









Post-exercise carbohydrate and energy availability induce independent effects on skeletal muscle cell signalling and bone turnover: implications for training adaptation

Kelly M. Hammond¹, Craig Sale² , William Fraser³, Jonathan Tang³, Sam O. Shepherd¹ , Juliette A. Strauss¹ , Graeme L. Close¹, Matt Cocks¹ , Julien Louis¹, Jamie Pugh¹ , Claire Stewart¹ , Adam P. Sharples¹  and James P. Morton¹ 

¹Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Tom Reilly Building, Byrom St Campus, Liverpool L3 3AF, UK

²Musculoskeletal Physiology Research Group, Sport, Health and Performance Enhancement Research Centre, School of Science and Technology, Nottingham Trent University, UK

³Norwich Medical School, University of East Anglia, Norfolk and Norwich University Hospital, Norfolk NR4 7UY, UK

Edited by: Michael Hogan & Karyn Hamilton

Linked articles: This article is highlighted in a Journal Club article by Diaz-Lara *et al.* To read this article, visit <https://doi.org/10.1113/JP279076>. It is also highlighted in a Journal Club article by Kucey *et al.* To read this article, visit <https://doi.org/10.1113/JP279020>.

Key points

- Reduced carbohydrate (CHO) availability before and after exercise may augment endurance training-induced adaptations of human skeletal muscle, as mediated via modulation of cell signalling pathways.
- However, it is not known whether such responses are mediated by CHO restriction, energy restriction or a combination of both.
- In recovery from a twice per day training protocol where muscle glycogen concentration is maintained within 200–350 mmol kg⁻¹ dry weight (dw), we demonstrate that acute post-exercise CHO and energy restriction (i.e. < 24 h) does not potentiate potent cell signalling pathways that regulate hallmark adaptations associated with endurance training.
- In contrast, consuming CHO before, during and after an acute training session attenuated markers of bone resorption, effects that are independent of energy availability.
- Whilst the enhanced muscle adaptations associated with CHO restriction may be regulated by absolute muscle glycogen concentration, the acute within-day fluctuations in CHO availability inherent to twice per day training may have chronic implications for bone turnover.

Abstract We examined the effects of post-exercise carbohydrate (CHO) and energy availability (EA) on potent skeletal muscle cell signalling pathways (regulating mitochondrial biogenesis and

Kelly Hammond is a PhD student at Liverpool John Moores University and also holds an MSc in Sports Physiology. She is also an accredited sports nutritionist with the UK Sport and Exercise Nutrition Register and is experienced in delivering nutrition support to athletes from team, endurance and weight restricted sports.



lipid metabolism) and indicators of bone metabolism. In a repeated measures design, nine males completed a morning (AM) and afternoon (PM) high-intensity interval (HIT) (8×5 min at $85\% \dot{V}_{O_{2peak}}$) running protocol (interspersed by 3.5 h) under dietary conditions of (1) high CHO availability (HCHO: CHO ~ 12 g kg^{-1} , EA ~ 60 kcal kg^{-1} fat free mass (FFM)), (2) reduced CHO but high fat availability (LCHF: CHO ~ 3 g kg^{-1} , EA ~ 60 kcal kg^{-1} FFM) or (3), reduced CHO and reduced energy availability (LCAL: CHO ~ 3 g kg^{-1} , EA ~ 20 kcal kg^{-1} FFM). Muscle glycogen was reduced to ~ 200 mmol kg^{-1} dw in all trials immediately post PM HIT ($P < 0.01$) and remained lower at 17 h (171, 194 and 316 mmol kg^{-1} dw) post PM HIT in LCHF and LCAL ($P < 0.001$) compared to HCHO. Exercise induced comparable p38MAPK phosphorylation ($P < 0.05$) immediately post PM HIT and similar mRNA expression (all $P < 0.05$) of PGC-1 α , p53 and CPT1 mRNA in HCHO, LCHF and LCAL. Post-exercise circulating β CTX was lower in HCHO ($P < 0.05$) compared to LCHF and LCAL whereas exercise-induced increases in IL-6 were larger in LCAL ($P < 0.05$) compared to LCHF and HCHO. In conditions where glycogen concentration is maintained within 200–350 mmol kg^{-1} dw, we conclude post-exercise CHO and energy restriction (i.e. < 24 h) does not potentiate cell signalling pathways that regulate hallmark adaptations associated with endurance training. In contrast, consuming CHO before, during and after HIT running attenuates bone resorption, effects that are independent of energy availability and circulating IL-6.

(Received 26 April 2019; accepted after revision 30 July 2019; first published online 31 July 2019)

Corresponding author J. Morton: Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Tom Reilly Building, Byrom St Campus, Liverpool L3 3AF, UK. Email: j.p.morton@ljmu.ac.uk

Introduction

The concept of deliberately promoting reduced carbohydrate (CHO) availability to enhance endurance training induced adaptations of skeletal muscle (i.e. the ‘train-low’ paradigm) is gaining increased acceptance amongst athletic populations (Bartlett *et al.* 2015; Marquet *et al.* 2016; Burke *et al.* 2018; Stellingwerff *et al.* 2019). Over the last decade, a multitude of research designs examining the train-low approach (e.g. fasted training, twice per day training, sleep low-train low etc.) have consistently demonstrated that commencing and/or recovering from a training session with reduced CHO availability activates potent cell signalling pathways that regulate many of the hallmark muscle adaptations inherent to endurance training (Yeo *et al.* 2008; Bartlett *et al.* 2013; Psilander *et al.* 2013). Accordingly, the strategic periodisation of reductions in endogenous and/or exogenous CHO availability during 3–10 weeks of training increases mitochondrial enzyme activity and protein content (Yeo *et al.* 2008; Morton *et al.* 2009; Van Proeyen *et al.* 2011), whole body (Yeo *et al.* 2008) and intramuscular (Hulston *et al.* 2010) lipid metabolism, and may improve exercise capacity (Hansen *et al.* 2005) and performance (Marquet *et al.* 2016).

The mechanisms underpinning the apparent enhanced training adaptation associated with CHO periodisation is more difficult to interpret in those experimental designs in which CHO restriction is simultaneously accompanied by energy restriction. Indeed, the performance improvements observed by Marquet *et al.* (2016) were

also associated with a 1 kg reduction in fat mass induced by the sleep-low model. Notwithstanding reductions in body mass, it is possible that many of the skeletal muscle adaptations associated with training-low are mediated by repeated and transient periods of energy restriction as opposed to CHO restriction *per se*. We recently observed that the post-exercise mRNA expression of peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α), p53, mitochondrial transcription factor A (Tfam) and peroxisome proliferator-activated receptor (PPAR) mRNA were increased with similar magnitude and time course when a low CHO and high fat diet was consumed *versus* an isoenergetic high CHO feeding strategy (Hammond *et al.* 2016). Such data conflict with previous observations from our laboratory (Bartlett *et al.* 2013) where simultaneous post-exercise CHO and calorie restriction augments the mRNA expression of many of the aforementioned genes. Given the similarities in metabolic adaptation to both CHO and calorie restriction (Meynet & Ricci, 2014), such data raise the question as to whether the acute and chronic muscle adaptations observed when training low are actually due to transient periods of calorie restriction (as mediated by a reduction in CHO intake) as opposed to CHO restriction *per se*. This point is especially relevant given that many endurance athletes present daily with transient periods of both CHO and calorie restriction due to multiple training sessions per day as well as longer term periods (i.e. weeks) of sub-optimal energy availability (Vogt *et al.* 2005; Fudge *et al.* 2006; Stellingwerff *et al.* 2019).

In addition to the potential effects of muscle glycogen concentration on skeletal muscle adaptations (Impey *et al.* 2018), it is also possible that the acute and transient within-day fluctuations in both CHO and energy availability (especially in the twice per day training model) can exert regulatory effects on other physiological systems, many of which are associated with the relative energy deficiency in sport (RED-S) syndrome (Mountjoy *et al.* 2014). Therefore, whilst train-low and/or energy restriction may be beneficial for certain aspects of training adaptation, the conscious or unconscious (as a default of the athlete's habitual training structure and daily pattern of feeding) manipulation of CHO and energy availability may have negative implications for indicators of the RED-S syndrome. As such, the acute regulation of bone turnover (i.e. alterations in resorption and formation, Sale *et al.* 2015), immune function (Nieman, 2007), endocrine function (Loucks *et al.* 1998; Hagmar *et al.* 2013) and appetite regulation (Considine *et al.* 1996) may be negatively impacted, the result of which could increase injury risk and impair athletic performance if performed chronically.

With this in mind, the aim of the present study was to examine the effects of post-exercise CHO and caloric restriction on the modulation of skeletal muscle cell signalling pathways associated with the regulation of mitochondrial biogenesis and lipid metabolism, as well as indicators of bone metabolism. We employed a repeated measures crossover design whereby male runners completed a twice per day exercise model consisting of a morning and afternoon high-intensity interval training session. Runners completed the training sessions under three dietary conditions comprising high CHO availability (HCHO) in the recovery period after both training sessions (i.e. usually considered as best practice recovery nutrition), reduced CHO but high fat availability (LCHF) or finally, reduced CHO and reduced energy intake (LCAL). In this way, our experimental design allowed us to evaluate the effects of post-exercise CHO restriction in modulating adaptive responses of muscle and bone in conditions of either sufficient or reduced energy availability. We hypothesised that post-exercise CHO restriction only modulates cell signalling, gene expression and bone metabolism when energy intake is simultaneously reduced.

Methods

Ethical approval

All participants gave written informed consent prior to participation after all experimental procedures and potential risks had been fully explained. The study was approved by the Ethics Committee of Liverpool John Moores University (Ethics No: 15 SPS 60) and conformed

to the standards set by the latest revision of the *Declaration of Helsinki* (except for registration in a database).

Participants

Nine male runners volunteered to participate in the study (mean \pm SD: age, 21 ± 1.9 years; body mass, 71.4 ± 7 kg; height, 1.78 ± 0.04 m; $\dot{V}_{O_{2peak}}$, 57 ± 5.1 ml $kg^{-1} min^{-1}$). None of the participants had any history of musculoskeletal or neurological disease, nor were they under any pharmacological treatment over the course of the testing period. Participants were instructed to refrain from any strenuous physical activity, alcohol and caffeine consumption in the 24 h prior to each experimental trial. On the basis of previous studies demonstrating that CHO availability can regulate skeletal muscle p38MAPK signalling (Cochran *et al.* 2010; mean paired differences of a 2-fold change between conditions and SD of differences of 1.5-fold), post-exercise PGC-1 α mRNA expression (Pilegaard *et al.* 2005; mean paired differences of a 3-fold change between conditions and SD of differences of 2-fold) and post-exercise AUC for circulating plasma CTx concentrations (Sale *et al.* 2015; mean paired differences of 5% changes between conditions and SD of differences of 3%), it was estimated that sample sizes of 7–9 were deemed appropriate to detect statistical significance at an alpha level of 0.05 and 80% power. Sample size estimation calculations were undertaken using Minitab software, version 19.1.

Design

In a repeated measures, randomised, cross-over design separated by 7 days, participants completed a twice per day exercise model under three different dietary conditions, consisting of either high CHO availability (HCHO) in the recovery period after both training sessions (i.e. best practice nutrition), reduced CHO but high fat availability (LCHF), or reduced CHO but reduced energy availability (LCAL). The twice per day exercise model comprised a morning (09.00–10.00 h) high-intensity interval (AM HIT) training session (8×5 min at 85% $\dot{V}_{O_{2peak}}$) followed by an afternoon (13.30–14.30 h) training session consisting of the same high-intensity interval exercise bout (PM HIT). To promote training compliance during the AM HIT protocol in all three trials, participants adhered to a standardised high CHO breakfast prior to this session. However, during the 3.5 h recovery between the HIT sessions and in the recovery period upon completion of the PM HIT exercise protocol until the subsequent morning, participants adhered to either the HCHO, LCHF, or LCAL feeding protocol. Muscle biopsies were obtained from the m. vastus lateralis muscle immediately before AM HIT, immediately after PM HIT and at 3 h and 17 h after

PM HIT. An overview of the experimental design and nutritional protocols are shown in Fig. 1.

Preliminary testing

At 7 days prior to the first main experimental trial, participants performed a combined running economy and maximal incremental running test to volitional fatigue on a motorised treadmill (h/p/Cosmos, Nussdorf-Traunstein, Germany). Both peak oxygen uptake and individual running speeds for the subsequent experimental trials were determined. Following a 10 min warm up at a self-selected treadmill speed, the running economy test commenced, beginning with a 3 min stage at a treadmill speed of 10 km h⁻¹. Running speed was then increased by 1 km h⁻¹ every 3 min thereafter until participants reached > 90% of their age predicted maximal heart rate. Participants were then given a 5 min rest period before commencing the maximal incremental test. This test began with a 2 min stage at a treadmill speed 2 km h⁻¹ slower than the final speed of the running economy test. Running speed was then increased by 2 km h⁻¹ every 2 min until a speed of 16 km h⁻¹ was reached, after which the treadmill inclined by 2% every 2 min until volitional exhaustion. $\dot{V}_{O_{2peak}}$ was defined as the highest \dot{V}_{O_2} value obtained during any 10 s period and was stated as being achieved by two of the following criteria: (1) heart rate was within 10 beats min⁻¹ (b.min⁻¹) of age-predicted maximum, (2) respiratory exchange ratio > 1.1, and (3) plateau of oxygen consumption despite increased workload. These measurements were recorded via breath-by-breath gas analysis obtained continuously throughout both tests using a CPX Ultima series online

gas analysis system (Medgraphics, MN, USA), with heart rate (Polar, Kempele, Finland) also recorded continuously during exercise.

Experimental protocols

AM HIT protocol. In the 24 h preceding each main experimental trial participants consumed a standardised high CHO diet in accordance with typical nutritional recommendations (Thomas *et al.* 2016) for endurance athletes (8 g kg⁻¹ CHO, 2 g kg⁻¹ protein, and 1 g kg⁻¹ fat) performing between 1–3 h of exercise per day. On the morning of each experimental trial participants reported to the laboratory between 7 and 7.15 am where a venous blood sample was collected from an antecubital vein in the anterior crease of the forearm. Participants were then given a standardised high-CHO breakfast (2 g kg⁻¹ CHO, 0.3 g kg⁻¹ protein, and 0.1 g kg⁻¹ fat; comprising breakfast cereal milk, orange juice, bread and jam), and 2 h after the meal a muscle biopsy sample was obtained from the vastus lateralis muscle. Participants were then fitted with a heart rate monitor and nude body mass (SECA, Hamburg, Germany) was recorded before commencing the first high intensity interval running (AM HIT) protocol which lasted ~1 h. The HIT protocol consisted of 8 × 5 min bouts running at a velocity corresponding to 85% $\dot{V}_{O_{2peak}}$ interspersed with 1 min of recovery at walking pace. The intermittent protocol started and finished with a 10 min warm up and cool down at a velocity corresponding to 50% $\dot{V}_{O_{2peak}}$, and a further venous blood sample was obtained immediately upon completion of the protocol. Water was consumed *ad libitum* throughout the duration of exercise with the pattern of intake recorded and

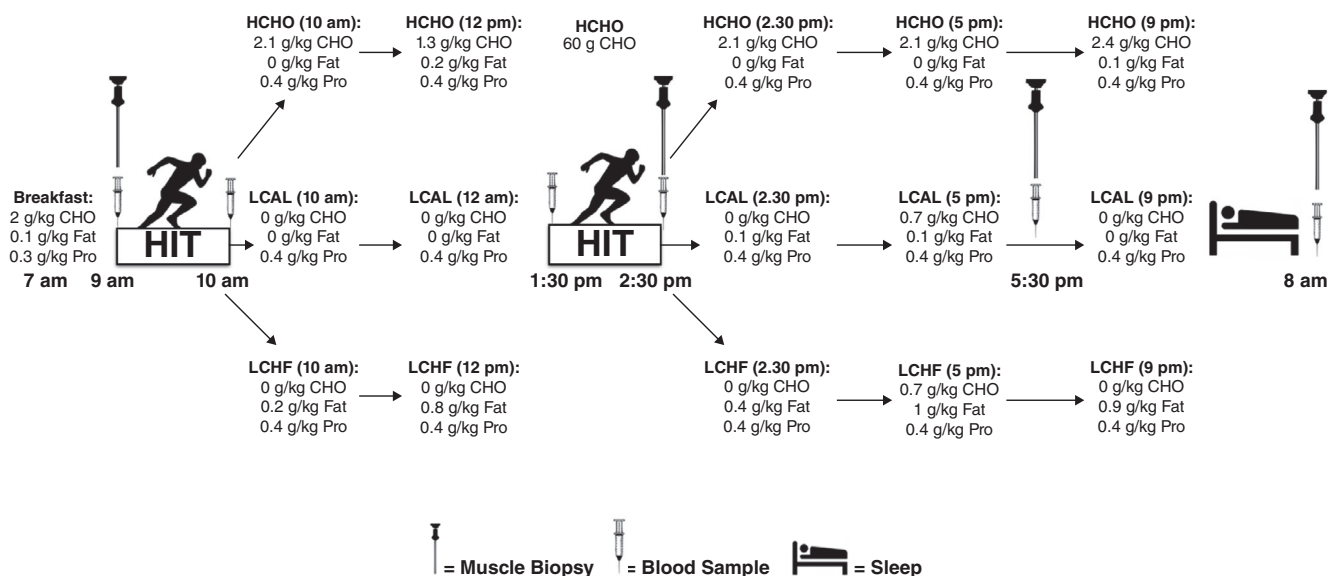


Figure 1. Overview of the experimental protocol employed in each trial

HIT = 8 × 5 min running at a workload equal to 85% $\dot{V}_{O_{2peak}}$ interspersed by 1 min recovery.

replicated for the subsequent experimental trials. Heart rate was measured continuously during exercise (Polar) and ratings of perceived exertion (RPE) (Borg, 1973) were obtained upon completion of each HIT bout. In order to determine substrate utilisation and energy expenditure during exercise (Jeukendrup & Wallis, 2005), expired gas was collected via a mouthpiece connected to an online gas analysis system (CPX Ultima, Medgraphics, MN, USA) for the final 2 min of each 5 min interval.

PM HIT protocol. During the 3.5 h recovery period between the AM HIT and PM HIT protocols, participants consumed either the HCHO (3.5 g kg⁻¹ CHO, 0.8 g kg⁻¹ protein, 0.2 g kg⁻¹ fat), LCHF (0 g kg⁻¹ CHO, 0.8 g kg⁻¹ protein, 1.2 g kg⁻¹ fat), or LCAL (0 g kg⁻¹ CHO, 0.8 g kg⁻¹ protein, 0.1 g kg⁻¹ fat) feeding protocols (the pattern and frequency of feeding is shown in Fig. 1). Following the recovery period, another venous blood sample was obtained immediately prior to commencing the afternoon HIT exercise protocol. After a 10 min warm up at a velocity corresponding to 50% $\dot{V}_{O_{2,peak}}$, participants subsequently commenced the PM HIT protocol. During exercise, participants also consumed 60 g h⁻¹ of CHO (SiS GO Isotonic Gels, Science in Sport, Blackburn, UK) in HCHO whereas no energy was consumed in the LCHF or LCAL trials. Water was consumed *ad libitum* throughout the duration of exercise with the pattern of intake recorded and replicated for the subsequent experimental trial. Expired gases were collected for the last 2 min of each 5 min interval throughout the exercise trial (CPX Ultima, Medgraphics) and substrate utilisation and energy expenditure was determined according to the method of Jeukendrup & Wallis (2005). Heart rate was measured continuously during exercise (Polar) and RPE (Borg, 1973) were obtained at the end of each HIT bout. Upon completion of the PM HIT protocol until sleep, participants consumed either the HCHO (6.4 g kg⁻¹ CHO, 1.3 g kg⁻¹ protein, 0.3 g kg⁻¹ fat), LCHF (0.8 g kg⁻¹ CHO, 1.3 g kg⁻¹ protein, 2.3 g kg⁻¹ fat), or LCAL (0.8 g kg⁻¹ CHO, 1.3 g kg⁻¹ protein, 0.1 g kg⁻¹ fat) feeding protocols where the pattern and frequency of feeding is shown in Fig. 1. Vastus lateralis muscle biopsies and venous blood samples were also collected immediately after and at 3 h and 17 h after completion (i.e. 08.00 h and in a fasted state) of the PM HIT exercise protocol. The total energy and estimated calcium intake across the whole trial period in HCHO was: ~12.6 g kg⁻¹ CHO, ~2.3 g kg⁻¹ protein, ~0.6 g kg⁻¹ fat, 1051 mg calcium, in LCHF was: ~3 g kg⁻¹ CHO, ~2.3 g kg⁻¹ protein, ~3 g kg⁻¹ fat, 1780 mg calcium (where both trials were matched for total energy intake), and in LCAL was ~3 g kg⁻¹ CHO, ~2.3 g kg⁻¹ protein, ~0.4 g kg⁻¹ fat, 865 mg calcium. Typical energy sources in the HCHO trial consisted of rice, bananas, sports drinks (e.g. Lucozade Original, UK; GO Energy and REGO, Science in Sport, UK), sports gels (GO Isotonic Energy

Gels, Science in Sport, UK) and confectionary products (e.g. Jaffa Cakes, McVities, UK) whereas increased dietary fat intake in the LCHF trial was reflective of increased intake of oily fish, avocado, butter, coconut oil, walnuts and cheese. In the LCAL trial, CHO intakes were limited to smaller absolute portions of rice and none of the aforementioned sources of dietary fat were consumed.

Energy availability

Energy availability was estimated for each experimental trial as calculated from: energy intake minus energy expenditure during AM HIT and PM HIT exercise/fat free mass (FFM) (Mountjoy *et al.* 2014). Whilst FFM was not directly measured, we based estimates of FFM on the assumption that all participants were ~15% body fat (as based on unpublished data from our laboratory examination of male participants of similar body mass, training history and maximal oxygen uptake, S. G. Impey *et al.*). The total energy expenditure from both AM HIT and PM HIT in HCHO was 1474 ± 107 kcal, in LCHF it was 1429 ± 142 kcal and in LCAL it was 1455 ± 190 kcal. Estimated energy availability across the whole trial period was 58 ± 5.3 kcal kg⁻¹ FFM in HCHO, 59 ± 6.4 kcal kg⁻¹ FFM in LCHF, and 20 ± 4.1 kcal kg⁻¹ FFM in LCAL.

Blood sampling and analysis

Venous blood samples were collected into vacutainers containing EDTA or lithium heparin and stored on ice until centrifugation at 1500 g for 15 min at 4°C. Following centrifugation, aliquots of plasma were stored in a freezer at -80°C for subsequent analysis. Samples were later analysed for plasma glucose, lactate, non-esterified fatty acids (NEFA), glycerol, and β -hydroxybutyrate using commercially available enzymatic spectrophotometric assays (RX Daytona Analyser, Randox, Co. Antrim, UK) as per the manufacturers' instructions. Samples were also analysed using commercially available solid phase enzyme-linked immunosorbent assays (ELISA) for insulin (DIAsource, Belgium), leptin (R&D systems, MN, USA) ghrelin (Invitrogen, Thermo Fisher Scientific, UK) and adiponectin (Abcam, Cambridge, UK) as per the manufacturer's instructions. Plasma β CTX and P1NP were measured by ECLIA on a fully automated COBAS e601 system (Roche Diagnostics, Mannheim, Germany). These markers were selected for use as they are the preferred markers of use as reference analytes for bone turnover markers in clinical studies (Vasikaran *et al.* 2011). The inter-assay CV for β CTX was ≤3% between 0.2 and 1.5 μ g l⁻¹, with a sensitivity of 0.01 μ g l⁻¹, and the inter-assay CV for P1NP was ≤3% between 20 and 600 μ g l⁻¹, with a sensitivity of 8 μ g l⁻¹. The assays used a sandwich test principle involving two incubations. Flow cytometric detection of IL-6 was

performed using a dual-laser FACSCalibur flow cytometer (BD biosciences, USA) calibrated for 2-colour analysis. Cytokine concentrations were measured using a cytometric bead array (CBA, BD Biosciences, San Diego, CA, USA) according to the manufacturer's instructions. Bead populations with distinct fluorescence intensities coated with phycoerythrin (PE)-conjugated capture antibodies specific for IL-6 were used. Following acquisition of sample data using the flow cytometer, the sample results were generated in graphical and tabular format using the BDA CBA Analysis Software.

Muscle biopsies

Muscle biopsy samples (~60 mg) were obtained from the lateral portion of the vastus lateralis muscle using a Bard Monopty Disposable Core Biopsy Instrument 12 gauge \times 10 cm length, (Bard Biopsy Systems, Tempe, AZ, USA). Consecutive biopsies were taken from alternate legs. Samples collected from the same limb were obtained from separate incision sites 2–3 cm apart under local anaesthesia (0.5% Marcaine) and immediately frozen in liquid nitrogen and stored at -80°C for later analysis.

Analysis of muscle glycogen

Muscle glycogen concentration was determined according to the methods described by Van Loon *et al* (2000). Approximately 3–5 mg of freeze-dried muscle was powdered and all visible blood and connective tissue removed. The freeze-dried sample was then hydrolysed by incubation in 500 μl of 1 M HCl for 3 h at 100°C . After cooling to room temperature for ~20 min, samples were neutralized by the addition of 250 μl 0.12 mol l^{-1} Tris/ 2.1 mol l^{-1} KOH saturated with KCl. Following centrifugation at 1500 RCF for 10 min at 4°C , 200 μl of the supernatant was analysed in duplicate for glucose concentration according to the hexokinase method using a commercially available kit (GLUC-HK, Randox Laboratories, Antrim, UK). Glycogen concentration is expressed as mmol kg^{-1} dry weight.

Analysis of phosphorylated p38 MAPK^{T180/Y182} phosphorylation

Flow cytometric detection of phosphoproteins was performed using a dual-laser BD C6 accuri flow cytometer (BD Biosciences). Phospho-p38 concentrations were measured using a cytometric bead array (CBA, BD Biosciences) according to the manufacturer's instructions. Bead populations with distinct fluorescence intensities coated with phycoerythrin (PE)-conjugated capture antibodies specific for phospho-p38 were used. Following acquisition of sample data using the flow cytometer, the sample results were generated in graphical and

tabular format using the BDA CBA Analysis Software (Hungary Software Ltd, for BD Biosciences) where median values were used. Briefly, muscle samples (~20 mg) were homogenised in 200 μl of denaturation buffer (BD biosciences, UK) in beaded tubes (Roche, UK) using a Roche MagnaLyser instrument. Samples were homogenised for 45 s at 6000 rpm \times 3 with 5 min on ice between runs. Protein concentrations were determined using a BCA protein assay, and samples added to the assay diluent provided (100 $\mu\text{g}/\text{sample}$). Standards were prepared by serial dilution of a stock protein in the BD CBA Cell Signalling Flex Set. All samples and standards were incubated with the capture beads conjugated to the p38MAPK^{T180/Y182} antibody for 3 h then with the phycoerythrin (PE) detection reagent for 1 h. Samples were then washed using the wash buffer provided and centrifuged at 300 g for 5 min before supernatant was removed and 300 μl of wash buffer was added for re-suspension of the capture beads. All samples were analysed on the flow cytometer with 300 events captured per sample.

RNA isolation and analysis

Muscle biopsy samples (~20 mg) were homogenised in 1 ml TRIzol reagent (Thermo Fisher Scientific, UK) in beaded tubes (Roche, UK) using a Roche MagnaLyser instrument. Samples were homogenized for 45 s at 6000 rpm \times 3 with 5 min on ice between runs. Total RNA was then isolated according to manufacturer's guidelines. High quality RNA was assessed by UV spectroscopy at ODs of 260 and 280 nm (mean \pm SD was 1.99 ± 0.05 for 260/280 nm ratios) using a Nanodrop 3000 (Fisher, Roskilde, Denmark), and 70 ng RNA was then used for each PCR reaction. Samples were analysed in duplicate.

Primers

Identification of primer sequences was enabled by Gene (NCBI, <http://www.ncbi.nlm.nih.gov/gene>) and primers designed using Primer-BLAST (NCBI, <http://www.ncbi.nlm.nih.gov/tools/primer-blast>). Specificity was ensured using sequence homology searches so the primers only matched the experimental gene with no unintended targets identified for primer sequences. In order to prevent amplification of gDNA, primers were ideally designed to yield products spanning exon-exon boundaries. Three or more GC bases in the last 5 bases at the 3' end, and secondary structure interactions (hairpins, self-dimer and cross dimer) within the primers were avoided so there would be no non-specific amplification. All primers were between 16 and 25 bp, and amplified a product between 141 and 244 bp. All primers were purchased from Sigma (Suffolk, UK) and sequences for each gene are shown in Table 1 for: peroxisome proliferator-activated γ receptor

Table 1. Primer sequences

Gene	Forward sequence	Reverse sequence	Product length (bp)
PGC-1 α	TGCTAAACGACTCCGAGAA	TGCAAAGTTCCTCTCTGCT	67
p53	ACCTATGGAAACTACTTCCTGAAA	CTGGCATTCTGGGAGCTTCA	141
Tfam	TGGCAAGTTGTCCAAAGAAACCTGT	GTTCCCTCCAACGCTGGGCA	135
PDK4	TGGTCCAAGATGCCTTTGAGT	GTTGCCCGCATTGCATTCTT	181
CPT1	GACAATACCTCGGAGCCTCA	AATAGGCCTGACGACACCTG	94
FAT/CD36	AGGACTTTCCTGCAGAATACCA	ACAAGCTCTGTTCTTATTCACA	199
SIRT1	CGGAAACAATACCTCCACCT	CACATGAAACAGACACCCCA	186
Parkin	TCCCAGTGGAGGTCGATTCT	GGAACCCCTGTCGCTTAG	73
GAPDH (NM.002046.5)	AAGACCTTGGGCTGGGACTG	TGGCTCGGCTGGCGAC	168

coactivator (PGC-1), tumour suppressor protein (p53), mitochondrial transcription factor A (Tfam), pyruvate dehydrogenase kinase isozyme 4 (PDK4), carnitine palmitoyltransferase (CPT1), fatty acid translocase (FAT/CD36), Sirtuin 1 (SIRT1), Parkin, and GAPDH.

Reverse transcriptase quantitative real-time polymerase chain reaction (rt-qRT-PCR)

Rt-qRT-PCR amplifications were performed using a QuantiFast SYBR Green RT-PCR one step kit on a Rotogene 3000Q (Qiagen, Crawley, UK) supported by Rotogene software (Hercules, CA, USA). The following rt-qTR-PCR cycling parameters were used: hold 50°C for 10 min (reverse transcription/cDNA synthesis), initial denaturation and transcriptase inactivation at 95°C for 5 min, followed by PCR steps: 40 cycles of denaturation at 95°C for 10 s, and annealing/extension at 60°C for 30 s. Upon completion, dissociation/melting curve analysis were performed to reveal and exclude non-specific amplification or primer-dimer issues (all melt analysis presented single reproducible peaks for each target gene suggesting amplification of a single product). Changes in mRNA content were calculated using the comparative $C_T(\Delta\Delta C_T)$ equation (Schmittgen & Livak, 2008) where relative mRNA expression was calculated as $2^{-\Delta\Delta C_T}$ and where C_T represents the threshold cycle. GAPDH was used as a reference gene and did not change significantly between groups or time points studied ($C_T = 24.2 \pm 1$), therefore a pooled reference gene C_T was used in the relative mRNA expression equation above. Furthermore, to enable calculation of mRNA expression values immediately after and 3 h after exercise, the calibrator condition in the $\Delta\Delta C_T$ equation was assigned to the pre-exercise condition.

Statistical analysis. All data were analysed using Statistical Package for the Social Scientist (SPSS version 21, IBM, USA). Metabolic responses (i.e. blood metabolites, muscle glycogen, kinase activity, mRNA data), physiological and perceptual responses (i.e. HR,

RPE and oxidation rates) were analysed using a two-way repeated-measures general linear model, where the within factors were time and condition. *Post hoc* LSD tests were used where significant main effects and interactions were observed in order to locate specific differences between time points and conditions. All data in text, figures and tables are presented as means \pm SD, with P values ≤ 0.05 indicating statistical significance.

Results

Physiological responses and substrate utilisation during exercise

Comparisons of subject's heart rate, RPE and substrate oxidation during both the AM HIT and PM HIT protocols are displayed in Fig. 2. Heart rate, RPE and lipid oxidation (all $P < 0.001$) progressively increased during both AM HIT and PM HIT exercise whereas CHO oxidation progressively decreased ($P < 0.001$) during both exercise protocols (see Fig. 2). In accordance with identical pre-exercise feeding in all trials prior to AM HIT, no significant differences were apparent in any of the aforementioned variables between HCHO, LCHF and LCAL (all $P > 0.05$). In contrast, CHO oxidation was significantly greater during PM HIT in HCHO compared to LCHF ($P = 0.001$) and LCAL ($P = 0.004$) whereas lipid oxidation was significantly greater during LCHF ($P = 0.014$) and LCAL ($P = 0.011$) compared to HCHO. No differences were observed in either CHO ($P = 0.595$) or lipid ($P = 0.403$) oxidation between LCAL and LCHF during PM HIT.

Plasma metabolite responses

Plasma glucose, lactate, NEFA, glycerol and β -hydroxybutyrate all displayed significant changes (all $P < 0.001$) over the sampling period (Fig. 3). However, in accordance with the provision of CHO in the HCHO trail, plasma glucose was significantly higher compared to LCHF ($P < 0.001$) and LCAL ($P = 0.003$)

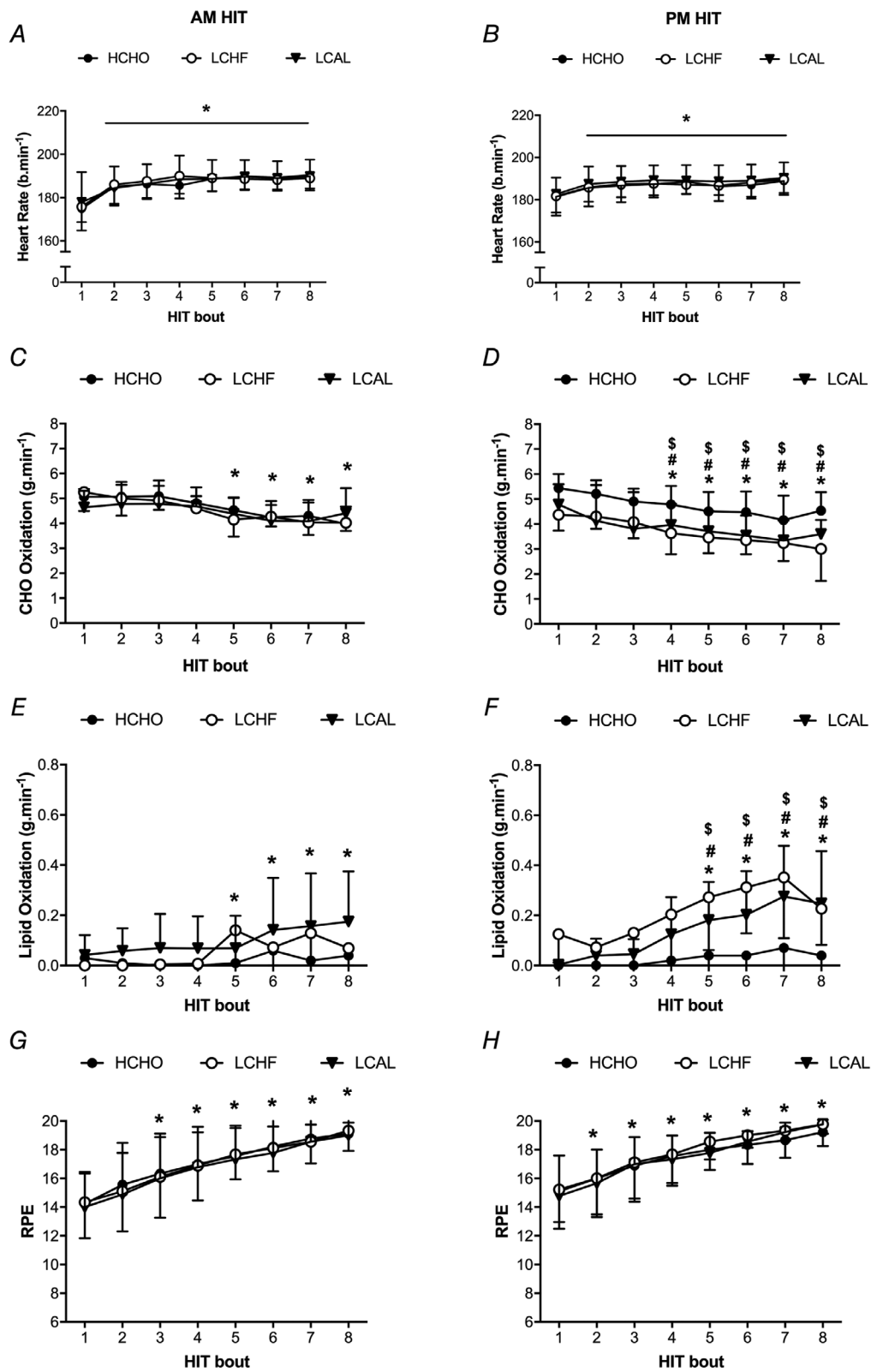


Figure 2. Heart rate, CHO oxidation, lipid oxidation and RPE responses during each HIT bout in the HCHO, LCHF and LCAL trials

A and B, heart rate; C and D, CHO oxidation; E and F, lipid oxidation; G and H, RPE. *Significant difference from HIT bout 1 ($P < 0.05$); #significant difference between HCHO and LCHF ($P < 0.05$); \$significant difference between HCHO and LCAL ($P < 0.05$).

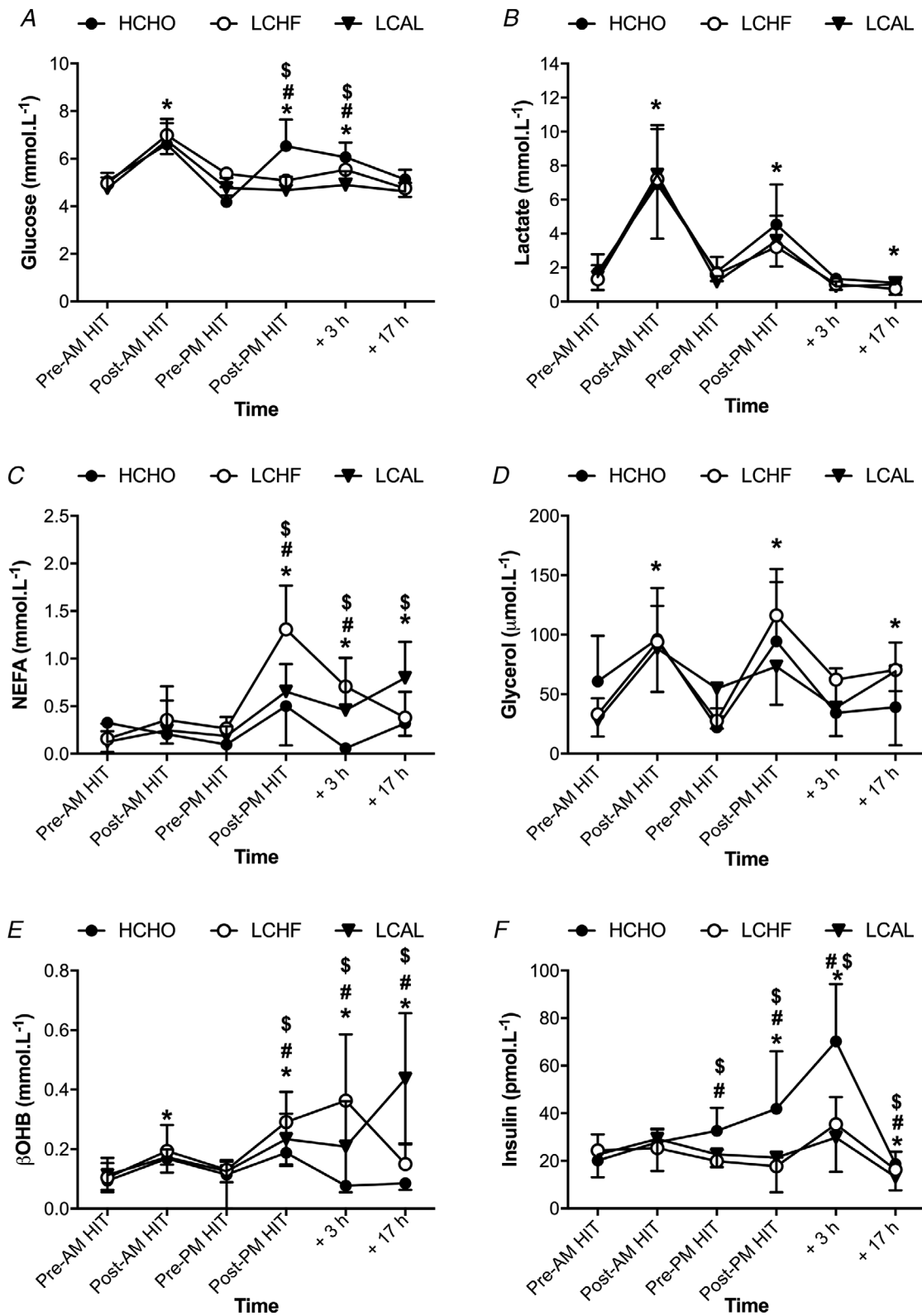


Figure 3. Plasma glucose, lactate, NEFA, glycerol, β-OHB and insulin before and after the AM HIT and PM HIT protocols

A, plasma glucose; B, lactate; C, NEFA; D, glycerol; E, β-OHB; F, insulin. *Significant difference from pre-exercise ($P < 0.05$); #significant difference between HCHO and LCHF ($P < 0.05$); \$significant difference between HCHO and LCAL ($P < 0.05$).

whereas restricting CHO and energy intake post-exercise induced significantly greater plasma NEFA and β -OHB in LCHF ($P = 0.001$ and 0.001) and LCAL ($P = 0.007$ and 0.001) compared to the HCHO trial. There was a tendency for higher circulating NEFA in LCHF compared to LCAL ($P = 0.06$) and β -OHB was also different ($P = 0.024$) between LCHF and LCAL. Plasma glycerol significantly increased in response to both AM HIT ($P = 0.001$) and PM HIT ($P < 0.001$), although this response was not different between trials ($P = 0.362$). Similarly, plasma lactate concentrations were significantly increased in response to AM HIT ($P < 0.001$) and PM HIT ($P = 0.004$), with no differences between feeding conditions ($P = 0.383$). Consistent with CHO feeding before and after PM HIT, plasma insulin concentration was significantly higher (all $P < 0.01$) immediately, 3 h and 17 h post exercise in HCHO compared to LCHF and LCAL (Fig. 3). Additionally, insulin concentration significantly changed over time ($P < 0.001$) such that significant differences from before AM HIT were evident immediately ($P < 0.001$) and 3 h after completion of the PM HIT session ($P = 0.024$).

Muscle glycogen

Exercise significantly decreased ($P < 0.01$) muscle glycogen immediately after PM HIT, though no differences were apparent between HCHO, LCHF and LCAL at this time point (see Fig. 4). However, in accordance with the provision of CHO after the PM HIT exercise protocol in HCHO, muscle glycogen re-synthesis was observed such that glycogen concentration was higher in HCHO compared to both LCHF ($P = 0.028$) and LCAL ($P = 0.002$) at 3 h and 17 h post exercise.

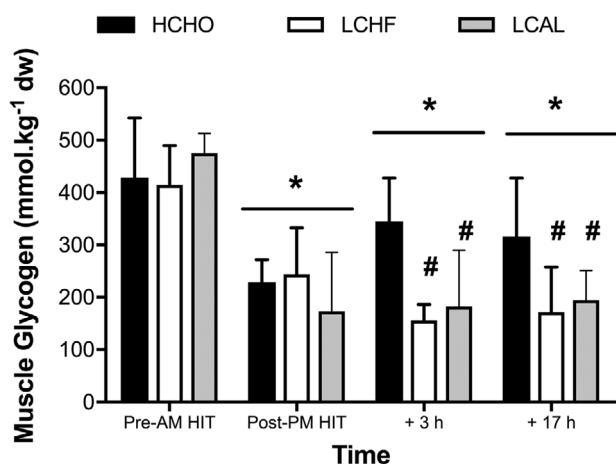


Figure 4. Skeletal muscle glycogen concentration before the AM HIT and after the PM HIT exercise protocols

*Significant difference from pre-exercise ($P < 0.05$); #significant difference from HCHO ($P < 0.05$).

p38MAPK^{T180/Y182} phosphorylation

Exercise induced significant increases in p38MAPK phosphorylation immediately post PM HIT ($P = 0.037$) though no differences ($P = 0.755$) were observed between trials (Fig. 5). At 17 h post PM HIT, p38MAPK phosphorylation had returned to basal levels in all three experimental trials.

Gene expression

Exercise increased the expression of PGC-1 α ($P < 0.001$), p53 ($P = 0.01$), and CPT1 mRNA ($P < 0.001$) although there were no differences (all $P > 0.05$) between trials (Fig. 6). In contrast, the exercise-induced increase ($P = 0.001$) in PDK4 mRNA was greater in LCHF ($P = 0.002$) and LCAL ($P = 0.034$) than in HCHO at 3 h post exercise (Fig. 6). Similarly, mRNA expression of SIRT1 was significantly greater in LCAL compared to HCHO ($P = 0.013$) at 17 h post exercise (Fig. 6). In contrast, neither exercise nor dietary condition significantly affected the mRNA expression of CD36 ($P = 0.901$ and $P = 0.206$, respectively), Parkin ($P = 0.25$ and 0.74 , respectively) or Tfam ($P = 0.17$ and $P = 0.38$, respectively).

Bone metabolism

Exercise significantly increased P1NP ($P < 0.01$) following both AM HIT ($P = 0.011$) and PM HIT ($P = 0.005$), with concentrations subsequently decreasing at 3 h ($P = 0.041$) and 17 h post exercise ($P = 0.002$) (Fig. 7). No differences were apparent between trials ($P = 0.633$). Exercise significantly reduced β CTX concentration following AM HIT ($P < 0.001$) whilst PM HIT significantly increased β CTX ($P < 0.001$) (Fig. 6). β CTX concentration was

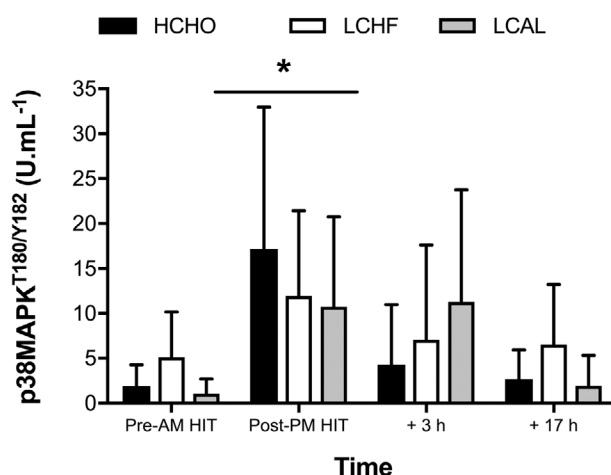


Figure 5. Skeletal muscle phosphorylated p38MAPK^{T180/Y182} before the AM HIT and after the PM HIT exercise protocols

*Significant difference from pre-exercise ($P < 0.05$).

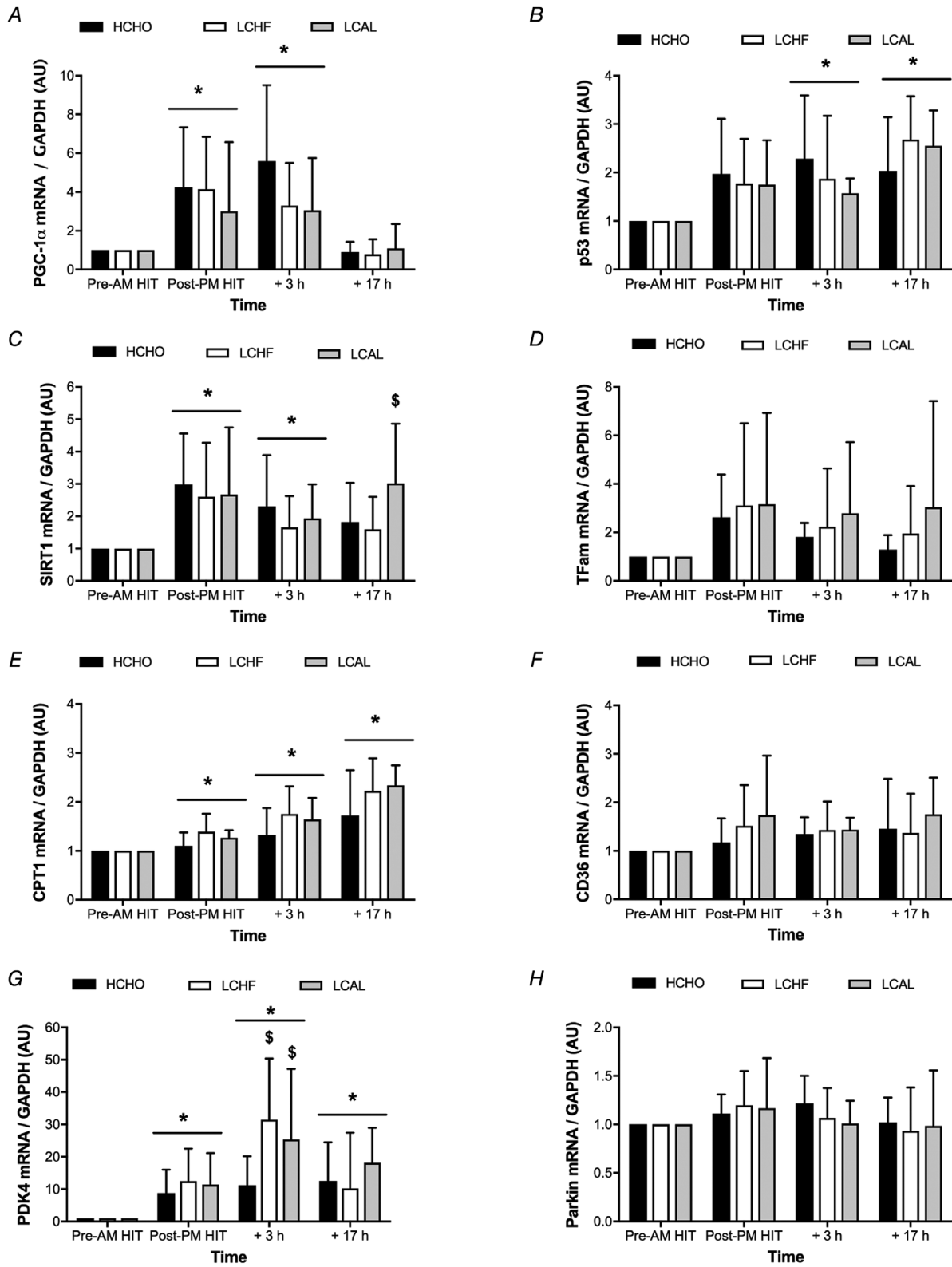


Figure 6. PGC-1 α , p53, SIRT1, Tfam, CPT1, CD36, PDK4 and Parkin mRNA before AM HIT exercise and after PM HIT exercise
 A, PGC-1 α ; B, p53; C, SIRT1; D, Tfam; E, CPT1; F, CD36; G, PDK4; H, Parkin. *Significant difference from pre-exercise ($P < 0.05$); #significant difference from HCHO and LCHF ($P < 0.05$); \$significant difference from HCHO ($P < 0.05$).

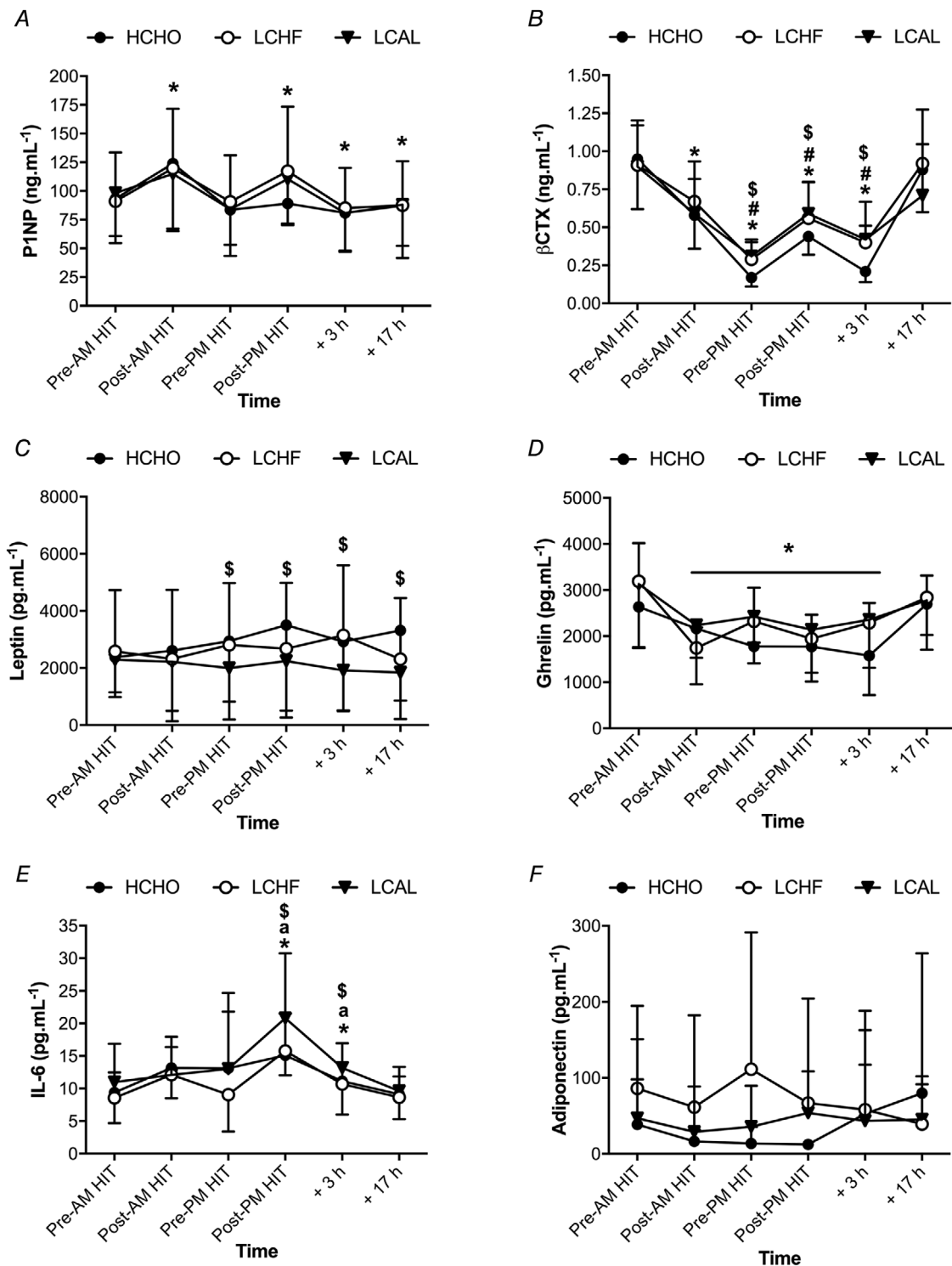


Figure 7. Plasma P1NP, β CTX, leptin, ghrelin, IL-6 and adiponectin before and after the AM HIT and PM HIT protocols

A, P1NP; B, β CTX; C, leptin; D, ghrelin; E, IL-6; F, adiponectin. *Significant difference from pre-exercise ($P < 0.05$); #significant difference between HCHO and LCHF ($P < 0.05$); \$significant difference between HCHO and LCAL ($P < 0.05$); a significant difference between LCHF and LCAL ($P < 0.05$).

significantly lower immediately before and immediately and 3 h after PM HIT in HCHO compared to LCHF ($P = 0.032$) and LCAL ($P = 0.035$). No differences were apparent in the response of β CTX between the LCHF and LCAL trials ($P = 0.178$).

Appetite hormones

Whilst exercise did not affect circulating leptin concentration ($P = 0.72$), leptin was significantly lower ($P = 0.041$) in LCAL compared to HCHO in the post-exercise sampling period (Fig. 7). There was a tendency for differences in leptin concentration between HCHO and LCHF ($P = 0.057$) though no differences were apparent between LCAL and LCHF ($P = 0.27$). Exercise significantly reduced ghrelin concentration to comparable levels in all three trials following both AM HIT ($P = 0.001$) and PM HIT ($P = 0.025$), with no significant differences between conditions ($P = 0.408$) (Fig. 7). Ghrelin concentration returned to baseline levels at 17 h post PM HIT in all conditions ($P = 0.392$). Neither exercise ($P = 0.524$) nor dietary condition ($P = 0.156$) significantly affected adiponectin concentration throughout the sampling period (Fig. 7).

Interleukin-6 (IL-6) responses

Exercise significantly increased IL-6 concentration immediately post PM HIT ($P = 0.016$) and at 3 h post PM HIT ($P = 0.009$), with the concentration being higher in LCAL compared to LCHF ($P = 0.021$) and HCHO ($P = 0.016$) (Fig. 7). There were no differences in post-exercise elevations in IL-6 concentration between LCHF and HCHO ($P = 0.145$). Plasma IL-6 concentration returned to baseline levels at 17 h post-PM HIT in all three experimental trials.

Discussion

In contrast to our hypothesis, we provide novel data by demonstrating that simultaneous post-exercise CHO and energy restriction did not augment the acute cell signalling (i.e. p38MAPK-PGC-1 α pathway) that is associated with mitochondrial biogenesis and lipid metabolism, as compared to conditions of high energy availability. We also report for the first time that consuming CHO before, during and after HIT running attenuates circulating β CTX concentrations in the hours after exercise, effects that are independent of energy availability. When taken together, our data suggest that in recovery conditions where muscle glycogen concentration remains within the range of 200–350 mmol kg⁻¹ dw, acute post-exercise CHO and energy restriction (i.e. < 24 h) does not potentiate the activation of key cell signalling responses that are

associated with the regulation of hallmark adaptations to endurance training. Nonetheless, the acute within-day fluctuation in CHO availability that is inherent in twice per day training protocols modulates bone resorption, though the chronic implications of these acute alterations remain to be determined.

Similar to the original train-low investigations (Hansen *et al.* 2005; Yeo *et al.* 2008; Morton *et al.* 2009), we employed a twice per day exercise protocol (considered representative of real-world practices often adopted by elite endurance athletes), whereby multiple training sessions are completed on the same day with limited recovery time between sessions (Fudge *et al.* 2006). When subjects adhered to a high CHO diet during the day prior to all three experimental trials and consumed a standardised high CHO breakfast before the trials, we observed no differences in cardiovascular strain, RPE, substrate availability or substrate oxidation during the AM HIT session. In accordance with the provision of CHO intake between training sessions during the HCHO trial, we subsequently observed higher rates of CHO oxidation during PM HIT compared to both the LCHF and LCAL trials, probably reflective of the higher pre-exercise muscle glycogen availability and the provision of exogenous CHO during exercise (Bartlett *et al.* 2013). Nonetheless, despite higher circulating NEFA levels in LCHF compared to the LCAL trial, we observed comparable lipid oxidation rates between the LCHF and LCAL trials, both of which were significantly higher than HCHO. These data are in agreement with classical studies demonstrating that in high-intensity exercise conditions, elevations in circulating free fatty acid (FFA) availability (i.e. >1 mmol l⁻¹) does not readily translate to increased rates of whole body lipid oxidation (Romijn *et al.* 1995), owing to limitations in FFA uptake across the mitochondrial membrane during conditions of high glycolytic flux (Sidossis *et al.* 1997).

It is, of course, difficult to draw accurate conclusions on rates of lipolysis based on measurements of circulating glycerol and NEFA *per se*. Nonetheless, on the basis of statistical differences in plasma NEFA concentrations between trials immediately post PM HIT and consistent with the well-documented effects of CHO feeding (Horowitz *et al.* 1997), it is assumed that lipolysis was suppressed in the HCHO trial compared to both the LCAL and LCHF trials. The fact that plasma glycerol did not display statistical differences between trials may also be potentially reflective of greater glycerol uptake by the liver in both the LCAL and LCHF trials in order to provide a gluconeogenic substrate to maintain plasma glucose concentration (Ahlborg *et al.* 1974; Jensen *et al.* 2001). Interestingly, the return of β -OHB towards basal levels at +17 h in the LCHF trial is consistent with our previously reported data utilising an almost identical dietary and exercise protocol (Hammond *et al.* 2016), whilst the

progressive increase in β -OHB at this time point in the LCAL trial is consistent with the well-documented effects of post-exercise calorie restriction on ketosis (Impey *et al.* 2016). In relation to the former, it is possible that the provision of dietary fat intake in the evening meal (i.e. 21.00 h) may result in an increase in liver uptake of NEFA in an attempt to preferentially stimulate gluconeogenesis (Chen *et al.* 1999), the result of which may actually suppress ketogenesis. We acknowledge, however, that further studies are now required to directly test this hypothesis.

In relation to upstream skeletal muscle cell signalling, the phosphorylation of p38MAPK^{T180/Y182} immediately post PM HIT was comparable between all trials. Despite the potentially increased pre-exercise muscle glycogen availability and utilisation during HCHO (owing to the consumption of CHO between sessions), such a comparable signalling response is not unexpected given that the absolute post-exercise glycogen concentration (i.e. approximately 200 mmol kg⁻¹ dw) was similar between trials. In accordance with comparable upstream signalling (Wright *et al.* 2007), we subsequently observed similar magnitudes of mRNA expression of PGC-1 α , p53 and CPT1 in the first 3 h of recovery from PM HIT. Furthermore, despite the sustained CHO and energy restriction for the subsequent 12 h recovery period, we observed that neither energy nor CHO availability had any further modulatory effect on mRNA expression (with the exception of both PDK4 and SIRT1). Such data contrast with those of Pilegaard *et al.* (2005), who observed that 8 h of post-exercise CHO restriction (0.5 g kg⁻¹ of CHO) enhanced PGC-1 α , CPT1 and CD36 mRNA compared to recovery conditions where 5 g kg⁻¹ of CHO was consumed in the initial 8 h post-exercise period. Such discrepancies between studies may be explained by the absolute glycogen concentrations achieved by the chosen feeding protocol. Indeed, whereas we achieved absolute muscle glycogen concentrations of approximately 350 (in the HCHO trial) *versus* 200 mmol kg⁻¹ dw (in the LCHF and LCAL trials) at 17 h post-completion of the PM HIT session, the feeding protocol adopted by Pilegaard *et al.* (2005) achieved glycogen concentrations of 550 *versus* 350 mmol kg⁻¹ dw in their conditions of high and low CHO availability, respectively. When considered this way, our data may lend further support to the glycogen threshold hypothesis (Impey *et al.* 2018), which proposes that the acute cell signalling (Gejl *et al.* 2017) and chronic training adaptations (Hansen *et al.* 2005; Yeo *et al.* 2008; Morton *et al.* 2009) associated with 'train-low' protocols typically only present when absolute glycogen concentration is depleted to <300 mmol kg⁻¹ dw. As such, it is possible that the acute manipulation of both CHO and energy availability in relation to the exercise protocols and participant training status in our study were probably not sufficient to elicit post-exercise metabolic

conditions that could be considered indicative of 'true' train-low conditions. Indeed, in a previous study from our laboratory where we utilised a sleep-low manipulation of muscle glycogen (achieved via evening glycogen depleting exercise followed by overnight CHO and energy restriction), we observed that differences in muscle glycogen availability of 400 *versus* 100 mmol kg⁻¹ dw induced marked differences in expression of PGC-1 α , CPT1 and Tfam mRNA (Bartlett *et al.* 2013). Future studies should therefore adopt experimental designs and feeding protocols that more readily achieve distinct differences in muscle glycogen availability.

Exercise training is largely considered anabolic to bone, as mediated by the mechanical signalling pathways inherent to the pattern of loading (Ozicvici *et al.* 2010; Thompson *et al.* 2012; Pagnotti *et al.* 2019). Nonetheless, the extent of increased bone formation and decreased bone resorption that may occur in response to exercise training (Fujimura *et al.* 1997) is, of course, dependent on genetics, age, exercise modality (i.e. loading pattern) and energy availability. In this way, bone mineral density (BMD) may differ markedly amongst elite athletic populations such as soccer players, swimmers, cyclists, runners and jockeys (Fredericson *et al.* 2007; Scofield & Hecht, 2012; Olmedillas *et al.* 2012; Gomez-Bruton *et al.* 2013; Wilson *et al.* 2018). In relation to the acute markers of bone metabolism studied here, we observed comparable responses of P1NP and β CTX following the AM HIT training session, probably due to the identical CHO intake in both the 24 h control diet and the pre-training breakfast. In contrast, subsequent ingestion of CHO in the 3 h recovery period prior to PM HIT caused a greater suppression in β CTX compared to those conditions where energy was restricted (LCAL) or matched by consuming the isocaloric high fat diet (LCHF). Similarly, when CHO was ingested during PM HIT and in the subsequent 3 h recovery period, there was a greater suppression in β CTX immediately and 3 h post exercise. The apparent suppression of β CTX with CHO feeding agrees with previously reported observations, albeit under resting conditions, where a reduction in β CTX in response to an oral glucose tolerance test has been reported (Bjarnason *et al.* 2002; Bergmann *et al.* 2019). The finding that CHO availability attenuates post-exercise circulating β CTX is also similar to previous research reporting that consuming an 8% CHO solution before and during a prolonged bout of running (120 min at 70% of $\dot{V}_{O_{2max}}$) induced a greater reduction in β CTX compared to a placebo solution (Sale *et al.* 2015). Furthermore, de Sousa *et al.* (2014) also observed a reduction in the bone resorption response (as determined by β CTX) to an acute exercise bout following an 8 day 'overload training programme' in individuals fed a higher CHO diet during training. The CHO group consumed an additional 1 g kg⁻¹ body mass of maltodextrin per hour of running during the

8 days of training compared to the control group. When specifically examining the effects of feeding during the post-exercise recovery period, Townsend *et al.* (2017) also demonstrated that consuming a mixed CHO and protein recovery drink immediately following an exhaustive run significantly suppressed β CTX compared to a placebo solution. When taken together, the present data therefore confirm and extend the previous observations given that we observed no differences in β CTX between the LCHF and LCAL trials, thus demonstrating that it is the energy from CHO, rather than overall energy availability, that is important in attenuating the bone resorption response to an acute training session. In contrast to β CTX, however, we observed no effect of dietary condition on markers of bone formation (i.e. P1NP). Such data contrast with those of Sale *et al.* (2015), who reported that CHO feeding before and during exercise also reduces post-exercise P1NP concentrations. Our data therefore suggest that the influence of CHO availability in the current study was confined (at least within the study time frame) to bone resorption. We acknowledge, however, the limitation of solely measuring β CTX and P1NP and suggest that future studies may benefit from measurement of additional markers of bone formation and resorption such as that associated with Wnt- β -catenin signalling and the RANKL-RANK-OPG pathway (Ozcivici *et al.* 2010). The assessment of renal function may also prove beneficial given the possibility that changes in renal function as a result of high-intensity exercise and energy restriction may influence the excretion rate of β CTX and hence, its relative concentration.

The potential mechanisms for acute suppression of bone resorption by CHO feeding are still not fully understood, although it has been suggested that the enteric hormones might play a part in mediating the effects of glucose on bone metabolism (Bjarnason *et al.* 2002; Henriksen *et al.* 2003). Indeed, when enteric hormone secretion was limited by somatostatin administration, the reduction in bone resorption following oral glucose ingestion was negated (Clowes *et al.* 2002). Accordingly, Bergmann *et al.* (2019) have also recently demonstrated that the gut derived incretin hormones, glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide 1 (GLP-1), could be involved in regulation of bone resorption during glucose administration, as evidenced by a suppression in β CTX in response to glucose infusion when both hormones are also simultaneously infused. Additionally, specific regulatory effects of insulin (Clowes *et al.* 2002), leptin (Thomas & Burguera, 2002; Holloway *et al.* 2002) and/or IL-6 (Sale *et al.* 2015) signalling have also been postulated. Support for leptin related signalling is also provided by the observation that we also observed higher circulating leptin concentrations in the HCHO trial when compared to both the LCAL ($P = 0.041$) and LCHF trials (albeit

$P = 0.057$), an effect that is likely to be secondary to the hyperinsulinaemia induced by CHO feeding (Kolaczynski *et al.* 1996; Gonzalez *et al.* 2019). In relation to IL-6, Sale *et al.* (2015) reported a strong correlation between changes in IL-6 and β CTX in the post-exercise period and therefore postulated that IL-6 may regulate bone turnover via stimulating osteoclastogenesis and bone resorption. It is noteworthy, however, that although we observed differences in insulin and leptin between trials, circulating IL-6 was not apparently affected by CHO availability. Indeed, the repeated bout of high intensity running in PM HIT significantly increased circulating IL-6 in all three experimental conditions, the magnitude of which was greater in LCAL compared to LCHF and HCHO. Whilst the effects of muscle glycogen (Steensberg *et al.* 2001; Keller *et al.* 2001; Chan *et al.* 2004) and exogenous (Nieman *et al.* 1998, 2003; Febbraio *et al.* 2003) CHO availability on the regulation of exercise-induced IL-6 production is well documented, the present data suggest that energy availability plays a more dominant regulatory role in mediating IL-6 responses, compared to CHO availability *per se*. In contrast, an alternative interpretation is that high fat availability in the LCHF trial (despite also being low in absolute CHO content) may have suppressed IL-6 production to levels that were comparable to the HCHO trial (Phillips *et al.* 2003; DiLorenzo *et al.* 2014; Badenhorst *et al.* 2016). Taken together, the present data suggest that energy availability (but not CHO availability) is the more dominant factor in modulating components of cytokine production when training under conditions representative of the twice per day train low model.

Whilst the clinical implications of such acute and transient alterations in bone resorption remain to be determined, it would seem sensible for athletes to avoid prolonged periods of low CHO availability in order to reduce the potentially detrimental effects on bone mass (Ihle & Loucks, 2004) and/or bone injury risk. The findings of the current study might also lend some further support to the requirement for post-exercise feeding (particularly of CHO) in order to attenuate the bone resorption response to intense exercise or high training volumes. That said, this must be carefully considered over time, given that these alterations in bone metabolism are also likely to be an important part of the bone adaptive response, which remains an area requiring significant future research. Given the acute nature of this study, we were, of course, not able to confirm whether the longer-term adherence to the training and dietary regimen studied here would result in morphological changes to bone. Indeed, as discussed previously, we were limited to the use of blood borne markers of bone metabolism and selected the preferred markers of use as reference analytes for bone turnover markers in clinical studies (Vasikaran *et al.* 2011). Nonetheless, we acknowledge that future studies would benefit from the use of scanning techniques (e.g. DXA, pQCT or HR-pQCT)

or bone biopsy analyses to determine whether our chosen exercise and dietary approach does indeed manifest as long-term morphological changes to bone.

In summary, we provide novel data demonstrating that in conditions where post-exercise muscle glycogen concentration is maintained within the range of 200–350 mmol kg⁻¹ dw, short-term periods of acute CHO and energy restriction (i.e. < 24 h) do not potentiate potent skeletal muscle signalling pathways associated with the regulation of mitochondrial biogenesis and lipid metabolism. In addition, promotion of high CHO availability before, during and in recovery from exercise may be of greater importance for the acute regulation of markers of bone metabolism when compared to energy intake *per se*. In relation to skeletal muscle adaptation, future studies should now examine the potential presence of a muscle glycogen threshold as an important regulator of adaptations to endurance training. Additionally, the long-term implications (in relation to other physiological systems) of the acute within-day fluctuations in both CHO and energy availability inherent to chronic training twice per day should also be examined, which would also allow the determination of any effects on bone mass and structure using bone scanning techniques.

References

- Ahlborg G, Felig P, Hagenfeldt L, Hendlar R & Wahren J (1974). Substrate turnover during prolonged exercise in man. Splanchnic and leg metabolism of glucose, free fatty acids, and amino acids. *J Clin Invest* **53**, 1080–1090.
- Bartlett JD, Hawley JA & Morton JP (2015). Carbohydrate availability and exercise training adaptation: too much of a good thing? *Eur J Sports Sci* **15**, 3–12.
- Bartlett JD, Louhelainen J, Iqbal Z, Cochran AJ, Gibala MJ, Gregson W & Morton JP (2013). Reduced carbohydrate availability enhances exercise-induced p53 signalling in human skeletal muscle: implications for mitochondrial biogenesis. *Am J Physiol Regul Integr Comp Physiol* **304**, R450–R458.
- Badenhorst CE, Dawson B, Cox GR, Sim M, Laarakkers CM, Swinkels dw & Peeling P (2016). Seven days of high carbohydrate ingestion does not attenuate post-exercise IL-6 and hepcidin levels. *Eur J Appl Physiol* **116**, 1715–1724.
- Bergmann NC, Lund A, Gasbjerg LS, Jørgensen NR, Jessen L, Hartmann B, Holst JJ, Christensen MB, Vilsbøll T & Knop FK (2019). Separate and combined effects of GIP and GLP-1 infusions on bone metabolism in overweight men without diabetes. *J Clin Endocrinol Metab* **104**, 2953–2960.
- Bjarnason NH, Henriksen EE, Alexandersen P, Christgau S, Henriksen DB & Christiansen C (2002). Mechanism of circadian variation in bone resorption. *Bone*, **30**, 307–313.
- Borg GA (1973). Perceived exertion: a note on 'history' and methods. *Med Sci Sports* **5**, 90–93.
- Burke LM, Hawley JA, Jeukendrup A, Morton JP, Stellingwerff T & Maughan RJ (2018). Toward a common understanding of diet-exercise strategies to manipulate fuel availability for training and competition preparation in endurance sports. *Int J Sports Nutr Exerc Metab* **28**, 451–463.
- Chan MHS, McGee SL, Watt MJ, Hargreaves M & Febbraio MA (2004). Altering dietary nutrient intake that reduces glycogen content leads to phosphorylation of p38MAP kinase in human skeletal muscle: Association with IL-6 gene transcription during contraction. *FASEB J* **18**, 1785–1787.
- Chen X, Iqbal N, Boden G (1999). The effects of free fatty acids on gluconeogenesis and glycogenolysis in normal subjects. *J Clin Invest* **10**, 365–372.
- Clowes J, Hannon R, Yap T, Hoyle N, Blumsohn A & Eastell R (2002). Effect of feeding on bone turnover markers and its impact on biological variability of measurements. *Bone* **30**, 886–890.
- Cochran AJR, Little JP, Tarnopolsky MA & Gibala MJ (2010). Carbohydrate feeding during recovery alters the skeletal muscle metabolic response to repeated sessions of high intensity interval exercise in humans. *J Appl Physiol* **108**, 628–636.
- Considine RV, Sinha MK & Heiman ML (1996). Serum immunoreactive-leptin concentrations in normal-weight and obese humans. *N Engl J Med* **334**, 292–295.
- de Sousa MV, Pereira RM, Fukui R, Caparbo VF & da Silva ME (2014). Carbohydrate beverages attenuate bone resorption markers in elite runners. *Metab* **63**, 1536–1541.
- DiLorenzo FM, Drager CJ & Rankin JW (2014). Docosahexaenoic acid affects markers of inflammation and muscle damage after eccentric exercise. *J Strength Cond Res* **28**, 2768–2774.
- Febbraio MA, Steensberg A, Keller C, Starkie RL, Nielsen HB, Krstrup P, Ott P, Secher NH & Pedersen BK (2003). Glucose ingestion attenuates interleukin-6 release from contracting skeletal muscle in humans. *J Physiol* **549**, 607–612.
- Fredericson M, Chew K, Ngo J, Cleek T, Kiratli J & Cobb K (2007). Regional bone mineral density in male athletes: a comparison of soccer players, runners and controls. *Br J Sports Med* **41**, 664–668.
- Fudge BW, Westerterp, KR, Kiplamai FK, Onywera VO, Boit MK, Kayser B & Pitsiladis YP (2006). Evidence of negative energy balance using doubly labelled water in elite Kenyan endurance runners prior to competition. *Br J Nutr* **95**, 59–66.
- Fujimura R, Ashizawa N, Watanabe M, Mukai N, Hitoshi A, Fukubayashi T, Hayashi K, Tokuyama K & Suzuki M (1997). Effect of resistance exercise training on bone formation and resorption in young male subjects assessed by biomarkers of bone metabolism. *J Bone Min Res* **12**, 656–662.
- Gejl KD, Tharns LB, Hansen M, Rokkedal-Lausch T, Plomgaard P, Nybo L, Larsen FJ, Cardinale DA, Jensen K, Holmberg HC, Vissing K & Ørtenblad N (2017). No superior adaptations to carbohydrate periodization in elite endurance athletes. *Med Sci Sports Exerc* **49**, 2486–2497.
- Gomez-Bruton A, Gonzalez-Aguero A, Gomez-Cabello A, Casajus JA & Vicente-Rodriguez G (2013). Is bone tissue really affected by swimming? A systematic review. *PLoS One* **8**, e70119.

- Gonzalez JT, Betts JA & Thompson D (2019). Carbohydrate availability as a regulator of energy balance with exercise. *Exerc Sport Sci Rev*, in press; <https://doi.org/10.1249/JES.000000000000196>.
- Hagmar M, Berglund B, Brismar K & Hirschberg AL (2013). Body composition and endocrine profile of male Olympic athletes striving for leanness. *Clin J Sports Med* **23**, 197–201.
- Hammond KM, Impey SG, Currell K, Mitchell N, Shepherd SO, Jeromson S, Hawley JA, Close GL, Hamilton LD, Sharples AP & Morton JP (2016). Postexercise high-fat feeding suppresses p70S6K1 activity in human skeletal muscle. *Med Sci Sport Exerc* **48**, 2108.
- Hansen AK, Fischer CP, Plomgaard P, Andersen JL, Saltin B & Pedersen BK (2005). Skeletal muscle adaptation: training twice every second day vs. training once daily. *J Appl Physiol* **98**, 93–99.
- Henriksen DB, Alexandersen P, Bjarnason NH, Vilsboll T, Hartmann B, Henriksen EE, Byrjalsen I, Krarup T, Holst JJ & Christiansen C (2003). Role of gastrointestinal hormones in postprandial reduction of bone resorption. *J Bone Miner Res* **18**, 2180–2189.
- Holloway WR, Collier FM, Aitken CJ, Myers DE, Hodge JM, Malakellis M, Gough TJ, Collier GR & Nicholson GC (2002). Leptin inhibits osteoclast generation. *J Bone Miner Res* **17**, 200–209.
- Horowitz JF, Mora-Rodriguez R, Byerley LO & Coyle EF (1997). Lipolytic suppression following carbohydrate ingestion limits fat oxidation during exercise. *Am J Physiol Endocrin Metab* **273**, E768–E775.
- Hulston CJ, Venables MC, Mann CH, Martin C, Philp A, Baar K & Jeukendrup AE (2010). Training with low muscle glycogen enhances fat metabolism in well-trained cyclists. *Med Sci Sports Exerc* **42**, 2046–2055.
- Ihle R & Loucks AB (2004). Dose-response relationships between energy availability and bone turnover in young exercising women. *J Bone Miner Res* **19**, 1231–1240.
- Impey SG, Hammond KM, Shepherd SO, Sharples AP, Stewart C, Limb M, Smith K, Philp A, Jeromson S, Hamilton DL, Close GL & Morton JP (2016). Fuel for the work required: a practical approach to amalgamating train-low paradigms for endurance athletes. *Phys Reports* **4**, e12803.
- Impey SG, Hearn MA, Hammond KM, Bartlett JD, Louis J, Close GL & Morton JP (2018). Fuel for the work required: a theoretical framework for carbohydrate periodization and the glycogen threshold hypothesis. *J Sports Med* **48**, 1031–1048.
- Jensen MD, Ekberg K & Landau BR (2001). Lipid metabolism during fasting. *Am J Physiol Endocrinol Metab* **281**, E789–E793.
- Jeukendrup AE & Wallis GA (2005). Measurement of substrate oxidation during exercise by means of gas exchange measurements. *Int J Sports Med* **1**, 28–37.
- Keller C, Steensberg A, Pilegaard H, Osada T, Saltin B, Pedersen BK & Neufer PD (2001). Transcriptional activation of the IL-6 gene in human contracting skeletal muscle: influence of muscle glycogen content. *FASEB J* **15**, 2748–2750.
- Kolaczynski JW, Considine RV, Ohannesian J, Marco C, Opentanova I, Nyce MR, Myint M & Caro JF (1996). Responses of leptin to short-term fasting and refeeding in humans: a link with ketogenesis but not ketones themselves. *Diabetes* **45**, 1511–1515.
- Loucks AB, Verdun M & Heath EM (1998). Low energy availability, not stress of exercise, alters LH pulsatility in exercising women. *J Appl Physiol* **84**, 37–46.
- Marquet LA, Brisswalter J, Louis J, Tiollier E, Burke LM, Hawley JA & Hausswirth C (2016). Enhanced endurance performance by periodization of CHO intake: “sleep low” strategy. *Med Sci Sports Exerc* **48**, 663–672.
- Meynet O & Ricci JE (2014). Caloric restriction and cancer: molecular mechanisms and clinical implications. *Trends Mol Med* **20**, 419–427.
- Morton JP, Croft L, Bartlett JD, Maclaren DPM, Reilly T, Evans L, McArdle A & Drust B (2009). Reduced carbohydrate availability does not modulate training-induced heat shock protein adaptations but does upregulated oxidative enzyme activity in human skeletal muscle. *J Appl Physiol* **106**, 1513–1521.
- Mountjoy M, Sundgot-Borgen J, Burke LM, Carter S, Constantini N, Lebrun C, Meyer N, Sherman R, Steffen K, Budgett R & Ljungqvist A (2014). The IOC consensus statement: beyond the female athlete triad—relative energy deficiency in sport (RED-S). *Br J Sports Med* **48**, 491–497.
- Nieman DC (2007). Marathon training and immune function. *Sports Med* **37**, 412–415.
- Nieman DC, Davis JM, Henson DA, Walberg-Rankin J, Shute M, Dumke CL, Utter AC, Vinci DM, Carson JA, Brown A, Lee WJ, McAnulty SR & McAnulty LS (2003). Carbohydrate ingestion influences skeletal muscle cytokine mRNA and plasma cytokine levels after a 3-h run. *J Appl Physiol* (1985) **94**, 1917–1925.
- Nieman DC, Nehlsen-Cannarella SL, Fagoaga OR, Henson DA, Utter A, Davis JM, Williams F & Butterworth DE (1998). Influence of mode and carbohydrate on the cytokine response to heavy exertion. *Med Sci Sports Exerc* **30**, 671–678.
- Olmedillas H, Gonzalez-Aguero A, Moreno LA, Casajus JA & Vicente-Rodriguez G (2012). Cycling and bone health: a systematic review. *BMC Med* **10**, 168.
- Ozicivici E, Luu YK, Adler BA, Qin Y-X, Rubin J, Judex S & Rubin CT (2010). Mechanical signals as anabolic agents in bone. *Nat Rev Rheumatol* **6**, 50–59.
- Pagnotti GM, Styner M, Uzer G, Patel VS, Wright LE, Ness KK, Guise TA, Rubin J & Rubin CT (2019). Combating osteoporosis and obesity with exercise: leveraging cell mechanosensitivity. *Nat Rev Endocrinol* **15**, 339–355.
- Phillips T, Childs AC, Dreon DM, Phinney S & Leeuwenburgh C (2003). A dietary supplement attenuates IL-6 and CRP after eccentric exercise in untrained males. *Med Sci Sports Exerc* **35**, 2032–2037.
- Pilegaard H, Osada T, Andersen LT, Helge JW, Saltin B & Neufer PD (2005). Substrate availability and transcriptional regulation of metabolic genes in human skeletal muscle during recovery from exercise. *Metab* **54**, 1048–1055.
- Psilander N, Frank P, Flockhart M & Sahlin K (2013). Exercise with low glycogen increases PGC-1 α gene expression in human skeletal muscle. *Eur J Appl Physiol* **113**, 951–963.
- Romijn JA, Coyle EF, Sidossis LS, Zhang XJ & Wolfe RR (1995). Relationship between fatty acid delivery and fatty acid oxidation during strenuous exercise. *J Appl Physiol* **79**, 1939–1945.

- Sale C, Varley I, Jones TW, James RM, Tang JC, Fraser WD & Greeves JP (2015). Effect of carbohydrate feeding on the bone metabolic response to running. *J Appl Physiol* (1985) **119**, 824–830.
- Schmittgen TD & Livak KJ (2008). Analyzing real-time PCR data by the comparative C_T method. *Nat Protoc* **3**, 1101–1108.
- Scofield KL & Hecht S (2012). Bone health in endurance athletes: runners, cyclists, and swimmers. *Curr Sports Med Rep* **11**, 328–334.
- Sidossis LS, Gastaldelli A, Klein, S & Wolfe RR (1997). Regulation of plasma fatty acid oxidation during low- and high-intensity exercise. *Am J Physiol Endocrinol Metab* **272**, E1065–E1070.
- Steensberg A, Febbraio MA, Osada T, Schjerling P, van Hall G, Saltin B & Pedersen BK (2001). Interleukin-6 production in contracting human skeletal muscle is influenced by pre-exercise muscle glycogen content. *J Physiol* **537**, 633–639.
- Stellingwerff T, Morton JP & Burke LM (2019). A framework for periodized nutrition for athletics. *Int J Sports Nutr Exerc Metab* **11**, 1–29.
- Thomas DT, Erdman KA & Burke LM (2016). Position of the academy of nutrition and dietetics, dietitians of Canada, and the American College of sports medicine: nutrition and athletic performance. *J Acad Nutr Diet* **116**, 501–528.
- Thomas T & Burguera B (2002). Is leptin the link between fat and bone mass? *J Bone Miner Res* **17**, 1563–1569.
- Thompson, WR, Rubin CT & Rubin J (2012). Mechanical regulation of signalling pathways in bone. *Gene* **503**, 179–193.
- Townsend R, Elliot-Sale KJ, Currell K, Tang J, Fraser WD & Sale C (2017). The effect of postexercise carbohydrate and protein ingestion on bone metabolism. *Med Sci Sports Exerc* **49**, 1209–1218.
- van Loon LJ, Saris WH, Kruijshoop M & Wagenmakers AJ (2000). Maximizing postexercise muscle protein synthesis: carbohydrate supplementation and the application of amino acid or protein hydrolysate mixtures. *Am J Clin Nutr* **72**, 106–110.
- Van Proeyen K, Szlufcik K, Nielens H, Ramaekers M & Hespel P (2011). Beneficial metabolic adaptations due to endurance exercise training in the fasted state. *J Appl Physiol* **110**, 236–245.
- Vasikaran SD, Morris HA, Cooper C, Kanis JA (2011). Standardising biochemical assessment of bone turnover in osteoporosis. *Clin Biochem* **44**, 1033–1034.
- Vogt S, Heinrich L, Schumacher YO, Grosshauser M, Blum A, Koing D, Berg A & Schmid A (2005). Energy intake and energy expenditure of elite cyclists during preseason training. *Int J Sports Med* **26**, 701–706.
- Wilson G, Martin D, Morton JP & Close GL (2018). Male flat jockeys do not display deteriorations in bone density or resting metabolic rate in accordance with race riding experience: implications for RED-S. *Int J Sport Nutr Exerc Metab* **28**, 434–439.
- Wright DC, Geiger PC, Han DH, Jones TE & Holloszy JO (2007). Calcium induces increases in peroxisome proliferator-activated receptor γ coactivator-1 α and mitochondrial biogenesis by a pathway leading to p38 mitogen-activated protein kinase activation. *J Biol Chem* **282**, 18793–18799.
- Yeo WK, Paton CD, Garnham AP, Burke LM, Carey AL & Hawley JA (2008). Skeletal muscle adaptation and performance responses to once a day versus twice every second day endurance training regimens. *J Appl Physiol* **105**, 1462–1470.

Additional information

Competing interests

J.P.M. is a consultant for Science in Sport (SiS), GlaxoSmithKline (GSK) and Lucozade Ribena Suntory (LRS) has funded his previous research on glycogen metabolism and exercise.

Author contributions

K.M.H., C.S., C.St., A.P.S., J.P.M.: substantial contributions to the conception or design of the work; All authors: the acquisition, analysis, or interpretation of data for the work; drafting the work or revising it critically for important intellectual content; All authors: final approval of the version to be published; agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Funding

This study was funded in part by the English Institute of Sport (research grant awarded to J.P.M.).

Acknowledgements

The authors acknowledge the technical assistance of Dean Morrey and Gemma Miller in their maintenance of the research physiology laboratory at LJMU.

Keywords

β CTX, caloric restriction, IL-6, PGC-1 α