Graded reductions in pre-exercise muscle glycogen concentration impairs exercise capacity but does not augment cell signalling regulating mitochondrial biogenesis: Implications for CHO periodisation strategies

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Running title: CHO restriction and cell signalling

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Abstract

We examined the effects of graded muscle glycogen on exercise capacity and modulation of skeletal muscle signalling pathways associated with the regulation of mitochondrial biogenesis. In a repeated measures design, eight males completed a sleep-low, train-low model comprising an evening glycogen depleting cycling protocol followed by an exhaustive exercise capacity test (8 x 3 min at 80% PPO, followed by 1 min efforts at 80% PPO until exhaustion) the subsequent morning. Following glycogen depleting exercise, subjects ingested a total of 0 g kg^{-1} (L-CHO), 3.6 g kg^{-1} (M-CHO) or 7.6 g kg^{-1} (H-CHO) of carbohydrate during a 6 h period prior to sleeping, such that exercise was commenced the next morning with graded ($P < 0.05$) muscle glycogen concentrations (Mean ± SD) (L-CHO: 88 ± 43, M-CHO: 185 ± 62, H-CHO: 278 ± 47 mmol kg^{-1} dw). Despite differences ($P < 0.05$) in exercise capacity at 80% PPO between trials (L-CHO: 18 ± 7, M-CHO: 36 ± 3, H-CHO: 44 ± 9 min) exercise induced comparable $AMPK^{Thr172}$ phosphorylation (~4 fold) and PGC-1α mRNA expression (~5 fold) post- and 3 h post-exercise, respectively. In contrast, exercise nor CHO availability affected the phosphorylation of $p38MAPK^{Thr180/Tyr182}$, $CaMKII^{Thr268}$ or mRNA expression of p53, Tfam, CPT-1, CD36 or PDK4 Data demonstrate that when exercise is commenced with muscle glycogen below 300 mmol kg^{-1} dw, further graded reductions of 100 mmol kg^{-1} dw impairs exercise capacity but does not augment skeletal muscle cell signaling.

Keywords: muscle glycogen, mitochondrial biogenesis, train low, exercise capacity

New & Noteworthy

We provide novel data demonstrating that when exercise is commenced with muscle glycogen below 300 mmol kg^{-1} dw (as achieved using the sleep-low, train-low model) further graded reductions in pre-exercise muscle glycogen of 100 mmol kg^{-1} dw reduces exercise capacity at 80% PPO by 20-50% but does not augment skeletal muscle cell signalling.

Introduction

Skeletal muscle glycogen is recognised as the predominant energy substrate used during endurance exercise (14) and plays an important role in regulating the capacity to sustain exercise at a given workload (4, 13, 16). Additionally, muscle glycogen acts as a regulatory molecule (32) that is able to modulate cell signalling and transcriptional responses to exercise and subsequently augment selected skeletal muscle markers of training adaptation (e.g succinate dehydrogenase (29), citrate synthase (12) and β-hydroxyacyl-CoA-dehydrogenase (18) enzyme activity and cytochrome c oxidase subunit IV content (47)). Most notably, exercise commenced with reduced muscle glycogen (as defined as a 'train-low' session (40)) augments the AMPK-PGC-1α signaling axis (3, 35, 39, 46) and results in the augmented expression of target genes with putative roles in the regulation of mitochondrial biogenesis and substrate utilisation (3, 25, 33). Whilst a multitude of research designs have been used to study the physiological and molecular responses to 'train-low' exercise, the recently developed 'sleep-low, train-low' model (which requires athletes to perform an evening training session, restrict carbohydrate (CHO) during overnight recovery, and then complete a fasted training session the subsequent morning) provides a potent strategy to augment mitochondrial related cell signalling (3, 5, 25). Furthermore, repeated bouts of sleep-low, train-low is the only train-low model shown to enhance performance in trained endurance athletes (27, 28).

Given the enhanced training response associated with the sleep-low, train-low model is potentially regulated by muscle glycogen availability, it is prudent to consider the absolute glycogen concentrations required to facilitate this response. In this regard, examination of available data demonstrate that the augmented signalling and transcriptional responses associated with train-low models are particularly apparent when absolute pre-exercise muscle glycogen concentrations are ≤300 mmol kg^{-1} dw (20). Such data suggest the presence of a muscle glycogen threshold, whereby a critical absolute level of glycogen must be surpassed in

order to induce the augmented cell signalling responses associated with the train-low model (33). In accordance with data derived from acute exercise protocols, the notion of a glycogen threshold is also apparent when investigating selected skeletal muscle markers of training adaptation (20). For example, train-low sessions commenced with glycogen concentrations < 300 mmol kg^{-1} dw (12, 29, 47) result in augmented oxidative enzyme activity and/or content following 3-10 weeks of training. In contrast, when 'train-low' sessions are commenced with markedly higher pre-exercise muscle glycogen concentrations (400-500 mmol kg^{-1} dw) skeletal muscle markers of training adaptation are not augmented (11). Nonetheless, whist training with low muscle glycogen augments selected signalling events, absolute training volume (19) and/or intensity (18, 24, 47) may be reduced due to a lack of muscle substrate and/or an impairment in the contractile apparatus of skeletal muscle (10, 30). When taken together, the challenge that exists is to therefore facilitate the pro-signalling environment whilst simultaneously maintaining the ability to complete the desired workload and intensity in order to promote training adaptation.

With this in mind, the aim of the present study was to examine the effects of graded pre-exercise glycogen concentrations on both exercise capacity and the modulation of selected skeletal muscle signalling pathways with putative roles in the regulation of mitochondrial biogenesis. Our model of graded pre-exercise muscle glycogen was achieved through a sleep-low, train-low model that adopted CHO intakes considered practically viable (within the time-course of sleep-low designs) and representative of real-world refeeding strategies. Whilst the use of such sleep-low, train-low models are primarily designed for athletic populations, the use of recreational populations allows for a greater understanding of the molecular events that occur in response to such 'train-low' designs, given the difficulties of collecting muscle biopsies from elite athletes. We hypothesised that the activation of skeletal muscle signalling pathways would be proportionally dependent on pre-exercise muscle glycogen concentrations.

Methods

Participants

Eight recreationally active males (mean ± SD: age, 22 ± 3 years; body mass 76.0 ± 12.7 kg; height, 177.9 ± 5.7 cm) took part in this study. Mean $\dot{V}O_{2peak}$ and peak power output (PPO) for the cohort were 48.9 ± 7.0 mL kg^{-1} min^{-1} and 273 ± 21 W, respectively. None of the subjects had any history of musculoskeletal or neurological disease nor were they under any pharmacological treatment during the course of the testing period. All subjects provided written informed consent and all procedures conformed to the standards set by the Declaration of Helsinki (2008). The study was approved by the local Research Ethics Committee of Liverpool John Moores University.

Experimental Design

Using a sleep-low, train-low model and a repeated measures design, with each experimental trial separated by a minimum of 7 days, subjects undertook an evening bout of glycogen depletion exercise followed by the consumption of graded quantities of CHO (L-CHO: 0 g, M-CHO: 3.6 g kg^{-1}, H-CHO: 7.6g kg^{-1}) across a 6 h period, so that exhaustive exercise was commenced the next morning with three different levels of pre-exercise muscle glycogen concentrations. Skeletal muscle biopsies were obtained from the vastus lateralis immediately before, post- and 3 h post-exercise. During the H-CHO and M-CHO trials, an additional muscle biopsy was obtained at a matched time point corresponding to the point of exhaustion in the L-CHO trial, allowing for work-matched comparison between trials. Consequently, all subjects completed the L-CHO trial first, whilst the subsequent M-CHO and H-CHO trials were completed in a randomised and counterbalanced order. An overview of the experimental protocol is shown in Figure 1.

Assessment of peak oxygen uptake

At least 7 days prior to experimental trials, all subjects were assessed for peak oxygen consumption ($\dot{V}O_{2peak}$) and peak power output (PPO) on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, Netherlands). Following the completion of a 10 min warm-up at 75 W, the test began at 100 W and consisted of 2 minute stages with 30 W increments in resistance until volitional exhaustion. $\dot{V}O_{2peak}$ was stated as being achieved by the following end-point criteria: (1) heart rate within 10 beats min^{-1} of age-predicted maximum, (2) respiratory exchange ratio > 1.1 and (3) plateau of oxygen consumption despite increased workload. Peak aerobic power was taken as the final stage completed during the incremental test.

Overview of sleep-low, train-low model

Phase 1: Glycogen depletion exercise

In the 24 h preceding glycogen depleting exercise (i.e. from 12 pm the day prior), subjects consumed a standardised high CHO diet (8 g kg^{-1} CHO, 2 g kg^{-1} protein and 1 g kg^{-1} fat) and refrained from alcohol and vigorous physical exercise for the previous 48 h. The standardised diet consisted of 3 main meals and 3 CHO rich snacks, with subjects required to stop eating 3 h prior to commencing glycogen depleting exercise. On the day of glycogen depleting exercise, subjects reported to the laboratory at approximately 3 pm to perform a bout of intermittent glycogen depleting cycling, as previously completed in our laboratory (19, 43). The pattern of exercise and total time to exhaustion in the subject's initial trial was recorded and replicated in all subsequent trials. Subjects were permitted to consume water *ad libitum* during exercise, with the pattern of ingestion replicated during subsequent trials.

Phase 2: Carbohydrate re-feeding strategy

To facilitate our overnight sleep-low model, subjects were fed 30 g of whey protein isolate (Science in Sport, Nelson, UK) mixed with 500 ml of water immediately following the

cessation of glycogen depleting exercise to reflect real-world practice as per current nutritional guidelines (44). Subjects in the L-CHO trial then refrained from eating for the remainder of the evening whereas subjects within the M-CHO and H-CHO trials were provided with a mixture of CHO drinks (Maltodextrin, Science in Sport, Nelson UK) and gels (GO isotonic energy gel, Science in Sport, Nelson UK) to be consumed at hourly intervals. In the M-CHO trial, subjects were provided with CHO at a rate of 1.2 g kg^{-1} h^{-1} for 3 h whereas subjects in the H-CHO trial were provided with 1.2 g kg^{-1} h^{-1} for 3 h followed by a high carbohydrate meal (4 g kg^{-1} CHO, 51 ± 1 g protein and 17 ± 1 g fat) consisting of bread, soup, rice, fresh juice, rice pudding and jam after 4 h of recovery. In this way, total CHO intakes in the L-CHO, M-CHO and H-CHO trials equated to 0, 3.6 and 7.6 g kg^{-1}, respectively, with fluid intake allowed *ad libitum.*

Phase 3: High intensity interval cycling and exercise capacity test

To facilitate our train-low exercise session, subjects arrived the subsequent morning between 8-9 am, in a fasted state, where a venous blood sample was collected from the antecubital vein and a muscle biopsy taken from the vastus lateralis. Subjects then completed the high-intensity interval (HIIT) cycling protocol, consisting of 8 x 3 min intervals at 80% PPO, interspersed with 1 min rest. During exercise, heart rate (HR) was continuously measured and the final HR for each 3 min interval was recorded, whilst ratings of perceived exertion (RPE) were recorded upon completion of each interval. Expired gas was collected via a mouthpiece connected to an online gas analysis system (CPX Ultima, Medgraphics, Minnesota, US) for the final 1.5 min of each interval and substrate utilisation was assessed using the equations of Jeukendrup & Wallis (22) given the validity of indirect calorimetry for the assessment of substrate utilisation at exercise intensities up to 80-85% $\dot{V}O_{2max}$ (37). Upon completion of the high-intensity cycling protocol, subjects were provided with 5 min of active recovery prior to commencing an exercise capacity test consisting of intermittent "1 min efforts" corresponding to 80% PPO interspersed with 1 min recovery periods at 40% PPO. This intermittent protocol was followed until the

subjects reached volitional exhaustion and has been previously utilised in our laboratory (19). Following the completion of the exercise capacity test and collection of the post-exercise biopsy, subjects were fed 30 g whey protein (Science in Sport, Nelson, UK) mixed with 500 ml of water.

Blood analysis

Venous blood samples were collected in vacutainers containing K_2EDTA, lithium heparin or serum separation tubes and stored on ice or at room temperature until centrifugation at 1500 g for 15 min at 4^{o}C. Samples were collected immediately prior to exercise, at the point of exhaustion (post exercise) and 3 h post exercise, whilst an additional sample was obtained at a time point corresponding to the point of exhaustion in the L-CHO trial during the M-CHO and H-CHO trials. Plasma was aliquoted and stored at -80^{o}C until analysis. Samples were later analysed for plasma glucose, lactate, non-esterified fatty acids (NEFA) and glycerol using commercially available enzymatic spectrophotometric assays (RX Daytona Analyser, Randox, UK) as per manufacturer instructions.

Muscle biopsies

Skeletal muscle biopsies (~20 mg) were obtained from the vastus lateralis immediately prior to exercise, at the point of exhaustion (post exercise) and 3 h post exercise. During the M-CHO and H-CHO trials, an additional muscle biopsy was obtained at a time point corresponding to the point of exhaustion in the L-CHO trial, thereby allowing for 'work-matched' comparison between trials. For the work-matched biopsy, subjects dismounted the cycle ergometer and were moved to the adjacent biopsy suite. Following collection of the biopsy sample (~5 min), subjects recommenced cycling exercise. Muscle biopsies were obtained from separate incision sites 2-3 cm apart using a Bard Monopty Disposable Core Biopsy Instrument (12 gauge x 10

246 cm length, Bard Biopsy Systems, Tempe, AZ, USA) under local anaesthesia (0.5% Marcaine)
247 and immediately frozen in liquid nitrogen and stored at -80ºC for later analysis.
248
249 **Muscle glycogen concentration**
250 Muscle glycogen concentrations were determined according to the acid hydrolysis method
251 described by Van Loon et al (26). Approximately 2-5 mg of freeze-dried tissue was powdered,
252 dissected of all visible blood and connective tissue and subsequently hydrolysed by incubation
253 in 500 µl of 1 M HCl for 3 h at 95ºC. After cooling to room temperature, samples were
254 neutralised by the addition of 250 µl 0.12 mol L^{-1} Tris/2.1 mol L^{-1} KOH saturated with KCl.
255 Following centrifugation, 200 µl of supernatant was analysed in duplicate for glucose
256 concentration according to the hexokinase method using a commercially available kit (GLUC-
257 HK; Randox Laboratories, Antrim, UK). Glycogen concentration is expressed as mmol kg^{-1}
258 dry weight and intra-assay coefficients of variation were <5%.
259
260 **RNA isolation and analysis**
261 Muscle samples (~20 mg) were homogenised in 1 ml TRIzol reagent (Thermo Fisher Scientific,
262 UK) and total RNA isolated according to the manufacturer's guidelines. Concentrations and
263 purity of RNA were assessed by UV spectroscopy at optical densities (OD's) of 260 and 280
264 nm, using a Nanodrop 3000 (Fisher, Rosklide, Denmark) with an average 260/280 ratio of 1.9
265 ± 0.1. A quantity of 70 ng RNA was used for each 20 µl PCR reaction.
266
267 **Reverse transcriptase quantitative Real-Time Polymerase Chain Reaction (rt-qRT-PCR)**
268 RT-PCR amplifications were performed using QuantiFast™ SYBR® Green RT-PCR one-step
269 kit on a Rotogene 300Q (Qiagen, Crawley, UK) supported by Rotogene software (Hercules,
270 CA). RT-qTR-PCR was performed as follows: hold 50ºC for 10 min (reverse
271 transcription/cDNA synthesis), 95ºC for 5 min (transcriptase inactivation and initial

denaturation step), and PCR steps of 40 cycles; 95ºC for 10 sec (denaturation), 60ºC for 30 sec (annealing and extension). Upon completion, dissociation/melting curve analyses were performed to reveal and exclude nonspecific amplification or primer-dimer issues (all melt analysis in this study presented single reproducible peaks for each target gene suggesting amplification of a single product). Following initial screening of suitable reference genes, GAPDH showed the most stable C_t values across all RT-PCR runs and subjects, regardless of experimental condition (25.3 ± 1.0) and was therefore selected as the reference gene in all RT-PCR assays. The average PCR efficiency for all RT-PCR runs (90 ± 2%) was similar for all genes across all time points and experimental conditions. As such, the relative gene expression levels were calculated using the comparative C_t ($^{\Delta\Delta}C_t$) equation (38) where the relative expression was calculated as $2^{-\Delta\Delta ct}$ where C_t represents the threshold cycle. mRNA expression for all target genes was calculated relative to the reference gene (GAPDH) within the same subject and condition and relative to the pre-exercise value in the H-CHO condition.

SDS page & Western blotting

Muscle samples (~20 mg) were powdered on dry ice and homogenised (FastPrep-24™ 5G Instrument) for 2 x 40 s at 6 $m.s^{-1}$ in 10-fold mass of ice-cold lysis homogenisation buffer (10% glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 7.5), 1% NP-40, 20 mM β-glycerphosphate, 10 mM NaF, 1 mM EDTA (pH 8.8), 1 mM EGTA (pH 8.8), 3 mM Benzamidine, 1 mM 1,4-Dithiothreitol, 1% Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich), 1% Phosphatase Inhibitor Cocktail 3 (Sigma), 4.8% complete Mini Protease Inhibitor Cocktail (Roche)). The resulting homogenate was centrifuged at 4°C for 10 min at 8000 g and the supernatant used for the determination of protein concentrations using the DC protein assay (Bio-Rad, California, USA). Samples were resuspended in 4X Laemlli buffer, boiled for 5 min and separated by SDS-PAGE before being transferred to nitrocellulose membranes (Pall Life Sciences, Pensacola, Florida, USA). Following transfer, membranes were stained for protein

with Ponceau S (Sigma-Aldrich, Gillingham, UK), blocked in TBS-Tween containing 3% non-fat milk for 1 h and incubated overnight in primary antibodies (AMPKα (2603), p-AMPK^{Thr172} (2531), ACC (3676), p-ACC^{Ser79} (3661), p38MAPK (9212), p-p38MAPK$^{Thr180/Tyr182}$ (4511), CaMKII (3362) and p-CaMKII^{Thr268} (12716) from Cell Signaling Technologies), before incubation in relevant secondary antibodies (anti-rabbit (7074) from Cell Signaling Technologies) for 1 h at room temperature. Proteins were detected via chemiluminescence (Millipore, Watford, UK) and quantified by densitometry using GeneTools software (Syngene, Cambridge, UK). Sufficient muscle was available for Western blot analysis for seven subjects. Data is reported as the phosphorylated protein of interest normalised to total protein and each timepoint is reported relative to the pre-exercise value in the H-CHO condition.

Statistical analysis

All statistical analyses were performed using Statistical Package for the Social Sciences (SPSS Version 24). Comparison of average physiological responses and exercise capacity were analysed using a one-way repeated-measures general linear model whereas changes in physiological and molecular responses between conditions (i.e. muscle glycogen, mRNA expression and activity of signalling molecules) were analysed using a two-way repeated measures general linear model, where the within factors were time and condition. Here, the post-exercise sampling point in the L-CHO trial was also used as the work-matched sampling point as this corresponded to the same sampling point and allowed for comparison between trials. Where a significant main effect was observed, pairwise comparisons were analysed according to Bonferroni post-hoc tests in order to locate specific differences. All data in text, figures and tables are presented as means ± SD with P values ≤ 0.05 indicating statistical significance.

Results

Skeletal muscle glycogen and exercise capacity

The exercise and nutritional strategy employed was successful in achieving graded levels of pre-exercise muscle glycogen (H-CHO; 278 ± 47, M-CHO; 185 ± 62, L-CHO; 88 ± 43 mmol kg^{-1} dw) such that exercise was commenced with three distinct levels of muscle glycogen (P = 0.016) (Figure 2A). Exhaustive exercise significantly reduced (P < 0.001) muscle glycogen concentration to comparable levels (<100 mmol kg^{-1} dw) with no difference between conditions (P = 0.11). In accordance with the observed differences in pre-exercise muscle glycogen concentration, total exercise time spent at 80% PPO in the H-CHO trial (44 ± 9 min) was significantly greater than both M-CHO (36 ± 3 min) (P = 0.037) and L-CHO (18 ± 6 min) trials (P < 0.001) whilst the M-CHO trial was significantly greater than the L-CHO trial (P < 0.001) (Figure 2B). Given the low pre-exercise muscle glycogen concentration of subjects in the L-CHO trial, 6 of the 8 subjects were unable to complete the prescribed HIIT protocol. As such, exercise capacity data is presented as the total amount of time spent at 80% PPO and is inclusive of the exercise performed during the prescribed HIIT protocol and subsequent capacity test and excludes all time spent at rest/recovery.

Physiological and metabolic responses to exercise

Subject's average heart rate (Figure 3A) across the HIIT session, when matched for work done (H-CHO; 163 ± 16, M-CHO; 167 ± 15, L-CHO; 171 ± 17 $beats.min^{-1}$) was significantly higher in the L-CHO trial compared with H-CHO (P = 0.031) only. Similarly, subject's average RPE (Figure 3B) across the HIIT session (H-CHO; 13 ± 1, M-CHO; 14 ± 1, L-CHO; 16 ± 1 $beats.min^{-1}$) was significantly higher in the L-CHO trial compared with both M-CHO (P = 0.041) and H-CHO (P = 0.012) trials, respectively. Exhaustive exercise resulted in a significant reduction in plasma glucose (P = 0.036), where plasma glucose was significantly lower in the L-CHO trial when compared with the H-CHO trial only (P = 0.015) (Figure 3C). Exhaustive exercise resulted in a significant increase in plasma lactate (P = 0.001), NEFA (P < 0.001) and

glycerol ($P = 0.012$) but did not display any significant differences between trials (Figures 3D, E and F, respectively). However, when matched for work done, plasma NEFA ($P = 0.01$) and plasma glycerol ($P = 0.017$) was increased to a significantly greater extent in the L-CHO trial when compared with the H-CHO trial only. In addition, subjects in the L-CHO trial oxidised significantly less CHO ($P = 0.048$) and greater amounts of lipid ($P = 0.004$) when compared with the H-CHO trial only (Figure 3G and H, respectively).

Regulation of mitochondrial biogenesis related cell signalling

Exhaustive exercise induced significant increases in $AMPK^{Thr172}$ phosphorylation ($P = 0.017$) but did not display any significant differences between trials ($P = 0.548$) (Figure 4A). Similarly, exhaustive exercise induced significant increases in ACC^{Ser79} phosphorylation ($P = 0.005$), although phosphorylation was higher in the M-CHO trial when compared with the L-CHO trial only ($P = 0.021$) (Figure 4B). When exercise duration was matched to the post-exercise sampling point in the L-CHO group, the increase in $AMPK^{Thr172}$ phosphorylation remained comparable between groups ($P = 0.269$) and the increase in ACC^{Ser79} phosphorylation still remained higher in the M-CHO trial when compared with the L-CHO trial ($P = 0.021$). In contrast, exhaustive exercise did not induce phosphorylation of $p38MAPK^{Thr180/Tyr182}$ ($P = 0.656$) (Figure 4C) or $CaMKII^{Thr286}$ ($P = 0.707$) (Figure 4D). Representative Western blots are shown in Figure 4E. With regard to exercise induced gene expression, exhaustive exercise induced a significant increase in PGC-1α mRNA expression at 3 h post-exercise ($P = 0.001$) but did not display any significant differences between trials (Figure 5A). In contrast, p53, Tfam, CPT-1, CD36 and PDK4 mRNA expression (Figures 5B, C, D, E, F, respectively) was unaffected by either glycogen availability or the exhaustive exercise protocol ($P > 0.05$).

Discussion

Using a sleep-low, train-low model, we examined the effects of three distinct levels of pre-exercise muscle glycogen on exercise capacity and the modulation of selected skeletal muscle signalling pathways with putative roles in mitochondrial biogenesis. We provide novel data by demonstrating that 1) graded reductions in pre-exercise muscle glycogen of 100 mmol kg^{-1} dw reduce exercise capacity at 80% PPO by ~20-50% and 2) despite significant differences in pre-exercise muscle glycogen availability, we observed comparable increases in $AMPK^{Thr172}$ phosphorylation and PGC-1α mRNA. In contrast to our hypothesis, these data suggest that graded levels of muscle glycogen below 300 mmol kg^{-1} dw do not augment skeletal muscle cell signalling, a finding that may be related to the fact that commencing exercise with <300 mmol kg^{-1} dw is already a critical level of absolute glycogen (as suggested by Impey et al. (20)) that is required to induce a metabolic milieu conducive to cell signalling. In relation to the goal of promoting cell signalling, our data therefore suggest that reducing pre-exercise glycogen concentrations below 300 mmol kg^{-1} dw does not confer any additional benefit within the context of the sleep-low, train-low model.

To achieve our intended model of graded glycogen concentrations, we adopted a sleep-low, train-low design whereby subjects performed an evening bout of glycogen depleting exercise and subsequently ingested three graded quantities of CHO that were practically viable within the time-course of the sleep-low model. This strategy was effective in achieving graded differences in pre-exercise muscle glycogen concentration (278 vs. 185 vs. 88 mmol kg^{-1} dw in H-CHO, M-CHO and L-CHO, respectively) and represent muscle glycogen resynthesis rates (approximately 30 mmol kg^{-1} h^{-1}) commonly observed with CHO feeding rates of 1-1.2 g kg^{-1} h^{-1} (21). A novel aspect of our chosen study design was that we employed a sampling point in both the H-CHO and M-CHO trials that was matched to the point of exhaustion in the L-CHO trial, thus allowing for the assessment of mitochondrial related signalling events at both work-matched and exhaustive exercise time points, whilst also allowing for the assessment of

401 exercise capacity. In accordance with differences in muscle glycogen, both NEFA availability
402 and lipid oxidation were greater in the L-CHO trial when compared with the H-CHO trial at
403 the work-matched sampling point. However, at the point of exhaustion, plasma NEFA and
404 glycerol were comparable between all conditions which is likely reflective of the post-exercise
405 muscle glycogen concentrations in all three conditions given the well documented effects of
406 muscle glycogen (2) on substrate utilisation during exercise.
407
408 Consistent with the well documented effects of muscle glycogen on exercise capacity (4, 16)
409 we observed that even small differences in pre-exercise muscle glycogen concentrations (~100
410 mmol kg^{-1} dw) can induce changes in exercise capacity at 80% PPO of between ~20-50% (8-
411 18 minutes). Whilst we acknowledge that the lack of blinding to each experimental condition
412 may have influenced exercise capacity (despite subjects receiving no feedback during
413 exercise), it is unclear whether prior knowledge of CHO intake alone would enhance exercise
414 performance (17). Nonetheless, these data are consistent with previous data (1, 6) that suggest
415 differences in muscle glycogen of 100-120 mmol kg^{-1} dw enhance exercise capacity at 70%
416 $\dot{V}O_{2max}$ by 5-12 minutes. As such, the 8 minute difference in exercise capacity between M-
417 CHO and H-CHO trials is likely more representative of changes in muscle glycogen
418 concentration. Whilst we consider that the present data may help to characterise what is
419 considered a worthwhile change in absolute muscle glycogen concentration in determining
420 exercise capacity, we acknowledge these changes should be considered in the context of each
421 individual given the interindividual variability between subjects in the present study.
422 Furthermore, as the capacity for glycogen storage is enhanced and its utilisation during exercise
423 reduced amongst well-trained populations (1, 15, 23) such small differences in muscle
424 glycogen (as observed within the present study) may allow for extended exercise times amongst
425 well-trained individuals.
426

In relation to post-exercise mitochondrial related signalling, it is widely accepted that commencing work-matched exercise protocols with reduced muscle glycogen induces greater skeletal muscle signalling (20). For example, $AMPK^{Thr172}$ phosphorylation (46), AMPK-α2 activity (45) and nuclear abundance (39) are all augmented when acute exercise is commenced with reduced pre-exercise muscle glycogen. In contrast, we observed no enhancement in $AMPK^{Thr172}$ or ACC^{Ser79} phosphorylation at our work-matched time point (i.e. following the completion of ~20 min high-intensity cycling) despite graded reductions in pre-exercise muscle glycogen concentrations. This apparent lack of augmented cell signalling may be explained by subjects already commencing exercise with pre-exercise glycogen concentrations below 300 mmol kg^{-1} dw, an absolute concentration that was previously suggested to facilitate the enhanced cell signalling responses associated with low glycogen availability (20). Indeed, our range of pre-exercise muscle glycogen concentrations are distinctly lower than previous work that report greater skeletal muscle signalling following work-matched exercise protocols. For example, high glycogen trials are commonly commenced with muscle glycogen concentrations between 400 and 600 mmol kg^{-1} dw (3, 36) and remain above 300 mmol kg^{-1} dw post-exercise (3, 36, 45). In such instances, these researchers observed attenuated (45) or abolished (3, 36) activation of cell signalling pathways. Interestingly, despite the completion of significantly more work in both the M-CHO and H-CHO trials, no further increases in $AMPK^{Thr172}$ phosphorylation were observed following exhaustive exercise. Whilst both AMPK activity and ACC phosphorylation are known to be regulated by exercise duration (41) these responses appear to be closely linked to changes in muscle glycogen concentrations (9, 41). With this in mind, the lack of augmented signalling in response to further exercise in the present study may be explained by the relatively small changes in muscle glycogen from the work-matched time point to exhaustion.

In contrast to AMPK and ACC, we did not observe any change in the phosphorylation status of p38MAPK$^{Thr180/Tyr182}$ or CAMKII ^{Thr286} either in response to exercise or muscle glycogen concentration, though we note the large inter-individual variability and recommend the use of larger sample sizes in future. These data are in agreement with previous work that demonstrate no change in p38MAPK or CAMKII phosphorylation using a variety of train-low methodologies, including sleep-low, train-low (3, 25), twice-per day training (46) and fasted training (42). Whilst augmented p38MAPK phosphorylation has been observed when pre-exercise muscle glycogen is reduced (163 vs. 375 mmol kg^{-1} dw), this is only apparent within the nucleus and not the cytoplasm (7). As such, further work should utilise cellular fractionation methodologies in order to investigate the cellular localisation of such exercise-inducible kinases.

Despite the observed augmented mRNA expression of PGC-1α within the post-exercise recovery period, exhaustive exercise did not augment the mRNA expression of other mitochondrial (p53 or Tfam) or substrate utilization related genes (PDK4, CPT1 or CD36). Although the time-course of mRNA expression for these genes is not well understood, the lack of change in mRNA expression in the present study may be explained by our chosen sampling points in accordance with our sleep-low, train-low exercise model. Indeed, given that our pre-exercise biopsy was sampled within ~14 h of glycogen depleting exercise, it is difficult to determine whether mRNA expression was already elevated at pre-exercise. For instance, time-course studies have revealed that the mRNA expression of Tfam (31), PDK4 and CPT1 (34) is enhanced for up to 24 h post-exercise which coincides with our pre-exercise sampling time point (~14 h between the two exercise bouts). However, given the time-course of phosphorylation of our chosen protein targets (8) it is highly unlikely that any of these proteins would be phosphorylated at pre-exercise as a result of the previous evenings glycogen depletion exercise.

Practically, these data suggest that in the context of the sleep-low, train-low model, where muscle glycogen is depleted to very low levels (~100 mmol kg^{-1} dw), insufficient time is available to restore muscle glycogen to normal levels. As such, individuals undertaking sleep-low, train-low models, that reduces muscle glycogen to very low levels, should consume CHO in accordance with the energetic requirements of the subsequent morning session, given that withholding CHO intake overnight appears to confer no additional benefit in relation to cell signalling but impairs exercise capacity. In contrast, it appears that when muscle glycogen is not depleted to such low levels (> 300 mmol kg^{-1} dw), withholding CHO intake in the post-exercise period may prolong the acute cell signalling and gene expression responses (25, 34). With this in mind, it should be noted that driving glycogen depletion below 300 mmol kg^{-1} dw would likely be more difficult and require considerably more work in well-trained individuals (11) given they display an enhanced capacity for glycogen storage and reduced utilisation during exercise (1, 15, 23). In practice, it appears that careful consideration of the individuals training status and the metabolic demands of each training session is required to ensure appropriate day-to-day periodisation of CHO in order to ensure absolute training intensity is not compromised whilst also creating a metabolic milieu conducive to facilitating the metabolic adaptations associated with 'train low'.

In summary, we provide novel data by demonstrating that graded reductions in pre-exercise muscle glycogen below 300 mmol kg^{-1} dw (as achieved using a sleep-low, train-low model) impairs exercise capacity but does not augment skeletal muscle cell signalling responses. Practically, our data suggest that, within the context of the sleep-low, train-low model (when muscle glycogen is depleted to very low levels) overnight CHO restriction is not required to augment skeletal muscle cell signalling, and thus, CHO should be consumed in accordance with the metabolic demands of the subsequent morning session. Future studies should

investigate step-wise reductions in pre-exercise muscle glycogen, within a wider range (i.e. 100-600 mmol kg^{-1} dw), in order to investigate the existence of a potential glycogen threshold (20) and allow for a better definition of its potential upper and lower limits.

Disclosures

No conflicts of interest, financial or otherwise, are declared by the authors

Author contributions

MAH, AS, JPM and JBL conception and design of research; MAH, KMH, RAS, SOS, APS, JPM, JBL performed experiments; MAH, BS, APS, JPM, JBL analyzed data; MAH, JPM and JBL interpreted results of experiments; MAH, JPM and JBL prepared the figures; MAH, JPM and JBL drafted the manuscript; MAH, KMH, RAS, BS, SOS, AP APS, JPM, JBL edited and revised the manuscript; MAH, KMH, RAS, BS, SOS, AP APS, JPM, JBL approved the final version of manuscript.

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Figure legends

Figure 1. Schematic overview of the experimental sleep-low, train-low protocol. Following 24 h of standardised dietary conditions, subjects completed an evening bout of glycogen depleting cycling exercise. Upon completion, subjects received three graded levels of CHO in order to manipulate pre-exercise muscle glycogen the subsequent morning. Following an overnight fast, subjects completed an exhaustive bout of cycling exercise. Muscle biopsies were obtained pre-exercise, at the point of exhaustion (post exercise) and 3 h post exercise. During H-CHO and M-CHO trials, an additional muscle biopsy was obtained at a time point corresponding to the point of exhaustion in the L-CHO trial, allowing for work-matched comparison between trials.

Figure 2. (A) Skeletal muscle glycogen concentration and (B) Exercise capacity at 80% PPO (reflective of set work protocol plus time to exhaustion). $^{\#}P < 0.05$, significantly different from pre-exercise, §$P < 0.05$, significantly different from H-CHO, ‡$P < 0.05$, significantly different from M-CHO. Data is presented as means and individual data points represent individual subjects. N=8

Figure 3. (A) Heart rate, (B) RPE and plasma (C) Glucose, (D) Lactate, (E) NEFA (F) glycerol pre-exercise, at work-matched time points and post-exercise, (E) Average CHO and (F) lipid oxidation during exercise. $^{\#}P < 0.05$, significantly different from pre-exercise, §$P < 0.05$, significantly different from H-CHO. Data is presented as mean ± SD (A-D) and individual data points represent individual subjects (E & F). N=8

Figure 4. (A) AMPK^{Thr172} phosphorylation, (B) ACC^{Ser79} phosphorylation, (C) p38$^{Thr180/Tyr182}$ phosphorylation, (D) CaMKII^{Thr286} phosphorylation and (E) representative Western blot images at pre-exercise, work-matched time point and post-exercise. $^{\#}P < 0.05$, significantly different from pre-exercise, *$P < 0.05$, significantly different from L-CHO. Data is presented as means and individual data points represent individual subjects. N=7

693 **Figure 5.** (A) PGC-1α, (B) p53, (C) Tfam, (D) CPT-1, (E) CD36 and (F) PDK4 mRNA
694 expression pre- and 3 h post-exercise. $^{\#}P < 0.05$, significantly different from pre-exercise. Data
695 is presented as means and individual data points represent individual subjects. N=8
696

Figure 1

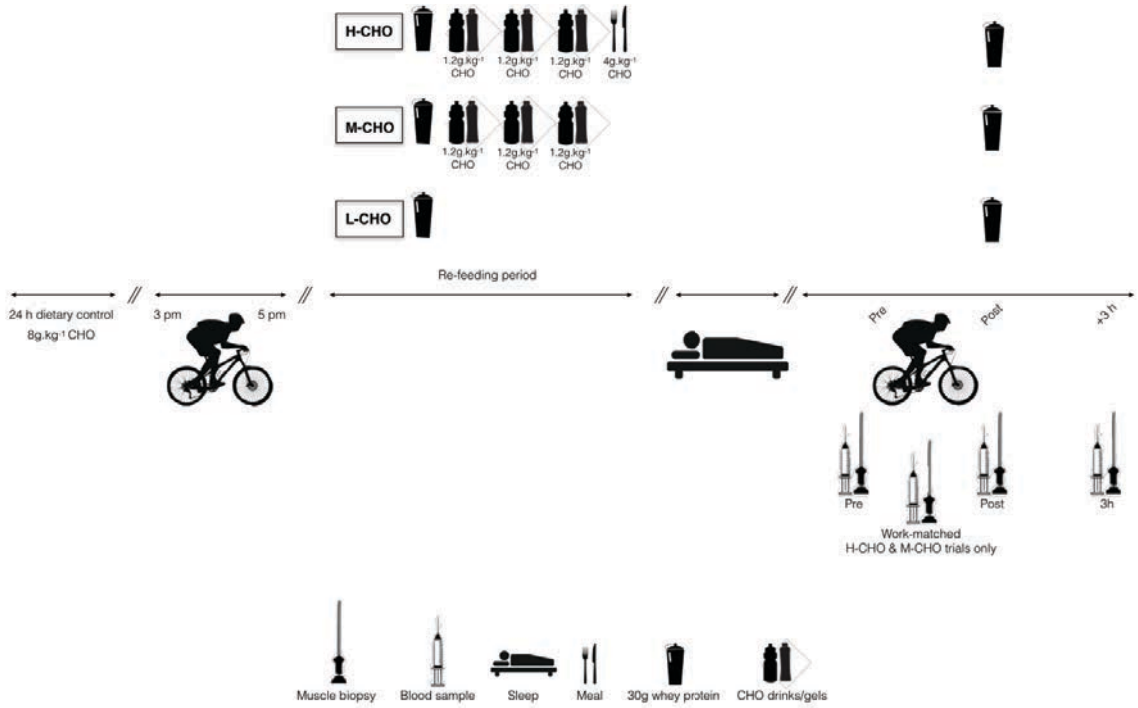


Figure 2

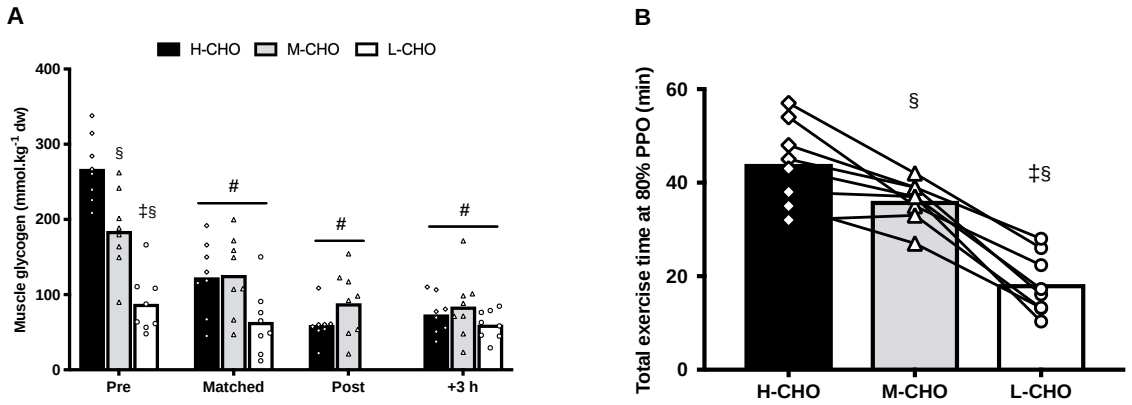


Figure 3

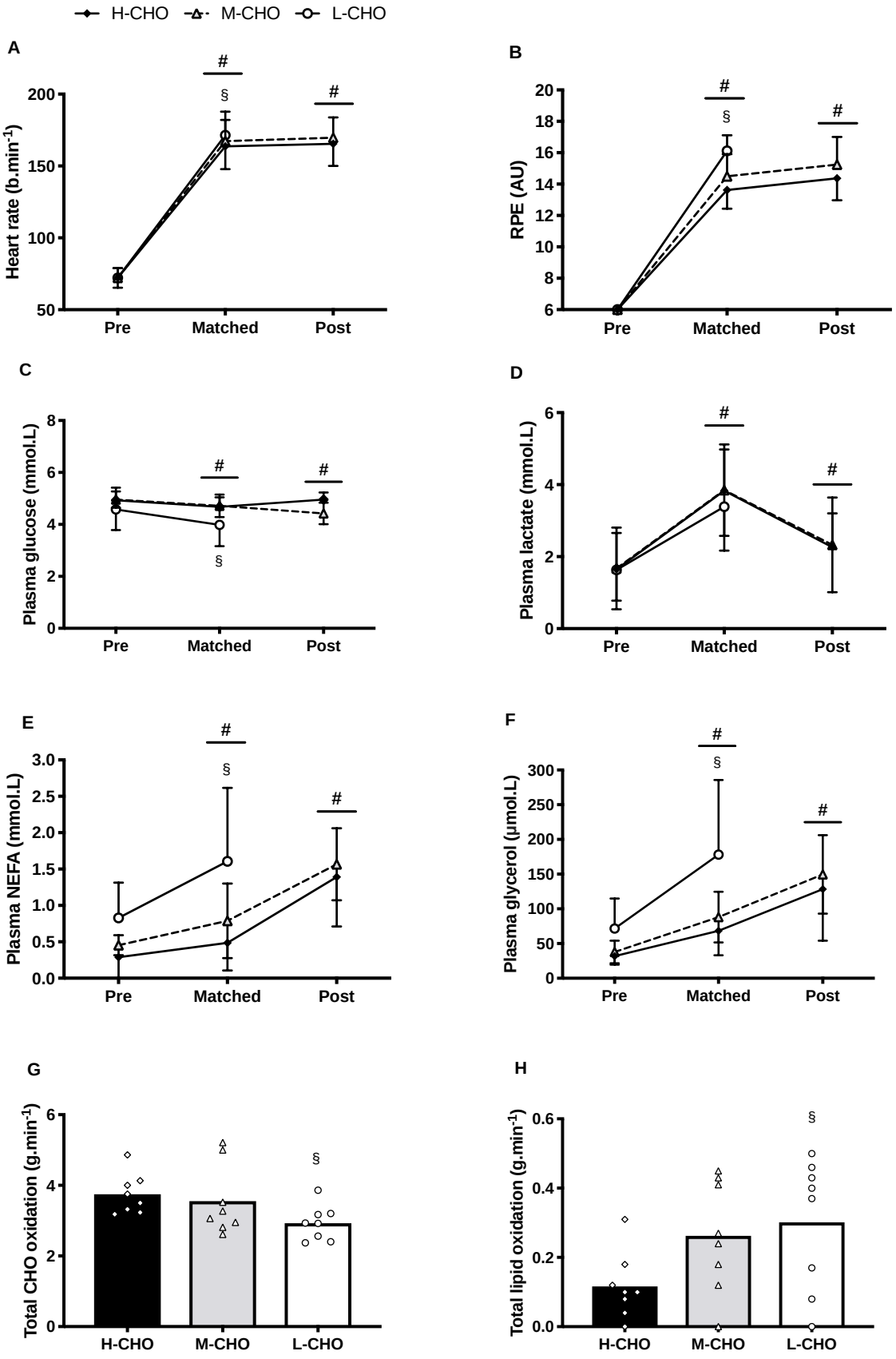

H-CHO
M-CHO
L-CHO
A
Heart rate (b.min-1)
B
RPE (AU)
C
Plasma glucose (mmol.L)
D
Plasma lactate (mmol.L)
E
Plasma NEFA (mmol.L)
F
Plasma glycerol (µmol.L)
G
Total CHO oxidation (g.min-1)
H
Total lipid oxidation (g.min-1)
Pre
Matched
Post
H-CHO
M-CHO
L-CHO
#
§

Figure 4

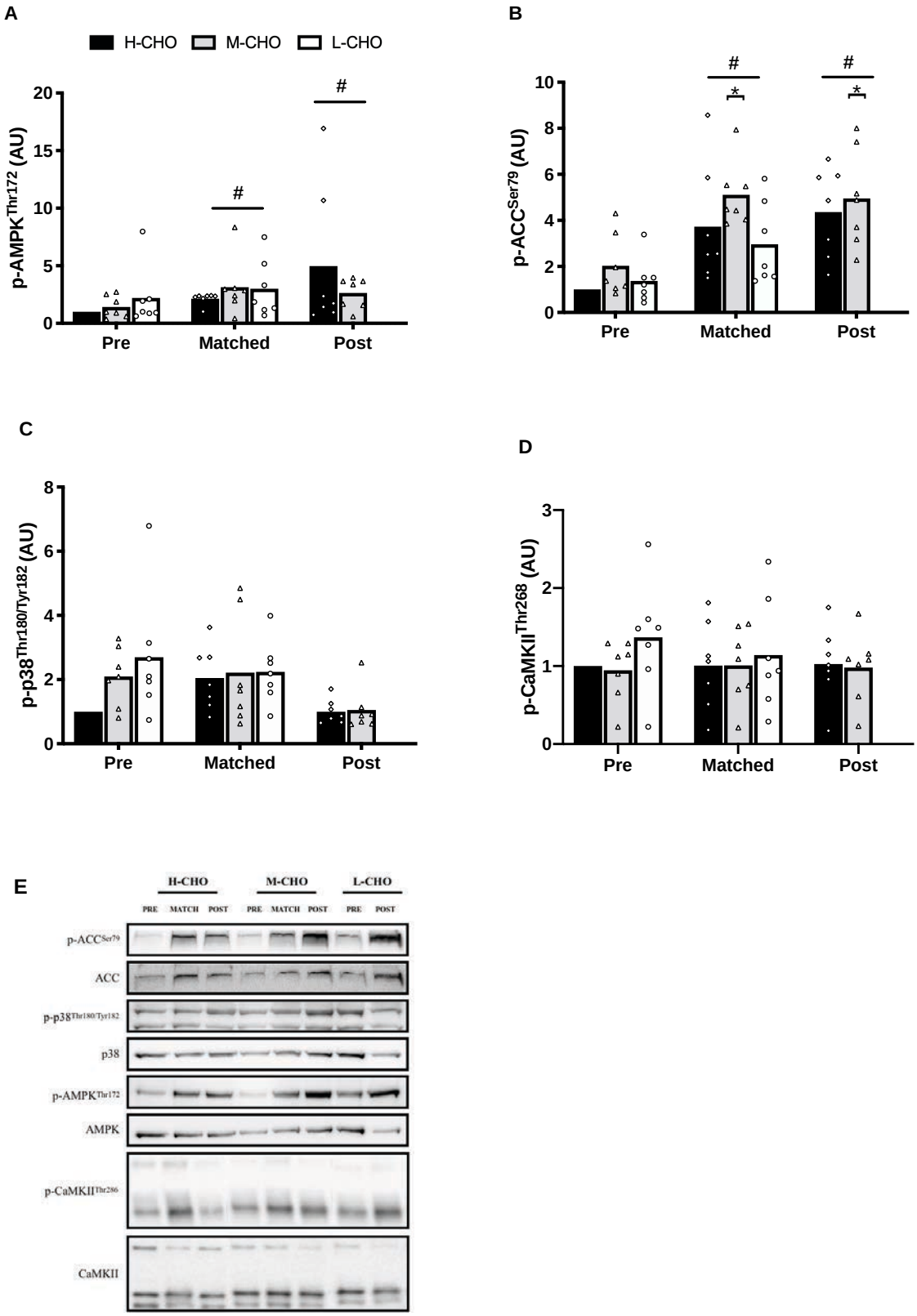
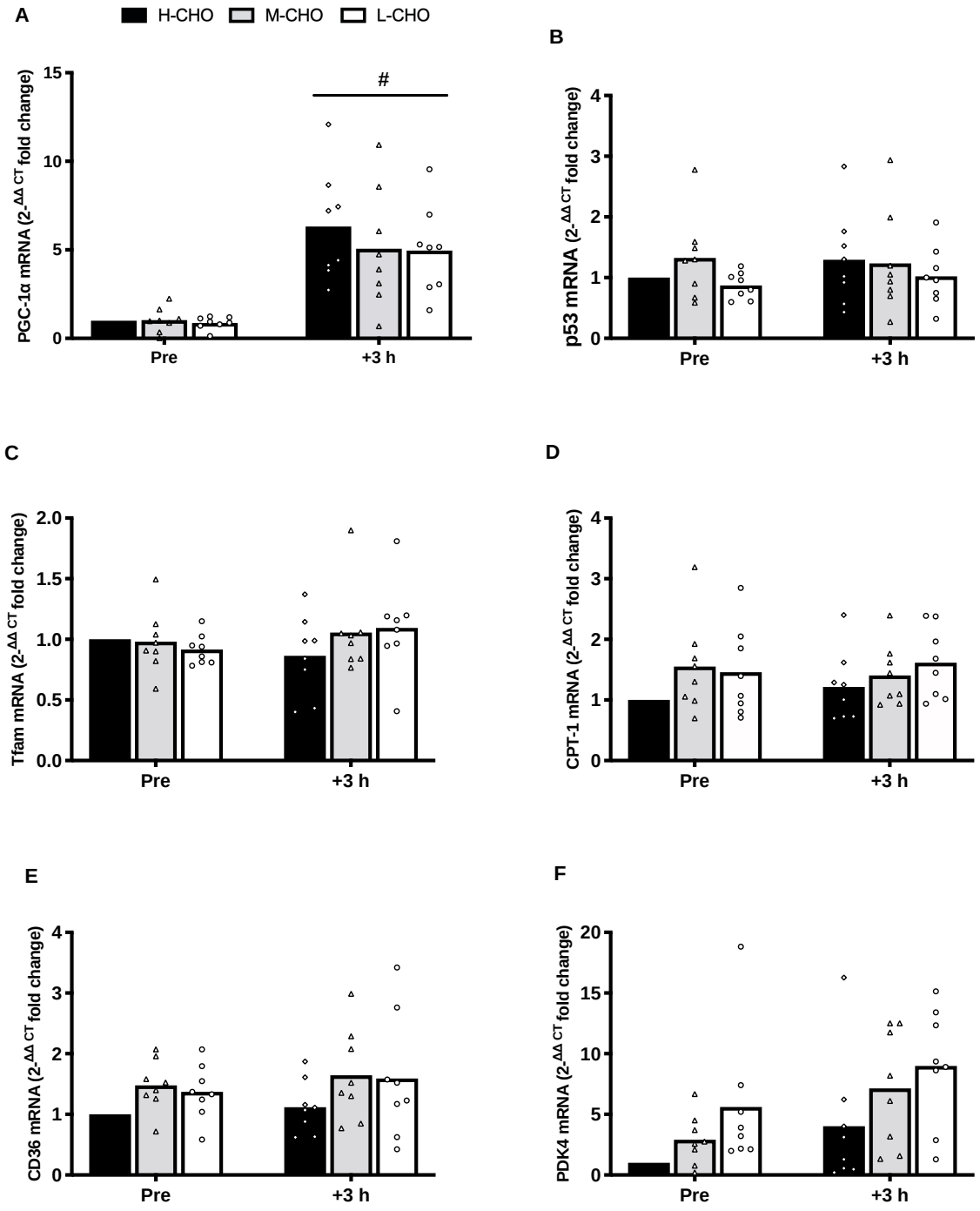

A
H-CHO
M-CHO
L-CHO
p-AMPK^{Thr172} (AU)
Pre
Matched
Post
B
p-ACC^{Ser79} (AU)
C
p-p38$^{Thr180/Tyr182}$ (AU)
D
p-CaMKII^{Thr268} (AU)
E
H-CHO
M-CHO
L-CHO
PRE
MATCH
POST
p-ACC^{Ser79}
ACC
p-p38$^{Thr180/Tyr182}$
p38
p-AMPK^{Thr172}
AMPK
p-CaMKII^{Thr286}
CaMKII

Figure 5


A
H-CHO
M-CHO
L-CHO
PGC-1α mRNA (2-ΔΔ CT fold change)
#
Pre
+3 h
B
p53 mRNA (2-ΔΔ CT fold change)
C
Tfam mRNA (2-ΔΔ CT fold change)
D
CPT-1 mRNA (2-ΔΔ CT fold change)
E
CD36 mRNA (2-ΔΔ CT fold change)
F
PDK4 mRNA (2-ΔΔ CT fold change)

728 **Table 1.** Primers used for real-time RT-PCR

Gene	Forward primer	Reverse primer
PGC-1	TGCTAAACGACTCCGAGAA	TGCAAAGTTCCCTCTCTGCT
p53	ACCTATGGAAACTACTTCCTGAAA	CTGGCATTCTGGGAGCTTCA
Tfam	TGGCAAGTTGTCCAAAGAAACCTGT	GTTCCCTCCAACGCTGGGCA
CD36	AGGACTTTCCTGCAGAATACCA	ACAAGCTCTGGTTCTTATTCACA
PDK4	TGGTCCAAGATGCCTTTGAGT	GTTGCCCGCATTGCATTCTT
CPT1	GACAATACCTCGGAGCCTCA	AATAGGCCTGACGACACCTG
GAPDH	AAGACCTTGGGCTGGGACTG	TGGCTCGGCTGGCGAC

729