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Abstract

Purpose: To examine the effects of reduced CHO but high post-exercise fat availability on cell signalling and expression of genes with putative roles in regulation of mitochondrial biogenesis, lipid metabolism and muscle protein synthesis (MPS). **Methods:** Ten males completed a twice per day exercise model (3.5 h between sessions) comprising morning high-intensity interval (HIT) (8 x 5-min at 85% VO_{2peak}) and afternoon steady-state (SS) running (60 min at 70% VO_{2peak}). In a repeated measures design, runners exercised under different isoenergetic dietary conditions consisting of high CHO (HCHO: 10 CHO, 2.5 Protein and 0.8 Fat $g.kg^{-1}$ per whole trial period) or reduced CHO but high fat availability in the post-exercise recovery periods (HFAT: 2.5 CHO, 2.5 Protein and 3.5 Fat $g.kg^{-1}$ per whole trial period). **Results:** Muscle glycogen was lower ($P<0.05$) at 3 (251 vs 301 $mmol.kg^{-1}dw$) and 15 h (182 vs 312 $mmol.kg^{-1}dw$) post-SS exercise in HFAT compared to HCHO. AMPK-α2 activity was not increased post-SS in either condition ($P=0.41$) though comparable increases (all $P<0.05$) in PGC-1α, p53, CS, Tfam, PPAR and ERRα mRNA were observed in HCHO and HFAT. In contrast, PDK4 ($P=0.003$), CD36 ($P=0.05$) and CPT1 ($P=0.03$) mRNA were greater in HFAT in the recovery period from SS exercise compared with HCHO. p70S6K activity was higher ($P=0.08$) at 3 h post-SS exercise in HCHO versus HFAT (72.7 ± 51.9 vs 44.7 ± 27 $fmol.min^{-1}$ mg^{-1}). **Conclusion:** Post-exercise high fat feeding does not augment mRNA expression of genes associated with regulatory roles in mitochondrial biogenesis though it does increase lipid gene expression. However, post-exercise p70S6K1 activity is reduced under conditions of high fat feeding thus potentially impairing skeletal muscle remodelling processes.

Keywords: AMPK-α2, PGC-1α, p53, glycogen, mitochondrial biogenesis

Introduction

Traditional nutritional strategies for endurance athletes have largely focused on ensuring high CHO availability before, during and after each training session (2). However, accumulating data from our laboratory (7, 29) and others (12, 16, 17, 23, 39) have demonstrated a potent effect of CHO restriction (the so-called *"train-low"* paradigm) in augmenting the adaptive responses inherent to endurance training. Indeed, reduced CHO availability before (33) during (1) and after (32) training sessions augments the acute cell signalling pathways and downstream gene expression responses associated with regulating training adaptation. Accordingly, reduced CHO availability during short-term periods of endurance training augments markers of mitochondrial biogenesis (16, 39, 29), increases both whole body (39) and intramuscular lipid metabolism (17) and also improves exercise capacity and performance (16, 24). In the context of nutrient-gene interactions, it is therefore apparent that the acute molecular regulation of cell signalling processes provides a theoretical basis for understanding the molecular mechanisms underpinning chronic training adaptations.

In addition to manipulation of CHO availability, many investigators have also demonstrated a modulatory role of high fat availability in augmenting components of training adaptation (10. For example, the acute elevation in circulating free fatty acid (FFA) availability during exercise regulates key cell signalling kinases and transcription factors that modulate the expression of genes regulating both lipid and CHO metabolism (31, 40). Additionally, 5-15 days of high fat feeding increases resting intramuscular triglyceride stores (38), hormone sensitive lipase (38), carnitine palmitoyltransferase (CPT1) (15), adenosine monophosphate activated protein kinase (AMPK)-α2 activity (38) and protein content of fatty acid translocase (FAT/CD36) (11). Such adaptations undoubtedly contribute to the enhanced rates of lipid oxidation observed during

exercise following “fat adaptation” protocols (10). Taken together, these data suggest carefully chosen periods of reduced CHO but concomitant high fat availability may therefore represent a strategic approach for which to maximise both training-induced skeletal muscle mitochondrial biogenesis and the enhanced capacity to utilise lipid sources as fuels during exercise.

However, such a feeding strategy is not without potential limitations especially if performed on consecutive days. Indeed, reduced CHO availability impairs acute training intensity (17, 39) and five days of high fat feeding reduces pyruvate dehydrogenase (PDH) activity (35), thus potentially leading to a de-training effect, reduced capacity to oxidise CHO and ultimately, impaired competition performance (17, 39). Moreover, although many endurance training-induced skeletal muscle adaptations are regulated at a transcriptional level, the turnover of myofibrillar (i.e. contractile) proteins are largely regulated through the translational machinery and the mechanistic target of rapamycin complex (mTOR) and ribosomal protein S6 kinase 1 (p70S6K) signalling axis (28). In this regard, recent data suggests high circulating FFA availability impairs muscle protein synthesis despite the intake of high quality protein, albeit examined via lipid and heparin fusion and euglycemic hyperinsulemic clamp conditions (36).

With this in mind, the aim of the present study was to examine the effects of reduced CHO but high post-exercise fat availability on the activation of key cell signalling kinases and expression of genes with putative roles in the regulation of mitochondrial biogenesis, lipid metabolism and muscle protein synthesis. In accordance with the original train-low investigations (16, 17, 29, 39), we employed a twice per day exercise model whereby trained male runners completed a morning high-intensity interval training session followed by an afternoon training session consisting of steady-state running. Runners completed the exercise protocols under two different dietary conditions (both energy and protein matched) consisting of high CHO availability

(HCHO) in the recovery period after both training sessions (i.e. best practice nutrition) or alternatively, reduced CHO but high fat availability in the post-exercise recovery periods (HFAT). We specifically hypothesised that our high fat feeding protocol would enhance cell signalling and the expression of those genes with putative roles in the regulation of mitochondrial biogenesis and lipid metabolism but would also impair the activity of muscle protein synthesis related signalling.

Methods

Subjects: Ten trained male runners volunteered to participate in the study (mean ± SD: age, 24 ± 1.5 years; body mass, 75.9 ± 6 kg; height, 177.3 ± 7.2 cm; VO_{2peak}, 60 ± 3.6 $ml \cdot kg^{-1} \cdot min^{-1}$). All subjects gave written informed consent prior to participation after all experimental procedures and potential risks had been fully explained. None of the subjects had any history of musculoskeletal or neurological disease, nor were they under any pharmacological treatment over the course of the testing period. Subjects were instructed to refrain from any strenuous physical activity, alcohol and caffeine consumption in the 48h prior to each experimental trial. The study was approved by the ethics committee of Liverpool John Moores University.

Design: In a repeated measures, randomised, cross-over design separated by 7 days, subjects completed a twice per day exercise model under two different dietary conditions (both energy and protein matched) consisting of high CHO availability (HCHO) in the recovery period after both training sessions (i.e. best practice nutrition) or alternatively, reduced CHO but high fat availability in the post-exercise recovery periods (HFAT). The twice per day exercise model comprised a morning (9-10 am) high-intensity interval (HIT) training session (8 x 5-min at 85%

VO_{2peak}) followed by an afternoon (130-230 pm) training session consisting of steady-state (SS) running (60 min at 70% VO_{2peak}). To promote training compliance during the HIT protocol in both the HCHO and HFAT trials, subjects adhered to a standardised high CHO breakfast prior to this session. However, during the 3.5 h recovery between the HIT and SS session and in the recovery period upon completion of the SS exercise protocol until the subsequent morning, subjects adhered to either a HCHO or HFAT feeding protocol. Muscle biopsies were obtained from the vastus lateralis muscle immediately pre-HIT, immediately post-SS and at 3 h and 15 h post-SS. An overview of the experimental design and nutritional protocols are shown in Figure 1.

***Preliminary testing*:** At least 7-10 days prior to the first main experimental trial, subjects performed a maximal incremental running test to volitional fatigue on a motorised treadmill (h/p/Cosmos, Nussdorf-Traunstein, Germany) in order to determine maximal oxygen uptake. Following a 10 minute warm up at a self-selected treadmill speed the maximal incremental test commenced, beginning with a 2-min stage at a treadmill speed of 10km/h. Running speed was then increased by 2km/h every 2-min until a speed of 16km/h was reached, after which the treadmill inclined by 2% every 2-min until volitional exhaustion. VO_{2peak} was defined as the highest VO_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} (bpm) of age-predicted maximum, 2) respiratory exchange ratio > 1.1, and 3) plateau of oxygen consumption despite increased workload. On their second visit to the laboratory (approx. 3 days later), subjects completed a running economy test in order to determine their individual running speeds for subsequent experimental trials. Following a warm up, the test began with a 5-min stage at a treadmill speed of 8km/h with 1% incline and speed was then increased by 1km/h every 5-mins thereafter . The test was stopped when >90% of the previously determined VO_{2peak} was reached. These

measurements were recorded via breath-by-breath gas measurements obtained continuously throughout both tests using a CPX Ultima series online gas analysis system (Medgraphics, Minnesota, US). The test-retest reliability of this system in our laboratory when quantified using 95% limits of agreement is 0.29 ± 2.4 $ml.kg^{-1}$ min^{-1} (data were compiled from comparison of the oxygen uptake during the HIT protocols in the HCHO and HFAT trials undertaken in the present study). Heart rate (Polar, Kempele, Finland) was also recorded continuously during exercise.

***Experimental protocols*:**

***HIT protocol*:** In the 24-h preceding each main experimental trial, subjects consumed a standardised high CHO diet in accordance with typical nutritional recommendations (8 $g.kg^{-1}$ CHO, 2 $g.kg^{-1}$ protein, and 1 $g.kg^{-1}$ fat). On the morning of each experimental trial, subjects reported to the laboratory at ~7 am where they were given a standardised high-CHO breakfast (2 $g.kg^{-1}$ CHO, 0.3 $g.kg^{-1}$ protein, and 0.1 $g.kg^{-1}$ fat). At 2-h post-prandial, a venous blood sample was then collected from an antecubital vein in the anterior crease of the forearm and a muscle biopsy sample taken from the vastus lateralis muscle. Subjects were then fitted with a heart rate monitor and nude body mass (SECA, Hamburg, Germany) was recorded before commencing the high intensity interval running (HIT) protocol which lasted ~1-h. The HIT protocol consisted of 8 x 5-min bouts running at a velocity corresponding to 85% VO_{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% $VO_{2peak,}$ and a further venous blood sample was obtained immediately upon completion of the protocol. Water was given ad libitum throughout the duration of exercise with the pattern of intake recorded and replicated for the subsequent experimental trial. Heart rate was measured continuously during exercise (Polar, Kempele, Finland) and ratings of perceived exertion (RPE, 9) were obtained upon completion of

each HIT bout. In order to determine substrate utilisation during exercise (20), expired gas was collected via a mouthpiece connected to an online gas analysis system (CPX Ultima, Medgraphics, Minnesota, US) for the final 2-mins of each 5-min interval.

***SS protocol*:** During the 3.5 h recovery period between the HIT and SS protocols, subjects consumed either the HCHO (2.5 $g.kg^{-1}$ CHO, 1 $g.kg^{-1}$ Protein, 0.3 $g.kg^{-1}$ Fat) or HFAT (0 $g.kg^{-1}$ CHO, 1 $g.kg^{-1}$ Protein, 1 $g.kg^{-1}$ Fat) feeding protocols (the pattern and frequency of feeding is shown in Figure 1). Following the recovery period, another venous blood sample was obtained immediately prior to commencing the afternoon SS exercise protocol. After a 5-min warm up at a self-selected treadmill speed, subjects subsequently commenced the 60-min steady state running (SS) protocol at a velocity corresponding to 70% VO_{2peak}. During exercise, subjects also consumed 60 $g.h^{-1}$ of CHO (SiS GO Istonic Gels, Science in Sport, Blackburn, UK) in HCHO whereas no form of energy was consumed in the HFAT trial. Water was given ad libitum throughout the duration of exercise with the pattern of intake recorded and replicated for the subsequent experimental trial. Expired gases were also collected for 5-mins at 15-min intervals throughout the exercise trial (CPX Ultima, Medgraphics, Minnesota, US) and substrate utilisation again determined according to Jeukendrup and Wallis (20). Heart rate was measured continuously during exercise (Polar, Kempele, Finland) and ratings of perceived exertion (RPE, 9) were obtained every 15 minutes during exercise. Upon completion of the SS protocol until sleep, subjects consumed either the HCHO (3.6 $g.kg^{-1}$ CHO, 1.5 $g.kg^{-1}$ Protein, 0.4 $g.kg^{-1}$ Fat) or HFAT (0.2 $g.kg^{-1}$ CHO, 1.5 $g.kg^{-1}$ Protein, 2.3 $g.kg^{-1}$ Fat) feeding protocols where the pattern and frequency of feeding is shown in Figure 1. Vastus lateralis muscle biopsies and venous blood samples were also collected immediately post- and at 3 h and 15 h post completion (i.e. ~8 am and in a fasted state) of the SS exercise protocol. The total energy intake across the whole trial

period (i.e 7 am – 9 pm) in HCHO was: ~10 $g.kg^{-1}$ CHO, ~2.5 $g.kg^{-1}$ Protein and ~0.8 $g.kg^{-1}$ Fat, and in HFAT was: ~2.5 $g.kg^{-1}$ CHO, ~2.5 $g.kg^{-1}$ Protein and ~3.5 $g.kg^{-1}$ Fat, where both trials were matched for total energy intake.

Blood sampling and analysis: Venous blood samples were collected into vacutainers containing EDTA or lithium heparin and stored on ice until centrifugation at 1500g for 15-mins at 4ºC. Following centrifugation, aliquots of plasma were stored in a freezer at -80ºC 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.

***Muscle biopsies*:** Muscle biopsy samples (~50 mg) were obtained from the lateral portion of the vastus lateralis muscle using a Bard Monopty Disposable Core Biopsy Instrument 12 gauge x 10 cm length, (Bard Biopsy Systems, Tempe, AZ, USA). Samples 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 -80ºC for later analysis.

Analysis of muscle glycogen: Muscle glycogen concentration was determined according to the methods described by van Loon et al (37). 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 1M HCl for 3 hours at 100°C. After cooling to room temperature for ~20-min, samples were neutralized by the addition of 250µl 0.12 $mol.L^{-1}$ Tris/2.1 $mol.L^{-1}$ KOH saturated with KCl. Following centrifugation at 1500 RCF for 10-mins at 4ºC, 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 and intra-assay coefficients of variation were <5%.

RNA isolation and analysis: Muscle biopsy samples (~20 mg) were homogenized in 1ml TRIzol reagent (Thermo Fisher Scientific, UK) and total RNA isolated according to manufacturer's guidelines. Concentrations and purity of RNA were assessed by UV spectroscopy at ODs of 260 and 280 nm using a Nanodrop 3000 (Fisher, Rosklide, Denmark). 70 ng RNA was then used for each PCR reaction. Samples were ran 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. 3 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 25bp, and amplified a product between 141 – 244bp. All primers were purchased from Sigma (Suffolk, UK) and sequences for each gene are shown in parentheses: peroxisome proliferator-activated γ receptor coactivator **(**PGC-1) (fwd: TGCTAAACGACTCCGAGAA, rev: TGCAAAGTTCCCTCTCTGCT), tumour suppressor protein (p53) (fwd: ACCTATGGAAACTACTTCCTGAAA, rev: CTGGCATTCTGGGAGCTTCA), mitochondrial transcription factor A (Tfam) (fwd: TGGCAAGTTGTCCAAAGAAACCTGT, rev: GTTCCCTCCAACGCTGGGCA), citrate synthase (CS) (fwd: CCTGCCTAATGACCCCATGTT, rev:

CATAATACTGGAGCAGCACCCC), estrogen related receptor (ERR)-α (fwd: TGCCAATTCAGACTCTGTGC, rev: CCAGCTTCACCCCATAGAAA), peroxisome proliferator-activated receptor (PPAR) (fwd: ATGGAGCAGCCACAGGAGGAAGCC, rev: GCATGAGGCCCCGTCACAGC), pyruvate dehydrogenase kinase, isozyme 4 (PDK4) (fwd: TGGTCCAAGATGCCTTTGAGT, rev: GTTGCCCGCATTGCATTCTT), Glucose transporter type 4 (GLUT4) (fwd: TCTCCAACTGGACGAGCAAC, rev: CAGCAGGAGGACCGCAAATA) carnitine palmitoyltransferase (CPT1) (fwd: GACAATACCTCGGAGCCTCA, rev: AATAGGCCTGACGACACCTG), fatty acid translocase (FAT/CD36) (fwd: AGGACTTTCCTGCAGAATACCA, rev: ACAAGCTCTGGTTCTTATTCACA), and GAPDH (fwd: AAGACCTTGGGCTGGGACTG, rev: TGGCTCGGCTGGCGAC).

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 10s, and annealing/extension at 60°C for 30s. 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 (34) where relative gene expression was calculated as $2^{-\Delta\Delta ct}$ and where 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±1), therefore a pooled reference gene C_t was used in the

relative gene expression equation above. Furthermore, to enable calculation of expression values immediately post and 3-h post exercise, the calibrator condition in the delta delta C_t equation was assigned to the pre-exercise condition.

[γ^{-32}P] ATP Kinase Assay: Approximately 10-20 mg of muscle tissue was used for the measurement of p70S6K1 and AMPKα2 activity as previously described (27).

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 (HCHO vs HFAT). 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 mean ± SD, with P values ≤0.05 indicating statistical significance.

Results

Physiological responses and substrate utilisation during exercise.

Comparisons of subjects' heart rate, RPE and substrate oxidation during the HIT and SS protocols are displayed in Table 1 and 2, respectively. Heart rate, RPE and lipid oxidation (all P<0.01) all displayed progressive increases during both HIT (see Table 1) and SS exercise (see Table 2) whereas CHO oxidation displayed a progressive decrease (P<0.01) during both exercise protocols. In accordance with identical pre-exercise feeding in HIT, no significant differences were apparent in any of the aforementioned variables between HCHO and HFAT (P=0.06, 0.19,

0.52 and 0.56, respectively). In contrast, however, during the SS exercise protocol CHO oxidation was significantly greater in HCHO compared to HFAT ($P<0.001$) whereas fat oxidation was significantly greater during HFAT compared to HCHO ($P < 0.001$).

***Plasma metabolite responses**:* Plasma glucose, lactate, NEFA, glycerol and β-hydroxybutyrate all displayed significant changes (all $P<0.01$) over the sampling period (see Table 3). However, in accordance with the provision of post-exercise CHO feeding in the HCHO trial, plasma glucose was significantly higher compared with HFAT ($P<0.01$) whereas post-exercise high fat feeding in HFAT induced significantly greater plasma NEFA, glycerol and β-OHB (all $P<0.01$) in HFAT compared with the HCHO trial.

***Muscle glycogen and exercise induced cell signalling**:* Exercise induced significant decreases ($P<0.01$) in muscle glycogen immediately post-SS though no differences were apparent between HCHO and HFAT at this time-point (see Figure 2A). However, in accordance with the provision of CHO after the SS exercise protocol in HCHO, muscle glycogen re-synthesis was observed such that significant differences between HCHO and HFAT ($P=0.01$) were observed at 3 h and 15 h post-SS exercise. Neither exercise ($P =0.407$) nor dietary condition ($P = 0.124$) affected AMPK-α2 activity at any time-point studied (see Figure 2B). In contrast, p70S6K1 activity was significantly increased 3 h post-SS exercise (30-mins post-feeding) ($P<0.01$), although this increase was supressed ($P=0.08$) in HFAT (see Figure 2C). Furthermore, p70S6K1 activity was significantly reduced at 15 h post-SS exercise when participants were fasted compared with pre-HIT when they were high CHO and protein fed ($P<0.01$).

***Gene expression**:* Exercise increased the expression of PGC-1α ($P<0.001$), p53 ($P=0.032$), CS ($P=0.05$), Tfam ($P=0.05$), PPAR ($P<0.01$) and ERRα ($P=0.01$) however, there were no differences (all $P>0.05$) between HFAT and HCHO trials (see Figure 3A-F). In contrast, the exercise-induced increase ($P=0.001$) in PDK4 mRNA was greater in HFAT versus HCHO ($P=0.003$). Similarly, mRNA expression of CD36 ($P=0.05$) and CPT1 ($P=0.02$) was significantly greater in HFAT in recovery from the SS exercise protocol (see Figure 3). In contrast, neither exercise ($P=0.12$) nor diet ($P=0.31$) significantly affected GLUT expression (see Figure 3).

Discussion

The aim of the present study was to examine the effects of reduced CHO but high post-exercise fat availability on the activation of key cell signalling kinases and expression of genes with putative roles in the regulation of mitochondrial biogenesis, lipid metabolism and muscle protein synthesis. When compared with high CHO availability, we observed that post-exercise high fat feeding had no modulatory affect on AMPK-α2 activity or the expression of those regulatory genes associated with mitochondrial biogenesis. Furthermore, although post-exercise high fat feeding augmented the expression of genes involved in lipid transport (i.e. FAT/CD36) and oxidation (i.e. CPT1), we also observed suppression of p70S6K1 activity despite sufficient post-exercise protein intake. This latter finding suggests that post-exercise high fat feeding may impair the regulation of muscle protein synthesis and skeletal muscle remodelling processes, thereby potentially causing maladaptive responses for training adaptation if performed long-term.

In accordance with the original train-low investigations examining cycling or knee extensor exercise (16, 17, 29, 39), we also employed a twice per day protocol, albeit consisting of

morning HIT and afternoon SS running exercise protocol. This model is practically relevant given that many elite endurance athletes (including runners) train multiple times per day with limited recovery time between training sessions (14). Given that reduced CHO availability impairs high-intensity training capacity (17, 39), we also chose to schedule the HIT session in the morning period after a standardised high CHO breakfast. As expected, no differences in cardiovascular strain, ratings of perceived exertion, substrate utilisation and plasma metabolite responses were observed between the HCHO and HFAT trials during the HIT session (see Table 1 and 3). Following completion of the HIT protocol, subjects then adhered to a HCHO or HFAT feeding protocol in the 3.5 h prior to commencing the afternoon SS exercise. Given that exogenous CHO feeding during exercise reduces oxidative adaptations even in the presence of reduced pre-exercise muscle glycogen (29), we also chose to feed exogenous CHO (at a rate of 60 g/h) during the afternoon SS protocol during the HCHO trial. Although we did not directly quantify muscle glycogen immediately prior to SS exercise, plasma metabolite and substrate utilisation during SS exercise were clearly suggestive of differences in both endogenous and exogenous CHO availability between the HCHO and HFAT trials. Indeed, plasma NEFA, glycerol, β-OHB and whole body lipid oxidation were all greater during SS exercise undertaken in the HFAT trial compared with the HCHO trial (see Table 2 and 3). On the basis of comparable muscle glycogen data post-SS exercise (see Figure 2A) and greater whole body CHO oxidation during the HCHO trial (see Table 2), we also suggest that exercise-induced muscle glycogen utilisation was greater during the SS exercise protocol when completed in the HCHO conditions (7).

Perhaps surprisingly, we observed that our SS exercise protocol did not increase AMPK-α2 activity in either the HCHO or HFAT trial. However, there are likely a number of

physiologically valid reasons to explain the apparent lack of AMPK mediated signalling. Indeed, exercise-induced AMPK activation is known to be intensity dependent where >70% VO_{2max} is likely required to induce metabolic perturbations sufficient to mediate a signalling response (13). Furthermore, the AMPK response to exercise is attenuated with exercise training (8), an effect that is especially relevant for the present investigation given the trained status of our chosen population and the low plasma lactate observed (approximately 2 $mmol.L^{-1}$) during SS exercise. Reduced absolute muscle fibre recruitment from the vastus lateralis, when compared with other lower extremity muscles recruited during walking and running (19), or when exercising at similar relative intensities during cycling (4) and where AMPK activation is typically reported (22) could also contribute, in part, to the lack of AMPK signalling observed here. Finally, although exercise-induced AMPK activity is also thought to be regulated, in part, via a glycogen binding domain on β-subunit of the AMPK heterotrimer (26), it is possible that our runners did not exceed a potential "muscle glycogen threshold" that is required to fully activate the AMPK complex during prolonged endurance exercise (30). Indeed, previous data from our laboratory also using running exercise protocols (6,7) have typically only observed AMPK related signalling when post-exercise whole muscle homogenate glycogen is <200 $mmol.kg^{-1}$ dw. Despite previous suggestions that train-low training sessions should be targeted to SS exercise protocols so as to not compromise training intensity (5), our data therefore suggest (at least for AMPK mediated signalling) that perhaps it is the actual completion of a high-intensity stimulus per se (especially in trained athletes) that is really required to create a metabolic milieu that is conducive to augmentation of necessary signalling networks.

In contrast to Yeo et al. (38), we also observed no modulatory effect of post-exercise high fat availability on resting AMPK-α2 activity. Indeed, these authors observed that 5 days of a fat

loading protocol increased resting AMPK-α2 activity as well as the exercise-induced phosphorylation of ACC^{Ser221}. Such discrepancies between studies are likely due to the differences in duration of high fat feeding in that we adopted an acute high fat feeding protocol (<24 h) whereas the latter authors adopted a 5 day "fat adaptation" protocol that also increased resting intramuscular triglyceride (IMTG) stores. In this regard, it is noteworthy that the magnitude of change in resting AMPK-α2 activity was positively correlated with the elevations in IMTG storage (38).

In contrast to our hypothesis, we also observed comparable 2-3 fold changes between trials in mRNA expression of those genes with key regulatory roles associated with mitochondrial biogenesis. For example, the expression of PGC-1α, p53, Tfam, PPAR and ERRα mRNA were all elevated with similar magnitude and time-course in recovery from the SS protocol in both the HCHO and HFAT trials. Such data conflict with previous observations from our laboratory (7) and others (32) where post-exercise CHO restriction (i.e. keeping muscle glycogen low) augments the expression of many of the aforementioned genes. However, in our previous report we simultaneously adopted a CHO but calorie restriction feeding protocol whereas the present design incorporated a reduced CHO but isocaloric and protein matched feeding protocol in our HFAT trial. Given the similarities in metabolic adaptation to both CHO and calorie restriction, such data raise the question as to whether the enhanced mitochondrial responses observed when "training low" are due to transient periods of CHO restriction, calorie restriction or indeed, a combination of both. This point is especially relevant from an applied perspective 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 of sub-optimal energy availability (14).

In agreement with multiple studies demonstrating a role of both acute elevations in FFA availability (7, 23) as well as high fat feeding protocols (11), we also observed that post-exercise expression of PDK4, FAT/CD36 and CPT1 mRNA expression were elevated in the HFAT trial versus the HCHO trial. However, unlike Arkinstall et al. (4), we did not detect any suppressive effects of high fat availability on GLUT4 mRNA expression though a longer and more severe period of CHO restriction utilised by these investigators (i.e. 48 h of absolute CHO intake <1 g/kg body mass resulting in muscle glycogen levels <150 mmol.kg^{-1} dw) may explain the discrepancy between studies. Nonetheless, the dietary protocol studied here clearly alters the expression of genes with potent regulatory roles in substrate utilisation and if performed long term, may increase the capacity to use lipids as a fuel but induce suppressive effects on CHO metabolism (through suppression of the PDH complex) thus potentially limiting high-intensity performance (35). Whilst we did not directly quantify the signalling mechanisms underpinning these responses (owing to a lack of a muscle tissue), we suggest both p38MAPK and PPAR mediated signalling are likely involved. Indeed, using a twice per day exercise model, Cochran et al. (12) also observed enhanced p38MAPK phosphorylation during the afternoon exercise protocol (despite similar pre-exercise muscle glycogen availability) that was associated with the enhanced circulating FFA availability during the afternoon exercise. Furthermore, pharmacological ablation of circulating FFA availability during exercise suppresses p38MAPK compared with control conditions (40). Additionally, FFA mediated signalling can also directly mediate PPAR binding to the CPT1 promoter thereby modulating CPT1 expression (31).

We also examined the effects of post-exercise fat feeding on the regulation of p70S6K activity, a key signalling kinase associated with regulating MPS. In relation to the effects of endurance exercise *per se*, the majority of studies are typically limited to measures of phosphorylation

status with some studies reporting increases (25) and others, no change. When examined quantitatively using the [γ^{-32}P] ATP kinase assay, our data agree with previous observations from Apro et al. (3) who also reported no change but yet, conflict with recent data from our group where we observed an exercise-induced suppression of p70S6K activity (18). Nonetheless, the exhaustive (a fatiguing cycling HIT protocol) and muscle glycogen depleting (<100 mmol.kg^{-1} dw) nature of the latter exercise protocol versus the moderate-intensity nature of the afternoon SS running protocol studied here, likely explains the discrepancy between studies.

In relation to the effects of post-exercise feeding, we also provide novel data by demonstrating that post-exercise high fat feeding was associated with a suppression of p70S6K activity (albeit P=0.08) at 3 h post-completion of the SS exercise protocol when compared with the elevated response observed in HCHO (when using both a mean difference and standard deviation of differences of 50 fmol.min^{-1}.mg^{-1}, we estimate a sample size of 12-13 would be required to achieve statistical significance with 90% power, as calculated using Minitab statistical software, version 17). Although we did not measure circulating insulin levels in this study, it is of course possible that the suppressed p70S6K response observed here may be due to reduced upstream insulin mediated activation of protein kinase B (PKB). Indeed, we recently observed post-exercise p70S6K activity to be suppressed in conditions of simultaneous carbohydrate and calorie restriction in a manner associated with reduced insulin and upstream signalling of PKB (18). Alternatively, the suppression of p70S6K observed here may be mediated through direct effects of post-exercise high fat feeding that are independent of CHO availability, energy availability and insulin. Indeed, Stephens et al. (36) observed infusion of Intralipid and heparin to elevate circulating FFA concentrations attenuates MPS in human skeletal muscle in response to ingesting 21g amino acids under euglycemic hyperinsulemic clamp conditions. Furthermore,

Kimball et al. (21) also reported that high fat feeding impairs MPS in rat liver in a manner associated with reduced p70S6K phosphorylation, an effect that may be induced through sestrin 2 and sestrin 3 mediated impairment of mTORC signalling. Clearly, further research is required to examine the effects of high fat feeding on direct measures (and associated regulatory sites) of MPS within the physiological context of the exercising human.

In summary, we provide novel data by concluding that post-exercise high fat feeding has no modulatory affect on AMPK-α2 activity or the expression of those genes associated with regulatory roles in mitochondrial biogenesis. Furthermore, although post-exercise high fat feeding augmented the expression of genes involved in lipid transport and oxidation, we also observed a suppression of p70S6K1 activity despite sufficient post-exercise protein intake. This latter finding suggests that post-exercise high fat feeding may impair the regulation of muscle protein synthesis and post-exercise muscle remodelling, thereby potentially causing maladaptive responses for training adaptation if performed long-term. Future studies should now examine the functional relevance of the signalling responses observed here, not only in terms of acute muscle protein synthesis but also the chronic skeletal muscle and performance adaptations induced by long-term use of this feeding strategy.

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Conflicts of interest

The authors report no conflict of interest. The results of the present study do not constitute endorsement by ACSM.

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FIGURE 1 - Overview of the experimental protocol employed in each trial. HIT = 8 x 5mins running at a workload equal to 85% VO_{2peak} interspersed by 1min recovery. SS = 1-hour steady state running at a workload equal to 70% VO_{2peak}.

FIGURE 2 – (A) Skeletal muscle glycogen content, (B) AMPK-α2 and (C) p70S6K1 activity before HIT exercise and after the SS exercise protocol. # denotes significant difference from Pre-HIT, P<0.05. * denotes significant difference between conditions, P<0.05. $ denotes difference between conditions, P=0.08.

FIGURE 3 – (A) PGC-1 α, (B) PPAR, (C) p53, (D) Tfam, (E) CS, (F) ERRα, (G) PDK4, (H) GLUT4, (I) CPT1 and (J) CD36 mRNA before HIT exercise and after the SS exercise protocol. # denotes significant difference from Pre-HIT, P<0.05. * denotes significant difference between conditions, P<0.05.

TABLE 1 – Heart rate, RPE and substrate oxidation responses during the HIT protocol in both the HCHO and HFAT trials. * denotes significant difference from HIT-1, P<0.05.

TABLE 2 – Heart rate, RPE and substrate oxidation during the SS protocol in both the HCHO and HFAT trials. * denotes significant difference from 15 min, P<0.05. # denotes significant difference between conditions, P<0.05.

TABLE 3 – Plasma (A) glucose, (B) lactate, (C) NEFA, (D) glycerol and (E) β-OHB before and after the HIT and SS exercise protocols. # denotes significant difference from Pre-HIT, P<0.05. * denotes significant difference between conditions, P<0.05.

Figure 1

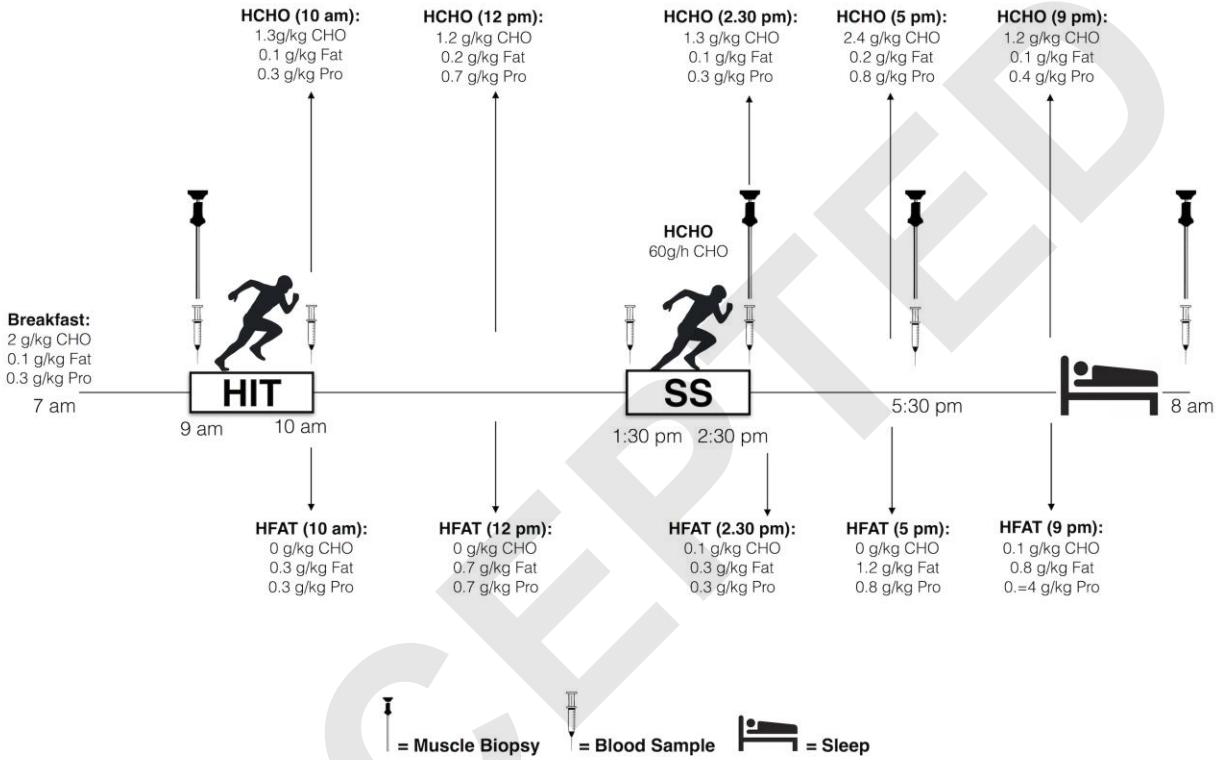

Figure 2

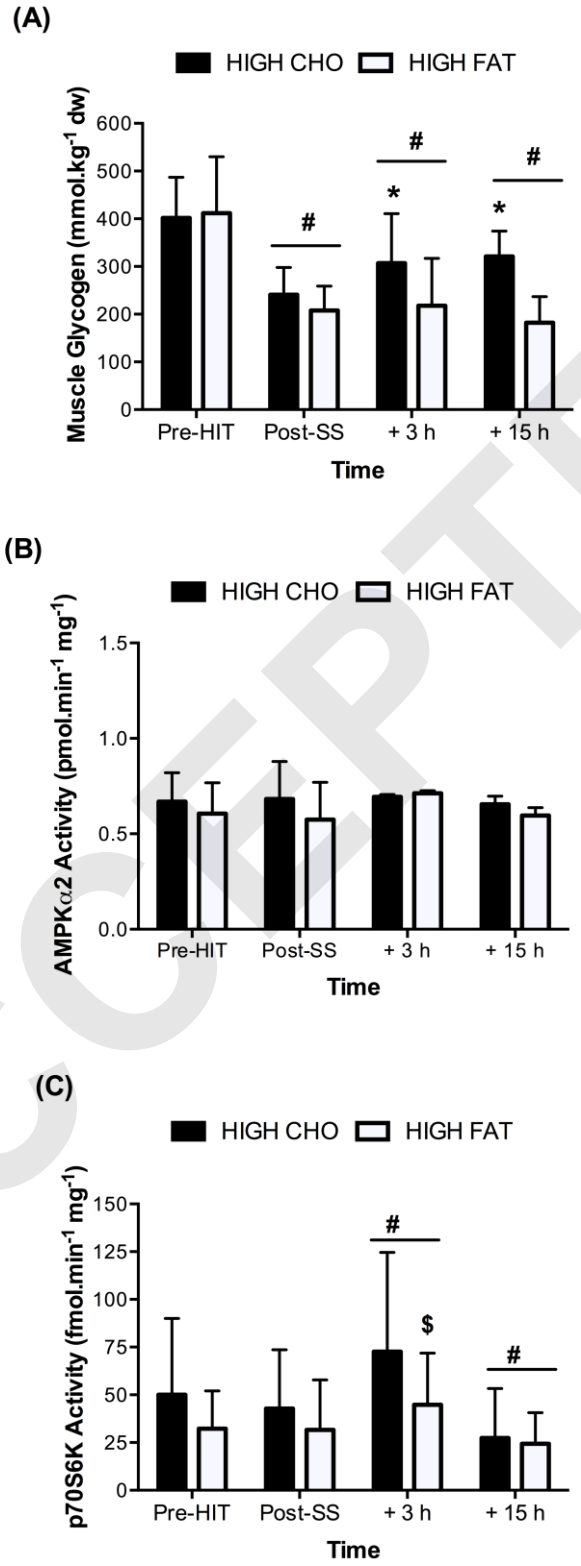

Figure 3

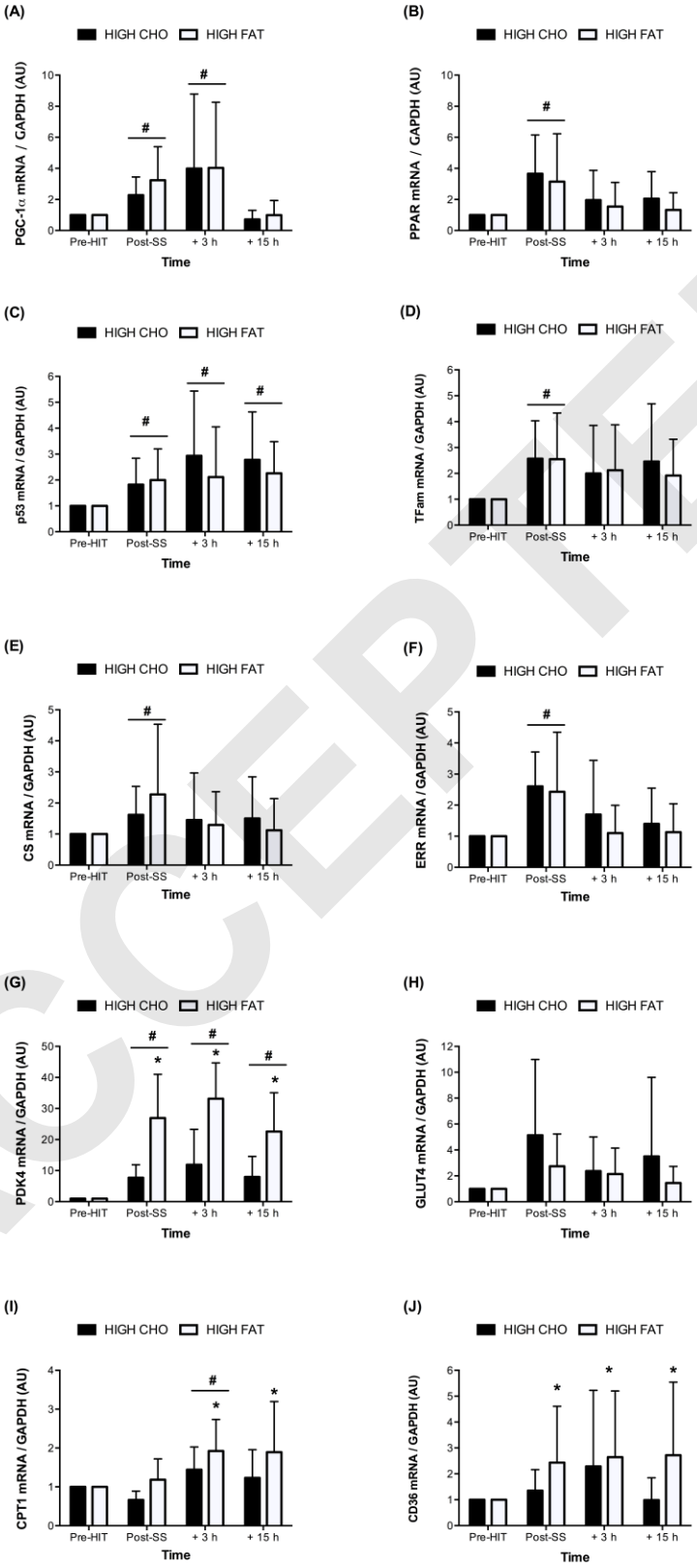

TABLE 1 – Heart rate, RPE and substrate oxidation responses during the HIT protocol in both the HCHO and HFAT trials. * denotes significant difference from HIT-1, P<0.05.

	HIT (Bout number)							
	1	2	3	4	5	6	7	8
Heart rate (b.min^{-1})								
HCHO	177 ± 9	181 ± 9	184 ± 8	185 ± 6*	186 ± 6*	185 ± 5	185 ± 5*	186 ± 7*
HFAT	173 ± 10	174 ± 8	180 ± 6	182 ± 7*	182 ± 7*	179 ± 6	182 ± 8*	184 ± 7*
RPE								
HCHO	14 ± 1.4	15 ± 1.5*	16 ± 2.0*	17 ± 1.6*	18 ± 0.9*	18 ± 1.0*	19 ± 0.6*	19 ± 0.7*
HFAT	14 ± 1.6	15 ± 1.8*	16 ± 1.9*	17 ± 1.1*	18 ± 1.2*	18 ± 1.0*	19 ± 0.9*	19 ± 1.03*
CHOoxidation (g.min^{-1})								
HCHO	5.5 ±2.6	5.1 ± 1.4	4.9 ± 1.4	4.4 ± 1.4	3.9 ± 2.1*	3.7 ± 1.9*	3.5 ± 2.3*	3.8 ± 2.7*
HFAT	5.5 ± 2.7	4.8 ± 1.8	4.5 ± 1.6	4.4 ± 1.5	4.1 ± 2.2*	3.6 ± 1.9*	3.6 ± 2.4*	3.7 ± 2.7*
Fat oxidation (g.min^{-1})								
HCHO	0.00 ± 0.29	0.00 ± 1.2	0.00 ± 1.4	0.06 ± 1.8	0.25 ± 2.2*	0.3 ± 2.5*	0.38 ± 3.1*	0.31 ± 3.5*
HFAT	0.00 ± 0.96	0.00 ± 1.1	0.03 ± 1.4	0.09 ± 1.8	0.19 ± 2.1*	0.3 ± 2.5*	0.37 ± 3.0*	0.34 ± 3.4*

TABLE 2 – Heart rate, RPE and substrate oxidation during the SS protocol in both the HCHO and HFAT trials. * denotes significant difference from 15 min, P<0.05. # denotes significant difference between conditions, P<0.05.

	Time (mins)			
	15	30	45	60
Heart rate				
(b.min^{-1})				
HCHO	166 ± 12	169 ± 11	170 ± 12*	172 ± 12*
HFAT	161 ± 11	165 ± 12	166 ± 10*	168 ± 10*
RPE				
HCHO	13 ± 1.3	14 ± 1.5*	15 ± 1.8	15 ± 1.8*
HFAT	13 ± 1.4	14 ± 1.8*	15 ± 2.1	16 ± 1.4*
CHO oxidation				
(g.min^{-1})				
HCHO	3.4 ±0.8	3.1 ± 1.2*#	3.5 ± 0.9*#	3.3 ± 0.6*#
HFAT	2.8 ±0.5	2.3 ± 0.4*#	2.1 ± 0.5*#	2.0 ± 0.6*#
Fat oxidation				
(g.min^{-1})				
HCHO	0.00 ± 0.30	0.13 ± 0.30*#	0.26 ± 0.31*#	0.35 ± 0.31*#
HFAT	0.32 ± 0.30	0.55 ± 0.20*#	0.65 ± 0.31*#	0.71 ± 0.40*#

TABLE 3 – Plasma glucose, lactate, NEFA, Glycerol and β-OHB before and after the HIT and SS exercise protocols. # denotes significant difference from Pre-HIT, P<0.05. * denotes significant difference between conditions, P<0.05.

		Pre HIT	Post HIT	Pre SS	Post SS	3-h Post SS	15-h Post SS
Glucose (mmol.L^{-1})							
	HCHO	5.2 ± 0.863	7.6 ± 0.74 #	4.7 ± 0.96*#	8.7 ± 1*#	5.9± 0.62 #	5.5 ± 0.3*
	HFAT	5.4 ± 0.84	7.6 ± 1.03 #	5.3 ± 0.34*#	5.6 ± 0.8*#	5.7 ± 0.6 #	5.1± 0.42*
Lactate (mmol.L^{-1})							
	HCHO	1.9 ± 0.42	7.8 ± 3.1 #	1.5 ± 0.3*#	1.6 ± 0.46 #	1.5 ± 0.16*#	1.1 ± 0.26 #
	HFAT	1.8 ± 0.41	7 ± 3.4 #	1.1 ± 0.22*#	1.3 ± 0.35 #	0.8 ± 0.21*#	0.9 ± 0.36 #
NEFA (mmol.L^{-1})							
	HCHO	0.01 ± 0.02	0.23 ± 0.27 #	0.09 ± 0.13 #	0.59 ± 0.56*#	0.09 ± 0.17*#	0.22 ± 0.22 #
	HFAT	0.05 ± 0.05	0.25 ± 0.28 #	0.32 ± 0.21 #	1.42 ± 0.74*#	0.48 ± 0.27*#	0.24 ± 0.14 #
Glycerol (µmol.L^{-1})							
	HCHO	14.8 ± 6.07	92.8 ± 27.1 #	22.9 ± 12*#	50.2 ± 37.4*#	13.2 ± 4.39*#	23.4 ± 20.1 #
	HFAT	12.9 ± 4.82	79.3 ± 25.4 #	33 ± 7.3*#	122.9 ± 57*#	40.8 ± 12*#	30.85 ± 12.6#
β-OHB (mmol.L^{-1})							
	HCHO	0.07 ± 0.02	0.14 ± 0.04 #	0.07 ± 0.02*#	0.15 ± 0.08*#	0.08 ± 0.02*#	0.08 ± 0.03
	HFAT	0.07 ± 0.02	0.14 ± 0.04 #	0.11 ± 0.05*#	0.33 ± 0.21*#	0.29 ± 0.2*#	0.19 ± 0.017