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

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

*These authors contributed equally to this work

[1]*Department of Sport and Health Sciences, College of Life and Environmental Sciences, University of Exeter, United Kingdom*

[2]*Department of Human Biology, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre*[+]*, 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

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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

New Findings

What is the central question of this study?

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

What is the main finding and its importance?

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

Abstract

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

Abstract word count: 250

Introduction

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

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

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

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

Methods

Ethical approval

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

Subjects

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

Experimental overview

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

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

Pre-testing

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

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

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

Dietary and physical activity control designs

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

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

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

Experimental visits

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

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

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

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

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

Continuous blood glucose monitoring system (CGMS)

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

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

Blood analyses

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

Skeletal muscle biopsy collection and analysis

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

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

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

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

Statistical analysis

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

Results

Standardised physical activity and muscle strength

The minutes of lying/sitting (551±114 min·d^{-1}), standing (312±103 min·d^{-1}), and walking (145±41 min·d^{-1}) during the CON visit were not different to the habitual activity measured during pre-testing (**Figure 2**; all P>0.05). All regular volitional exercise was successfully replicated during the CON days in terms of intensity/workload and duration. Maximal leg strength did not change following the CON (from 83.7±17.7 to 83.3±18.6 kg), BR-B (84.0±17.7 to 80.3±19.1 kg), or BR-M (84.0±17.7 to 79.9±19.2 kg) visits (interaction and time effect: both P>0.05).

Whole-body substrate utilisation

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

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

Insulin sensitivity and glycaemic control

Fasting blood glucose concentrations prior to and following the 24 h experimental periods were not different between the CON (from 4.59±0.30 to 4.56±0.27 mmol·L^{-1}), BR-B (from 4.52±0.35 to 4.56±0.33 mmol·L^{-1}), and BR-M (from 4.49±0.31 to 4.62±0.25 mmol·L^{-1}) visits, although a trend for a condition*time interaction (P=0.065) was observed. None of the interventions affected fasting serum insulin concentrations. As a result, HOMA-IR (Matthews *et al.*, 1985) (**Figure 4A**) showed a trend for a condition*time interaction (P=0.096), due to a trend for an increase in BR-M only (P=0.061).

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

Skeletal muscle gene expression

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

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

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

Discussion

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

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

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

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

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

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

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

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

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

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

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

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

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

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Tables

Table 1: Subjects' characteristics

Age (y)	25 ± 3
Body mass (kg)	79.0 ± 7.4
Height (m)	1.77 ± 0.06
BMI ($kg \cdot m^{-2}$)	25.3 ± 2.5
Body fat (%)	13.6 ± 5.0
RMR ($MJ \cdot d^{-1}$)	8.9 ± 1.0
Habitual physical activity	
Light activity ($min \cdot d^{-1}$)	114 ± 41
Moderate activity ($min \cdot d^{-1}$)	122 ± 42
Vigorous activity ($min \cdot d^{-1}$)	18 ± 15
MVPA ($min \cdot d^{-1}$)	139 ± 48
Total physical activity ($min \cdot d^{-1}$)	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.

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