

# A single day of bed rest, irrespective of energy balance, does not affect skeletal muscle gene expression or insulin sensitivity

Marlou L Dirks<sup>1\*</sup>, Francis B Stephens<sup>1\*</sup>, Sarah R Jackman<sup>1</sup>, Jesús Galera Gordo<sup>1</sup>, David Machin<sup>1</sup>, Richard M Pulsford<sup>1</sup>, Luc JC van Loon<sup>2</sup>, and Benjamin T Wall<sup>1</sup>

\*These authors contributed equally to this work

<sup>1</sup>*Department of Sport and Health Sciences, College of Life and Environmental Sciences, University of Exeter, United Kingdom*

<sup>2</sup>*Department of Human Biology, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre<sup>+</sup>, the Netherlands*

## Corresponding author:

Benjamin T. Wall, PhD  
Department of Sport and Health Sciences  
College of Life and Environmental Sciences  
St Luke's Campus, Heavitree Road  
University of Exeter  
Exeter, EX1 2LU  
UK  
Tel: +44 (0)1392 724774  
Email: [b.t.wall@exeter.ac.uk](mailto:b.t.wall@exeter.ac.uk)

**Running title:** Acute transcriptional responses with bed rest

**Keywords:** Disuse, glucose tolerance, insulin resistance, nutrition, transcriptional response

**Word count:** 6654

## **Abbreviations**

AUC, area under the curve; BR-B, bed rest-balanced; BR-M, bed rest-matched; CGMS, continuous glucose monitoring system; CHO, carbohydrate; CON, control; Dm, dry matter; HOMA-IR, Homeostatic Model Assessment of Insulin Resistance; MVPA, moderate-to-vigorous physical activity; OGTT, oral glucose tolerance test; RER, respiratory exchange ratio; RMR, resting metabolic rate; 1RM, one-repetition maximum

1 **New Findings**

2

3 *What is the central question of this study?*

4 What are the initial metabolic and molecular events that underpin bed rest-induced skeletal muscle  
5 deconditioning, and what is the contribution of energy balance?

6 *What is the main finding and its importance?*

7 A single day of bed rest, irrespective of energy balance, did not lead to overt changes in skeletal muscle  
8 gene expression or insulin sensitivity. More than one day of physical inactivity is required to observe  
9 the insulin resistance and robust skeletal muscle transcriptional responses associated with bed rest and  
10 consequent alterations in energy balance.

11 **Abstract**

12 The initial metabolic and molecular events that underpin disuse-induced skeletal muscle  
13 deconditioning, and the contribution of energy balance, remain to be investigated. Ten young, healthy  
14 males (age:  $25 \pm 1$  y; BMI:  $25.3 \pm 0.8$  kg m<sup>-2</sup>) underwent three 24 h laboratory-based experimental periods  
15 in a randomized, crossover manner: 1) controlled habitual physical activity with an energy-balanced  
16 diet (CON); 2) strict bed rest with a diet to maintain energy balance (BR-B); and 3) strict bed rest with  
17 a diet identical to CON, consequently resulting in positive energy balance. Continuous glucose  
18 monitoring was performed throughout each visit, with vastus lateralis muscle biopsies and an oral  
19 glucose tolerance test performed before and after. In parallel with muscle samples collected from a  
20 previous 7-day bed rest study, biopsies were used to examine expression of genes associated with the  
21 regulation of muscle mass and insulin sensitivity. A single day of bed rest, irrespective of energy  
22 balance, did not lead to overt changes in whole-body substrate oxidation, indices of insulin sensitivity  
23 (i.e. HOMA-IR (BR-B: from  $2.7 \pm 1.7$  to  $3.1 \pm 1.5$ ,  $P > 0.05$ ), Matsuda (BR-B: from  $5.9 \pm 3.3$  to  $5.2 \pm 2.9$ ,  
24  $P > 0.05$ )), or 24 h glycaemic control/variability compared to CON. Seven days of bed rest led to ~30-  
25 55% lower expression of genes involved in insulin signalling, lipid storage/oxidation, and muscle  
26 protein breakdown, whereas no such changes were observed after one day of bed rest. In conclusion,  
27 more than one day of physical inactivity is required to observe the insulin resistance and robust skeletal  
28 muscle transcriptional responses associated with bed rest and consequent alterations in energy balance.

29

30 Abstract word count: 250

## 31 **Introduction**

32 The recovery from illness or rehabilitation after injury often requires a period of local (e.g. limb  
33 immobilization) or whole-body (e.g. bed rest) muscle disuse. Even in otherwise healthy humans, a  
34 single bout of muscle disuse leads to rapid skeletal muscle loss of approximately 0.5-0.6% per day  
35 (Deitrick, 1948; Gibson *et al.*, 1987; Wall & van Loon, 2013). This muscle atrophy is accompanied by  
36 a decrease in functional capacity (Deitrick, 1948; Gibson *et al.*, 1987), insulin sensitivity (Stuart *et al.*,  
37 1988; Mikines *et al.*, 1991; Dirks *et al.*, 2016), glucose tolerance (Yanagibori *et al.*, 1994; Dirks *et al.*,  
38 2016), and basal metabolic rate (Haruna *et al.*, 1994). However, the underlying mechanisms responsible  
39 for disuse-induced skeletal muscle deconditioning remain incompletely understood.

40 We (Dirks *et al.*, 2016) and others (Stuart *et al.*, 1988; Mikines *et al.*, 1991; Bienso *et al.*, 2012) have  
41 previously shown that muscle atrophy observed following one week of bed rest is associated with a  
42 ~30% decrease in insulin sensitivity. However, it has been demonstrated that changes in glucose  
43 homeostasis and insulin sensitivity already occur after 3-5 days of muscle disuse (Yanagibori *et al.*,  
44 1994; Reidy *et al.*, 2017), which suggests these processes may be evident even sooner. A major health  
45 consequence of impaired insulin sensitivity is reduced blood clearance of ingested carbohydrates and,  
46 as a result, greater frequency and magnitude of hyperglycaemic excursions throughout the day (van  
47 Dijk & van Loon, 2015), which is also linked to the development of metabolic inflexibility (i.e. reduced  
48 ability to appropriately switch between the oxidation of fat and carbohydrate depending on prandial  
49 status (Kelley & Mandarino, 2000)). Aside from the development of insulin resistance and metabolic  
50 inflexibility, disuse-induced deconditioning also includes an imbalance between muscle protein  
51 synthesis and breakdown (Wall *et al.*, 2013) and the accumulation of lipid in skeletal muscle tissue  
52 (Cree *et al.*, 2010; Bergouignan *et al.*, 2011). Although it generally takes several days or weeks for these  
53 changes to occur, the identification of early transcriptional changes that occur prior to (or coincide with)  
54 the onset of insulin resistance could point at mechanisms responsible for the muscle deconditioning that  
55 is observed later on. Consequently, the primary aim of this study was to establish which aspects of  
56 glucose intolerance occur first, if at all, and how these are related to the early skeletal muscle  
57 transcriptional response that could account for the well-established deconditioning in muscle  
58 metabolism and the development of insulin resistance during muscle disuse.

59 A consideration of muscle disuse research is how to isolate the impact of physical inactivity *per se*, and  
60 not the influence of a concomitant (due to altered energy demands) change in energy intake. That is to  
61 say, changes in energy balance and/or macronutrient intake can impact on insulin sensitivity and muscle  
62 mass independently of physical activity status (Hulston *et al.*, 2015; Backx *et al.*, 2016). For example,  
63 maintaining a habitual diet during a period of physical inactivity will impose a positive energy balance  
64 and would therefore exacerbate the impact of muscle disuse on insulin sensitivity and muscle atrophy  
65 (Biolo *et al.*, 2008; Peterson *et al.*, 2017). However, decreasing energy intake during physical inactivity,  
66 in line with reduced energy demands, inevitably leads to changes in absolute or relative protein intake,  
67 factors which have been shown to accelerate muscle disuse atrophy (Biolo *et al.*, 2007). As such, a  
68 secondary aim of this study was to investigate the impact of reducing (according to reduced energy  
69 requirements) or maintaining (matched to an individual's habitual diet) energy intake on the muscle  
70 transcriptional response and glucose homeostasis during a single day of bed rest.

71 In the present study we hypothesised that a single day of bed rest would lead to a coordinated  
72 transcriptional response within skeletal muscle tissue associated with alterations in the expression of  
73 genes relating to muscle mass and insulin sensitivity, which would be accompanied by the onset of  
74 whole-body insulin resistance and impaired blood glucose homeostasis. To address the relative impact  
75 of muscle disuse *per se* vs alterations in diet, we applied a fully controlled diet under three separate  
76 conditions: 1) maintaining energy balance in a control condition (habitual physical activity; CON), 2)  
77 maintaining energy balance during bed rest (BR-B), 3) matching the CON diet but under bed rested  
78 conditions (i.e. resulting in a positive energy balance; BR-M). Furthermore, to design a true control  
79 condition that could represent habitual, 'free-living' physical activity, but be performed under  
80 laboratory conditions, we used free-living accelerometry data to design individualised 24 h activity  
81 patterns for each volunteer.

## 82 **Methods**

83

### 84 *Ethical approval*

85 All subjects were informed on the nature and risks of the experiment before written informed consent  
86 was obtained. The study was approved by the Department of Sport and Health Sciences, University of  
87 Exeter's Ethical Committee (proposal reference number 141203/B/09) in accordance with the  
88 Declaration of Helsinki (version October 2013). This study was registered at clinicaltrials.gov as  
89 NCT02981927.

90

### 91 *Subjects*

92 Ten healthy young men (age:  $25\pm 1$  y; BMI:  $25.3\pm 0.8$  kg·m<sup>-2</sup>) were included in the present study.  
93 Subjects' characteristics are presented in **Table 1**. Prior to inclusion in the study, subjects completed a  
94 routine medical screening to ensure suitability for acceptance onto the study. During the screening,  
95 subjects completed a general health questionnaire and height, weight, and resting blood pressure were  
96 determined. Exclusion criteria were: a BMI below 18.5 or above 30 kg·m<sup>-2</sup>, regular smoker, a (family)  
97 history of thrombosis, type-2 diabetes mellitus and any major current back, knee or shoulder complaints.  
98 Furthermore, subjects who had been involved in structured and prolonged resistance-type exercise  
99 training during the six months prior to the study were excluded. Subjects were also instructed to cease  
100 taking any nutritional supplements for two weeks prior to the study until all study visits were completed.

101

### 102 *Experimental overview*

103 In a randomized, crossover design, subjects participated in three 24 h metabolic test days each including  
104 an overnight stay within the laboratory (**Figure 1**). During the control (CON) visit, subjects maintained  
105 a supervised, pre-designed 24 h period of habitual physical activity within the confines of the  
106 laboratories and University campus and were provided a diet designed to maintain energy balance. On  
107 the two other visits, subjects underwent a 24 h period of strict bed rest and were provided with either a  
108 diet with reduced energy content designed to maintain energy balance during bed rest (bed rest-balance;  
109 BR-B) or the same diet as during the control visit, which was 133% of energy requirements (bed rest-

110 matched; BR-M). At the beginning and end of study visit a muscle biopsy was obtained, an oral glucose  
111 tolerance test (OGTT) was performed, and resting energy expenditure and whole body substrate  
112 oxidation were determined under fasting conditions. Muscle strength was assessed prior to the first  
113 experimental visit, and immediately following the three visits. In addition, interstitial continuous  
114 glucose monitoring was applied during the test days.

115

### 116 *Pre-testing*

117 Following acceptance onto the study, two pre-testing visits were conducted. During the first pre-testing  
118 visit, subjects arrived at the laboratory in the overnight fasted state where body composition (body fat  
119 and lean mass) was determined by Air Displacement Plethysmography (Bodpod; Life Measurement,  
120 Inc., Concord, CA, USA). Thereafter, resting metabolic rate and whole body substrate oxidation were  
121 determined using expired gas collections through a facemask for indirect calorimetry (Cortex Metalyzer  
122 2R gas analyser, Cortex, Leipzig, Germany). First, subjects were asked to lie quiet and still in a supine  
123 position for 30 min, after which a 30 min measurement period commenced. The last 10 min of this  
124 measurement period was used to obtain average  $\dot{V}O_2$  and  $\dot{V}CO_2$  values to determine substrate oxidation  
125 rates according to the non-protein stoichiometric equations detailed by Frayn (Frayn, 1983). Total  
126 energy expenditure during this period was then calculated as the sum of energy production from fat and  
127 carbohydrate, assuming that the oxidation of 1 g of triacylglycerol ( $862 \text{ g mol}^{-1}$ ) liberates 39.4 kJ and 1  
128 g of glucose ( $180 \text{ g mol}^{-1}$ ) liberates 15.6 kJ of energy. This was then used to calculate resting 24 h energy  
129 expenditure.

130 Subjects were then given a list of all food products in the standardized diet, and were asked to indicate  
131 if they had any intolerances or allergies, or if there were products they could not eat. Based on this, a  
132 standardized diet was created that consisted of breakfast, lunch, mid afternoon snack, dinner, and pre  
133 bedtime snack. The nutritional content and macronutrient composition of the diet was calculated using  
134 specific nutritional software (Nutritics Professional Nutritional Analysis Software, Swords, Co. Dublin,  
135 Ireland). Subjects were then issued with two accelerometers that were worn simultaneously for three  
136 days. An activPAL™ (PAL Technologies Ltd, Glasgow, UK) was adhered to the anterior aspect of the  
137 mid-thigh using a waterproof dressing in order to measure daily minutes of sitting, standing, walking.



138 A GENEActiv accelerometer (ActivInsights, Kimbolton, Cambridge, UK) was worn on the left wrist  
139 to classify daily physical activity by intensity. Physical activity data from the GENEActiv monitors was  
140 processed in R using the GGIR package (<http://cran.r-project.org>) and activity intensity estimated using  
141 previously validated cut points (Esliger *et al.*, 2011). Finally, unilateral maximal leg extension strength  
142 (one-repetition maximum, 1-RM) was estimated for both legs separately. All subjects warmed up  
143 briefly (10 min cycling at 100 W) and were then instructed and familiarized with safe lifting technique  
144 for the leg extension exercise. Maximum strength was estimated using the multiple repetitions testing  
145 procedure for each leg separately (Mayhew *et al.*, 1995). At least two days later, subjects attended the  
146 laboratory to confirm single leg one repetition maximum (1RM; (Kraemer & Fry, 1995)). Briefly, the  
147 load was set at 97.5% of the estimated 1-RM from the previous visit, and increased after each successful  
148 lift until failure. Two minute rest periods were allowed between lifts. A repetition was considered valid  
149 when the subject used proper form and was able to complete the entire lift in a controlled manner  
150 without assistance. The maximum strength obtained during this visit was taken as their baseline for the  
151 duration of the study.

152

### 153 *Dietary and physical activity control designs*

154 Two diets were designed for each subject individually containing their selected meals and snacks (*see*  
155 *above*). The two diets differed only in their energy content (amount of each chosen meal provided) and  
156 therefore their macronutrient compositions were identical, i.e. 56 en% carbohydrate; 32 en% fat; 12  
157 en% protein (**Table 2**). The first diet was designed to provide energy balance during a day of habitual  
158 physical activity (for the CON and BR-M visits), and was calculated as 24 h resting energy expenditure  
159 multiplied by an activity factor of 1.6. The second diet was designed to provide energy balance during  
160 a day of bed rest (BR-B visit) and was calculated as 24 h resting energy expenditure multiplied by an  
161 activity factor of 1.2. As a result, during the BR-M visits, subjects' energy intake was 33% greater than  
162 during BR-B.

163 For the CON visit, a day mimicking habitual free-living physical activity was individually designed for  
164 each subject based upon physical activity diaries and accelerometer data collected during the pre-testing  
165 period. Average values for minutes spent sitting, standing and walking were computed across

166 measurement days for the following periods: 11:00-14:00, 14:00-17:00, 17:00-20:00 and 20:00-23:00.  
167 Within each corresponding period during the control visit, the total minutes in each behaviour and the  
168 distribution of any prolonged bouts of activity were replicated as far as the experimental protocols  
169 would allow. In addition, all regular volitional exercise reported in the physical activity diary was  
170 replicated in terms of mode, intensity/workload and duration. Any walking activity and volitional  
171 exercise habitually undertaken prior to 11:00 was replicated between 11:00 and 14:00. An example plan  
172 for the CON visit can be seen in **Figure 2**.

173

#### 174 *Experimental visits*

175 Following inclusion and pre-testing, subjects participated in three experimental test days after having  
176 refrained from strenuous physical activity and alcohol for 2 days prior. To standardise pre-trial dietary  
177 intake, subjects were asked to record their food intake on the evening prior to the first experimental  
178 visit, and to replicate this prior to the following two visits. Subjects arrived at the laboratory at 07.00 in  
179 the fasted state, voided their bladder and rested semi supine on a hospital bed. A single muscle biopsy  
180 was taken from *m. vastus lateralis*. A cannula was placed into an antecubital vein in their forearm and  
181 a facemask was applied for 30 min of expired gas collections, with the final 10 min being used to  
182 determine metabolic rate and whole body substrate oxidation via indirect calorimetry (Cortex Metalyzer  
183 2R gas analyser, Cortex, Leipzig, Germany; *calculations described above*). A baseline venous blood  
184 sample was obtained (8 mL) and subjects then ingested 75 g glucose dissolved in 300 mL water  
185 (Dextrose, BulkPowders.com), signifying the beginning of a 120 min OGTT period. A venous blood  
186 sample was drawn from the cannula every 15 min for the measurement of glucose and insulin, and  
187 indirect calorimetry was performed throughout the 120 min OGTT period.

188 For the CON visit, the end of the OGTT signified the beginning of the day of pre-determined physical  
189 activity. For each three hour period (11:00-14:00, 14:00-17:00, 17:00-20:00 and 20:00-23:00) time  
190 spent (minutes) sitting, standing and walking, and any exercise behaviour were prescribed in order to  
191 mimic habitual free-living behaviour. Subjects were monitored throughout the day by members of the  
192 research team, and constant feedback was provided to ensure the required amount of each activity was  
193 undertaken in each 3 h block as far as possible. All the conditions were performed within the university

194 campus and subjects were accompanied by a researcher at all times. Lying time was considered as a  
195 sitting condition. Subjects returned to bed and asked to sleep at 23:00.

196 For the BR-B and BR-M visits, following the biopsy and OGTT subjects remained (semi)supine for the  
197 rest of the day and night, with an optional pillow under their head. All toilet activities were performed  
198 using a bedpan, and meals were served in bed and consumed in a supine position to ensure no weight  
199 bearing or upright posturing occurred for the 24 h period. Subjects were accompanied by a member of  
200 the research team continuously through the day and evening, and laptops, TV/DVDs, books and games  
201 were available for entertainment. At night, subjects could communicate with the researchers who were  
202 available in an adjacent room by the use of baby monitors (also serving to ensure no major movements  
203 were taking place in the researchers' absence). Lights were switched off and subjects requested to sleep  
204 at 23:00.

205 For all visits, meals were provided following metabolic testing at identical times: 11:00 (breakfast),  
206 14:00 (lunch), 16:30 (snack), 19:00 (dinner), and 21:00 (snack). The next morning, subjects were woken  
207 up at 7:00 and an indirect calorimetry and OGTTs were performed in the same manner as the previous  
208 day. Thereafter, subjects were provided with breakfast and walked to the gym to perform the 1-RM test  
209 in an identical manner as during the second pre-testing visit.

210

#### 211 *Continuous blood glucose monitoring system (CGMS)*

212 Glucose sensors (Enlite®, Medtronic Inc, Northridge, CA, USA) were placed subcutaneously at the  
213 side of the abdomen and attached to a continuous glucose monitoring system (CGMS; iPRO2®,  
214 Medtronic Inc, Northridge, CA, USA) on the day prior to the experimental visit. Within several hours  
215 after placing the probe, the device started collecting a blood glucose reading every 5 min. Data were  
216 collected from the start of the pre-OGTT until after the post-OGTT, after which the probe was removed.  
217 Such an approach has been shown to generate accurate glucose readings representative of plasma  
218 glucose levels for at least 6 days (Keenan *et al.*, 2012). The recorded data was analysed for glycaemic  
219 control (i.e. average 24 h glucose, and glucose area under the curve (AUC)) and glycaemic variability  
220 (SD, CONGA1, CONGA2). The CONGA1 and CONGA2 indices were determined by calculating the

221 difference between each glucose concentration and the reading 1 (CONGA1) or 2 (CONGA2) hours  
222 earlier, and taking the SD of those differences (McDonnell *et al.*, 2005).

223

#### 224 *Blood analyses*

225 Blood samples (5 mL) were collected for determination of plasma glucose and serum insulin  
226 concentrations prior to and during the OGTT. A small part (1 mL) was immediately analysed for whole  
227 blood glucose concentrations (YSI 2300 PLUS, Yellow Springs, OH, USA). The second part was  
228 collected in BD vacutainers (SST II) which were left to clot at room temperature for at least 30 min and  
229 then centrifuged at 3000 x g and 21°C for 15 min to obtain serum samples. These were used to determine  
230 insulin concentrations using a commercially available kit (human insulin ELISA kit, DX-EIA-2935,  
231 Oxford Biosystems Ltd, Milton Park, UK). Glucose and insulin concentrations were used to calculate  
232 the HOMA-IR (Matthews *et al.*, 1985) and Matsuda (Matsuda & DeFronzo, 1999) indices.

233

#### 234 *Skeletal muscle biopsy collection and analysis*

235 Muscle biopsies were taken in the morning prior to the pre- and post-OGTTs, in the overnight-fasted  
236 state. Percutaneous muscle biopsy samples were collected from *m. vastus lateralis*, ~15 cm above the  
237 patella with the Bergstrom technique (Bergstrom, 1975). The collected muscle was freed from any  
238 visible non-muscle tissue, snap-frozen in liquid nitrogen, and stored at -80°C until further analysis.

239 Muscle glycogen content was determined spectrophotometrically, as described elsewhere (Harris *et al.*,  
240 1974).

241 Skeletal muscle mRNA expression of 48 metabolic genes was analysed as published previously  
242 (Tsintzas *et al.*, 2013). In brief, total RNA was extracted from ~15-20 mg frozen muscle tissue  
243 (Chomczynski & Sacchi, 1987), and quantification and reverse transcription of RNA was carried out  
244 (Tsintzas *et al.*, 2006). Taqman low-density custom array cards (ABI Applied Biosystems, Foster City,  
245 CA, USA) were used for the relative quantification of expression of genes involved in muscle mass  
246 regulation and metabolic health. Each card allowed for eight samples to be run in parallel against 48  
247 Taqman gene expression assay targets that were pre-loaded into each of the wells on the card  
248 (**Supplemental Table 1**). In short, 50 µL Taqman Universal PCR master mix (2x; ABI Applied

249 Biosystems) was added to 200 ng of RNA equivalent of cDNA into an RNase free Eppendorf tube, and  
250 RNase free water was added to make the total reaction volume up to 100  $\mu$ L. The reaction mixture was  
251 mixed, centrifuged and loaded into one of the fill reservoir of the Micro Fluidic card, after which the  
252 cards were centrifuged (MULTIFUGE 3 S-R; Heraeus) and ran on a 7900HT Fast Real-Time PCR  
253 System (ABI Applied Biosystems). Relative quantification of the genes of interest was performed using  
254 the comparative *Ct* method. Data was normalised to the geometric mean of ACTA1 (Stephens *et al.*,  
255 2010), GAPDH (Mahoney *et al.*, 2004), and HMBS (Wall *et al.*, 2012) to minimize variation in the  
256 expression of individual housekeeping genes. Muscle biopsies from  $n=7$  subjects in this study (age  
257  $25\pm 4$ , height  $1.76\pm 0.06$ , BMI  $25.1\pm 2.4$ ) were compared to biopsies from  $n=7$  age-, height-, and BMI-  
258 matched subjects (age  $23\pm 2$ , height  $1.82\pm 0.08$ , BMI  $23.6\pm 2.8$ , all  $P>0.05$  when compared to the current  
259 study) who underwent seven days of strict bed rest in our previous study (Dirks *et al.*, 2016), to assess  
260 the early (BR-B) vs late (7 DAYS; i.e. serving as a positive control) effects of bed rest on muscle mRNA  
261 expression. Samples from both studies were extracted and analysed at the same time, using the same  
262 reagents.

263

#### 264 *Statistical analysis*

265 All data are expressed as means $\pm$ SD. Paired samples t-tests were used to examine differences in planned  
266 versus observed minutes of sitting, standing and walking during the control day. Data was analysed  
267 using a Repeated Measures ANOVA with condition (CON vs BR-B vs BR-M) and, when appropriate,  
268 time (pre vs post) and feeding status (fasted vs fed), all as within-subjects factors. To assess the effect  
269 of bed rest duration on muscle gene expression (BR-B vs 7 DAYS), an independent samples t-test was  
270 used. For all ANOVA's when a significant interaction was detected, paired sample t tests were applied  
271 to test the effect within the different conditions. Statistical significance was set at  $P<0.05$ . All *P*-values  
272 are reported as  $<0.05$  or  $>0.05$ ; however, *P*-values between 0.05 and 0.10 are considered trends and  
273 depicted as an exact number. All calculations were performed by using SPSS version 23.0 (IBM Corp,  
274 Armonk, NY, USA).

275 **Results**

276

277 *Standardised physical activity and muscle strength*

278 The minutes of lying/sitting ( $551\pm114$  min·d<sup>-1</sup>), standing ( $312\pm103$  min·d<sup>-1</sup>), and walking ( $145\pm41$   
279 min·d<sup>-1</sup>) during the CON visit were not different to the habitual activity measured during pre-testing  
280 (**Figure 2**; all  $P>0.05$ ). All regular volitional exercise was successfully replicated during the CON days  
281 in terms of intensity/workload and duration. Maximal leg strength did not change following the CON  
282 (from  $83.7\pm17.7$  to  $83.3\pm18.6$  kg), BR-B ( $84.0\pm17.7$  to  $80.3\pm19.1$  kg), or BR-M ( $84.0\pm17.7$  to  
283  $79.9\pm19.2$  kg) visits (interaction and time effect: both  $P>0.05$ ).

284

285 *Whole-body substrate utilisation*

286 During both pre and post OGTTs, carbohydrate ingestion led to an increase in the relative contribution  
287 of carbohydrate oxidation to energy expenditure (**Figure 3**;  $P<0.01$ ). This was accompanied by a  
288 resultant increase in respiratory exchange ratio (RER) and energy expenditure (both  $P<0.01$ ).

289 Following all three 24 h visits, RER was higher in the fasting state and following carbohydrate ingestion  
290 when compared to pre-measurements ( $P<0.01$ ), which was due to an increase in the relative contribution  
291 of carbohydrate oxidation to energy expenditure ( $P<0.05$ ). A trend for a time\*feeding interaction was  
292 found for the contribution of carbohydrate oxidation to energy expenditure ( $P=0.066$ ), suggesting an  
293 impaired capacity to switch from fat to carbohydrate oxidation after carbohydrate ingestion following  
294 all three visits.

295

296 *Insulin sensitivity and glycaemic control*

297 Fasting blood glucose concentrations prior to and following the 24 h experimental periods were not  
298 different between the CON (from  $4.59\pm0.30$  to  $4.56\pm0.27$  mmol·L<sup>-1</sup>), BR-B (from  $4.52\pm0.35$  to  
299  $4.56\pm0.33$  mmol·L<sup>-1</sup>), and BR-M (from  $4.49\pm0.31$  to  $4.62\pm0.25$  mmol·L<sup>-1</sup>) visits, although a trend for a  
300 condition\*time interaction ( $P=0.065$ ) was observed. None of the interventions affected fasting serum  
301 insulin concentrations. As a result, HOMA-IR (Matthews *et al.*, 1985) (**Figure 4A**) showed a trend for  
302 a condition\*time interaction ( $P=0.096$ ), due to a trend for an increase in BR-M only ( $P=0.061$ ).

303 Peripheral insulin sensitivity, as indicated by the Matsuda index (Matsuda & DeFronzo, 1999) (**Figure**  
304 **4B**,  $P>0.05$ ), was not affected by time or intervention. Mean 24 h interstitial glucose concentrations  
305 during the 24 h experimental periods were  $5.7\pm 0.4$ ,  $6.0\pm 0.4$ , and  $5.7\pm 0.4$   $\text{mmol}\cdot\text{L}^{-1}$  in the CON, BR-B,  
306 and BR-M conditions, respectively (data from  $n=7$ ). Analyses of the 24 h glucose traces, depicted in  
307 **Figure 4C**, showed no difference in 24 h glucose area under the curve (AUC; inset in **Figure 4C**,  
308  $P>0.05$ ). There was no difference in glycaemic variability, expressed as standard deviation (SD),  
309 CONGA1, or CONGA2 (McDonnell *et al.*, 2005), between the three conditions (all  $P>0.05$ ). Muscle  
310 glycogen content also did not change as a result of the interventions (**Figure 5**;  $P>0.05$ ).

311

### 312 *Skeletal muscle gene expression*

313 Relative expression of genes involved in insulin signalling and glucose metabolism is depicted in  
314 **Figure 6**. PRKCQ expression (PKC $\theta$ ; **6A**) showed a tendency for an effect ( $P=0.088$ ) due to a threefold  
315 increase following both 24 h bed rest visits. This effect was no longer visible after seven days of bed  
316 rest, when mRNA expression was ~twofold lower than during one day of energy-balanced bed rest  
317 ( $P<0.01$ ). GLUT4 (**6C**) expression was not different following the three 24 h visits ( $P=0.229$ ), but was  
318 approximately 60% lower following seven days of bed rest ( $P<0.01$ ). PDP1 (**6F**) showed a twofold  
319 lower expression following seven days of bed rest ( $P<0.05$ ), while a similar, although not significant  
320 ( $P=0.065$ ), effect was observed for PDK2 (**6G**). No changes in mRNA expression of PI3K (**6B**),  
321 SNAP23 (**6D**), HK2 (**6E**), and PDK4 (**6H**) were observed.

322 Skeletal muscle mRNA expression of genes involved in lipid storage and oxidation is presented in  
323 **Figure 7**. No difference in expression of these genes was found between the three 24 h experimental  
324 visits. However, seven days of bed rest led to a lower expression of ACADM (**7J**; ~30% lower than  
325 BR-B;  $P<0.05$ ), ACAT1 (**7L**; ~55% lower than BR-B;  $P<0.01$ ), and PPARA (**7M**; ~45% lower than  
326 BR-B;  $P<0.05$ ). A tendency for lower mRNA expression following seven days of bed rest was observed  
327 for PLIN5 (**7B**; ~55% lower than BR-B,  $P=0.065$ ) and HADBH (**7K**; ~30% lower than BR-B,  
328  $P=0.085$ ). No differences in expression of PLIN2 (**7A**), PNPLA2 (ATGL; **7C**), DGAT1 (**7D**), DGAT2  
329 (**7E**), LPIN1 (**7F**), ACACB (**7G**), CPT1 (**7H**), CPT2 (**7I**), DGKD (**7N**), FASN (**7O**), SCD (**7P**),  
330 SPTLC1 (**7Q**), GPAM (**7R**), UCP3 (**7S**), and TFAM (**7T**) were observed.

331 Skeletal muscle mRNA expression of genes involved in the regulation of muscle mass is displayed in  
332 **Figure 8**. None of the genes involved in muscle protein synthesis (panel **A-G**) were different between  
333 three 24 h visits or between one and seven days of bed rest. Of the genes involved in muscle protein  
334 breakdown (panel **H-O**), TRIM63 (MURF-1; **8K**) was twofold lower following seven days energy-  
335 balanced bed rest than following one day ( $P<0.05$ ). Moreover, tendencies for a 40% lower expression  
336 of FOXO3 (**8I**;  $P=0.053$ ) and 30% lower expression of BECN1 (**8O**;  $P=0.083$ ) were observed.  
337 Expression of FOXO1 (**8H**), FBO32 (MAFBx, **8J**), MSTN (**8L**), GDF11 (**8M**), and ULK1 (**8N**) was  
338 not different between the three conditions or following seven days of bed rest. SLC7A5 (LAT1) and  
339 LIPE (HSL) were undetectable, and are therefore not presented.



## 340 **Discussion**

341 We have previously demonstrated that short periods (5-7 days) of muscle disuse lead to substantial  
342 muscle atrophy, which is accompanied by impaired muscle protein synthesis rates and the development  
343 of insulin resistance. In the present study we report that seven days of bed rest leads to transcriptional  
344 responses associated with impairments in insulin signalling, the regulation of lipid storage/oxidation,  
345 and muscle protein breakdown. However, these molecular changes are not yet pronounced after a single  
346 day of bed rest, nor is any striking insulin resistance or disturbance in blood glucose homeostasis evident  
347 at this early stage. Moreover, none of these factors were modulated by energy balance during a single  
348 day of bed rest.

349 It is established that periods of physical inactivity lead to rapid loss of skeletal muscle mass (Deitrick,  
350 1948; Gibson *et al.*, 1987; Wall & van Loon, 2013) and the development of insulin resistance of glucose  
351 metabolism (Stuart *et al.*, 1988; Mikines *et al.*, 1991; Dirks *et al.*, 2016), although the mechanisms  
352 underlying this skeletal muscle deconditioning are currently unclear. Here, we investigated the effect  
353 of a single day of bed rest on muscle transcriptional responses when compared to seven days of bed  
354 rest, as well as glucose homeostasis and markers of insulin sensitivity. To study if the effect of bed rest  
355 was due to physical inactivity *per se* or associated changes in energy balance, we also examined the  
356 impact of positive energy balance (33% calorie excess, **Table 2**) on our outcome measures. Previous  
357 work from Biensø and colleagues reported that seven days of bed rest leads to a 22% decline in insulin  
358 sensitivity, measured as leg glucose uptake during a hyperinsulinaemic-euglycaemic clamp, which was  
359 due to a direct effect on intracellular insulin signalling pathways (Bienso *et al.*, 2012). Specifically, they  
360 reported reduced muscle GLUT4, hexokinase, and Akt protein levels, and a concomitant blunted ability  
361 of insulin to phosphorylate Akt and activate glycogen synthase (Bienso *et al.*, 2012). However,  
362 associated (and preceding) muscle transcriptional responses, taking into account nutritional status, have  
363 not been reported. To develop this understanding, we applied custom-designed, low density qRT-PCR  
364 array cards to assess how the expression of various genes encoding proteins involved in insulin  
365 signalling and glucose metabolism were affected by a single day, when compared to seven days (serving  
366 as a positive control insofar as we know this duration leads to the development of insulin resistance  
367 (Dirks *et al.*, 2016)), of bed rest (**Figure 6**). We observed that protein kinase C theta (PRKCQ/PKCθ)

368 gene expression was lower after seven days of bed rest, whereas it had increased following both 24 h  
369 bed rest visits (**6A**). Increased PKC $\theta$  activation has been associated with insulin resistance, especially  
370 under conditions of lipid oversupply (Griffin *et al.*, 1999). PKC is activated via diacylglycerol (DAG;  
371 (Szendroedi *et al.*, 2014)) and our previous work showed an increase in several DAG species following  
372 one week of bed rest (Dirks *et al.*, 2016), implying that an early upregulation of PKC $\theta$  gene expression  
373 may be implicated in the development of bed rest-induced insulin resistance irrespective of energy  
374 balance. Worthy of note, PKC acts via the inhibition of IRS1 (Li *et al.*, 2004) and subsequent  
375 impairment of PI3K and Akt activity, and no effect on the expression of these genes was observed.  
376 Although this may seem contradictory, this can likely be explained by the fact that their activity is  
377 regulated via (de)phosphorylation. Upstream of PKC $\theta$ , GLUT4 gene expression showed a similar  
378 pattern by showing a non-significant increase with 24 h of bed rest and subsequent downregulation after  
379 one week of bed rest (**6C**). Although GLUT4 mRNA expression does not necessarily represent GLUT4  
380 protein content (or translocation to the plasma membrane), here we show a ~60% decrease in GLUT4  
381 mRNA (**6C**) expression following seven days, but not a single day, of bed rest, which is in line with a  
382 decrease in GLUT4 protein expression (Bienso *et al.*, 2012) and muscle glucose uptake (Bienso *et al.*,  
383 2012; Dirks *et al.*, 2016). As we have previously shown that an increase in insulin sensitivity and muscle  
384 glycogen synthesis is accompanied by an increase in GLUT4 expression (Stephens *et al.*, 2010), it is  
385 expected that the decrease in GLUT4 expression following seven days of bed rest is preceded by the  
386 onset of insulin resistance and a reduction in muscle glucose flux.

387 The pyruvate dehydrogenase complex (PDC) plays a key role in determining muscle fuel selection as  
388 the rate limiting step of glucose oxidation (van Loon *et al.*, 2001), and is covalently regulated by a  
389 phosphatase (PDP) and a kinase (PDK). We have previously shown that inhibition of PDC activation  
390 results in a diversion of disposed glucose from oxidation to storage (Stephens *et al.*, 2006;  
391 Chokkalingam *et al.*, 2007), which was associated with a selective upregulation of PDK4, but not  
392 PDK2, mRNA expression (Chokkalingam *et al.*, 2007). Here we show that the expression of PDP1 (**6F**)  
393 and PDK2 (**6G**) was downregulated following seven days of bed rest, whereas previous work has  
394 reported unaltered fasting PDC activation following such periods of muscle disuse in young volunteers  
395 (Wall *et al.*, 2015; Vigelso *et al.*, 2016). Surprisingly, both studies report an increased fasting PDC

396 activation in older volunteers (Wall *et al.*, 2015; Vigelso *et al.*, 2016), possibly caused by a more  
397 pronounced and/or rapid shift from fat to carbohydrate oxidation in response to disuse. In contrast, in  
398 the present work, 24 h of bed rest and alterations in energy balance did not change the expression of  
399 genes encoding proteins that control the activation status of PDC (**6F-H**), despite an increased reliance  
400 on carbohydrate oxidation and therefore muscle glucose flux (**Figure 3**). However, since only basal  
401 gene expression was assessed in this study, it cannot be ruled out that bed rest and alterations in energy  
402 balance had a more profound impact on insulin-stimulated gene expression. Overall, our data suggest  
403 that disuse-induced alterations in fuel metabolism precede related transcriptional responses, which seem  
404 to be a molecular compensatory reaction rather than a causative metabolic mechanism. With the  
405 exception of an early increase in PKC $\theta$  expression, such transcriptional changes do not yet manifest  
406 following a single day of bed rest, suggesting that the absence of muscle contraction and associated  
407 changes in metabolism must persist for longer than 24 h before transcriptional changes occur.

408 Excess lipid storage and impaired/incomplete lipid oxidation are associated with the development of  
409 insulin resistance during muscle disuse (Cree *et al.*, 2010; Bergouignan *et al.*, 2011). Here, various  
410 genes involved in lipid storage and lipolysis (i.e. PLIN2, PNPLA2 (ATGL), DGAT-1, DGAT-2,  
411 LPIN1; **Figure 7**) were not affected by 24 h or seven days of bed rest. However, perilipin 5 (PLIN5),  
412 present on the coating of lipid droplets and involved in regulating fatty acid utilization (Wolins *et al.*,  
413 2006), was lower following seven days of bed rest. This extends on previous animal research, showing  
414 overexpression of PLIN5 protects against both high-fat diet-induced (Bosma *et al.*, 2013) and fasting-  
415 induced (Gemink *et al.*, 2016) insulin resistance, and supporting PLIN5 as a responsible candidate for  
416 mediating a disuse-induced decrease in insulin sensitivity after seven days. Similarly, we demonstrate  
417 that various genes involved in fatty acid oxidation (i.e. CPT1, CPT2, ACADM, HADBDH, ACAT-1,  
418 PPAR $\alpha$ ) consistently show a downregulation following seven days. This downregulation, which  
419 occurred in parallel with a decrease in fasting fat oxidation (Dirks *et al.*, 2016), may be, at least in part,  
420 the cause for muscle lipid accumulation that occurs following more prolonged disuse (i.e. > 1 week).

421 We have previously shown that muscle disuse leads to considerable skeletal muscle atrophy within a  
422 few days (Wall *et al.*, 2014). Although altered muscle protein synthesis rates have been reported as a  
423 feature of muscle disuse atrophy (Gibson *et al.*, 1987; Wall *et al.*, 2016), the post-translational control

424 of muscle protein synthesis makes it unsurprising that we did not observe any bed rest related alterations  
425 in gene expression of components of the mTOR/P70S6K/4E-BP1/GSK/eIF pathway (**Figure 8A-G**).  
426 As such, measures of protein expression and phosphorylation status would likely increase insight in  
427 mechanisms underlying the loss of muscle mass and quality. Muscle protein breakdown rates are  
428 technically challenging to measure in humans and therefore seldom reported (Symons *et al.*, 2009), so  
429 gene expression of one or more components of proteolytic pathways are often used as a proxy.  
430 Quantitatively, the main proteolytic systems in skeletal muscle is the ubiquitin proteasome pathway  
431 (UPP; (Bodine *et al.*, 2001)). The autophagy-lysosome pathway also contributes to mass breakdown  
432 (Mizushima & Klionsky, 2007), and also facilitates UPP breakdown via ‘pre-processing’ myofibrillar  
433 proteins. Here we show that seven days of bed rest leads to a decrease in gene expression of key  
434 components of the UPP, FOXO3 (**8I**) and MuRF1 (**8K**), presumably reflecting a lower net protein  
435 turnover of muscle at this time point. In line, a key target of autophagy-signalling, Beclin 1 (Sanchez *et al.*,  
436 2012), followed a similar pattern with a tendency to decrease following one week of bed rest (**8O**).  
437 In contrast, a single day of bed rest did not alter key genes in the regulation of muscle protein  
438 breakdown, nor did energy balance influence this (**Figure 8H-O**). Collectively, our data suggest that  
439 the early and transient rise in muscle protein breakdown, which has been thought to occur in the first  
440 days following the onset of muscle disuse and contribute to muscle atrophy (Urso *et al.*, 2006; Tesch *et al.*,  
441 2008; Abadi *et al.*, 2009), is not yet visible following a single day of bed rest, or is not best reflected  
442 by transcriptional responses.

443 Given the previously observed potent effect of bed rest on insulin resistance (Stuart *et al.*, 1988; Mikines  
444 *et al.*, 1991; Dirks *et al.*, 2016), we assessed the impact of a single day of bed rest on insulin sensitivity  
445 and glycaemic control. Here we demonstrate that a single day of bed rest does not significantly affect  
446 whole body insulin sensitivity, irrespective of energy balance (**Figure 3A and B**). However, a trend for  
447 impaired insulin sensitivity as determined by HOMA-IR was observed ( $P=0.096$ ), with a tendency  
448 present to suggest maintaining habitual energy intake exacerbated this effect. Our data are in line with  
449 previous work demonstrating an increase in HOMA-IR following a similar 1-day overfeeding protocol  
450 (Magkos *et al.*, 2014), and is in agreement with previous observations during more prolonged bed rest  
451 protocols showing that surplus energy intake exacerbates the negative effect of bed rest on liver insulin

452 sensitivity (Kiilerich *et al.*, 2011; Dirks *et al.*, 2016), possibly via an impaired suppression of nocturnal  
453 hepatic glucose production (Magkos *et al.*, 2014). The lack of a statistically significant effect of bed  
454 rest on the Matsuda index, a proxy of peripheral insulin sensitivity (Matsuda & DeFronzo, 1999), is in  
455 contrast to the general consensus that prolonged bed rest-induced insulin resistance is predominantly  
456 present peripherally (Stuart *et al.*, 1988; Mikines *et al.*, 1991; Cree *et al.*, 2010; Dirks *et al.*, 2016).  
457 Although a visual decline in Matsuda index was seen in both bed rest conditions, it seems that any effect  
458 of a single day of bed rest on peripheral insulin sensitivity is modest. A methodological consideration  
459 here was the collection of venous (rather than arterial or arterialed venous) blood to calculate glucose  
460 tolerance and insulin sensitivity. Recent data demonstrate that the quantification of insulin sensitivity  
461 during an OGTT when muscle contraction level is manipulated can vary depending on whether venous  
462 or arterialed venous blood is used (Edinburgh *et al.*, 2017). However, within the present study, insulin  
463 sensitivity was calculated and compared consistently from venous blood, and we anticipate that any  
464 differences if arterialed blood had been obtained would be minimal and not alter the direction of our  
465 findings. Rather than simply insulin resistance *per se*, numerous epidemiological studies have  
466 demonstrated a clear and independent link between post-prandial hyperglycaemia and cardiovascular  
467 co-morbidities (de Vegt *et al.*, 1999; Cavalot *et al.*, 2006), and more specifically the prevalence of  
468 hyperglycaemia and glycaemic excursions across an entire day. To this end, we applied continuous  
469 interstitial glucose monitoring methodology and observed similar glycaemic variability throughout all  
470 three visits and a close to identical 24 h, and nocturnal, average interstitial glucose concentration and  
471 interstitial glucose area under the curve (inset in **Figure 3C**). These data are in line with our insulin  
472 sensitivity indices implying that the impaired glucose homeostasis, to be expected with prolonged  
473 muscle disuse, does not yet manifest within 24 h. Similarly, a 24 h time period did not lead to changes  
474 in muscle glycogen content (**Figure 4**), which likely takes longer to occur (Kiilerich *et al.*, 2011; Bienso  
475 *et al.*, 2012). As such, at least in healthy humans, there appears to be a brief (albeit short lived) window  
476 for resisting the deleterious metabolic effects of physical inactivity.

477 A final consideration of the present work is our novel approach towards designing a representative  
478 control condition. The severity and invasiveness of an experimental bed rest intervention has often  
479 required researchers to rely on a pre/post comparison only (i.e. no true control condition, e.g. (Mikines

480 *et al.*, 1991; Cree *et al.*, 2010; Dirks *et al.*, 2016)), or utilise a non-laboratory based control condition  
481 (e.g. (LeBlanc *et al.*, 1992)). We attempted to address this by proposing a novel quantitative method,  
482 employing precise objective measures to individually assess each participant's habitual physical  
483 activity, taking into account their time spent sitting, standing, and walking, as well as the type, volume,  
484 and intensity of any exercise they typically perform. In this way, each subject was able to undertake an  
485 individualised control day, mimicking their habitual physical activity behaviours as closely as possible,  
486 under laboratory and nutritionally controlled conditions identical to the interventions (**Figure 2**). This  
487 obviated any potential order effects, washout periods, or lack of full control of physical activity, and  
488 provided us with a 'true' control condition allowing the detection of small, but physiologically relevant,  
489 changes in metabolic parameters. For instance, this additional control allowed us to accurately  
490 discriminate changes in the expression of certain genes as a direct result of disuse and/or diet from those  
491 which are seemingly an artefact of repeated biopsies/test days (Van Thienen *et al.*, 2014). Due to the  
492 nature and complexity of *in vivo* human studies, such approaches as included herein result in largely  
493 descriptive data rather than isolating definitive mechanisms such as are more regularly described using  
494 animal and/or pharmacological approaches which can allow reductionism to the level of a metabolic  
495 process or even individual molecule. However, we view the novel habitual physical activity control  
496 condition and the dietary approach in the present study as a steps forward in our approach to identifying  
497 subtle, *in vivo* mechanisms of disuse induced deconditioning in human volunteer studies.

498 In conclusion, we demonstrate that a single day of bed rest does not substantially change the skeletal  
499 muscle expression of genes involved in the regulation of muscle mass, insulin sensitivity, and/or fuel  
500 oxidation. Although prolonged bed rest leads to overt decreases in insulin sensitivity and glycaemic  
501 control, such effects only modestly manifest following a single day of bed rest. Importantly, the muscle  
502 transcriptional responses that did occur were irrespective of whether volunteers were in energy balance  
503 or maintaining their habitual diet (i.e. modest overfeeding), and can therefore be attributed directly to  
504 the withdrawal of muscle contraction.

**Competing interests**

None of the authors disclose any conflicts of interest.

**Author contributions**

FBS and BTW designed the study. SRJ, JGG, DM, and BTW organized and carried out the clinical experiments. FBS, SRJ, and BTW performed the laboratory analyses. MLD performed the (statistical) analyses. MLD, FBS, and BTW interpreted the primary data. RMP analysed the accelerometer data and designed the habitual activity laboratory days. MLD drafted, and FBS and BTW edited and revised the manuscript. All authors approved the final version.

**Funding**

BTW received internal funding from the College of Life and Environmental Sciences, University of Exeter, to support this project. None of the other authors received funding from any funding agency in the public, commercial or not-for-profit sectors to conduct this research.

**Acknowledgements**

The authors would like to thank Dr Emma J. Cockcroft for her assistance in the experimental physiology testing and serum insulin analyses.

## References

- Abadi A, Glover EI, Isfort RJ, Raha S, Safdar A, Yasuda N, Kaczor JJ, Melov S, Hubbard A, Qu X, Phillips SM & Tarnopolsky M (2009). Limb immobilization induces a coordinate down-regulation of mitochondrial and other metabolic pathways in men and women. *PLoS One* 4, e6518. <https://doi.org/10.1371/journal.pone.0006518>
- Backx EM, Tieland M, Borgonjen-van den Berg KJ, Claessen PR, van Loon LJ & de Groot LC (2016). Protein intake and lean body mass preservation during energy intake restriction in overweight older adults. *Int J Obes (Lond)* 40, 299-304. <https://doi.org/10.1038/ijo.2015.182>
- Bergouignan A, Rudwill F, Simon C & Blanc S (2011). Physical inactivity as the culprit of metabolic inflexibility: evidence from bed-rest studies. *J Appl Physiol (1985)* 111, 1201-1210. <https://doi.org/10.1152/jappphysiol.00698.2011>
- Bergstrom J (1975). Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest* 35, 609-616.
- Bienso RS, Ringholm S, Kiilerich K, Aachmann-Andersen NJ, Krogh-Madsen R, Guerra B, Plomgaard P, van Hall G, Treebak JT, Saltin B, Lundby C, Calbet JA, Pilegaard H & Wojtaszewski JF (2012). GLUT4 and glycogen synthase are key players in bed rest-induced insulin resistance. *Diabetes* 61, 1090-1099. <https://doi.org/10.2337/db11-0884>
- Biolo G, Agostini F, Simunic B, Sturma M, Torelli L, Preiser J, Deby-Dupont G, Magni P, Strollo F, di Prampero P, Guarnieri G, Mekjavic I, Pisot R & Narici M (2008). Positive energy balance is associated with accelerated muscle atrophy and increased erythrocyte glutathione turnover during 5 wk of bed rest. *Am J Clin Nutr.* 88, 950-958.
- Biolo G, Ciochi B, Stulle M, Bosutti A, Barazzoni R, Zanetti M, Antonione R, Lebenstedt M, Platen P, Heer M & Guarnieri G (2007). Calorie restriction accelerates the catabolism of lean body mass during 2 wk of bed rest. *Am J Clin Nutr* 86, 366-372.
- Bodine SC, Latres E, Baumhueter S, Lai VK, Nunez L, Clarke BA, Poueymirou WT, Panaro FJ, Na E, Dharmarajan K, Pan ZQ, Valenzuela DM, DeChiara TM, Stitt TN, Yancopoulos GD & Glass DJ (2001). Identification of ubiquitin ligases required for skeletal muscle atrophy. *Science* 294, 1704-1708. <https://doi.org/10.1126/science.1065874>
- Bosma M, Sparks LM, Hooiveld GJ, Jorgensen JA, Houten SM, Schrauwen P, Kersten S & Hesselink MK (2013). Overexpression of PLIN5 in skeletal muscle promotes oxidative gene expression and intramyocellular lipid content without compromising insulin sensitivity. *Biochim Biophys Acta* 1831, 844-852. <https://doi.org/10.1016/j.bbali.2013.01.007>
- Cavalot F, Petrelli A, Traversa M, Bonomo K, Fiora E, Conti M, Anfossi G, Costa G & Trovati M (2006). Postprandial blood glucose is a stronger predictor of cardiovascular events than fasting blood glucose in type 2 diabetes mellitus, particularly in women: lessons from the San Luigi Gonzaga Diabetes Study. *J Clin Endocrinol Metab* 91, 813-819. <https://doi.org/10.1210/jc.2005-1005>



- Chokkalingam K, Jewell K, Norton L, Littlewood J, van Loon LJ, Mansell P, Macdonald IA & Tsintzas K (2007). High-fat/low-carbohydrate diet reduces insulin-stimulated carbohydrate oxidation but stimulates nonoxidative glucose disposal in humans: An important role for skeletal muscle pyruvate dehydrogenase kinase 4. *J Clin Endocrinol Metab* 92, 284-292. <https://doi.org/10.1210/jc.2006-1592>
- Chomczynski P & Sacchi N (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162, 156-159. <https://doi.org/10.1006/abio.1987.9999>
- Cree MG, Paddon-Jones D, Newcomer BR, Ronsen O, Aarsland A, Wolfe RR & Ferrando A (2010). Twenty-eight-day bed rest with hypercortisolemia induces peripheral insulin resistance and increases intramuscular triglycerides. *Metabolism* 59, 703-710. <https://doi.org/10.1016/j.metabol.2009.09.014>
- de Vegt F, Dekker J, Ruhé H, Stehouwer C, Nijpels G, Bouter L & Heine R (1999). Hyperglycaemia is associated with all-cause and cardiovascular mortality in the Hoorn population: the Hoorn Study. *Diabetologia* 42, 926-931. <https://doi.org/10.1007/s001250051249>
- Deitrick JE (1948). The effect of immobilization on metabolic and physiological functions of normal men. *Bull N Y Acad Med* 24, 364-375.
- Dirks ML, Wall BT, van de Valk B, Holloway TM, Holloway GP, Chabowski A, Goossens GH & van Loon LJ (2016). One Week of Bed Rest Leads to Substantial Muscle Atrophy and Induces Whole-Body Insulin Resistance in the Absence of Skeletal Muscle Lipid Accumulation. *Diabetes* 65, 2862-2875. <https://doi.org/10.2337/db15-1661>
- Edinburgh RM, Hengist A, Smith HA, Betts JA, Thompson D, Walhin JP & Gonzalez JT (2017). Prior exercise alters the difference between arterialised and venous glycaemia: implications for blood sampling procedures. *Br J Nutr* 117, 1414-1421. <https://doi.org/10.1017/S0007114517001362>
- Esliger DW, Rowlands AV, Hurst TL, Catt M, Murray P & Eston RG (2011). Validation of the GENE Accelerometer. *Med Sci Sports Exerc* 43, 1085-1093. <https://doi.org/10.1249/MSS.0b013e31820513be>
- Frayn KN (1983). Calculation of substrate oxidation rates in vivo from gaseous exchange. *J Appl Physiol Respir Environ Exerc Physiol* 55, 628-634. <https://doi.org/10.1152/jappl.1983.55.2.628>
- Gemmink A, Bosma M, Kuijpers HJ, Hoeks J, Schaart G, van Zandvoort MA, Schrauwen P & Hesselink MK (2016). Decoration of intramyocellular lipid droplets with PLIN5 modulates fasting-induced insulin resistance and lipotoxicity in humans. *Diabetologia* 59, 1040-1048. <https://doi.org/10.1007/s00125-016-3865-z>

- Gibson JN, Halliday D, Morrison WL, Stoward PJ, Hornsby GA, Watt PW, Murdoch G & Rennie MJ (1987). Decrease in human quadriceps muscle protein turnover consequent upon leg immobilization. *Clin Sci (Lond)* 72, 503-509.
- Griffin ME, Marcucci MJ, Cline GW, Bell K, Barucci N, Lee D, Goodyear LJ, Kraegen EW, White MF & Shulman GI (1999). Free fatty acid-induced insulin resistance is associated with activation of protein kinase C theta and alterations in the insulin signaling cascade. *Diabetes* 48, 1270-1274.
- Harris RC, Hultman E & Nordesjo LO (1974). Glycogen, glycolytic intermediates and high-energy phosphates determined in biopsy samples of musculus quadriceps femoris of man at rest. Methods and variance of values. *Scand J Clin Lab Invest* 33, 109-120.
- Haruna Y, Suzuki Y, Kawakubo K, Yanagibori R & Gunji A (1994). Decremental reset in basal metabolism during 20-days bed rest. *Acta Physiol Scand Suppl* 616, 43-49.
- Hulston C, Churnside A & Venables M (2015). Probiotic supplementation prevents high-fat, overfeeding-induced insulin resistance in human subjects. *Br J Nutr* 113, 596-602. <https://doi.org/10.1017/S0007114514004097>
- Keenan D, Mastrototaro J, Zisser H, Cooper K, Raghavendhar G, Lee S, Yusi J, Bailey T, Brazg R & Shah R (2012). Accuracy of the Enlite 6-day glucose sensor with guardian and Veo calibration algorithms. *Diabetes Technol Ther* 14, 225-231. <https://doi.org/10.1089/dia.2011.0199>
- Kelley DE & Mandarino LJ (2000). Fuel selection in human skeletal muscle in insulin resistance: a reexamination. *Diabetes* 49, 677-683.
- Kiilerich K, Ringholm S, Bienso RS, Fisher JP, Iversen N, van Hall G, Wojtaszewski JF, Saltin B, Lundby C, Calbet JA & Pilegaard H (2011). Exercise-induced pyruvate dehydrogenase activation is not affected by 7 days of bed rest. *J Appl Physiol (1985)* 111, 751-757. <https://doi.org/10.1152/jappphysiol.00063.2011>
- Kraemer W & Fry A (1995). *Strength testing: Development and evaluation of methodology.*, vol. P. Maud and C. Foster (Eds.) Leeds: Human Kinetics.
- LeBlanc AD, Schneider VS, Evans HJ, Pientok C, Rowe R & Spector E (1992). Regional changes in muscle mass following 17 weeks of bed rest. *J Appl Physiol (1985)* 73, 2172-2178. <https://doi.org/10.1152/jappl.1992.73.5.2172>
- Li Y, Soos TJ, Li X, Wu J, Degennaro M, Sun X, Littman DR, Birnbaum MJ & Polakiewicz RD (2004). Protein kinase C Theta inhibits insulin signaling by phosphorylating IRS1 at Ser(1101). *J Biol Chem* 279, 45304-45307. <https://doi.org/10.1074/jbc.C400186200>

- Magkos F, Smith GI, Reeds DN, Okunade A, Patterson BW & Mittendorfer B (2014). One day of overfeeding impairs nocturnal glucose but not fatty acid homeostasis in overweight men. *Obesity (Silver Spring)* 22, 435-440. <https://doi.org/10.1002/oby.20562>
- Mahoney DJ, Carey K, Fu MH, Snow R, Cameron-Smith D, Parise G & Tarnopolsky MA (2004). Real-time RT-PCR analysis of housekeeping genes in human skeletal muscle following acute exercise. *Physiol Genomics* 18, 226-231. 10.1152/physiolgenomics.00067.2004
- Matsuda M & DeFronzo RA (1999). Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care* 22, 1462-1470.
- Matthews DR, Hosker JP, Rudenski AS, Naylor BA, Treacher DF & Turner RC (1985). Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man. *Diabetologia* 28, 412-419.
- Mayhew JL, Prinster JL, Ware JS, Zimmer DL, Arabas JR & Bemben MG (1995). Muscular endurance repetitions to predict bench press strength in men of different training levels. *J Sports Med Phys Fitness* 35, 108-113.
- McDonnell CM, Donath SM, Vidmar SI, Werther GA & Cameron FJ (2005). A novel approach to continuous glucose analysis utilizing glycemic variation. *Diabetes Technol Ther* 7, 253-263. <https://doi.org/10.1089/dia.2005.7.253>
- Mikines KJ, Richter EA, Dela F & Galbo H (1991). Seven days of bed rest decrease insulin action on glucose uptake in leg and whole body. *J Appl Physiol (1985)* 70, 1245-1254. <https://doi.org/10.1152/jappl.1991.70.3.1245>
- Mizushima N & Klionsky DJ (2007). Protein turnover via autophagy: implications for metabolism. *Annu Rev Nutr* 27, 19-40. <https://doi.org/10.1146/annurev.nutr.27.061406.093749>
- Peterson CM, Zhang B, Johannsen DL & Ravussin E (2017). Eight weeks of overfeeding alters substrate partitioning without affecting metabolic flexibility in men. *Int J Obes (Lond)* 41, 887-893. <https://doi.org/10.1038/ijo.2017.58>
- Reidy PT, Lindsay CC, McKenzie AI, Fry CS, Supiano MA, Marcus RL, LaStayo PC & Drummond MJ (2017). Aging-related effects of bed rest followed by eccentric exercise rehabilitation on skeletal muscle macrophages and insulin sensitivity. *Exp Gerontol*. <https://doi.org/10.1016/j.exger.2017.07.001>
- Sanchez AM, Csibi A, Raibon A, Cornille K, Gay S, Bernardi H & Candau R (2012). AMPK promotes skeletal muscle autophagy through activation of forkhead FoxO3a and interaction with Ulk1. *J Cell Biochem* 113, 695-710. <https://doi.org/10.1002/jcb.23399>
- Stephens FB, Constantin-Teodosiu D, Laithwaite D, Simpson EJ & Greenhaff PL (2006). An acute increase in skeletal muscle carnitine content alters fuel metabolism in resting human skeletal muscle. *J Clin Endocrinol Metab* 91, 5013-5018. <https://doi.org/10.1210/jc.2006-1584>

- Stephens FB, Norton L, Jewell K, Chokkalingam K, Parr T & Tsintzas K (2010). Basal and insulin-stimulated pyruvate dehydrogenase complex activation, glycogen synthesis and metabolic gene expression in human skeletal muscle the day after a single bout of exercise. *Exp Physiol* 95, 808-818. <https://doi.org/10.1113/expphysiol.2009.051367>
- Stuart CA, Shangraw RE, Prince MJ, Peters EJ & Wolfe RR (1988). Bed-rest-induced insulin resistance occurs primarily in muscle. *Metabolism* 37, 802-806.
- Symons TB, Sheffield-Moore M, Chinkes DL, Ferrando AA & Paddon-Jones D (2009). Artificial gravity maintains skeletal muscle protein synthesis during 21 days of simulated microgravity. *J Appl Physiol (1985)* 107, 34-38. <https://doi.org/10.1152/jappphysiol.91137.2008>
- Szendroedi J, Yoshimura T, Phielix E, Koliaki C, Marcucci M, Zhang D, Jelenik T, Muller J, Herder C, Nowotny P, Shulman GI & Roden M (2014). Role of diacylglycerol activation of PKC $\theta$  in lipid-induced muscle insulin resistance in humans. *Proc Natl Acad Sci U S A* 111, 9597-9602. <https://doi.org/10.1073/pnas.1409229111>
- Tesch PA, von Walden F, Gustafsson T, Linnehan RM & Trappe TA (2008). Skeletal muscle proteolysis in response to short-term unloading in humans. *J Appl Physiol (1985)* 105, 902-906. <https://doi.org/10.1152/jappphysiol.90558.2008>
- Tsintzas K, Jewell K, Kamran M, Laithwaite D, Boonsong T, Littlewood J, Macdonald I & Bennett A (2006). Differential regulation of metabolic genes in skeletal muscle during starvation and refeeding in humans. *J Physiol* 575, 291-303. <https://doi.org/10.1113/jphysiol.2006.109892>
- Tsintzas K, Norton L, Chokkalingam K, Nizamani N, Cooper S, Stephens F, Billeter R & Bennett A (2013). Independent and combined effects of acute physiological hyperglycaemia and hyperinsulinaemia on metabolic gene expression in human skeletal muscle. *Clin Sci (Lond)* 124, 675-684. <https://doi.org/10.1042/CS20120481>
- Urso ML, Scrimgeour AG, Chen YW, Thompson PD & Clarkson PM (2006). Analysis of human skeletal muscle after 48 h immobilization reveals alterations in mRNA and protein for extracellular matrix components. *J Appl Physiol (1985)* 101, 1136-1148. <https://doi.org/10.1152/jappphysiol.00180.2006>
- van Dijk J & van Loon L (2015). Exercise strategies to optimize glycemic control in type 2 diabetes: a continuing glucose monitoring perspective. *Diabetes Spectr* 28, 24-31. <https://doi.org/10.2337/diaspect.28.1.24>
- van Loon LJ, Greenhaff PL, Constantin-Teodosiu D, Saris WH & Wagenmakers AJ (2001). The effects of increasing exercise intensity on muscle fuel utilisation in humans. *J Physiol* 536, 295-304.
- Van Thienen R, D'Hulst G, Deldicque L & Hespel P (2014). Biochemical artifacts in experiments involving repeated biopsies in the same muscle. *Physiol Rep* 2, e00286. <https://doi.org/10.14814/phy2.286>

- Vigelso A, Gram M, Dybboe R, Kuhlman AB, Prats C, Greenhaff PL, Constantin-Teodosiu D, Birk JB, Wojtaszewski JF, Dela F & Helge JW (2016). The effect of age and unilateral leg immobilization for 2 weeks on substrate utilization during moderate-intensity exercise in human skeletal muscle. *J Physiol* 594, 2339-2358. <https://doi.org/10.1113/JP271712>
- Wall BT, Dirks ML, Snijders T, Senden JM, Dolmans J & van Loon LJ (2014). Substantial skeletal muscle loss occurs during only 5 days of disuse. *Acta Physiol (Oxf)* 210, 600-611. <https://doi.org/10.1111/apha.12190>
- Wall BT, Dirks ML, Snijders T, Stephens FB, Senden JM, Verscheijden ML & van Loon LJ (2015). Short-term muscle disuse atrophy is not associated with increased intramuscular lipid deposition or a decline in the maximal activity of key mitochondrial enzymes in young and older males. *Exp Gerontol* 61, 76-83. <https://doi.org/10.1016/j.exger.2014.11.019>
- Wall BT, Dirks ML, Snijders T, van Dijk JW, Fritsch M, Verdijk LB & van Loon LJ (2016). Short-term muscle disuse lowers myofibrillar protein synthesis rates and induces anabolic resistance to protein ingestion. *Am J Physiol Endocrinol Metab* 310, E137-147. <https://doi.org/10.1152/ajpendo.00227.2015>
- Wall BT, Dirks ML & van Loon LJ (2013). Skeletal muscle atrophy during short-term disuse: implications for age-related sarcopenia. *Ageing Res Rev* 12, 898-906. <https://doi.org/10.1016/j.arr.2013.07.003>
- Wall BT, Dirks ML, Verdijk LB, Snijders T, Hansen D, Vranckx P, Burd NA, Dendale P & van Loon LJ (2012). Neuromuscular electrical stimulation increases muscle protein synthesis in elderly type 2 diabetic men. *Am J Physiol Endocrinol Metab* 303, E614-623. [10.1152/ajpendo.00138.2012](https://doi.org/10.1152/ajpendo.00138.2012)
- Wall BT & van Loon LJ (2013). Nutritional strategies to attenuate muscle disuse atrophy. *Nutr Rev* 71, 195-208. <https://doi.org/10.1111/nure.12019>
- Wolins NE, Quaynor BK, Skinner JR, Tzekov A, Croce MA, Gropler MC, Varma V, Yao-Borengasser A, Rasouli N, Kern PA, Finck BN & Bickel PE (2006). OXPAT/PAT-1 is a PPAR-induced lipid droplet protein that promotes fatty acid utilization. *Diabetes* 55, 3418-3428. <https://doi.org/10.2337/db06-0399>
- Yanagibori R, Suzuki Y, Kawakubo K, Makita Y & Gunji A (1994). Carbohydrate and lipid metabolism after 20 days of bed rest. *Acta Physiol Scand Suppl* 616, 51-57.

## Tables

**Table 1:** Subjects' characteristics

---

<b>Age (y)</b>	25 ± 3
<b>Body mass (kg)</b>	79.0 ± 7.4
<b>Height (m)</b>	1.77 ± 0.06
<b>BMI (kg·m<sup>-2</sup>)</b>	25.3 ± 2.5
<b>Body fat (%)</b>	13.6 ± 5.0
<b>RMR (MJ·d<sup>-1</sup>)</b>	8.9 ± 1.0
<b>Habitual physical activity</b>	
<b>Light activity (min·d<sup>-1</sup>)</b>	114 ± 41
<b>Moderate activity (min·d<sup>-1</sup>)</b>	122 ± 42
<b>Vigorous activity (min·d<sup>-1</sup>)</b>	18 ± 15
<b>MVPA (min·d<sup>-1</sup>)</b>	139 ± 48
<b>Total physical activity (min·d<sup>-1</sup>)</b>	252 ± 88

---

Values are presented as mean±SD. MVPA, moderate-to-vigorous physical activity; RMR, resting metabolic rate

**Table 2:** Dietary intake during the control (CON), bed rest-matched (BR-M) and bed rest-balanced (BR-B) visit.

	<b>CON and BR-M</b>	<b>BR-B</b>
<b>Energy (kcal·d<sup>-1</sup>)</b>	3409 ± 377	2558 ± 282 *
<b>Protein (g·kg body weight<sup>-1</sup>·d<sup>-1</sup>)</b>	1.30 ± 0.15	0.98 ± 0.11 *
<b>Protein (g·d<sup>-1</sup>)</b>	103 ± 12	77 ± 9 *
<b>Carbohydrates (g·d<sup>-1</sup>)</b>	479 ± 55	359 ± 41 *
<b>Fat (g·d<sup>-1</sup>)</b>	122 ± 14	91 ± 10 *
<b>Protein (en%)</b>	12 ± 0	12 ± 0
<b>Carbohydrate (en%)</b>	56 ± 0	56 ± 0
<b>Fat (en%)</b>	32 ± 0	32 ± 0

Values represent means±SD. \* Significantly different from CON/BR-M ( $P<0.001$ )

**Table 3:** Names and symbols of gene expression assay targets that were pre-loaded on to the microfluidic cards

Gene name (human skeletal muscle)	Symbol
Acyl-CoA dehydrogenase medium chain	ACADM
Acetyl-CoA carboxylase (beta)	ACACB
Acetyl-CoA acetyltransferase 1	ACAT1
Actin, alpha 1, skeletal muscle	ACTA1
AKT serine/threonine kinase 1	AKT1
Beclin 1	BECN1
Carnitine palmitoyl transferase 1	CPT1
Carnitine palmitoyl transferase 2	CPT2
DNA damage inducible transcript 4	DDIT4 (REDD-1)
Diacylglycerol O-acyltransferase 1	DGAT1
Diacylglycerol O-acyltransferase 2	DGAT2
Diacylglycerol kinase (delta)	DGKD
Eukaryotic translation initiation factor 4E binding protein 1	EIF4EBP1 (4E-BP1)
Fatty acid synthase	FASN
F-box protein 32	FBXO32 (MAFBx)
Forkhead box protein 1	FOXO1
Forkhead box protein 3	FOXO3
Glyceraldehyde 3-phosphate dehydrogenase	GAPDH
Growth differentiation factor 11	GDF11
Solute carrier family 2 member 4	GLUT4
Glycerol-3-phosphate acyltransferase 1	GPAM
Glycogen synthase kinase 3 beta	GSK3B
Hydroxyacyl-CoA dehydrogenase trifunctional multienzyme complex	HADHB
Hexokinase 2	HK2
Hydroxymethylbilane synthase	HMBS
Lipase E, hormone sensitive	LIPE (HSL)
Lipin 1	LPIN1
Myostatin	MSTN
Mechanistic target of rapamycin kinase	MTOR
Pyruvate dehydrogenase kinase 2	PDK2
Pyruvate dehydrogenase kinase 4	PDK4
Pyruvate dehydrogenase phosphatase catalytic subunit 1	PDP1
Phosphatidylinoditol 3-kinase	PI3K
Perilipin 2	PLIN2
Perilipin 5	PLIN5
Patatin like phospholipase domain containing 2	PNPLA2 (ATGL)
Peroxisome proliferator activated receptor alpha	PPARA
Protein kinase C theta	PRKCQ (PKCθ)
Ribosomal protein S6 kinase B1	RPS6KB1 (P70S6K)
Stearoyl-CoA desaturase	SCD
Solute carrier family 7 member 5	SLC7A5 (LAT1)
Solute carrier family 36 member 1	SLC36A1 (PAT1)
Synaptosome associated protein 23	SNAP23
Serine palmitoyltransferase long chain base subunit 1	SPTLC1
Transcription factor A, mitochondrial	TFAM
Tripartite motif containing 63	TRIM63 (MURF-1)
Uncoupling protein 3	UCP3
Unc-51 like autophagy activating kinase 1	ULK1

Symbols between brackets represent gene synonyms.



## Figure legends

**Figure 1:** Experimental overview of the study. BR-B, bed rest-balanced; BR-M, bed rest-matched; CON, controlled habitual physical activity.

**Figure 2:** Example of habitual lying/sitting, standing, and walking (measured across five consecutive free-living days) and this activity recreated during the control day for a representative subject. Data for lying/sitting, standing, and walking are presented for the whole day, as well as proportions of each time period.

**Figure 3:** Relative contribution of carbohydrate (CHO) and fat oxidation to energy expenditure in the overnight fasted state and during a 2 h oral glucose tolerance test (OGTT; 75 g carbohydrate ingestion) before (pre) and after (post) three experimental conditions ( $n=10$ ): 24 h of laboratory controlled habitual physical activity with a diet designed to maintain energy balance (CON), 24 h bed rest with a diet designed to maintain energy balance (i.e. reduced energy intake compared with CON; BR-B), and 24 h bed rest with a diet matched to CON (i.e. positive energy balance; BR-M). A three-way Repeated Measures ANOVA demonstrated that the relative contribution of carbohydrate oxidation to energy expenditure increased with carbohydrate ingestion ( $P<0.01$ ) and following all experimental visits ( $P<0.05$ ), while a trend for a time\*feeding interaction ( $P=0.066$ ) suggests an impairment to switch from fat to carbohydrate oxidation following all three experimental visits.

**Figure 4:** Insulin sensitivity before and after, and glycaemic control during three 24 h experimental laboratory visits involving controlled habitual physical activity with a diet designed to maintain energy balance (CON), bed rest with a diet designed to maintain energy balance (i.e. reduced energy intake compared with CON; BR-B), and bed rest with a diet matched to CON (i.e. positive energy balance; BR-M). Panel A-C and D-F display the plasma glucose and insulin concentrations during the Oral Glucose Tolerance Test (OGTT), respectively. The insets in these graphs represent the corresponding area under the curve (AUC). The experimental visits had no significant effect on the Homeostatic Model

Assessment of Insulin Resistance (HOMA-IR, **G**) or Matsuda (**H**) indices (both  $P>0.05$ ), calculated from the OGTT. Panel **I** displays glucose traces from a representative subject, measured every 5 min during the 24 h experimental visits using a continuous glucose monitoring system (CGMS). The inset in panel **I** represents the 24 h AUC for the total cohort ( $n=7$ ). Dashed vertical lines represent the beginning and end of the night.

**Figure 5:** Muscle glycogen before (pre) and after (post) a 24 h experimental visit involving standardised physical activity with a diet to maintain energy balance (CON), a day of bed rest with a diet designed to maintain energy balance (i.e. reduced energy intake compared with CON; BR-B), and a day of bed rest with a diet matched to CON (i.e. positive energy balance; BR-M) in  $n=10$  healthy, young males. Dm, dry matter.

**Figure 6:** Skeletal muscle mRNA expression of genes involved in insulin signalling and glucose metabolism in healthy, young men ( $n=7$ ) who underwent three 24 h interventions in a crossover manner: standardised physical activity combined with a diet to maintain energy balance (CON), bed rest with a diet designed to maintain energy balance (i.e. reduced energy intake compared with CON; BR-B), and bed rest with a diet matched to that in the CON visit (i.e. positive energy balance; BR-M). Data were compared to  $n=7$  healthy men who have undergone seven days of strict bed rest in a previous study (7 DAYS, (Dirks *et al.*, 2016)). Data were analysed using a Repeated Measures ANOVA including CON vs BR-B vs BR-M, and an independent samples t-test including BR-B vs 7 DAYS. \* Significantly different from BR-B ( $P<0.05$ ). # Trend for a significant difference ( $P<0.10$ ).

**Figure 7:** Skeletal muscle mRNA expression of genes involved in lipid metabolism and fat oxidation in  $n=7$  healthy males undergoing controlled physical activity and an energy-balanced diet (CON), bed rest with a diet designed to maintain energy balance (i.e. reduced energy intake compared with CON; BR-B), and bed rest with a diet matched to that in the CON visit (i.e. positive energy balance; BR-M) in a crossover manner. Data were compared to group of healthy males ( $n=7$ ) who underwent seven days of strict bed rest in energy balanced conditions (Dirks *et al.*, 2016). Data were analysed using a Repeated

Measures ANOVA including CON vs BR-B vs BR-M, and an independent samples t-test including BR-B vs 7 DAYS. \* Significantly different from BR-B ( $P<0.05$ ). # Trend for a difference versus BR-B ( $P<0.10$ ).

**Figure 8:** Skeletal muscle mRNA expression of genes involved in muscle mass regulation (**A-G**: muscle protein synthesis; **H-O**: muscle protein breakdown) in  $n=7$  healthy males undergoing controlled physical activity under energy-balanced conditions (CON), bed rest with a diet designed to maintain energy balance (i.e. reduced energy intake compared with CON; BR-B), and bed rest with a diet matched to that in the CON visit (i.e. positive energy balance; BR-M) in a crossover manner. Data were compared to  $n=7$  healthy males who underwent seven days of strict bed rest in energy balanced conditions (Dirks *et al.*, 2016). Data were analysed using a Repeated Measures ANOVA including CON vs BR-B vs BR-M, and an independent samples t-test including BR-B vs 7 DAYS. \* Significantly different from BR-B ( $P<0.05$ ). # Trend for a significant difference versus BR-B ( $P<0.10$ ).