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1 **Graded reductions in pre-exercise muscle glycogen concentration impairs**
2 **exercise capacity but does not augment cell signalling regulating mitochondrial**
3 **biogenesis: Implications for CHO periodisation strategies**
4

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45

46 **Abstract**

47 We examined the effects of graded muscle glycogen on exercise capacity and modulation of
48 skeletal muscle signalling pathways associated with the regulation of mitochondrial biogenesis.
49 In a repeated measures design, eight males completed a sleep-low, train-low model comprising
50 an evening glycogen depleting cycling protocol followed by an exhaustive exercise capacity
51 test (8 x 3 min at 80% PPO, followed by 1 min efforts at 80% PPO until exhaustion) the
52 subsequent morning. Following glycogen depleting exercise, subjects ingested a total of 0 g
53 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
54 prior to sleeping, such that exercise was commenced the next morning with graded ($P < 0.05$)
55 muscle glycogen concentrations (Mean \pm SD) (L-CHO: 88 ± 43 , M-CHO: 185 ± 62 , H-CHO:
56 278 ± 47 mmol kg⁻¹ dw). Despite differences ($P < 0.05$) in exercise capacity at 80% PPO
57 between trials (L-CHO: 18 ± 7 , M-CHO: 36 ± 3 , H-CHO: 44 ± 9 min) exercise induced
58 comparable AMPK^{Thr172} phosphorylation (~ 4 fold) and PGC-1 α mRNA expression (~ 5 fold)
59 post- and 3 h post-exercise, respectively. In contrast, exercise nor CHO availability affected
60 the phosphorylation of p38MAPK^{Thr180/Tyr182}, CaMKII^{Thr268} or mRNA expression of p53, Tfam,
61 CPT-1, CD36 or PDK4 Data demonstrate that when exercise is commenced with muscle
62 glycogen below 300 mmol kg⁻¹ dw, further graded reductions of 100 mmol kg⁻¹ dw impairs
63 exercise capacity but does not augment skeletal muscle cell signaling.

64

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

66 **New & Noteworthy**

67 We provide novel data demonstrating that when exercise is commenced with muscle glycogen
68 below 300 mmol kg⁻¹ dw (as achieved using the sleep-low, train-low model) further graded
69 reductions in pre-exercise muscle glycogen of 100 mmol kg⁻¹ dw reduces exercise capacity at
70 80% PPO by 20-50% but does not augment skeletal muscle cell signalling.

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92 **Introduction**

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

110

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

118 order to induce the augmented cell signalling responses associated with the train-low model
119 (33). In accordance with data derived from acute exercise protocols, the notion of a glycogen
120 threshold is also apparent when investigating selected skeletal muscle markers of training
121 adaptation (20). For example, train-low sessions commenced with glycogen concentrations <
122 300 mmol kg⁻¹ dw (12, 29, 47) result in augmented oxidative enzyme activity and/or content
123 following 3-10 weeks of training. In contrast, when 'train-low' sessions are commenced with
124 markedly higher pre-exercise muscle glycogen concentrations (400-500 mmol kg⁻¹ dw) skeletal
125 muscle markers of training adaptation are not augmented (11). Nonetheless, whilst training with
126 low muscle glycogen augments selected signalling events, absolute training volume (19) and/or
127 intensity (18, 24, 47) may be reduced due to a lack of muscle substrate and/or an impairment
128 in the contractile apparatus of skeletal muscle (10, 30). When taken together, the challenge that
129 exists is to therefore facilitate the pro-signalling environment whilst simultaneously
130 maintaining the ability to complete the desired workload and intensity in order to promote
131 training adaptation.

132

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

144 **Methods**

145 **Participants**

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

154

155 **Experimental Design**

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

168

169 **Assessment of peak oxygen uptake**

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

179

180 **Overview of sleep-low, train-low model**

181 *Phase 1: Glycogen depletion exercise*

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

192 *Phase 2: Carbohydrate re-feeding strategy*

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

195 cessation of glycogen depleting exercise to reflect real-world practice as per current nutritional
196 guidelines (44). Subjects in the L-CHO trial then refrained from eating for the remainder of the
197 evening whereas subjects within the M-CHO and H-CHO trials were provided with a mixture
198 of CHO drinks (Maltodextrin, Science in Sport, Nelson UK) and gels (GO isotonic energy gel,
199 Science in Sport, Nelson UK) to be consumed at hourly intervals. In the M-CHO trial, subjects
200 were provided with CHO at a rate of $1.2 \text{ g kg}^{-1} \text{ h}^{-1}$ for 3 h whereas subjects in the H-CHO trial
201 were provided with $1.2 \text{ g kg}^{-1} \text{ h}^{-1}$ for 3 h followed by a high carbohydrate meal (4 g kg^{-1} CHO,
202 $51 \pm 1 \text{ g}$ protein and $17 \pm 1 \text{ g}$ fat) consisting of bread, soup, rice, fresh juice, rice pudding and
203 jam after 4 h of recovery. In this way, total CHO intakes in the L-CHO, M-CHO and H-CHO
204 trials equated to 0, 3.6 and 7.6 g kg^{-1} , respectively, with fluid intake allowed *ad libitum*.

205

206 ***Phase 3: High intensity interval cycling and exercise capacity test***

207 To facilitate our train-low exercise session, subjects arrived the subsequent morning between
208 8-9 am, in a fasted state, where a venous blood sample was collected from the antecubital vein
209 and a muscle biopsy taken from the vastus lateralis. Subjects then completed the high-intensity
210 interval (HIIT) cycling protocol, consisting of 8 x 3 min intervals at 80% PPO, interspersed
211 with 1 min rest. During exercise, heart rate (HR) was continuously measured and the final HR
212 for each 3 min interval was recorded, whilst ratings of perceived exertion (RPE) were recorded
213 upon completion of each interval. Expired gas was collected via a mouthpiece connected to an
214 online gas analysis system (CPX Ultima, Medgraphics, Minnesota, US) for the final 1.5 min
215 of each interval and substrate utilisation was assessed using the equations of Jeukendrup &
216 Wallis (22) given the validity of indirect calorimetry for the assessment of substrate utilisation
217 at exercise intensities up to 80-85% $\dot{V}O_{2\max}$ (37). Upon completion of the high-intensity cycling
218 protocol, subjects were provided with 5 min of active recovery prior to commencing an exercise
219 capacity test consisting of intermittent “1 min efforts” corresponding to 80% PPO interspersed
220 with 1 min recovery periods at 40% PPO. This intermittent protocol was followed until the

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

225

226 **Blood analysis**

227 Venous blood samples were collected in vacutainers containing K₂EDTA, lithium heparin or
228 serum separation tubes and stored on ice or at room temperature until centrifugation at 1500 g
229 for 15 min at 4°C. Samples were collected immediately prior to exercise, at the point of
230 exhaustion (post exercise) and 3 h post exercise, whilst an additional sample was obtained at a
231 time point corresponding to the point of exhaustion in the L-CHO trial during the M-CHO and
232 H-CHO trials. Plasma was aliquoted and stored at -80°C until analysis. Samples were later
233 analysed for plasma glucose, lactate, non-esterified fatty acids (NEFA) and glycerol using
234 commercially available enzymatic spectrophotometric assays (RX Daytona Analyser, Randox,
235 UK) as per manufacturer instructions.

236

237 **Muscle biopsies**

238 Skeletal muscle biopsies (~20 mg) were obtained from the vastus lateralis immediately prior
239 to exercise, at the point of exhaustion (post exercise) and 3 h post exercise. During the M-CHO
240 and H-CHO trials, an additional muscle biopsy was obtained at a time point corresponding to
241 the point of exhaustion in the L-CHO trial, thereby allowing for 'work-matched' comparison
242 between trials. For the work-matched biopsy, subjects dismounted the cycle ergometer and
243 were moved to the adjacent biopsy suite. Following collection of the biopsy sample (~5 min),
244 subjects recommenced cycling exercise. Muscle biopsies were obtained from separate incision
245 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 -80°C 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 95°C. 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, Roskilde, 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 QuantiFastTM 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 50°C for 10 min (reverse
271 transcription/cDNA synthesis), 95°C for 5 min (transcriptase inactivation and initial

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

285

286 **SDS page & Western blotting**

287 Muscle samples (~20 mg) were powdered on dry ice and homogenised (FastPrep-24™ 5G
288 Instrument) for 2 x 40 s at 6 m.s⁻¹ in 10-fold mass of ice-cold lysis homogenisation buffer (10%
289 glycerol, 20 mM Na-pyrophosphate, 150 mM NaCl, 50 mM HEPES (pH 7.5), 1% NP-40, 20
290 mM β -glycerphosphate, 10 mM NaF, 1 mM EDTA (pH 8.8), 1 mM EGTA (pH 8.8), 3 mM
291 Benzamidine, 1 mM 1,4-Dithiothreitol, 1% Phosphatase Inhibitor Cocktail 2 (Sigma-Aldrich),
292 1% Phosphatase Inhibitor Cocktail 3 (Sigma), 4.8% complete Mini Protease Inhibitor Cocktail
293 (Roche)). The resulting homogenate was centrifuged at 4°C for 10 min at 8000 g and the
294 supernatant used for the determination of protein concentrations using the DC protein assay
295 (Bio-Rad, California, USA). Samples were resuspended in 4X Laemlli buffer, boiled for 5 min
296 and separated by SDS-PAGE before being transferred to nitrocellulose membranes (Pall Life
297 Sciences, Pensacola, Florida, USA). Following transfer, membranes were stained for protein

298 with Ponceau S (Sigma-Aldrich, Gillingham, UK), blocked in TBS-Tween containing 3% non-
299 fat milk for 1 h and incubated overnight in primary antibodies (AMPK α (2603), p-AMPK^{Thr172}
300 (2531), ACC (3676), p-ACC^{Ser79} (3661), p38MAPK (9212), p-p38MAPK^{Thr180/Tyr182} (4511),
301 CaMKII (3362) and p-CaMKII^{Thr268} (12716) from Cell Signaling Technologies), before
302 incubation in relevant secondary antibodies (anti-rabbit (7074) from Cell Signaling
303 Technologies) for 1 h at room temperature. Proteins were detected via chemiluminescence
304 (Millipore, Watford, UK) and quantified by densitometry using GeneTools software (Syngene,
305 Cambridge, UK). Sufficient muscle was available for Western blot analysis for seven subjects.
306 Data is reported as the phosphorylated protein of interest normalised to total protein and each
307 timepoint is reported relative to the pre-exercise value in the H-CHO condition.

308

309 **Statistical analysis**

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

322

323 **Results**

324 **Skeletal muscle glycogen and exercise capacity**

325 The exercise and nutritional strategy employed was successful in achieving graded levels of
326 pre-exercise muscle glycogen (H-CHO; 278 ± 47 , M-CHO; 185 ± 62 , L-CHO; 88 ± 43 mmol
327 kg^{-1} dw) such that exercise was commenced with three distinct levels of muscle glycogen ($P =$
328 0.016) (Figure 2A). Exhaustive exercise significantly reduced ($P < 0.001$) muscle glycogen
329 concentration to comparable levels (<100 mmol kg^{-1} dw) with no difference between
330 conditions ($P = 0.11$). In accordance with the observed differences in pre-exercise muscle
331 glycogen concentration, total exercise time spent at 80% PPO in the H-CHO trial (44 ± 9 min)
332 was significantly greater than both M-CHO (36 ± 3 min) ($P = 0.037$) and L-CHO (18 ± 6 min)
333 trials ($P < 0.001$) whilst the M-CHO trial was significantly greater than the L-CHO trial ($P <$
334 0.001) (Figure 2B). Given the low pre-exercise muscle glycogen concentration of subjects in
335 the L-CHO trial, 6 of the 8 subjects were unable to complete the prescribed HIIT protocol. As
336 such, exercise capacity data is presented as the total amount of time spent at 80% PPO and is
337 inclusive of the exercise performed during the prescribed HIIT protocol and subsequent
338 capacity test and excludes all time spent at rest/recovery.

339

340 **Physiological and metabolic responses to exercise**

341 Subject's average heart rate (Figure 3A) across the HIIT session, when matched for work done
342 (H-CHO; 163 ± 16 , M-CHO; 167 ± 15 , L-CHO; 171 ± 17 beats. min^{-1}) was significantly higher
343 in the L-CHO trial compared with H-CHO ($P = 0.031$) only. Similarly, subject's average RPE
344 (Figure 3B) across the HIIT session (H-CHO; 13 ± 1 , M-CHO; 14 ± 1 , L-CHO; 16 ± 1
345 beats. min^{-1}) was significantly higher in the L-CHO trial compared with both M-CHO ($P =$
346 0.041) and H-CHO ($P = 0.012$) trials, respectively. Exhaustive exercise resulted in a significant
347 reduction in plasma glucose ($P = 0.036$), where plasma glucose was significantly lower in the
348 L-CHO trial when compared with the H-CHO trial only ($P = 0.015$) (Figure 3C). Exhaustive
349 exercise resulted in a significant increase in plasma lactate ($P = 0.001$), NEFA ($P < 0.001$) and

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

356

357 **Regulation of mitochondrial biogenesis related cell signalling**

358 Exhaustive exercise induced significant increases in AMPK^{Thr172} phosphorylation ($P = 0.017$)
359 but did not display any significant differences between trials ($P = 0.548$) (Figure 4A). Similarly,
360 exhaustive exercise induced significant increases in ACC^{Ser79} phosphorylation ($P = 0.005$),
361 although phosphorylation was higher in the M-CHO trial when compared with the L-CHO trial
362 only ($P = 0.021$) (Figure 4B). When exercise duration was matched to the post-exercise
363 sampling point in the L-CHO group, the increase in AMPK^{Thr172} phosphorylation remained
364 comparable between groups ($P = 0.269$) and the increase in ACC^{Ser79} phosphorylation still
365 remained higher in the M-CHO trial when compared with the L-CHO trial ($P = 0.021$). In
366 contrast, exhaustive exercise did not induce phosphorylation of p38MAPK^{Thr180/Tyr182} ($P =$
367 0.656) (Figure 4C) or CaMKII^{Thr286} ($P = 0.707$) (Figure 4D). Representative Western blots are
368 shown in Figure 4E. With regard to exercise induced gene expression, exhaustive exercise
369 induced a significant increase in PGC-1 α mRNA expression at 3 h post-exercise ($P = 0.001$)
370 but did not display any significant differences between trials (Figure 5A). In contrast, p53,
371 Tfam, CPT-1, CD36 and PDK4 mRNA expression (Figures 5B, C, D, E, F, respectively) was
372 unaffected by either glycogen availability or the exhaustive exercise protocol ($P > 0.05$).

373

374 **Discussion**

375 Using a sleep-low, train-low model, we examined the effects of three distinct levels of pre-
376 exercise muscle glycogen on exercise capacity and the modulation of selected skeletal muscle
377 signalling pathways with putative roles in mitochondrial biogenesis. We provide novel data by
378 demonstrating that 1) graded reductions in pre-exercise muscle glycogen of $100 \text{ mmol kg}^{-1} \text{ dw}$
379 reduce exercise capacity at 80% PPO by $\sim 20\text{-}50\%$ and 2) despite significant differences in pre-
380 exercise muscle glycogen availability, we observed comparable increases in AMPK^{Thr172}
381 phosphorylation and PGC-1 α mRNA. In contrast to our hypothesis, these data suggest that
382 graded levels of muscle glycogen below $300 \text{ mmol kg}^{-1} \text{ dw}$ do not augment skeletal muscle
383 cell signalling, a finding that may be related to the fact that commencing exercise with <300
384 $\text{mmol kg}^{-1} \text{ dw}$ is already a critical level of absolute glycogen (as suggested by Impey et al. (20))
385 that is required to induce a metabolic milieu conducive to cell signalling. In relation to the goal
386 of promoting cell signalling, our data therefore suggest that reducing pre-exercise glycogen
387 concentrations below $300 \text{ mmol kg}^{-1} \text{ dw}$ does not confer any additional benefit within the
388 context of the sleep-low, train-low model.

389

390 To achieve our intended model of graded glycogen concentrations, we adopted a sleep-low,
391 train-low design whereby subjects performed an evening bout of glycogen depleting exercise
392 and subsequently ingested three graded quantities of CHO that were practically viable within
393 the time-course of the sleep-low model. This strategy was effective in achieving graded
394 differences in pre-exercise muscle glycogen concentration (278 vs. 185 vs. $88 \text{ mmol kg}^{-1} \text{ dw}$
395 in H-CHO, M-CHO and L-CHO, respectively) and represent muscle glycogen resynthesis rates
396 (approximately $30 \text{ mmol kg}^{-1} \text{ h}^{-1}$) commonly observed with CHO feeding rates of $1\text{-}1.2 \text{ g kg}^{-1}$
397 h^{-1} (21). A novel aspect of our chosen study design was that we employed a sampling point in
398 both the H-CHO and M-CHO trials that was matched to the point of exhaustion in the L-CHO
399 trial, thus allowing for the assessment of mitochondrial related signalling events at both work-
400 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

427 In relation to post-exercise mitochondrial related signalling, it is widely accepted that
428 commencing work-matched exercise protocols with reduced muscle glycogen induces greater
429 skeletal muscle signalling (20). For example, AMPK^{Thr172} phosphorylation (46), AMPK- α 2
430 activity (45) and nuclear abundance (39) are all augmented when acute exercise is commenced
431 with reduced pre-exercise muscle glycogen. In contrast, we observed no enhancement in
432 AMPK^{Thr172} or ACC^{Ser79} phosphorylation at our work-matched time point (i.e. following the
433 completion of ~20 min high-intensity cycling) despite graded reductions in pre-exercise muscle
434 glycogen concentrations. This apparent lack of augmented cell signalling may be explained by
435 subjects already commencing exercise with pre-exercise glycogen concentrations below 300
436 mmol kg⁻¹ dw, an absolute concentration that was previously suggested to facilitate the
437 enhanced cell signalling responses associated with low glycogen availability (20). Indeed, our
438 range of pre-exercise muscle glycogen concentrations are distinctly lower than previous work
439 that report greater skeletal muscle signalling following work-matched exercise protocols. For
440 example, high glycogen trials are commonly commenced with muscle glycogen concentrations
441 between 400 and 600 mmol kg⁻¹ dw (3, 36) and remain above 300 mmol kg⁻¹ dw post-exercise
442 (3, 36, 45). In such instances, these researchers observed attenuated (45) or abolished (3, 36)
443 activation of cell signalling pathways. Interestingly, despite the completion of significantly
444 more work in both the M-CHO and H-CHO trials, no further increases in AMPK^{Thr172}
445 phosphorylation were observed following exhaustive exercise. Whilst both AMPK activity and
446 ACC phosphorylation are known to be regulated by exercise duration (41) these responses
447 appear to be closely linked to changes in muscle glycogen concentrations (9, 41). With this in
448 mind, the lack of augmented signalling in response to further exercise in the present study may
449 be explained by the relatively small changes in muscle glycogen from the work-matched time
450 point to exhaustion.

451

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

463

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

478

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

496

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

504 investigate step-wise reductions in pre-exercise muscle glycogen, within a wider range (i.e.
505 100-600 mmol kg⁻¹ dw), in order to investigate the existence of a potential glycogen threshold
506 (20) and allow for a better definition of its potential upper and lower limits.

507

508

509 **Disclosures**

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

511

512 **Author contributions**

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

519

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- 664

665 **Figure legends**

666
667 **Figure 1.** Schematic overview of the experimental sleep-low, train-low protocol. Following 24
668 h of standardised dietary conditions, subjects completed an evening bout of glycogen depleting
669 cycling exercise. Upon completion, subjects received three graded levels of CHO in order to
670 manipulate pre-exercise muscle glycogen the subsequent morning. Following an overnight fast,
671 subjects completed an exhaustive bout of cycling exercise. Muscle biopsies were obtained pre-
672 exercise, at the point of exhaustion (post exercise) and 3 h post exercise. During H-CHO and
673 M-CHO trials, an additional muscle biopsy was obtained at a time point corresponding to the
674 point of exhaustion in the L-CHO trial, allowing for work-matched comparison between trials.

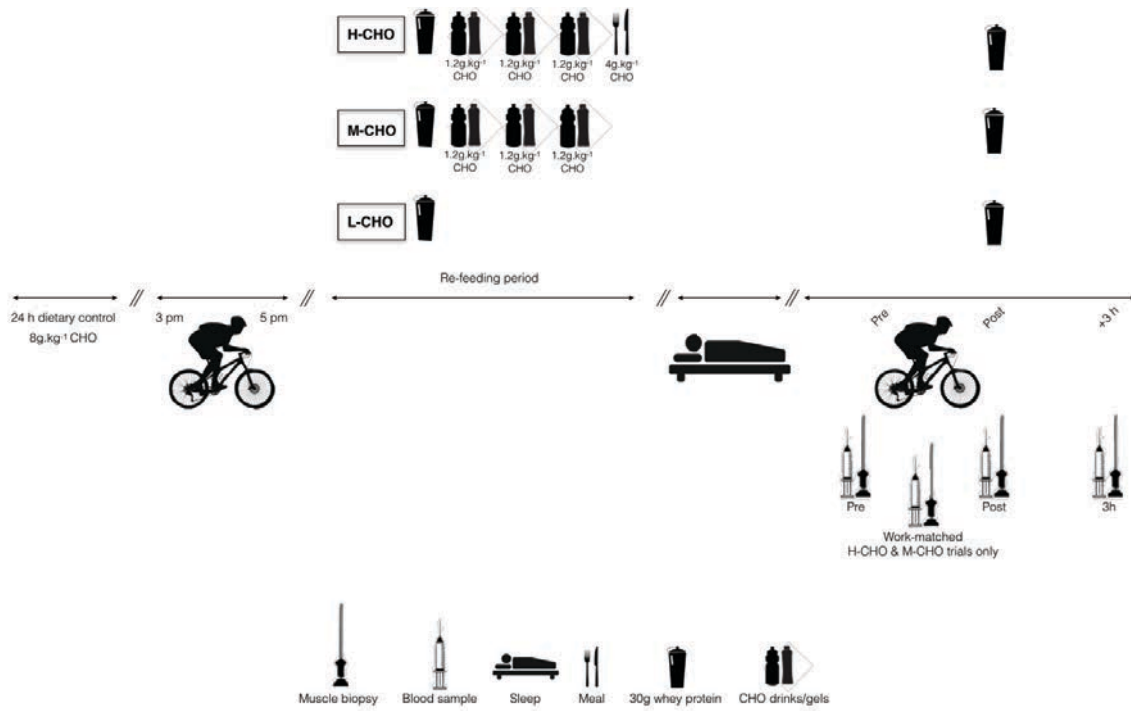
675
676 **Figure 2.** (A) Skeletal muscle glycogen concentration and (B) Exercise capacity at 80% PPO
677 (reflective of set work protocol plus time to exhaustion). $^{\#}P < 0.05$, significantly different from
678 pre-exercise, $^{\S}P < 0.05$, significantly different from H-CHO, $^{\ddagger}P < 0.05$, significantly different
679 from M-CHO. Data is presented as means and individual data points represent individual
680 subjects. N=8

681
682 **Figure 3.** (A) Heart rate, (B) RPE and plasma (C) Glucose, (D) Lactate, (E) NEFA (F) glycerol
683 pre-exercise, at work-matched time points and post-exercise, (E) Average CHO and (F) lipid
684 oxidation during exercise. $^{\#}P < 0.05$, significantly different from pre-exercise, $^{\S}P < 0.05$,
685 significantly different from H-CHO. Data is presented as mean \pm SD (A-D) and individual data
686 points represent individual subjects (E & F). N=8

687
688 **Figure 4.** (A) AMPK^{Thr172} phosphorylation, (B) ACC^{Ser79} phosphorylation, (C) p38^{Thr180/Tyr182}
689 phosphorylation, (D) CaMKII^{Thr286} phosphorylation and (E) representative Western blot
690 images at pre-exercise, work-matched time point and post-exercise. $^{\#}P < 0.05$, significantly
691 different from pre-exercise, $^*P < 0.05$, significantly different from L-CHO. Data is presented
692 as means and individual data points represent individual subjects. N=7

693 **Figure 5.** (A) PGC-1 α , (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
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697 **Figure 1**

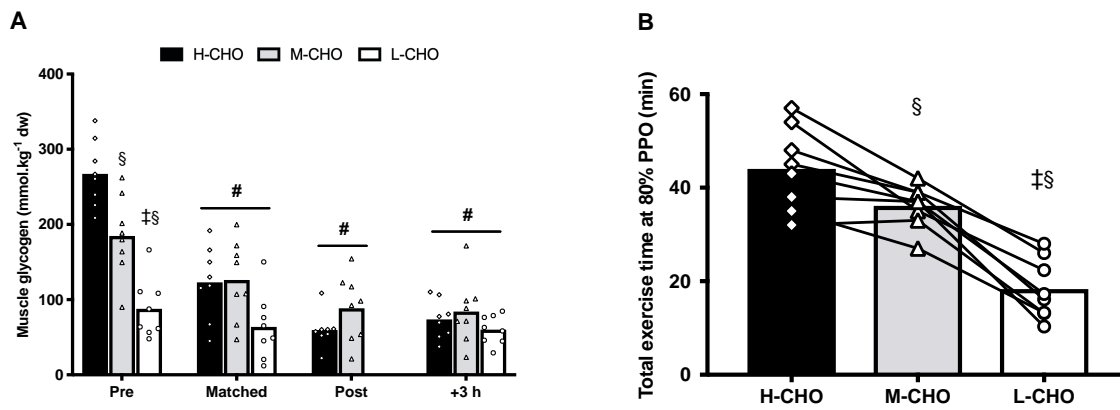


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701 **Figure 2**



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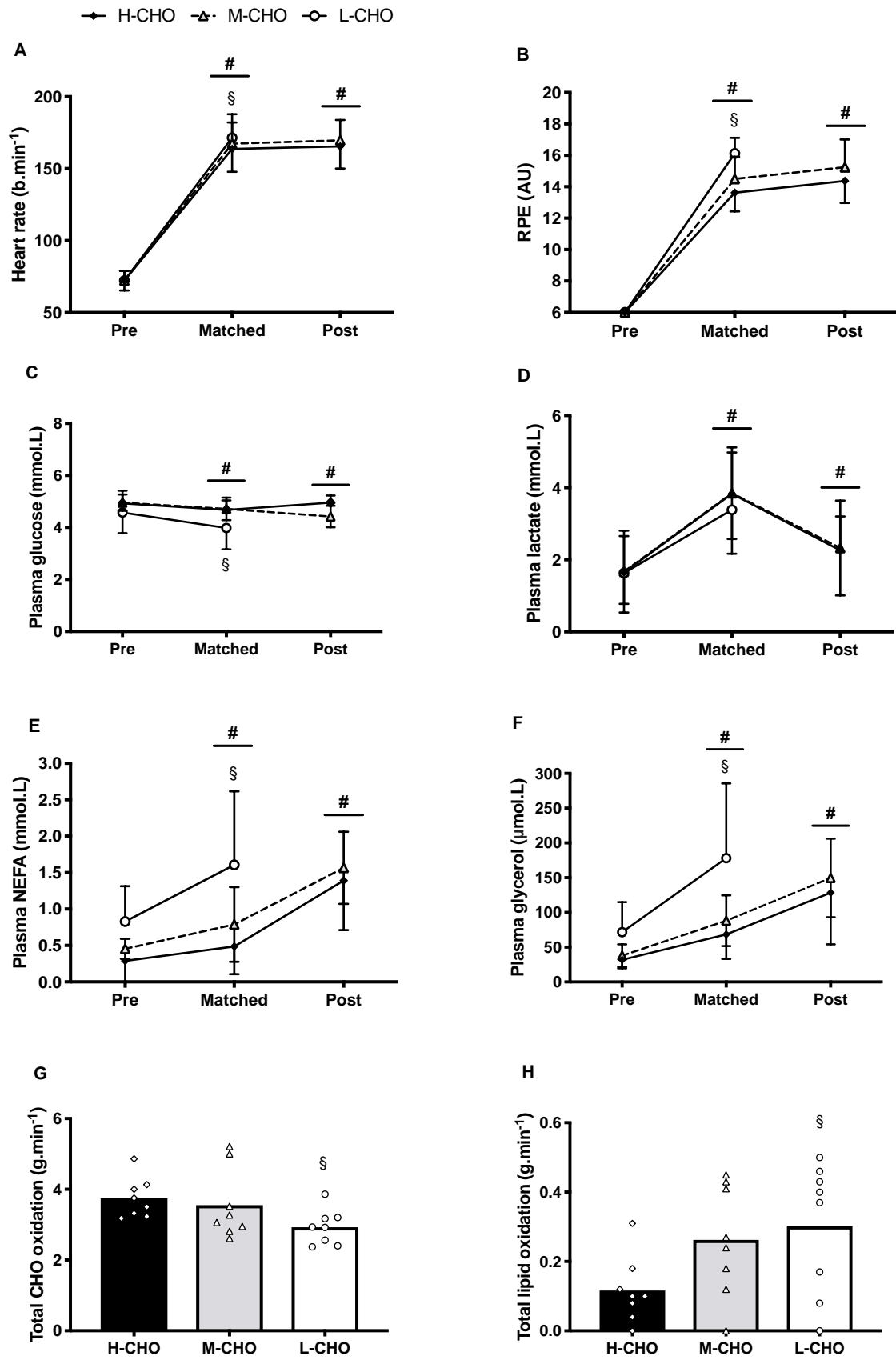
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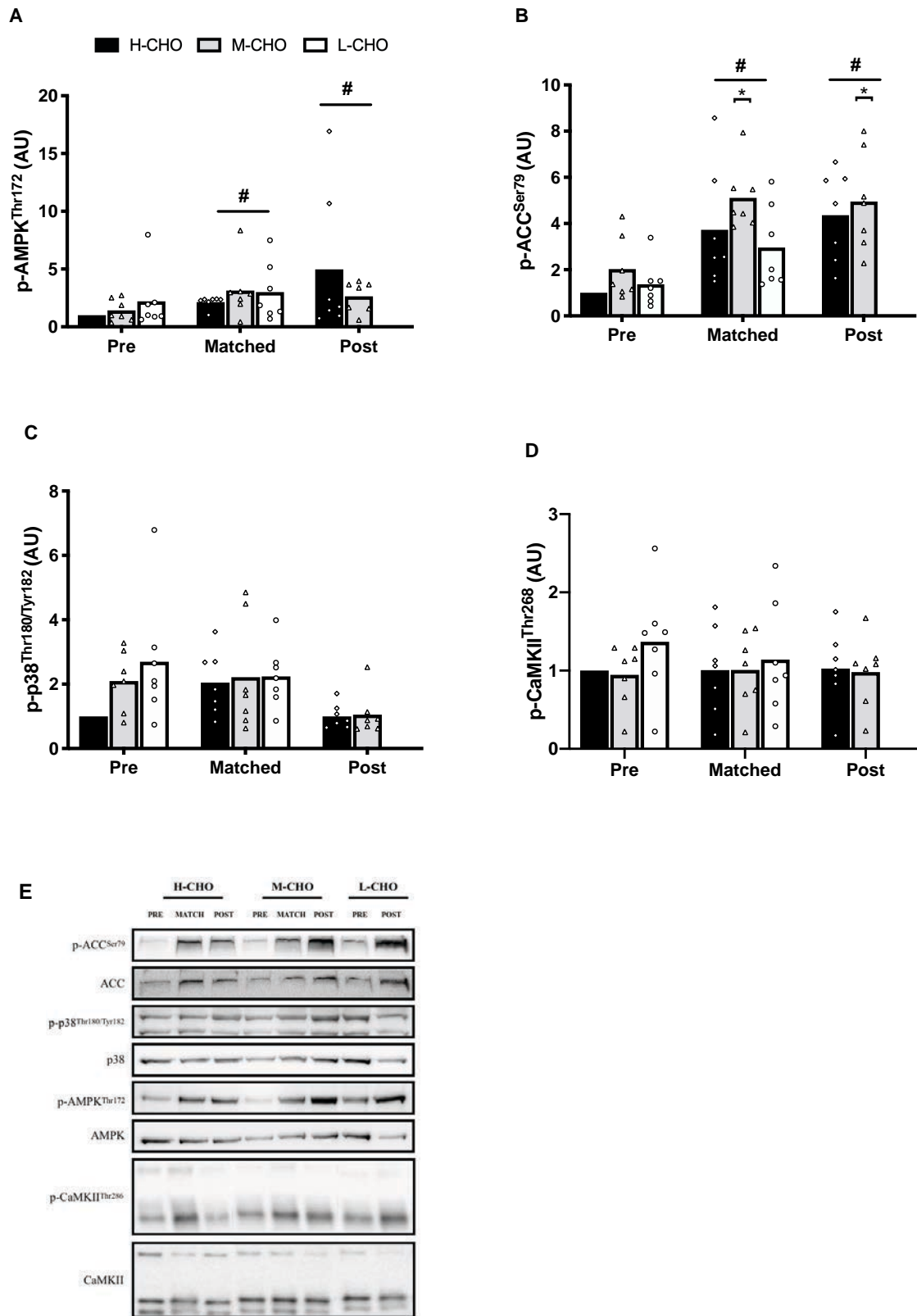
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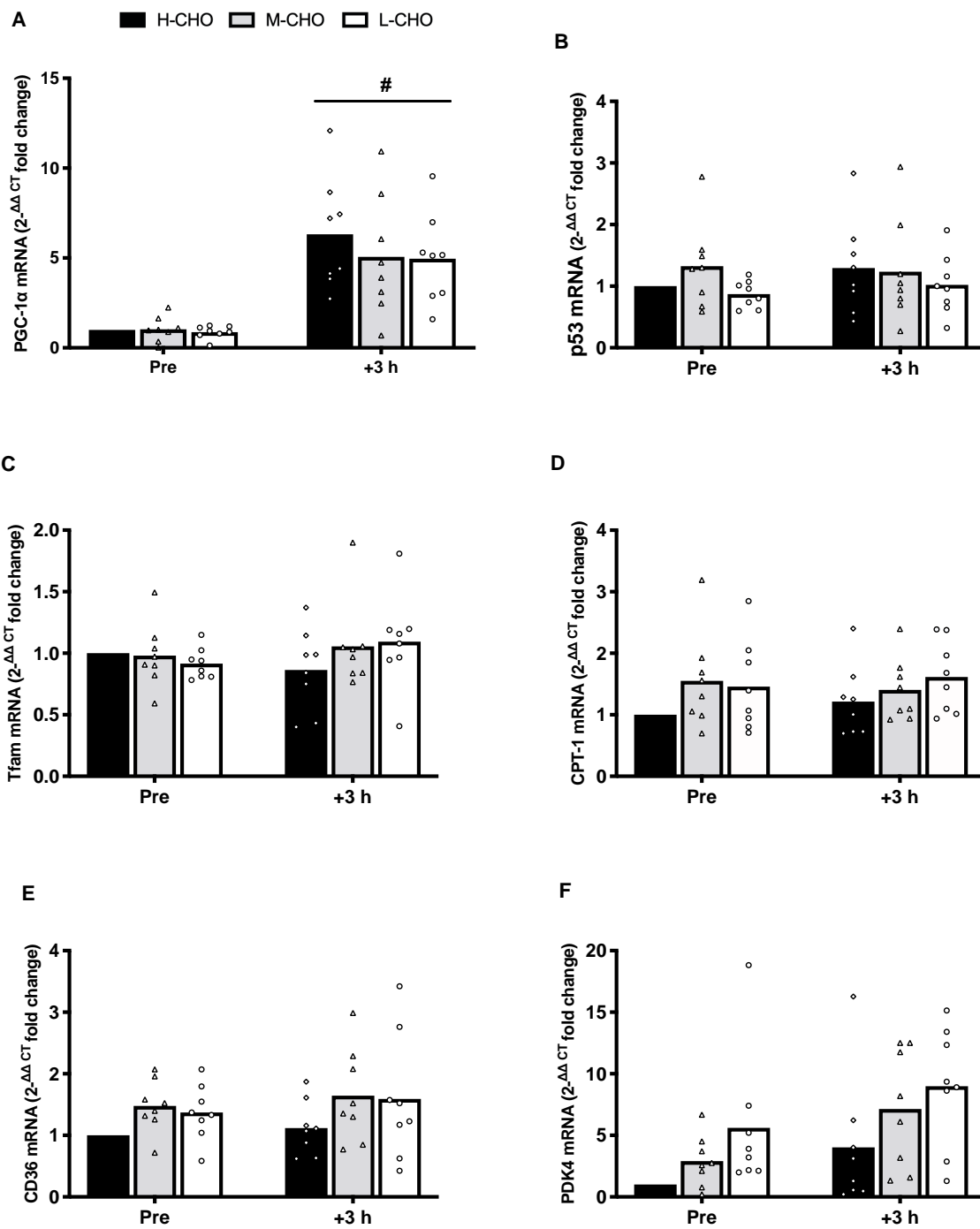


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Table 1. Primers used for real-time RT-PCR

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