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1 **Post exercise high-fat feeding supresses p70S6K1 activity in human skeletal**
2 **muscle**

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43 **Running head: High-fat feeding and training adaptation**

44 **Abstract**

45 **Purpose:** To examine the effects of reduced CHO but high post-exercise fat availability on
46 cell signalling and expression of genes with putative roles in regulation of mitochondrial
47 biogenesis, lipid metabolism and muscle protein synthesis (MPS). **Methods:** Ten males
48 completed a twice per day exercise model (3.5 h between sessions) comprising morning high-
49 intensity interval (HIT) (8 x 5-min at 85% $\dot{V}O_{2peak}$) and afternoon steady-state (SS) running
50 (60 min at 70% $\dot{V}O_{2peak}$). In a repeated measures design, runners exercised under different
51 isoenergetic dietary conditions consisting of high CHO (HCHO: 10 CHO, 2.5 Protein and 0.8
52 Fat $g \cdot kg^{-1}$ per whole trial period) or reduced CHO but high fat availability in the post-exercise
53 recovery periods (HFAT: 2.5 CHO, 2.5 Protein and 3.5 Fat $g \cdot kg^{-1}$ per whole trial period).
54 **Results:** Muscle glycogen was lower ($P < 0.05$) at 3 (251 vs 301 $mmol \cdot kg^{-1} dw$) and 15 h (182
55 vs 312 $mmol \cdot kg^{-1} dw$) post-SS exercise in HFAT compared to HCHO. AMPK- $\alpha 2$ activity
56 was not increased post-SS in either condition ($P = 0.41$) though comparable increases (all
57 $P < 0.05$) in PGC-1 α , p53, CS, Tfam, PPAR and ERR α mRNA were observed in HCHO and
58 HFAT. In contrast, PDK4 ($P = 0.003$), CD36 ($P = 0.05$) and CPT1 ($P = 0.03$) mRNA were
59 greater in HFAT in the recovery period from SS exercise compared with HCHO. p70S6K
60 activity was higher ($P = 0.08$) at 3 h post-SS exercise in HCHO versus HFAT (72.7 ± 51.9 vs
61 44.7 ± 27 $fmol \cdot min^{-1} mg^{-1}$). **Conclusion:** Post-exercise high fat feeding does not augment
62 mRNA expression of genes associated with regulatory roles in mitochondrial biogenesis
63 though it does increase lipid gene expression. However, post-exercise p70S6K1 activity is
64 reduced under conditions of high fat feeding thus potentially impairing skeletal muscle
65 remodelling processes.

66 **Keywords:** AMPK- $\alpha 2$, PGC-1 α , p53, glycogen, mitochondrial biogenesis

67

68 **Introduction**

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

82 In addition to manipulation of CHO availability, many investigators have also demonstrated a
83 modulatory role of high fat availability in augmenting components of training adaptation (10).
84 For example, the acute elevation in circulating free fatty acid (FFA) availability during
85 exercise regulates key cell signalling kinases and transcription factors that modulate the
86 expression of genes regulating both lipid and CHO metabolism (31, 40). Additionally, 5-15
87 days of high fat feeding increases resting intramuscular triglyceride stores (38), hormone
88 sensitive lipase (38), carnitine palmitoyltransferase (CPT1) (15), adenosine monophosphate
89 activated protein kinase (AMPK)- α 2 activity (38) and protein content of fatty acid translocase
90 (FAT/CD36) (11). Such adaptations undoubtedly contribute to the enhanced rates of lipid
91 oxidation observed during exercise following “fat adaptation” protocols (10). Taken together,
92 these data suggest carefully chosen periods of reduced CHO but concomitant high fat

93 availability may therefore represent a strategic approach for which to maximise both training-
94 induced skeletal muscle mitochondrial biogenesis and the enhanced capacity to utilise lipid
95 sources as fuels during exercise.

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

108 With this in mind, the aim of the present study was **to examine** the effects of reduced CHO
109 but high post-exercise fat availability on the activation of key cell signalling kinases and
110 expression of genes with putative roles in the regulation of mitochondrial biogenesis, lipid
111 metabolism and muscle protein synthesis. In accordance with the original train-low
112 investigations (16, 17, 29, 39), we employed a twice per day exercise model whereby trained
113 male runners completed a morning high-intensity interval training session followed by an
114 afternoon training session consisting of steady-state running. Runners completed the exercise
115 protocols under two different dietary conditions (both energy and protein matched) consisting
116 of high CHO availability (HCHO) in the recovery period after both training sessions (i.e. best
117 practice nutrition) or alternatively, reduced CHO but high fat availability in the post-exercise

118 recovery periods (HFAT). We specifically hypothesised that our high fat feeding protocol
119 would enhance cell signalling and the expression of those genes with putative roles in the
120 regulation of mitochondrial biogenesis and lipid metabolism but would also impair the
121 activity of muscle protein synthesis related signalling.

122

123 **Methods**

124 **Subjects:** Ten trained male runners volunteered to participate in the study (mean \pm SD: age,
125 24 ± 1.5 years; body mass, 75.9 ± 6 kg; height, 177.3 ± 7.2 cm; $\text{VO}_{2\text{peak}}$, 60 ± 3.6 ml·kg⁻¹·min⁻¹). All subjects gave written informed consent prior to participation after all
126 experimental procedures and potential risks had been fully explained. None of the subjects
127 had any history of musculoskeletal or neurological disease, nor were they under any
128 pharmacological treatment over the course of the testing period. Subjects were instructed to
129 refrain from any strenuous physical activity, alcohol and caffeine consumption in the 48h
130 prior to each experimental trial. The study was approved by the ethics committee of
131 Liverpool John Moores University.

133 **Design:** In a repeated measures, randomised, cross-over design separated by 7 days, subjects
134 completed a twice per day exercise model under two different dietary conditions (both energy
135 and protein matched) consisting of high CHO availability (HCHO) in the recovery period
136 after both training sessions (i.e. best practice nutrition) or alternatively, reduced CHO but
137 high fat availability in the post-exercise recovery periods (HFAT). The twice per day
138 exercise model comprised a morning (9-10 am) high-intensity interval (HIT) training session
139 (8 x 5-min at 85% $\text{VO}_{2\text{peak}}$) followed by an afternoon (130-230 pm) training session
140 consisting of steady-state (SS) running (60 min at 70% $\text{VO}_{2\text{peak}}$). To promote training
141 compliance during the HIT protocol in both the HCHO and HFAT trials, subjects adhered to

142 a standardised high CHO breakfast prior to this session. However, during the 3.5 h recovery
143 between the HIT and SS session and in the recovery period upon completion of the SS
144 exercise protocol until the subsequent morning, subjects adhered to either a HCHO or HFAT
145 feeding protocol. Muscle biopsies were obtained from the vastus lateralis muscle immediately
146 pre-HIT, immediately post-SS and at 3 h and 15 h post-SS. An overview of the experimental
147 design and nutritional protocols are shown in Figure 1.

148 **Preliminary testing:** At least 7-10 days prior to the first main experimental trial, subjects
149 performed a maximal incremental running test to volitional fatigue on a motorised treadmill
150 (h/p/Cosmos, Nussdorf-Traunstein, Germany) in order to determine maximal oxygen uptake.
151 Following a 10 minute warm up at a self-selected treadmill speed the maximal incremental
152 test commenced, beginning with a 2-min stage at a treadmill speed of 10km/h. Running speed
153 was then increased by 2km/h every 2-min until a speed of 16km/h was reached, after which
154 the treadmill inclined by 2% every 2-min until volitional exhaustion. $\dot{V}O_{2peak}$ was defined as
155 the highest $\dot{V}O_2$ value obtained during any 10-s period and was stated as being achieved by
156 **two of** the following criteria: 1) heart rate was within 10 beats.min⁻¹ (bpm) of age-predicted
157 maximum, 2) respiratory exchange ratio > 1.1, and 3) plateau of oxygen consumption despite
158 increased workload. On their second visit to the laboratory (approx. 3 days later), subjects
159 completed a running economy test in order to determine their individual running speeds for
160 subsequent experimental trials. Following a warm up, the test began with a 5-min stage at a
161 treadmill speed of 8km/h with 1% incline and speed was then increased by 1km/h every 5-
162 mins thereafter . The test was stopped when >90% of the previously determined $\dot{V}O_{2peak}$ was
163 reached. These measurements were recorded via breath-by-breath gas measurements obtained
164 continuously throughout both tests using a CPX Ultima series online gas analysis system
165 (Medgraphics, Minnesota, US). **The test-retest reliability of this system in our laboratory**
166 **when quantified using 95% limits of agreement is $0.29 \pm 2.4 \text{ ml.kg}^{-1} \text{ min}^{-1}$ (data were**

167 compiled from comparison of the oxygen uptake during the HIT protocols in the HCHO and
168 HFAT trials undertaken in the present study). Heart rate (Polar, Kempele, Finland) was also
169 recorded continuously during exercise.

170 **Experimental protocols:**

171 **HIT protocol:** In the 24-h preceding each main experimental trial, subjects consumed a
172 standardised high CHO diet in accordance with typical nutritional recommendations (8 g.kg⁻¹
173 CHO, 2 g.kg⁻¹ protein, and 1 g.kg⁻¹ fat). On the morning of each experimental trial, subjects
174 reported to the laboratory at ~7 am where they were given a standardised high-CHO breakfast
175 (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
176 sample was then collected from an antecubital vein in the anterior crease of the forearm and a
177 muscle biopsy sample taken from the vastus lateralis muscle. Subjects were then fitted with a
178 heart rate monitor and nude body mass (SECA, Hamburg, Germany) was recorded before
179 commencing the high intensity interval running (HIT) protocol which lasted ~1-h. The HIT
180 protocol consisted of 8 x 5-min bouts running at a velocity corresponding to 85% $\dot{V}O_{2peak}$
181 interspersed with 1-min of recovery at walking pace. The intermittent protocol started and
182 finished with a 10-min warm up and cool down at a velocity corresponding to 50% $\dot{V}O_{2peak}$,
183 and a further venous blood sample was obtained immediately upon completion of the
184 protocol. Water was given ad libitum throughout the duration of exercise with the pattern of
185 intake recorded and replicated for the subsequent experimental trial. Heart rate was measured
186 continuously during exercise (Polar, Kempele, Finland) and ratings of perceived exertion
187 (RPE, 9) were obtained upon completion of each HIT bout. In order to determine substrate
188 utilisation during exercise (20), expired gas was collected via a mouthpiece connected to an
189 online gas analysis system (CPX Ultima, Medgraphics, Minnesota, US) for the final 2-mins
190 of each 5-min interval.

191 **SS protocol:** During the 3.5 h recovery period between the HIT and SS protocols, subjects
192 consumed either the HCHO (2.5 g.kg⁻¹ CHO, 1 g.kg⁻¹ Protein, 0.3 g.kg⁻¹ Fat) or HFAT (0
193 g.kg⁻¹ CHO, 1 g.kg⁻¹ Protein, 1 g.kg⁻¹ Fat) feeding protocols (the pattern and frequency of
194 feeding is shown in Figure 1). Following the recovery period, another venous blood sample
195 was obtained immediately prior to commencing the afternoon SS exercise protocol. After a 5-
196 min warm up at a self-selected treadmill speed, subjects subsequently commenced the 60-min
197 steady state running (SS) protocol at a velocity corresponding to 70% $\dot{V}O_{2peak}$. During
198 exercise, subjects also consumed 60 g.h⁻¹ of CHO (SiS GO Istonic Gels, Science in Sport,
199 Blackburn, UK) in HCHO whereas no form of energy was consumed in the HFAT trial.
200 Water was given ad libitum throughout the duration of exercise with the pattern of intake
201 recorded and replicated for the subsequent experimental trial. Expired gases were also
202 collected for 5-mins at 15-min intervals throughout the exercise trial (CPX Ultima,
203 Medgraphics, Minnesota, US) and substrate utilisation again determined according to
204 Jeukendrup and Wallis (20). Heart rate was measured continuously during exercise (Polar,
205 Kempele, Finland) and ratings of perceived exertion (RPE, 9) were obtained every 15
206 minutes during exercise. Upon completion of the SS protocol until sleep, subjects consumed
207 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
208 CHO, 1.5 g.kg⁻¹ Protein, 2.3 g.kg⁻¹ Fat) feeding protocols where the pattern and frequency of
209 feeding is shown in Figure 1. Vastus lateralis muscle biopsies and venous blood samples
210 were also collected immediately post- and at 3 h and 15 h post completion (i.e. ~8 am and in
211 a fasted state) of the SS exercise protocol. The total energy intake across the whole trial
212 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
213 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
214 trials were matched for total energy intake.

215 **Blood sampling and analysis:** Venous blood samples were collected into vacutainers
216 containing EDTA or lithium heparin and stored on ice until centrifugation at 1500g for 15-
217 mins at 4°C. Following centrifugation, aliquots of plasma were stored in a freezer at -80°C
218 for subsequent analysis. Samples were later analysed for plasma glucose, lactate, non-
219 esterified fatty acids (NEFA), glycerol, and β -hydroxybutyrate using commercially available
220 enzymatic spectrophotometric assays (RX Daytona Analyser, Randox, Co. Antrim, UK) as
221 per the manufacturers' instructions.

222 **Muscle biopsies:** Muscle biopsy samples (~50 mg) were obtained from the lateral portion of
223 the vastus lateralis muscle using a Bard Monopty Disposable Core Biopsy Instrument 12
224 gauge x 10 cm length, (Bard Biopsy Systems, Tempe, AZ, USA). Samples were obtained
225 from separate incision sites 2-3 cm apart under local anaesthesia (0.5% Marcaine) and
226 immediately frozen in liquid nitrogen and stored at -80°C for later analysis.

227 **Analysis of muscle glycogen:** Muscle glycogen concentration was determined according to
228 the methods described by van Loon et al (37). Approximately 3-5 mg of freeze dried muscle
229 was powdered and all visible blood and connective tissue removed. The freeze dried sample
230 was then hydrolysed by incubation in 500 μ l of 1M HCl for 3 hours at 100°C. After cooling
231 to room temperature for ~20-min, samples were neutralized by the addition of 250 μ l 0.12
232 mol.L⁻¹ Tris/2.1 mol.L⁻¹ KOH saturated with KCl. Following centrifugation at 1500 RCF for
233 10-mins at 4°C, 200 μ l of the supernatant was analysed in duplicate for glucose concentration
234 according to the hexokinase method using a commercially available kit (GLUC-HK, Randox
235 Laboratories, Antrim, UK). Glycogen concentration is expressed as mmol.kg⁻¹ dry weight
236 and intra-assay coefficients of variation were <5%.

237 **RNA isolation and analysis:** Muscle biopsy samples (~20 mg) were homogenized in 1ml
238 TRIzol reagent (Thermo Fisher Scientific, UK) and total RNA isolated according to

239 manufacturer's guidelines. Concentrations and purity of RNA were assessed by UV
240 spectroscopy at ODs of 260 and 280 nm using a Nanodrop 3000 (Fisher, Roskilde,
241 Denmark). 70 ng RNA was then used for each PCR reaction. Samples were ran in duplicate.

242

243 **Primers:** Identification of primer sequences was enabled by Gene
244 (NCBI, <http://www.ncbi.nlm.nih.gov/gene>) and primers designed using Primer-BLAST
245 (NCBI, <http://www.ncbi.nlm.nih.gov/tools/primer-blast>). Specificity was ensured using
246 sequence homology searches so the primers only matched the experimental gene with no
247 unintended targets identified for primer sequences. In order to prevent amplification of
248 gDNA, primers were ideally designed to yield products spanning exon-exon boundaries. 3 or
249 more GC bases in the last 5 bases at the 3 end, and secondary structure interactions (hairpins,
250 self-dimer and cross dimer) within the primers were avoided so there would be no non-
251 specific amplification. All primers were between 16 and 25bp, and amplified a product
252 between 141 – 244bp. All primers were purchased from Sigma (Suffolk, UK) and sequences
253 for each gene are shown in parentheses: **peroxisome proliferator-activated γ receptor**
254 **coactivator** (PGC-1) (fwd: TGCTAAACGACTCCGAGAA, rev:
255 TGCAAAGTTCCTCTCTGCT), **tumour suppressor protein (p53)** (fwd:
256 ACCTATGGAACTACTTCCTGAAA, rev: CTGGCATTCTGGGAGCTTCA), **mitochondrial**
257 **transcription factor A (Tfam)** (fwd: TGGCAAGTTGTCCAAAGAAACCTGT, rev:
258 GTTCCCTCCAACGCTGGGCA), **citrate synthase (CS)** (fwd: CCTGCCTAATGACCCCATGTT,
259 rev: CATAATACTGGAGCAGCACCCC), **estrogen related receptor (ERR)- α** (fwd:
260 TGCCAATTCAGACTCTGTGC, rev: CCAGCTTCACCCCATAGAAA), **peroxisome proliferator-**
261 **activated receptor (PPAR)** (fwd: ATGGAGCAGCCACAGGAGGAAGCC, rev:
262 GCATGAGGCCCGTTCACAGC), **pyruvate dehydrogenase kinase, isozyme 4 (PDK4)** (fwd:
263 **TGGTCCAAGATGCCTTTGAGT**, rev: **GTTGCCCGCATTGCATTCTT**), **Glucose transporter type**

264 4 (GLUT4) (fwd: TCTCCAACCTGGACGAGCAAC, rev: CAGCAGGAGGACCGCAAATA)
265 carnitine palmitoyltransferase (CPT1) (fwd: GACAATACCTCGGAGCCTCA, rev:
266 AATAGGCCTGACGACACCTG), fatty acid translocase (FAT/CD36) (fwd:
267 AGGACTTTCCTGCAGAATACCA, rev: ACAAGCTCTGGTTCTTATTCACA), and GAPDH
268 (fwd: AAGACCTTGGGCTGGGACTG, rev: TGGCTCGGCTGGCGAC).

269 **Reverse transcriptase quantitative Real-Time Polymerase Chain Reaction (rt-qRT-PCR):**

270 rt-qRT-PCR amplifications were performed using a QuantiFast™ SYBR® Green RT-PCR
271 one step kit on a Rotogene 3000Q (Qiagen, Crawley, UK) supported by Rotogene software
272 (Hercules, CA, USA). The following rt-qTR-PCR cycling parameters were used: hold 50°C
273 for 10 min (reverse transcription/cDNA synthesis), initial denaturation and transcriptase
274 inactivation at 95°C for 5 min, followed by PCR steps: 40 cycles of denaturation at 95°C for
275 10s, and annealing/extension at 60°C for 30s. Upon completion, dissociation/melting curve
276 analysis were performed to reveal and exclude non-specific amplification or primer-dimer
277 issues (all melt analysis presented single reproducible peaks for each target gene suggesting
278 amplification of a single product). Changes in mRNA content were calculated using the
279 comparative C_t ($\Delta\Delta C_t$) equation (34) where relative gene expression was calculated as $2^{-\Delta\Delta C_t}$
280 and where represents the threshold cycle. GAPDH was used as a reference gene and did
281 not change significantly between groups or time points studied ($C_t = 24.2 \pm 1$), therefore a
282 pooled reference gene C_t was used in the relative gene expression equation above.
283 Furthermore, to enable calculation of expression values immediately post and 3-h post
284 exercise, the calibrator condition in the delta delta C_t equation was assigned to the pre-
285 exercise condition.

286 **[γ -³²P] ATP Kinase Assay:** Approximately 10-20 mg of muscle tissue was used for the
287 measurement of p70S6K1 and AMPK α 2 activity as previously described (27).

288 **Statistical analysis:** All data were analysed using Statistical Package for the Social Scientist
289 (SPSS version 21, IBM, USA). Metabolic responses (i.e. blood metabolites, muscle glycogen,
290 kinase activity, mRNA data), physiological and perceptual responses (i.e. HR, RPE, and
291 oxidation rates) were analysed using a two-way repeated-measures general linear model,
292 where the within factors were time and condition (HCHO vs HFAT). Post hoc LSD tests
293 were used where significant main effects and interactions were observed in order to locate
294 specific differences between time points and conditions. All data in text, figures and tables
295 are presented as mean \pm SD, with P values ≤ 0.05 indicating statistical significance.

296

297 **Results**

298 **Physiological responses and substrate utilisation during exercise.**

299 Comparisons of subjects' heart rate, RPE and substrate oxidation during the HIT and SS
300 protocols are displayed in Table 1 and 2, respectively. Heart rate, RPE and lipid oxidation
301 (all $P < 0.01$) all displayed progressive increases during both HIT (see Table 1) and SS
302 exercise (see Table 2) whereas CHO oxidation displayed a progressive decrease ($P < 0.01$)
303 during both exercise protocols. In accordance with identical pre-exercise feeding in HIT, no
304 significant differences were apparent in any of the aforementioned variables between HCHO
305 and HFAT ($P = 0.06, 0.19, 0.52$ and 0.56 , respectively). In contrast, however, during the SS
306 exercise protocol CHO oxidation was significantly greater in HCHO compared to HFAT
307 ($P < 0.001$) whereas fat oxidation was significantly greater during HFAT compared to HCHO
308 ($P < 0.001$).

309

310 **Plasma metabolite responses:** Plasma glucose, lactate, NEFA, glycerol and β -
311 hydroxybutyrate all displayed significant changes (all $P < 0.01$) over the sampling period (see

312 **Table 3**). However, in accordance with the provision of post-exercise CHO feeding in the
313 HCHO trial, plasma glucose was significantly higher compared with HFAT ($P<0.01$) whereas
314 post-exercise high fat feeding in HFAT induced significantly greater plasma NEFA, glycerol
315 and β -OHB (all $P<0.01$) in HFAT compared with the HCHO trial.

316 **Muscle glycogen and exercise induced cell signalling:** Exercise induced significant
317 decreases ($P<0.01$) in muscle glycogen immediately post-SS though no differences were
318 apparent between HCHO and HFAT at this time-point (see Figure 2A). However, in
319 accordance with the provision of CHO after the SS exercise protocol in HCHO, muscle
320 glycogen re-synthesis was observed such that significant differences between HCHO and
321 HFAT ($P=0.01$) were observed at 3 h and 15 h post-SS exercise. Neither exercise ($P=0.407$)
322 nor dietary condition ($P=0.124$) affected AMPK- $\alpha 2$ activity at any time-point studied (see
323 Figure 2B). In contrast, p70S6K1 activity was significantly increased 3 h post-SS exercise
324 (30-mins post-feeding) ($P<0.01$), although this increase was suppressed ($P=0.08$) in HFAT
325 (see Figure 2C). Furthermore, p70S6K1 activity was significantly reduced at 15 h post-SS
326 exercise when participants were fasted compared with pre-HIT when they were high CHO
327 and protein fed ($P<0.01$).

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

336 Discussion

337 The aim of the present study was to examine the effects of reduced CHO but high post-
338 exercise fat availability on the activation of key cell signalling kinases and expression of
339 genes with putative roles in the regulation of mitochondrial biogenesis, lipid metabolism and
340 muscle protein synthesis. When compared with high CHO availability, we observed that
341 post-exercise high fat feeding had no modulatory affect on AMPK- α 2 activity or the
342 expression of those regulatory genes associated with mitochondrial biogenesis. Furthermore,
343 although post-exercise high fat feeding augmented the expression of genes involved in lipid
344 transport (i.e. FAT/CD36) and oxidation (i.e. CPT1), we also observed suppression of
345 p70S6K1 activity despite sufficient post-exercise protein intake. This latter finding suggests
346 that post-exercise high fat feeding may impair the regulation of muscle protein synthesis and
347 skeletal muscle remodelling processes, thereby potentially causing maladaptive responses for
348 training adaptation if performed long-term.

349 In accordance with the original train-low investigations examining cycling or knee extensor
350 exercise (16, 17, 29, 39), we also employed a twice per day protocol, albeit consisting of
351 morning HIT and afternoon SS running exercise protocol. This model is practically relevant
352 given that many elite endurance athletes (including runners) train multiple times per day with
353 limited recovery time between training sessions (14). Given that reduced CHO availability
354 impairs high-intensity training capacity (17, 39), we also chose to schedule the HIT session in
355 the morning period after a standardised high CHO breakfast. As expected, no differences in
356 cardiovascular strain, ratings of perceived exertion, substrate utilisation and plasma
357 metabolite responses were observed between the HCHO and HFAT trials during the HIT
358 session (see Table 1 and 3). Following completion of the HIT protocol, subjects then adhered
359 to a HCHO or HFAT feeding protocol in the 3.5 h prior to commencing the afternoon SS
360 exercise. Given that exogenous CHO feeding during exercise reduces oxidative adaptations

361 even in the presence of reduced pre-exercise muscle glycogen (29), we also chose to feed
362 exogenous CHO (at a rate of 60 g/h) during the afternoon SS protocol during the HCHO trial.
363 Although we did not directly quantify muscle glycogen immediately prior to SS exercise,
364 plasma metabolite and substrate utilisation during SS exercise were clearly suggestive of
365 differences in both endogenous and exogenous CHO availability between the HCHO and
366 HFAT trials. Indeed, plasma NEFA, glycerol, β -OHB and whole body lipid oxidation were
367 all greater during SS exercise undertaken in the HFAT trial compared with the HCHO trial
368 (see Table 2 and 3). On the basis of comparable muscle glycogen data post-SS exercise (see
369 Figure 2A) and greater whole body CHO oxidation during the HCHO trial (see Table 2), we
370 also suggest that exercise-induced muscle glycogen utilisation was greater during the SS
371 exercise protocol when completed in the HCHO conditions (7).

372 Perhaps surprisingly, we observed that our SS exercise protocol did not increase AMPK- α 2
373 activity in either the HCHO or HFAT trial. However, there are likely a number of
374 physiologically valid reasons to explain the apparent lack of AMPK mediated signalling.
375 Indeed, exercise-induced AMPK activation is known to be intensity dependent where $>70\%$
376 VO_{2max} is likely required to induce metabolic perturbations sufficient to mediate a signalling
377 response (13). Furthermore, the AMPK response to exercise is attenuated with exercise
378 training (8), an effect that is especially relevant for the present investigation given the trained
379 status of our chosen population and the low plasma lactate observed (approximately 2
380 $mmol.L^{-1}$) during SS exercise. **Reduced absolute muscle fibre recruitment from the vastus**
381 **lateralis, when compared with other lower extremity muscles recruited during walking and**
382 **running (19), or when exercising at similar relative intensities during cycling (4) and where**
383 **AMPK activation is typically reported (22) could also contribute, in part, to the lack of**
384 **AMPK signalling observed here.** Finally, although exercise-induced AMPK activity is also
385 thought to be regulated, in part, via a glycogen binding domain on β -subunit of the AMPK

386 heterotrimer (26), it is possible that our runners did not exceed a potential “muscle glycogen
387 threshold” that is required to fully activate the AMPK complex during prolonged endurance
388 exercise (30). Indeed, previous data from our laboratory also using running exercise
389 protocols (6,7) have typically only observed AMPK related signalling when post-exercise
390 whole muscle homogenate glycogen is $<200 \text{ mmol.kg}^{-1} \text{ dw}$. Despite previous suggestions
391 that train-low training sessions should be targeted to SS exercise protocols so as to not
392 compromise training intensity (5), our data therefore suggest (at least for AMPK mediated
393 signalling) that perhaps it is the actual completion of a high-intensity stimulus per se
394 (especially in trained athletes) that is really required to create a metabolic milieu that is
395 conducive to augmentation of necessary signalling networks.

396 In contrast to Yeo et al. (38), we also observed no modulatory effect of **post-exercise** high fat
397 availability on resting AMPK- $\alpha 2$ activity. Indeed, these authors observed that 5 days of a fat
398 loading protocol increased resting AMPK- $\alpha 2$ activity as well as the exercise-induced
399 phosphorylation of ACC^{Ser221}. Such discrepancies between studies are likely due to the
400 differences in duration of high fat feeding in that we adopted an acute high fat feeding
401 protocol ($<24 \text{ h}$) whereas the latter authors adopted a 5 day “fat adaptation” protocol that also
402 increased resting intramuscular triglyceride (IMTG) stores. In this regard, it is noteworthy
403 that the magnitude of change in resting AMPK- $\alpha 2$ activity was positively correlated with the
404 elevations in IMTG storage (38).

405 In contrast to our hypothesis, we also observed comparable 2-3 fold changes between trials in
406 mRNA expression of those genes with key regulatory roles associated with mitochondrial
407 biogenesis. For example, the expression of PGC-1 α , p53, Tfam, PPAR and ERR α mRNA
408 were all elevated with similar magnitude and time-course in recovery from the SS protocol in
409 both the HCHO and HFAT trials. Such data conflict with previous observations from our
410 laboratory (7) and others (32) where post-exercise CHO restriction (i.e. keeping muscle

411 glycogen low) augments the expression of many of the aforementioned genes. However, in
412 our previous report we simultaneously adopted a CHO but calorie restriction feeding protocol
413 whereas the present design incorporated a reduced CHO but isocaloric and protein matched
414 feeding protocol in our HFAT trial. Given the similarities in metabolic adaptation to both
415 CHO and calorie restriction, such data raise the question as to whether the enhanced
416 mitochondrial responses observed when “training low” are due to transient periods of CHO
417 restriction, calorie restriction or indeed, a combination of both. This point is especially
418 relevant from an applied perspective given that many endurance athletes present daily with
419 transient periods of both CHO and calorie restriction due to multiple training sessions per day
420 as well as longer term periods of sub-optimal energy availability (14).

421 In agreement with multiple studies demonstrating a role of both acute elevations in FFA
422 availability (7, 23) as well as high fat feeding protocols (11), we also observed that post-
423 exercise expression of PDK4, FAT/CD36 and CPT1 mRNA expression were elevated in the
424 HFAT trial versus the HCHO trial. However, unlike Arkinstall et al. (4), we did not detect
425 any suppressive effects of high fat availability on GLUT4 mRNA expression though a longer
426 and more severe period of CHO restriction utilised by these investigators (i.e. 48 h of
427 absolute CHO intake <1 g/kg body mass resulting in muscle glycogen levels <150 mmol.kg⁻¹
428 dw) may explain the discrepancy between studies. Nonetheless, the dietary protocol studied
429 here clearly alters the expression of genes with potent regulatory roles in substrate utilisation
430 and if performed long term, may increase the capacity to use lipids as a fuel but induce
431 suppressive effects on CHO metabolism (through suppression of the PDH complex) thus
432 potentially limiting high-intensity performance (35). Whilst we did not directly quantify the
433 signalling mechanisms underpinning these responses (owing to a lack of a muscle tissue), we
434 suggest both p38MAPK and PPAR mediated signalling are likely involved. Indeed, using a
435 twice per day exercise model, Cochran et al. (12) also observed enhanced p38MAPK

436 phosphorylation during the afternoon exercise protocol (despite similar pre-exercise muscle
437 glycogen availability) that was associated with the enhanced circulating FFA availability
438 during the afternoon exercise. Furthermore, pharmacological ablation of circulating FFA
439 availability during exercise suppresses p38MAPK compared with control conditions (40).
440 Additionally, FFA mediated signalling can also directly mediate PPAR binding to the CPT1
441 promoter thereby modulating CPT1 expression (31).

442 We also examined the effects of post-exercise fat feeding on the regulation of p70S6K
443 activity, a key signalling kinase associated with regulating MPS. In relation to the effects of
444 endurance exercise per se, the majority of studies are typically limited to measures of
445 phosphorylation status with some studies reporting increases (25) and others, no change.
446 When examined quantitatively using the [γ - 32 P] ATP kinase assay, our data agree with
447 previous observations from Apro et al. (3) who also reported no change but yet, conflict with
448 recent data from our group where we observed an exercise-induced suppression of p70S6K
449 activity (18). Nonetheless, the exhaustive (a fatiguing cycling HIT protocol) and muscle
450 glycogen depleting (<100 mmol.kg⁻¹ dw) nature of the latter exercise protocol versus the
451 moderate-intensity nature of the afternoon SS running protocol studied here, likely explains
452 the discrepancy between studies.

453 In relation to the effects of post-exercise feeding, we also provide novel data by
454 demonstrating that post-exercise high fat feeding was associated with a suppression of
455 p70S6K activity (albeit P=0.08) at 3 h post-completion of the SS exercise protocol when
456 compared with the elevated response observed in HCHO (when using both a mean difference
457 and standard deviation of differences of 50 fmol.min⁻¹.mg⁻¹, we estimate a sample size of 12-
458 13 would be required to achieve statistical significance with 90% power, as calculated using
459 Minitab statistical software, version 17). Although we did not measure circulating insulin
460 levels in this study, it is of course possible that the suppressed p70S6K response observed

461 here may be due to reduced upstream insulin mediated activation of protein kinase B (PKB).
462 Indeed, we recently observed post-exercise p70S6K activity to be suppressed in conditions of
463 simultaneous carbohydrate and calorie restriction in a manner associated with reduced insulin
464 and upstream signalling of PKB (18). Alternatively, the suppression of p70S6K observed
465 here may be mediated through direct effects of post-exercise high fat feeding that are
466 independent of CHO availability, energy availability and insulin. Indeed, Stephens et al. (36)
467 observed infusion of Intralipid and heparin to elevate circulating FFA concentrations
468 attenuates MPS in human skeletal muscle in response to ingesting 21g amino acids under
469 euglycemic hyperinsulemic clamp conditions. Furthermore, Kimball et al. (21) also reported
470 that high fat feeding impairs MPS in rat liver in a manner associated with reduced p70S6K
471 phosphorylation, an effect that may be induced through sestrin 2 and sestrin 3 mediated
472 impairment of mTORC signalling. Clearly, further research is required to examine the
473 effects of high fat feeding on direct measures (and associated regulatory sites) of MPS within
474 the physiological context of the exercising human.

475 In summary, we provide novel data by concluding that post-exercise high fat feeding has no
476 modulatory affect on AMPK- α 2 activity or the expression of those genes associated with
477 regulatory roles in mitochondrial biogenesis. Furthermore, although post-exercise high fat
478 feeding augmented the expression of genes involved in lipid transport and oxidation, we also
479 observed a suppression of p70S6K1 activity despite sufficient post-exercise protein intake.
480 This latter finding suggests that post-exercise high fat feeding may impair the regulation of
481 muscle protein synthesis and post-exercise muscle remodelling, thereby potentially causing
482 maladaptive responses for training adaptation if performed long-term. Future studies should
483 now examine the functional relevance of the signalling responses observed here, not only in
484 terms of acute muscle protein synthesis but also the chronic skeletal muscle and performance
485 adaptations induced by long-term use of this feeding strategy.

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490

491 **Conflicts of interest**

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

494

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618

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

621

622

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

626

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

630

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

634

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

639

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

644