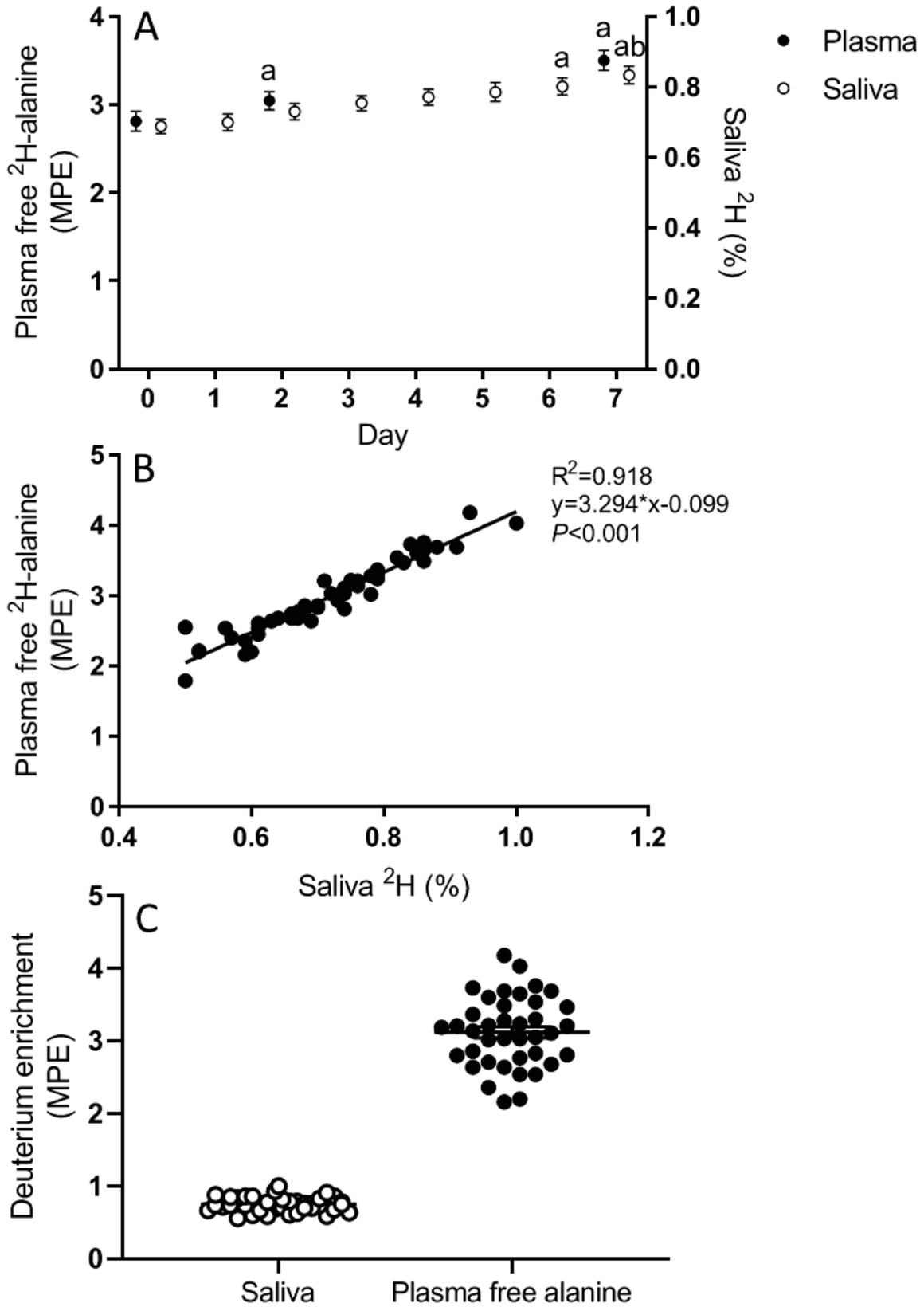


Figure 3

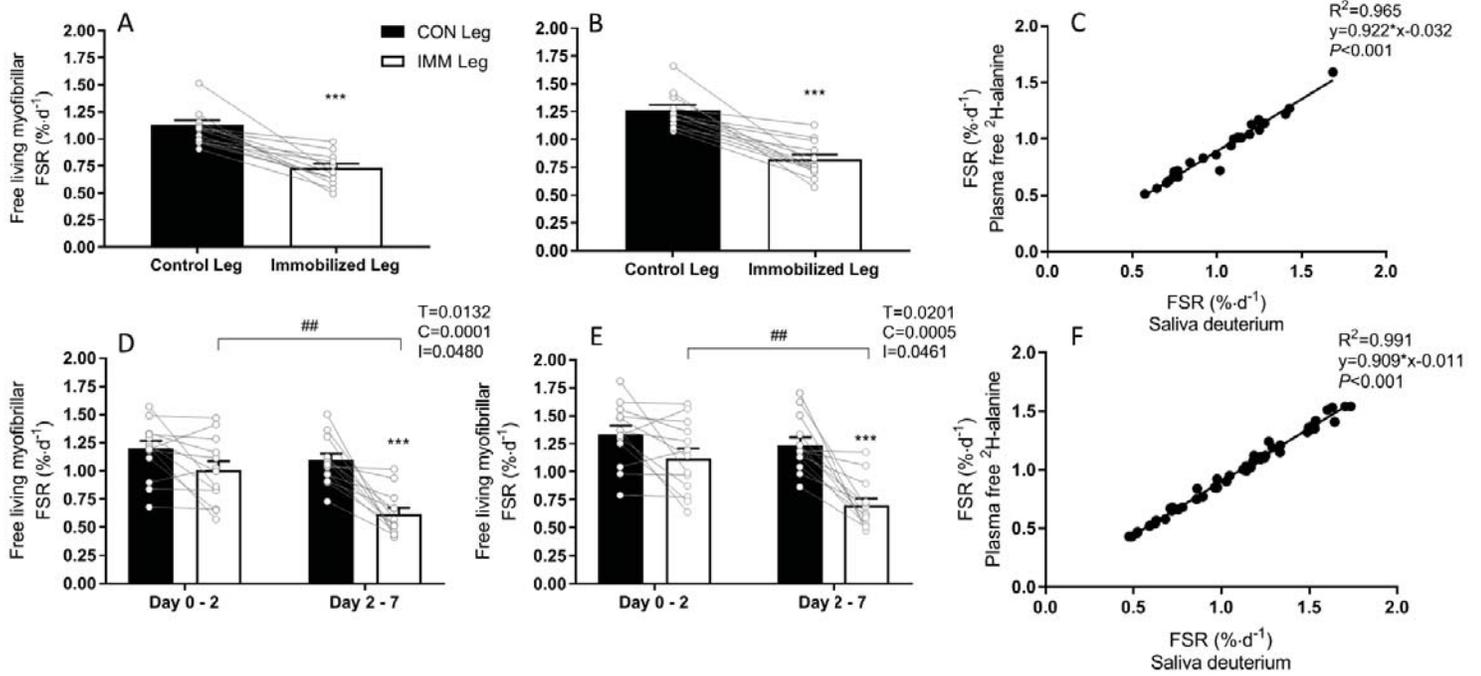
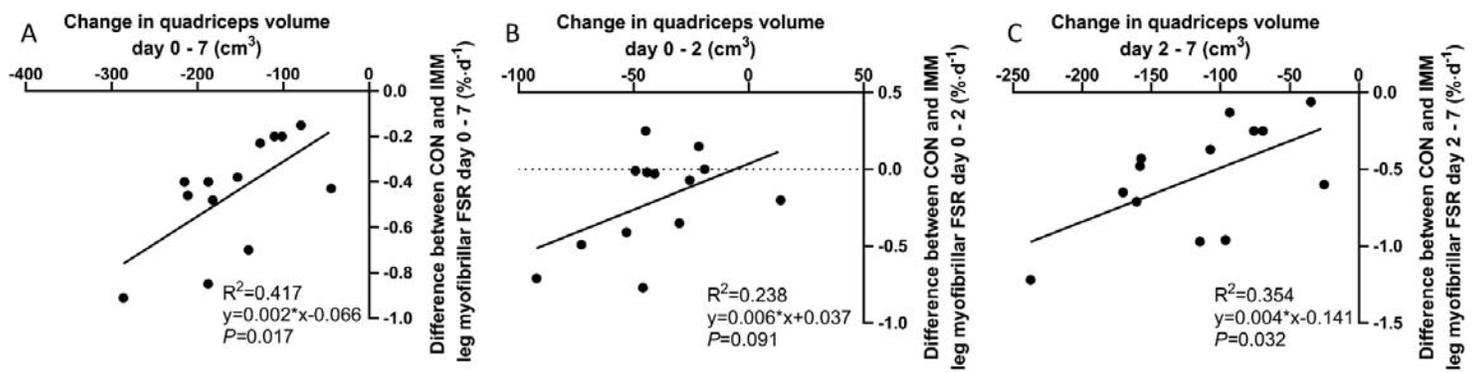
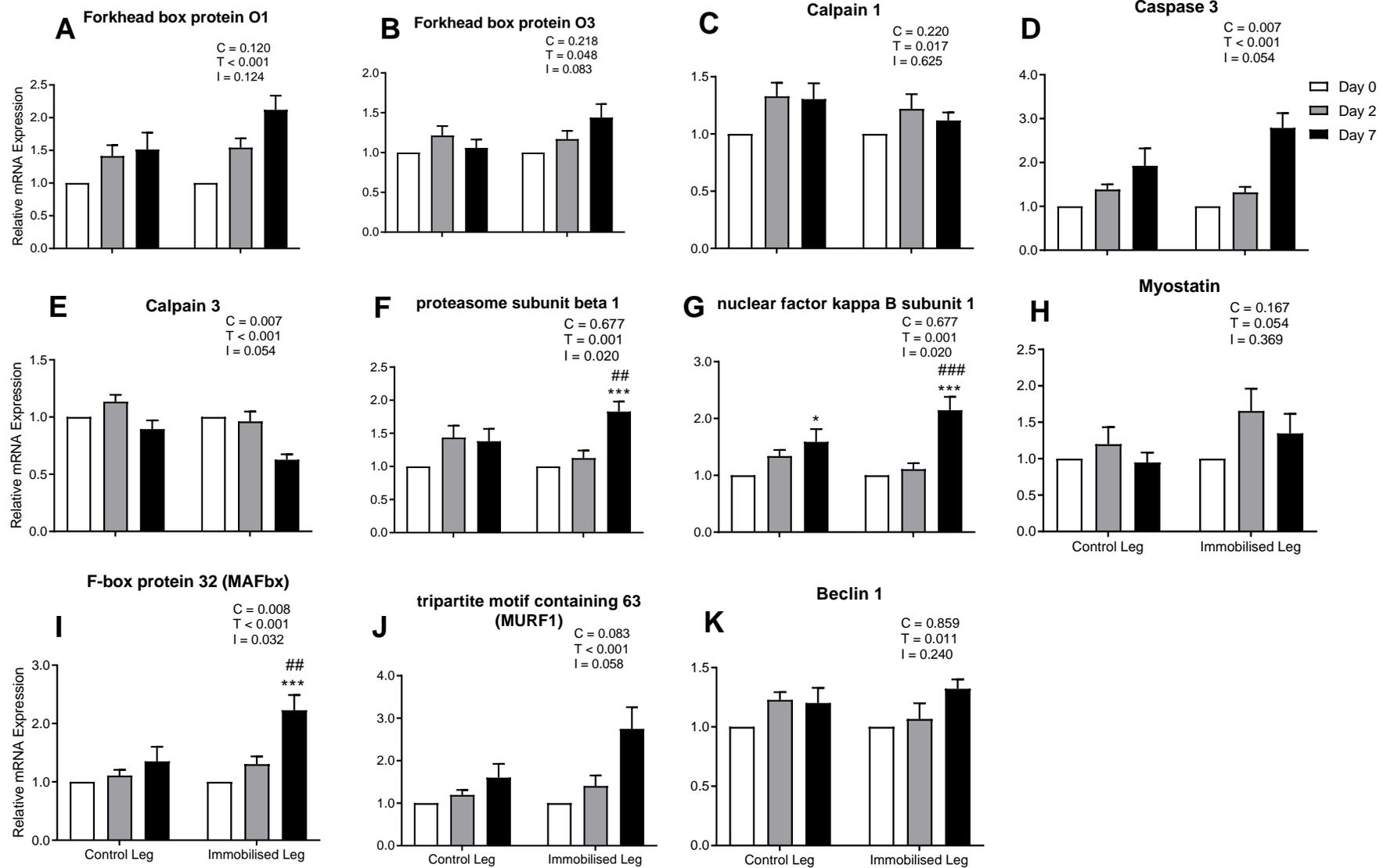


Figure 4

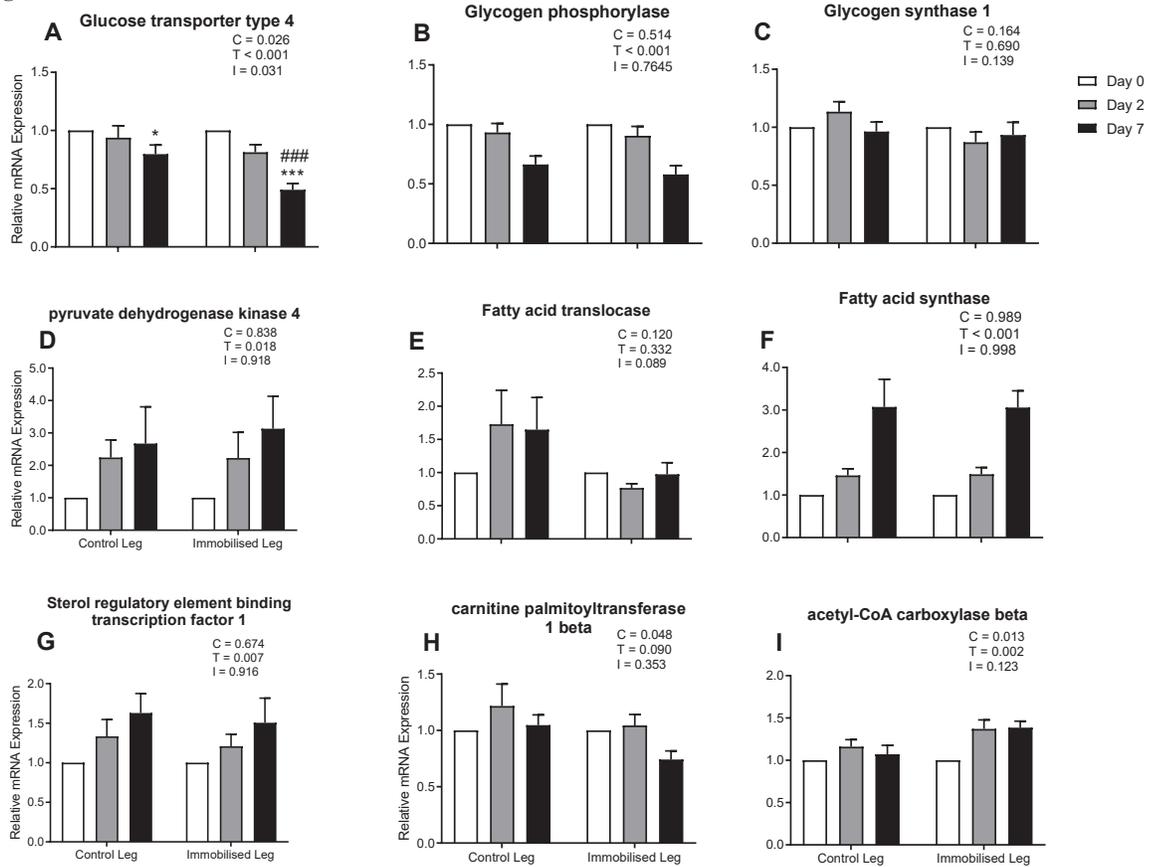




**Figure 6**



**Figure 7**



**Figure 8**

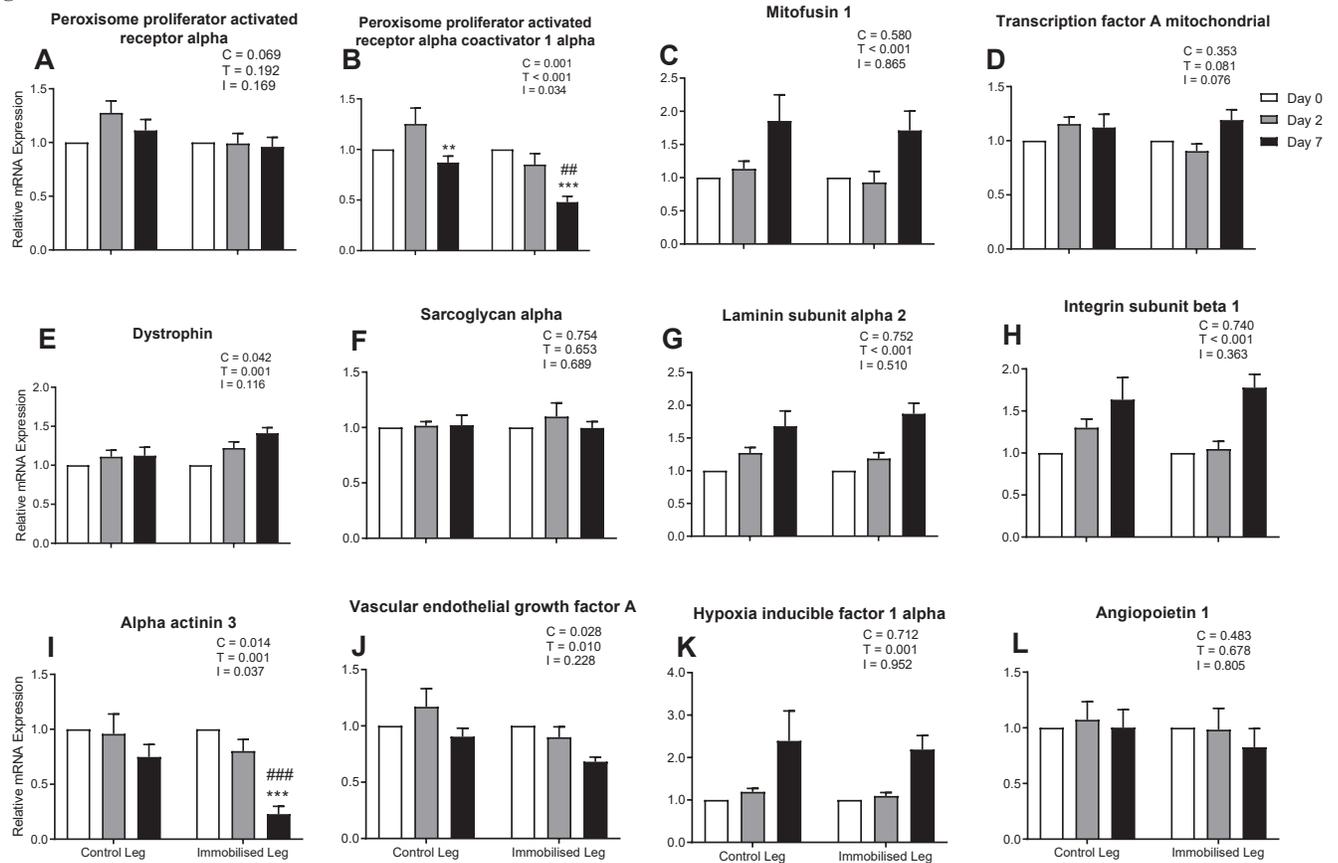


Figure 9

