Effects of sleeping with reduced carbohydrate availability on acute training responses

Stephen C. Lane¹, Donny M. Camera², David Gray Lassiter³, Jose´ L. Areta¹, Stephen R. Bird¹, Wee Kian Yeo⁴, Nikki A. Jeacocke², Anna Krook⁶, Juleen R. Zierath³,⁶, Louise M. Burke⁵, John A. Hawley²,⁷.

¹Exercise & Nutrition Research Group, School of Medical Sciences, RMIT University, Bundoora, VIC, Australia; ²Centre for Exercise & Nutrition, Mary MacKillop Health Research Institute, Australian Catholic University, VIC 3065, Australia; ³Molecular Medicine and Surgery, Karolinska Institutet, Stockholm, Sweden; ⁴National Sports Institute of Malaysia, Kuala Lumpur, Malaysia; ⁵Sports Nutrition, Australian Institute of Sport, Belconnen, ACT, Australia; ⁶Physiology and Pharmacology, Karolinska Institutet, Stockholm, Sweden ⁷Institute for Sport and Exercise Sciences, Liverpool John Moores University, Liverpool, United Kingdom

Running Head: Sleeping with reduced muscle glycogen.

Address for correspondence John A. Hawley, Ph.D.
Centre for Exercise & Nutrition
Mary MacKillop Health Research Institute
Australian Catholic University
VIC 3065, Australia

Email: john.hawley@acu.edu.au
Abstract

We determined the effects of ‘periodized nutrition’ on skeletal muscle and whole-body responses to a bout of prolonged exercise the following morning. Seven cyclists completed two trials receiving isoenergetic diets differing in the timing of ingestion: they consumed either 8 g·kg\(^{-1}\) BM of CHO before undertaking an evening session of high-intensity training (HIT) and slept without eating (FASTED), or consumed 4 g·kg\(^{-1}\) BM of CHO before HIT then 4 g·kg\(^{-1}\) BM of CHO before sleeping (FED). The next morning subjects completed 2 h cycling (120SS) while overnight fasted. Muscle biopsies were taken on day 1 (D1) before and 2 h after HIT and on Day 2 (D2) pre-, post-, and 4 h after 120SS. Muscle [glycogen] was higher in FED at all times post-HIT (P < 0.001). HIT increased PGC1α mRNA (P < 0.01) while PDK4 mRNA was elevated to a greater extent in FASTED (P < 0.05). Resting phosphorylation of AMPK\(^{Thr172}\), p38MAPK\(^{Thr180/Tyr182}\) and p-ACC\(^{Ser79}\) (D2) was greater in FASTED (P < 0.05). Fat oxidation during 120SS was higher in FASTED (P = 0.01) coinciding with increases in ACC\(^{Ser79}\) and CPT1, as well as mRNA expression of CD36 and FABP3 (P < 0.05). Methylation on the gene promoter for COX4I1 and FABP3 increased 4 h after 120SS in both trials, while methylation of the PPARδ promoter increased only in FASTED. We provide evidence for shifts in DNA methylation that correspond with inverse changes in transcription for metabolically adaptive genes, although delaying post-exercise feeding failed to augment markers of mitochondrial biogenesis.

Key words: Cycling; HIT; Train-low; Sleep-low, Muscle glycogen.
Introduction

Commencing endurance exercise with low muscle glycogen stores (so-called “train-low”) results in a greater transcriptional activation of enzymes involved in carbohydrate metabolism, including the adenosine 5′-monophosphate-activated protein kinase (AMPK), GLUT4 and the pyruvate dehydrogenase (PDH) complex, compared to when glycogen content is normal (6, 25, 33). Restricting carbohydrate (CHO) availability during early (1-5 h) post-exercise recovery has also been shown to acutely up regulate various markers of substrate metabolism and endurance training adaptation in skeletal muscle (6, 27). Because the time course of transcriptional activation for many exercise-induced genes occurs during the first few hours of recovery (18), returning to basal values within 24 h (34), such events may be linked by common signaling and/or regulatory mechanisms, such as the restoration of muscle energy stores, predominantly glycogen. These early adaptive responses to acute exercise may be orchestrated through epigenetic modifications involving DNA methylation (2). Exercise-induced changes in DNA methylation are inversely associated with activation of some, but not all genes underpinning the adaptive response to exercise (2, 21) and appear to be dependent on work intensity (2) and substrate availability (1).

The original “train-low” protocol advocated twice-a-day training sessions in which only the second exercise session was undertaken with low glycogen availability (8). A direct consequence of this strategy was that the maximal self-selected training intensity of the second session was substantially reduced when it was commenced with low, compared to normal glycogen levels (14, 36). Such an outcome is counterintuitive for the preparation of competitive athletes for whom high-intensity workouts are a critical component of a periodized training program (12). Against this background, we have formulated a novel approach in which we can prolong the duration of low CHO (i.e., muscle and liver glycogen)...
availability, thereby potentially enhancing and extending the time course of transcriptional
activation of metabolic genes and their target proteins, while simultaneously conserving the
training intensity of the initial session and hence the training ‘impulse’ to the working
muscles. We have termed this strategy “train-high, sleep-low” and here for the first time have
simultaneously measured gene, protein and methylation responses in skeletal muscle in
response to this protocol. We hypothesized that in comparison to the effects of rapid post-
exercise CHO intake (i.e., current sport nutrition guidelines), delaying CHO intake and
thereby extending the period during which an individual remains in a low glycogen state
would enhance the acute responses of selected genes and proteins with putative roles in
training adaptation.

Methods

Subjects

Seven male competitive endurance trained cyclists with a history of >3 yr endurance training
and who were riding (values mean ± SD) an average of 406 ± 59 km·wk⁻¹ (range 285-455
km·wk⁻¹), in the 6 wk prior to commencement of the study, volunteered to participate in these
trials. The subjects’ age, body mass (BM), peak oxygen uptake (VO₂peak) and peak power
output (PPO) were 29 ± 5 yr, 76.9 ± 9.1 kg, 67 ± 4.0 mL·kg⁻¹·min⁻¹ and 422 ± 39 W. Prior to
giving their written consent, all subjects were informed of the possible risks of all procedures.
The study was approved by the RMIT Human Research Ethics Committee.

Study overview

Each subject completed two experimental trials in a randomized cross-over design. In each
trial they performed two exercise bouts: the first bout (high-intensity training, HIT) was
undertaken in the evening of the first day, and the second bout (120 minute, steady state ride; 120SS) on the morning of the second day. In one trial, subjects consumed their total daily energy intake throughout the day (i.e., 0600-1800 h) before undertaking the HIT session in the evening (1900-2000 h). Following this session they consumed no food and remained fasted overnight (FASTED). In the other trial, subjects ate half of their energy intake prior to the evening HIT session, consuming the remainder immediately after HIT (FED). In both trials subjects slept in the laboratory overnight and then completed 120 minutes of steady state cycling (120SS) commencing at 0700 h the following morning. One hour after the completion of 120SS, all subjects consumed a standardized breakfast and remained in the laboratory until the completion of the trial at ~1300 h. Skeletal muscle biopsies were obtained before and 2 h post HIT (day 1 – D1) and at rest, immediately post 120SS and after 4 h recovery (day 2 – D2). These were analyzed for selected markers of training adaptation.

Pretesting: Incremental cycle test

Approximately 2 wk prior to commencing their first experimental trial, subjects underwent an incremental cycling test to exhaustion on an electronically braked cycle ergometer (Lode Excallibur Sport, Groningen, The Netherlands), as previously described (13). During this maximal test, subjects breathed through a Hans Rudolph two-way non-rebreathing valve and mouth piece attached to a calibrated online gas system (TrueOne 2400, Parvomedics, Utah, USA) interfaced to a computer that calculated the instantaneous rates of O₂ consumption ( \( \dot{V}_O_2 \) ), CO₂ production ( \( \dot{V}_C_O_2 \) ), minute ventilation (VE<sub>STPD</sub>), and the respiratory exchange ratio (RER). Before each test, analyzers were calibrated with commercially available gasses of known O₂ and CO₂ content. \( \dot{V}_O_2^{peak} \) was defined as the highest uptake a subject attained during any 30 s of the test while PPO was calculated from the last completed work rate plus the fraction of time spent in the final non-completed work rate (13). The maximal test and all
experimental trials were conducted under standardized laboratory conditions (18-22°C, 40-50% relative humidity) and subjects were fan cooled during all exercise sessions. Each individual’s PPO recorded during the incremental test was used to determine their prescribed cycling intensities (W) during the subsequent experimental trials.

Standardized Diet/Exercise control
Subjects consumed a pre-packaged standardized diet for the 24 h period prior to commencing an experimental trial (15). Dietary goals for this period were 8 g·kg\(^{-1}\) BM CHO; 1.5 g·kg\(^{-1}\) BM protein; 1.5 g·kg\(^{-1}\) BM fat; for a total energy intake of ~220 kJ·kg\(^{-1}\) BM for the 24 h period. Subjects were instructed to avoid any strenuous physical activity, as well as alcohol and caffeine consumption for the 24 h prior to a trial. Subjects were provided with all foods and drinks in portion controlled packages for consumption during the dietary control period and were given verbal and written instructions on how to follow the diet. Checklists were used to record each menu item as it was consumed and to note any deviations from the menu. Each subject’s food checklists were reviewed and clarified for compliance to the standardization protocols by the primary researcher.

Experimental Diet
For the experimental trials, subjects were provided with all food and fluid to be consumed prior to reporting to the laboratory at 1700 h. Subjects received one of two isoenergetic diets (containing 8 g·kg\(^{-1}\) BM CHO; 1.5 g·kg\(^{-1}\) BM protein; 1.5 g·kg\(^{-1}\) BM fat; ~220 kJ·kg\(^{-1}\) BM energy) that only differed in the timing of consumption (Figure 1). During one trial (FASTED), food was portioned such that subjects consumed 6 g·kg\(^{-1}\) BM CHO, 1.25 g·kg\(^{-1}\) BM protein and 1.25 g·kg\(^{-1}\) BM of fat throughout the day with their final days’ meal (2 g·kg\(^{-1}\) BM CHO, 0.25 g·kg\(^{-1}\) BM protein and 0.25 g·kg\(^{-1}\) BM of fat) being consumed upon arrival at
the laboratory (1700 h). During the other trial (FED), food was portioned so that subjects consumed 2 g·kg\(^{-1}\) BM CHO, 0.65 g·kg\(^{-1}\) BM protein and 0.35 g·kg\(^{-1}\) BM of fat before 1700 h, a further 2 g·kg\(^{-1}\) BM CHO, 0.25 g·kg\(^{-1}\) BM protein and 0.25 g·kg\(^{-1}\) BM of fat meal upon arrival in the lab (1700 h), with the remainder of that day’s intake (4 g·kg\(^{-1}\) BM CHO, 0.6 g·kg\(^{-1}\) BM protein and 0.9 g·kg\(^{-1}\) BM of fat) consumed after the HIT session at 2000 h. On day two, a breakfast containing 2 g·kg\(^{-1}\) BM CHO, 0.2 g·kg\(^{-1}\) BM protein and 0.2 g·kg\(^{-1}\) BM of fat was consumed 1 h after completion of the 120SS ride in both trials.

**Blood and tissue collection and analysis**

Seventeen blood samples were collected during each trial with a total 9 mL of whole blood obtained at each sampling time point (Figure 1). Six mL of blood was collected in tubes containing EDTA. Twenty-five μL of blood was then immediately analyzed for glucose concentration (YSI, Yellow Springs, Ohio, USA), while the remaining sample was centrifuged at 4ºC at 4000 rev·min\(^{-1}\) for 10 min with the resulting plasma transferred to 1.5 mL tubes and stored at -80ºC for subsequent analyses of plasma insulin and catecholamine concentrations. At each time point, a further 3 mL of blood was collected in a tube containing EGTA, which was then centrifuged and the resulting plasma frozen and stored (as described above) for later analyses of free fatty acids (FFA). Catecholamine concentrations were analyzed using a commercially available enzyme immunoassay (Bi-CAT EIA 17-BCTHU-E02.1, ALPCO, Salem NH), while plasma insulin concentrations were determined via ELISA (80-INSHU-E01.1, E10.1, ALPCO, Salem NH). Plasma FFA concentrations were determined using an enzymatic colorimetric method (NEFAC code 279-75401, Wako, Tokyo Japan).

A total of five biopsies were collected during each of the experimental trials from the *vastus lateralis* using a 5 mm Bergström needle adapted for manual suction. Samples were
immediately washed in 0.9 % saline solution then snap frozen in liquid nitrogen and stored at -80°C until later analysis. The sampling points were before and 2 h after HIT (day 1) and then at rest, immediately post 120SS and after 4 h recovery (day 2). Biopsies were subsequently analyzed for gene expression and protein abundance of markers of training adaptation (described subsequently).

High Intensity Interval Session (HIT)

On the evening of the first day of an experimental trial, subjects completed a HIT session on a Velotron cycle ergometer (Racermate, Seattle, WA, USA). After a standardized warm up (10 min at 60% PPO) subjects undertook HIT, which consisted of 8 x 5 min work bouts at 82.5% of individual PPO with 1 min active recovery (100 W) between work bouts. This protocol was chosen as the physiological demands have previously been characterized and in well-trained cyclists, the session has been demonstrated to reduce muscle glycogen by ~ 50% of starting values (31). During HIT, ratings of perceived exertion (RPE) were recorded at the end of each work bout while heart rate was averaged for each 5 min repetition.

120min Steady State ride (120SS)

On the morning of the second day of each trial, subjects completed a standardized, steady state cycling bout (120SS) at a fixed submaximal work rate that was the same for both trials. During this ride, subjects cycled at 50% PPO (~60% of VO2peak). RER was recorded as a 5 min average commencing at 10, 45, 80, 115 min, while heart rate (HR) and RPE were recorded at the end of each 5 min collection point. Whole body rates of CHO and fat oxidation (g·min⁻¹) were calculated from the respiratory data collected during the 120SS ride. The calculations were made from VCO2 and VO2 measurements, assuming a non-protein RER (23).
Fluid intake

During the first experimental trial (including the 24 h standardized dietary control), subjects were allowed water *ad libitum*. The volume of fluid consumed was recorded and then replicated during the subsequent trial.

Muscle glycogen concentration

Muscle glycogen concentration was analyzed as previously described (4). In brief, approximately 10-15 mg of muscle was freeze-dried and powdered, with all visible blood and connective tissue removed under magnification. The freeze-dried muscle sample was then extracted and glycogen concentration determined via enzymatic analyses.

DNA Methylation

Bisulfite conversion is a chemical treatment of genomic DNA utilized to convert non-methylated cytosines into uracils: the bisulfite treatment, however, does not convert methylated cytosines. Subsequent sequencing of bisulfite-converted DNA reveals cytosine methylation at the resolution of a single nucleotide. We utilized the bisulfite-sequencing method to interrogate DNA methylation in our targets of interest. This method is considered the “gold standard” in DNA methylation analysis, largely due to the base-pair resolution afforded by the technique (5, 32). To assay DNA methylation in known regulatory regions for PPARGC1A, PDK4, TFAM, PPARD, SLC2A4, COX4I1, and FABP3 we used the Qiagen Q24 Advanced Pyromark System (Qiagen, Venlo, the Netherlands, Cat No 9002270).

Genomic DNA was extracted from 2-23 mg (average 18 mg) of previously flash-frozen muscle biopsies using the Qiagen DNeasy Blood & Tissue kit (Qiagen, Venlo, the Netherlands, Cat No 69506). Genomic DNA was then bisulfite converted using the Qiagen Epitect Fast kit (Qiagen, Venlo, the Netherlands, Cat No 59826). After bisulfite treatment, the
DNA was amplified by PCR (primer sets are listed in Table 1). Finally, the DNA was pyrosequenced and the percentage of methylated cytosines at variable loci were determined.

For FABP3 and GLUT4, specific regions of interest were selected based on previously identified regions subject to changes in DNA methylation (22, 37). The UCSC Genome Browser permits users to add “tracks” which shows the genomic addresses where dynamic methylation has been previously observed. In the absence of specific literature to direct our search for methylation changes in the gene promoters of PPARδ and COX4I1, we designed our assays around such sites observed on the UCSC genome browser (Feb. 2009 GRCh37/hg19 Assembly).

RNA Extraction and Quantification
Approximately 20 mg of skeletal muscle was homogenized in TRIzol and chloroform added to form an aqueous RNA phase. This RNA phase was then precipitated by mixing with isopropanol alcohol and the resulting pellet was washed and resuspended in 50 µL of RNase-free water. Extracted RNA was quantified using a QUANT-iT analyser kit (Invitrogen, Melbourne, Australia, Cat No Q32852) and on a NanoDrop 1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA) by measuring absorbance at 260 nm and 280 nm with a 260/280 ratio of ~ 1.88 recorded for all samples.

Reverse Transcription and Real-Time PCR
First-strand complementary DNA (cDNA) synthesis was performed using commercially available TaqMan Reverse Transcription Reagents (Invitrogen, Melbourne, Australia) in a final reaction volume of 20 µL. All RNA and negative control samples were reverse transcribed to cDNA in a single run from the same reverse transcription master mix. Serial
dilutions of a template RNA (AMBION; Cat No AM7982) were included to ensure efficiency of reverse transcription and for calculation of a standard curve for real-time quantitative polymerase chain reaction (RT-PCR). Quantification (in duplicate) was performed using a Rotor-Gene 3000 Centrifugal Real-Time Cycler (Corbett Research, Mortlake, Australia). Taqman-FAM-labelled primer/probes for PGC-1α (Cat No. Hs01016719), TFAM (Cat No. Hs00273372_s1), COX4I1 (Cat No. Hs00971639_m1), PPARδ (Cat No. Hs04187066_g1), CD36 (Cat No. Hs01567185_m1), FABP3 (Cat No. Hs00997360_m1), PDK4 (Cat No. Hs01037712_m1), and GLUT-4 (Cat No. Hs00168966_m1) were used in a final reaction volume of 20 µL. PCR treatments were 2 min at 50°C for UNG activation, 10 min at 95°C, then 40 cycles at 95°C for 15 s and 60°C for 60 s. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cat No. Hs Hs99999905) was used as a housekeeping gene and expression was not different at any time point or between treatments (data not shown). The relative amounts of mRNAs were calculated using the relative quantification (ΔΔCT) method (17).

Western Blot Analysis
Muscle samples (~15 mg) were homogenized in ice-cold buffer containing 50 mM of Tris–HCl, pH 7.5, 1 mM of EDTA, 1 mM of EGTA, 10% glycerol, 1% Triton X-100, 50 mM of NaF, 5 mM of sodium pyrophosphate, 1 mM of DTT, 10 µg·mL⁻¹ of trypsin inhibitor, 2 µg·mL⁻¹ of aprotinin, 1 mM of benzamidine, and 1 mM PMSF using a motorized pellet pestle (Sigma-Aldrich, St. Louis, MO) with 5 s pulses. The lysate was kept on ice at all times and was then centrifuged at 12,000g for 20 min at 4°C. The supernatant was transferred to a sterile tube and was subsequently aliquoted for determination of protein concentration using a BCA protein assay (Pierce, Rockford, IL). The supernatant was then resuspended in Laemmli sample buffer and separated by sodium dodecyl sulfate–polyacrylamide gel electrophoresis before being transferred to polyvinylidene fluoride membranes and incubated with primary
antibody (1:1,000) overnight at 4°C and secondary antibody (1:2,000). Proteins were detected via chemiluminescence (Amersham Biosciences, Buckinghamshire, UK; Pierce Biotechnology, Rockford, IL) and quantified by densitometry (Chemidoc, BioRad, Gladesville, Australia). All sample (50 µg) time points for each subject were run on the same gel. Polyclonal anti-phospho AMPKα Thr172 (no. 2531), -ACC Ser79 (no. 3661P), -HSL Ser660 (no. 4126S), monoclonal anti-phospho p38MAPK Thr180/Tyr182 (no. 4511) and monoclonal total ATGL (no. 2439S) and CPT1A (no. 12252S) were purchased from Cell Signaling Technology (Danvers, USA). Data are expressed relative to α-tubulin (no. 3873, Cell Signaling Technology, Danvers, USA) in arbitrary units.

Statistical Analysis

Respiratory, physiological, blood, muscle glycogen and PCR data were analyzed using SPSS software package (version 21). Western blot data were analysed using Sigma Stat (version 3.1). All data were checked for sphericity using Mauchly's Test and normality using Kolomogorov-Smirnov tests. Where mRNA or DNA methylation data violated assumptions of sphericity or normality, data were natural log transformed before further analyses. To compare the responses during the experimental trials, data were analyzed using two-way Analyses of Variance (Trial by Time) with repeated measures (RM) (α = 0.05). Least Significant Difference and paired ‘t’ tests were used post-hoc. Results and statistics represent seven subjects unless otherwise indicated. All values are expressed as mean ± SD unless otherwise indicated.
Results

High Intensity Intervals (HIT)

During HIT, two subjects were unable to complete the prescribed session during their first trial (subject 4 at interval 4 and subject 8 at interval 5). As a consequence, their exercise intensity was reduced by 10 W for subsequent intervals to allow the subjects to complete the remaining work bouts and an identical set of work bouts was repeated for their second trial. Average power output sustained for HIT sessions was 346W ± 31W. There were no differences in average RPE or HR between trials (Table 2).

120min Steady State ride

During the standardized submaximal 120SS on the morning of day 2, RER was lower in FASTED at 10-15, 45-50 and 115-120 min time points compared with FED (Table 2). During both trials there was a main effect of time (P < 0.01), whereby there was a steady decline in RER throughout the ride. There were no differences in HR between trials at any time point. HR increased throughout 120SS, although there was no main effect of time for trials (P = 0.056). In the FED trial only, HR was statistically higher at 115-120 min when compared to 10-15 min (P = 0.01). There were no differences in RPE between trials at any point with all subjects being able to complete the training bout independent of overnight dietary status. Similarly, there was no effect of time on RPE during trials.

Muscle Glycogen Concentration

Subjects commenced the HIT session with similar muscle glycogen concentrations. Two hours post-HIT, muscle glycogen concentration was reduced by ~45% in the FASTED trial and by ~30% in FED compared to REST 1. Resting muscle glycogen concentrations on the
morning of day 2 (REST 2) remained lower than REST 1 (P < 0.05) and consequently the 120SS bout was commenced with lower glycogen concentration in the FASTED trial compared to FED (349 ± 141 vs. 459 ± 159 mmol·kg⁻¹ dry wt; P < 0.01). 120SS further reduced glycogen concentration in both trials by approximately 25%, with a greater absolute reduction in the FED (121 ± 42 mmol·kg⁻¹ dry wt) versus FASTED (83 ± 39 mmol·kg⁻¹ dry wt) trial. The meal consumed 1 h post the 120SS ride elevated glycogen concentration 3 h post feeding (P < 0.001) to a similar extent in both trials (~25 mmol·kg⁻¹ dry wt). Additionally, muscle glycogen concentration in the FASTED trial remained below that of the FED trial (P < 0.05).

Blood glucose concentration

At REST 1, immediately before the HIT exercise bout and 2 h post the standardized meal blood glucose concentrations were slightly higher in the FASTED trial compared to the FED trial (Figure 3A, P < 0.05), reflecting the greater overall CHO intake in the FASTED trial to this point in the day. Immediately post HIT, blood glucose concentration did not differ between the trials. The meal consumed immediately post-HIT in the FED trial increased blood glucose concentration with levels peaking at HIT + 30 (Figure 3A). Immediately prior to 120SS ride on the morning of day 2 (REST 2), immediately after the ride and then throughout recovery, blood glucose concentration in both trials displayed similar profiles: initially increasing as a consequence of the meal that was consumed 1 h after 120SS and then declining until the end of the trials. In both trials blood glucose concentrations peaked 40 min after the meal (120SS + 100) and declined to REST 1 concentration over the subsequent 3 h, with a small difference between trials 150 min post-meal (120SS + 210 min), at which point the blood glucose concentration in the FASTED trial was slightly higher than that in the FED trial.
At REST 1, plasma insulin was higher in FASTED compared to FED (Figure 3B), reflecting the larger daily CHO intake in FASTED prior to this point. The meal consumed in the FED trial immediately post-HIT significantly elevated plasma insulin values compared to the Post-HIT concentration and although starting to decline, these were still elevated at HIT + 120min. Insulin concentrations in FASTED trial remained at Post-HIT concentrations at all times points measured post-HIT. The following morning at REST 2 and immediately post the 120SS bout, the insulin concentration was similar between trials and near resting concentrations. Following the post 120SS meal (eaten 60 min post exercise in both trials), plasma insulin concentration peaked 40 min post ingestion (120SS + 100min) in the FED trial and 60 min post ingestion (120SS + 120min) in the FASTED trial. Plasma insulin concentrations then declined in a similar manner in both trials returning to the REST 1 concentrations within 3 h post ingestion.

There were no differences in plasma FFA concentrations between trials at REST 1 or immediately post HIT (Figure 3C). In FASTED, plasma FFA concentration continued to increase above REST 1 and compared to FED became greater 90 and 120 min after HIT. Resting plasma FFA concentration after the overnight sleep had declined from the previously elevated concentration in the FASTED trial, but was still above REST 1 concentration. There were no differences in plasma FFA level between trials at this time. The 120 min SS ride was associated with elevated plasma FFAs (P < 0.01), with no difference observed between trials (P = 0.179). Following breakfast, plasma FFA concentration continued to decline in both trials, returning to REST 2 concentration 40 min post ingestion (120SS + 100).
Catecholamine concentrations

Plasma concentrations of adrenaline and noradrenaline were similar between trials at REST 1 and at all other time point throughout the trials. Levels were elevated post-HIT (P < 0.01), but had returned to REST 1 concentration within 60 min of completing HIT. After the 120SS ride on day 2, plasma noradrenaline was elevated in both trials, but to a lesser magnitude than following the HIT session (P = 0.02). However, the plasma noradrenaline concentration was still slightly above REST 2 concentrations 60 min post exercise (P = 0.02). Plasma adrenaline concentration displayed a similar profile to the noradrenaline response and immediately post 120SS were above REST 2 concentration, but this only reached statistical significance in the FASTED trial (P = 0.005), and had returned to REST 2 concentration within 60 min post-exercise in both trials.

Substrate utilization during 120SS

Total CHO oxidation during 120SS was greater in FED (223 ± 42 g) than FASTED (168 ± 28 g) (P = 0.01), while total fat oxidation was greater in FASTED (111 ± 25 g) compared to FED (88 ±17 g) (P = 0.01, Figure 5).

Protein data

Signaling proteins

Baseline values for p-AMPK\textsuperscript{Thr172}, p-p38MAPK\textsuperscript{Thr180/Tyr182} and p-ACC\textsuperscript{Ser79} were similar at rest on day 1. At 2 h post-HIT, p-AMPK\textsuperscript{Thr172} tended to be higher in FASTED compared to the FED trial (P = 0.058). The following morning (REST 2), phosphorylation of AMPK\textsuperscript{Thr172}, p38MAPK\textsuperscript{Thr180/Tyr182} and ACC\textsuperscript{Ser79} was greater in the FASTED compared to the FED trial, while p-ACC\textsuperscript{Ser79} was also elevated compared to REST 1 (P < 0.05). The 120SS bout did not increase (post 120SS) p-AMPK\textsuperscript{Thr172} or p-p38MAPK\textsuperscript{Thr180/Tyr182} above REST 2. However, p-
ACCSer\textsuperscript{79} further increased in FASTED at Post 120SS at post 120SS (P < 0.05), but had returned to the resting value by 120SS + 4h. p-AMPK\textsuperscript{Thr172} showed a similar pattern.

\textit{Lipolysis and fat transport proteins}

There were no differences between the two trials for CPT1, ATGL or p-HSL\textsuperscript{Ser 660} at rest on day 1. At HIT + 2h, ATGL in the FASTED trial was elevated compared to REST 1 (P < 0.05). At post 120SS, CPT1 was higher in FASTED compared to FED (P < 0.01) and was elevated compared to both time points from the previous day (P < 0.01). CPT1 remained elevated above these levels at 120SS + 4h in FASTED (P < 0.01). ATGL was higher at 120SS + 4h in FASTED compared to all prior time points (P < 0.05), but despite being substantially elevated, protein abundance was not significantly different to FED (P = 0.06), possibly due to a lack of statistical power (n = 4).

\textit{Mitochondrial genes (Figure 8)}

PGC1\textalpha, TFAM and COX4I1 mRNA were not different between trials at REST 1. After HIT, PGC1\textalpha mRNA was increased in both trials (P < 0.05, ~6 fold-change). TFAM mRNA was increased only in FED (P < 0.01). At REST 2, PGC1\textalpha mRNA had declined in both trials, but remained slightly elevated in FED compared to REST 1 (P < 0.05) while TFAM was still elevated in FED and increased in FASTED compared to REST 1. POST 120SS, PGC1\textalpha and TFAM remained elevated compared to REST 1. Further increases in response to 120SS were only evident for PGC1\textalpha in the FED trial, which also further increased at 120SS + 4h reaching statistically higher levels compared to FASTED (P < 0.05). There were no differences between trials or within trials between time points for COX4I1 mRNA abundance. COX4I1 promoter methylation was higher in FASTED at 120SS + 4h compared to REST 1 and POST 120SS.
Lipolysis and fat transport genes (Figure 9)

There were no differences between trials at REST 1 for mRNA expression of PPARδ, FABP3, or CD36. At HIT + 2h, PPARδ mRNA was elevated in both trials (FED ~2 fold, P = 0.002 and FASTED ~1.5 fold, P = 0.038) but values for both trials had returned to pre-HIT levels by REST 2. FABP3 and CD36 mRNA increased overnight in FASTED (P < 0.05) with CD36 being higher compared to FED (P = 0.02). After 120SS, CD36 mRNA remained elevated in FASTED with FABP also becoming significantly higher in FASTED compared to FED. DNA methylation of FABP3 at 120SS + 4h was greater than at REST 2, which coincided with a trend for reduced mRNA abundance. At 120SS + 4h, PPARδ mRNA tended to increase in both trials, with levels only in the FED state reaching significantly higher than previous values. Also at 120SS + 4h, DNA methylation of the PPARδ promoter was greater in FASTED than FED (P < 0.05).

CHO oxidation genes

There were no differences between trials at REST 1 for mRNA of either PDK4 or GLUT4. At HIT +2, PDK4 mRNA was elevated in both trials (P < 0.01), with mRNA being elevated to a greater extent in FASTED (~65 fold) compared to FED (~10 fold difference) (P = 0.03). Overnight, PDK4 mRNA remained elevated in FASTED compared to FED (P = 0.02) and REST 1 (P < 0.01). The 120SS bout evoked further increases in PDK4 mRNA in both trials, which persisted until the final time point 4 h post 120SS (P < 0.05). Additionally, the PDK4 mRNA was significantly higher in the FASTED trial (P < 0.05), compared to the FED trial at both time points (POST 120SS and 120SS + 4 h).
At HIT +2h, GLUT4 mRNA did not differ from REST 1, but the following morning it was elevated in both trials compared to HIT +2 (P < 0.05). POST 120SS GLUT4 mRNA was elevated above REST 1 and HIT +2 in both trials (P < 0.05).

Discussion

Skeletal muscle adaptation to exercise training is a consequence of repeated contraction-induced increases in gene expression that lead to the accumulation of functional proteins whose role is to blunt the homeostatic perturbations generated by contraction-induced increases in energy demand and substrate turnover. The development of a specific “exercise phenotype” is the result of new, augmented steady-state mRNA and protein levels that stem from the training stimulus (24), which can be modified by the prevailing energy availability (11). The four fundamental cellular processes involved in gene expression are transcription, mRNA degradation, translation and protein degradation, with each step of this cascade controlled by gene-regulatory events (30). Recent evidence also suggests that acute gene activation is associated with a dynamic change in DNA methylation in skeletal muscle and that DNA hypomethylation is an early event in contraction-induced gene activation (2). Here for the first time we have simultaneously measured gene, protein and methylation status in skeletal muscle in response to a novel exercise-nutrient intervention in which athletes undertook an intense bout of endurance training late in the day and then slept with reduced CHO availability. Specifically, we periodized the timing of nutrient intake such that cyclists performed an evening bout of HIT with high-CHO availability, then restricted CHO intake so that they slept with low CHO availability before undertaking a standardized bout of submaximal exercise in the fasted state the following morning. We found that when feeding was withheld overnight and subjects slept with reduced energy availability, AMPK^{Thr172}, p38MAPK^{Thr180/Tyr182} and p-ACC^{Ser79} were upregulated to a greater extent the following
morning, compared to when subjects were fed a high CHO meal early in recovery. We also showed that when a second prolonged, steady-state training session was commenced after ‘sleeping low’, the expression of selected genes and abundance of phosphorylated signaling proteins with putative roles in lipid oxidation and transport were higher, compared to when a post-exercise meal was consumed and glycogen availability was partially restored.

A major aim of this study was to circumvent the previously observed impairment in maximal self-selected training intensity when athletes perform two bouts of training within several hours, the second session commenced with reduced muscle glycogen content (14, 36). Under such conditions, power output is reduced by ~8% (14, 36), even when caffeine is ingested in an attempt to offset this decline (16). By undertaking HIT in the evening and then withholding feeding overnight, athletes were able to complete HIT and still ‘train-low’ the following morning without compromising the total ‘training impulse’ to the working muscles. In the present study HIT reduced glycogen content by ~50% which is consistent with previous investigations using the same protocol and athletes of comparable training status (31, 35). However, the glycogen content of the well-trained cyclists in the current study was higher than our previous work (~600 mmol·kg\(^{-1}\) dry wt; range ~400 to ~900 mmol·kg\(^{-1}\) dry wt) and its relative utilization during HIT was similar to values we have previously reported (~50%) (31, 35) hence it resulted in a substantial amount of glycogen remaining in the muscle after the HIT session (~360 mmol·kg\(^{-1}\) dry wt; Figure 2). As such, athletes slept with reduced, but not low muscle glycogen levels and commenced the next morning’s training session with higher than anticipated glycogen availability. Of note is that the glycogen content attained in the current study after the HIT session is higher than concentrations reported by others who subsequently observed significant up-regulation of several training-induced signaling responses (3, 20, 25, 29, 35). Notwithstanding such differences in exercise-
induced glycogen utilization among studies, we observed significant increases in PGC1α mRNA expression several hours after HIT (Figure 8) consistent with a variety of glycogen-depleting protocols (6, 7, 19, 28). PDK4 mRNA expression was elevated compared to rest at 2 h post-HIT in both trials (Figure 10). However, the consumption of a high CHO meal immediately after HIT blunted the rise in PDK4 mRNA such that levels in the FASTED trial were ~6 fold greater than in the FED trial, with differences between trials persisting at all subsequent time points.

The effects of sleeping with reduced muscle glycogen content can be assessed by examining markers of training adaptation/substrate availability in the resting tissue samples obtained on the morning of day two of the experiment. As might be expected after withholding energy intake overnight, the abundance or phosphorylated AMPK<sup>Thr172</sup>, p38MAPK<sup>Thr180/Tyr182</sup> and p-ACC<sup>Ser79</sup> protein was elevated to a greater extent in the FASTED versus FED trial (Figure 6). Nevertheless, protein abundance and mRNA expression of several downstream targets, including TFAM and COX4I1 (Figure 8) and PPARδ (Figure 9) did not follow the same temporal pattern. For example, compared to 2 h post HIT the prior evening, PGC1α mRNA expression declined by the morning of day 2 (some 10 h later), irrespective of whether or not a high CHO meal was consumed. The second bout of prolonged, submaximal exercise failed to elicit further increases in the abundance of phosphorylated AMPK<sup>Thr172</sup> or p38MAPK<sup>Thr180/Tyr182</sup> protein, irrespective of whether training commenced after sleeping with low muscle glycogen or following an evening meal that resulted in the replenishment of some muscle glycogen (Figure 6). However, as might be expected from the greater oxidation of fat-compared to CHO-based fuels during this second exercise bout (Figure 5), sleeping with low muscle glycogen did induce a greater increase in ACC<sup>Ser79</sup> phosphorylation. Fasting may promote COX4I1 gene expression since a transient decrease in mRNA was observed after the
steady-state exercise only when participants were fed post-exercise. Furthermore, methylation of the COX4I1 promoter was increased after 4 h of recovery from steady-state exercise, which supports the notion that exercise induces transient changes in DNA methylation (2).

In contrast to COX4I1, the responses of some other genes with roles in mitochondrial biogenesis (PGC1α and TFAM) were not substantially altered by either dietary condition in response to the second exercise bout (Figure 8). We observed an exercise and diet-induced elevation in PGC1α mRNA expression in the FED, but not FASTED trial several hours after the completion of exercise, and at a time when carbohydrate availability was high for both conditions. This response is difficult to explain, but suggests that withholding carbohydrate intake immediately post-exercise may influence the adaptive responses to a subsequent training session undertaken in close proximity (i.e., within a 12 h window or even the same day). Conversely, when muscle glycogen was depleted by prior exercise and a subsequent exercise bout is commenced 14 h later with low (~170 mmol·kg⁻¹ dry wt) muscle glycogen availability, PGC1α mRNA expression was elevated to a greater extent than when carbohydrate was consumed during the recovery period (29). Differences in results between the current and earlier (29) study are hard to reconcile. PPARδ mRNA expression increased in the hours after exercise, but only to a significant extent during the FED trial. Correspondingly, greater methylation of PPARδ was observed in the FASTED trial 4 hours after the steady-state exercise, which may underlie the comparatively reduced mRNA expression. Since high-, but not low-intensity exercise led to hypomethylation of the PPARδ gene and increased gene transcription (2), exercising in a fasted state may preclude the necessary glycolytic flux to induce these adaptive responses at the genomic level.
While sleeping with reduced muscle CHO availability failed to augment selected markers of training adaptation, lack of nutrient availability resulted in marked increases in mRNA and/or abundance of proteins involved in lipid utilization. Compared to FED, FASTED resulted in a greater elevation in levels of FABP3 and CD36 mRNA content the following morning, with levels being further elevated and becoming significantly greater than the FED trial at POST 120SS. DNA methylation of the FABP3 gene tended to be inversely related to mRNA expression, implicating a role for transcriptional repression by this epigenetic mark. Taken together, exercise may induce an increase in methylation of the FABP3 gene in the first few hours after exercise, resulting in impaired mRNA transcription, which may be partially rescued by fasting. Immediately and 4 h after the second bout of exercise undertaken on day 2, PDK4 mRNA abundance was elevated to a greater extent in the FASTED compared to the FED trial. Given the differences in the contribution of fat and carbohydrate fuels to this exercise bout (Figure 5), this is unsurprising and highlights the sensitivity of PDK4 to substrate availability and its role in down-regulating CHO oxidation (26). One of the main responses to withholding CHO availability post-exercise, as well as commencing subsequent exercise with reduced muscle glycogen, is a marked elevation in fat transport and oxidation. Our findings corroborate previous studies in which post-exercise feeding was withheld (27), as well as commencing exercise with reduced muscle glycogen availability (3, 25). These approaches result in greater rates of fat oxidation measured using tracer derived techniques (14), as well as increasing markers of training adaptation when incorporated into a periodized training program (14, 36)

By measuring a time-course of gene, protein and methylation events in skeletal muscle we wished to gain insight into the temporal relationship between these cellular markers in response to our novel diet-exercise intervention. Disappointingly, we observed only small
increases in mRNA for most of the genes under investigation that, in many cases, were discordant from both protein and methylation responses. As protein synthesis is bioenergetically costly, any gene-protein responses are likely to be exaggerated under conditions of energy constraint (i.e. low-CHO availability). However, given that only ~40% of the variance in protein levels is likely to be explained by changes in mRNA levels (30) our results suggest that most of the mRNAs and proteins we measured are relatively stable and that the cellular perturbations induced by our diet-exercise intervention did not require rapid transcriptional/translational regulation. While we provide evidence for shifts in DNA methylation that correspond with inverse changes in transcription for metabolically adaptive genes, the minimal changes in mRNA we observed would certainly not be expected to trigger new steady-state protein levels and alter muscle phenotype over the longer term.

While our sleeping with reduced muscle glycogen protocol was specifically designed to prolong the time during which subjects were exposed to low CHO availability, we also expected to observe concomitant increases in systemic factors (i.e., elevated circulating FFA and catecholamine concentrations) with putative roles in the adaptive processes (9, 10). However, despite a substantially greater contribution from lipid-based fuels to total energy expenditure during the prolonged steady-state ride on the morning of day 2, circulating FFA and catecholamine concentrations were similar under both conditions (Figures 3 and 4). These results do not concur with other studies in which elevated adrenergic responses have been reported when exercise is commenced with reduced muscle glycogen (8). The discrepancy between these studies may be due to a combination of the higher starting muscle glycogen levels for the second exercise bout in the present study, as well as differences in exercise mode (i.e., single-limb kicking versus cycle ergometry).
In conclusion, delayed feeding after an intense evening training session, so that cyclists sleep with lowered CHO availability, results in a greater up-regulation of several exercise responsive signaling markers with roles in lipid oxidation the following morning compared to when an evening meal was consumed (i.e., high overnight CHO availability). Commencing prolonged, steady-state exercise after sleeping with reduced muscle glycogen promoted greater rates of whole-body fat oxidation, compared to sleeping with at least partially replenished muscle glycogen, but failed to elicit a greater upregulation of cellular markers of mitochondrial biogenesis. We also provide evidence for shifts in DNA methylation, which correspond with inverse changes in transcription for metabolically adaptive genes. Whether our novel delayed post-exercise feeding and sleeping with reduced muscle glycogen protocol when incorporated into a periodized training program undertaken over several weeks could provide an additional stimulus to enhance the normal adaptive responses to training remains to be determined. Our results suggest that critical absolute ‘thresholds’ for both pre-exercise glycogen concentration and training intensity exist if specific nutrient-exercise interactions are to augment the normal training response-adaptation. Future studies using unbiased –omic methodology may uncover novel insights into different exercise and diet regimens that can be implemented to optimize metabolic adaptations to training.
Acknowledgments

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Grants

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References


Figure 1: Experimental design. FED (A total of 4g CHO kg\(^{-1}\) BM prior to HIT and 4g CHO kg\(^{-1}\) BM post HIT), FASTED (A total of 8g CHO kg\(^{-1}\) BM prior to HIT and remained fasted during sleep and throughout 120SS).

Figure 2: Skeletal muscle glycogen concentration. (*) difference between trials
(a) different to REST 1, (b) different to HIT +2h, (c) different to REST 2, (d) different to POST 120SS, Values are mean ± SD; P < 0.05.

Figure 3: (A) Blood glucose concentration, (B) Plasma insulin concentration, (C) Plasma FFA concentration. * difference between trials (a) different to REST 1, (c) different to REST 2, (d) different to POST 120SS, (i) different to REST 1 in FED only (j) different to REST 1 in FASTED only, (k) different to 120SS + 60 in FED only, (l) different to 120SS + 60, (m) different to REST 2 in FED only, (Ex) Exercise, (M1) Meal in FED; 4 g·kg\(^{-1}\) BM CHO, (M2) Meal in both trials; 2 g·kg\(^{-1}\) BM CHO, Values are mean ± SD; P < 0.05.

Figure 4: Plasma Catecholamine concentrations, (A) Noradrenaline concentration, (B) Adrenaline concentration, (a) different to REST 1, (c) different to REST 2, (d) different POST 120SS, (o) Adrenaline different to REST 2 in FASTED only, Values are mean ± SD; P < 0.05.
Figure 5: Total carbohydrate and fat oxidation during the 120SS bout. (A) Total carbohydrate oxidation, (B) Total fat oxidation. (*) difference between trials, Values are mean ± SD; P < 0.05.

Figure 6: Signaling proteins. (A) Phosphorylation of 5’ adenosine monophosphate-activated protein kinase (p-AMPK<sup>Thr172</sup>), (B) Phosphorylation of p38 mitogen-activated protein kinase (p-p38MAPK<sup>Thr180/Tyr182</sup>), (C) Phosphorylation Acetyl-CoA carboxylase (p-ACC<sup>Ser79</sup>). (*) difference between trials, (a) different to REST 1, (b) different to HIT +2h, (c) different to REST 2, (e) different to 120SS + 4h; for all proteins at POST 120SS n = 6, Values are normalized to REST 1 and are expressed as mean ± SD; P < 0.05.

Figure 7: Lipolysis and fat transport proteins. (A) Carnitine palmitoyltransferase I (CPT1), (B) Adipose triglyceride lipase (ATGL), (C) Phosphorylation of Hormone sensitive lipase (p-HSL<sup>Ser660</sup>), *difference between trials, (a) different to REST 1, (b) different to HIT +2h, (c) different to REST 2, (d) different to POST 120SS; at all time-points CPT1 n = 6, ATGL n=4, HSL n=5; Values are normalized to REST 1 and are expressed as mean ± SD; P < 0.05.
Figure 8: Mitochondrial genes, (A) mRNA expression of peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α), (B) mRNA expression of mitochondrial transcription factor A (TFAM), (C-D) mRNA expression and DNA methylation of cytochrome c oxidase subunit IV (COX IV). For COX4I1 mRNA at 120SS +4h n=6 and DNA methylation all time-points n=4. *difference between trials, (a) different to REST 1, (b) different to HIT +2h, (c) different to REST 2, (d) different to POST 120SS; Values are normalized to REST 1 and are expressed as mean ± SD; P < 0.05.

Figure 9: Lipolysis and fat transport genes, (A-B) mRNA expression and DNA methylation of peroxisome proliferator-activated receptor delta (PPARδ), (C-D) mRNA expression and DNA methylation of fatty acid binding protein (FABP3), (E) mRNA expression of cluster of differentiation 36 (CD36). For mRNA 120SS +4h n=6 and DNA methylation all time-points n=4. *difference between trials, (a) different to REST 1, (b) different to HIT +2h, (c) different to REST 2, (d) different to POST 120SS; Values are normalized to REST 1 and are expressed as mean ± SD; P < 0.05.

Figure 10: CHO oxidation genes. (A) Pyruvate dehydrogenase kinase 4 (PDK4), (B) Glucose transporter 4 (GLUT4), (*) difference between trials, (a) different to REST 1, (b) different to HIT +2h, (c) different to REST 2, (d) different to POST 120SS. Values are normalized to REST 1 and are expressed as mean ± SD; P < 0.05.

Table 1: Target genes for DNA methylation.

Table 2: Physiological and respiratory response to HIT and 120SS. Values are mean ± SD, P < 0.05; (f) different to 10-15min, (g) different to 45-50 min, (h) different to 80-85 min. (*) different to FED.
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### HIT (8 x 5min Intervals at 82.5% Peak Power Output)

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### 120SS (120min at 50% Peak Power Output)

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