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1 **Title:** Effects of short-term graded dietary carbohydrate intake
2 on intramuscular and whole-body metabolism during
3 moderate-intensity exercise
4
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51 **Abstract**

52 Altering dietary carbohydrate (CHO) intake modulates fuel utilization during
53 exercise. However, there has been no systematic evaluation of metabolic
54 responses to graded changes in short-term (< 1 week) dietary CHO intake.
55 Thirteen active men performed interval running exercise combined with
56 isocaloric diets over 3 days before evaluation of metabolic responses to 60-
57 min running at 65% $\dot{V}O_2$ max on three occasions. Diets contained lower (LOW,
58 2.40 ± 0.66 g CHO \cdot kg $^{-1}\cdot$ d $^{-1}$, $21.3 \pm 0.5\%$ of energy intake [EI]), moderate
59 (MOD, 4.98 ± 1.31 g CHO \cdot kg $^{-1}\cdot$ d $^{-1}$, $46.3 \pm 0.7\%$ EI), or higher (HIGH, $6.48 \pm$
60 1.56 g CHO \cdot kg $^{-1}\cdot$ d $^{-1}$, $60.5 \pm 1.6\%$ EI) CHO. Pre-exercise muscle glycogen
61 content was lower in LOW (54.3 ± 26.4 mmol \cdot kg $^{-1}$ wet weight [ww]) compared
62 to MOD (82.6 ± 18.8 mmol \cdot kg $^{-1}$ ww) and HIGH (80.4 ± 26.0 mmol \cdot kg $^{-1}$ ww,
63 $P < 0.001$; MOD vs. HIGH, $P = 0.85$). Whole-body substrate oxidation, systemic
64 responses, and muscle substrate utilization during exercise indicated
65 increased fat and decreased CHO metabolism in LOW (RER: 0.81 ± 0.01)
66 compared to MOD (RER 0.86 ± 0.01 , $P = 0.0005$) and HIGH (RER: $0.88 \pm$
67 0.01 , $P < 0.0001$; MOD vs. HIGH, $P = 0.14$). Higher basal muscle expression of
68 genes encoding proteins implicated in fat utilization was observed in LOW. In
69 conclusion, muscle glycogen availability and subsequent metabolic responses
70 to exercise were resistant to increases in dietary CHO intake from ~ 5.0 to
71 ~ 6.5 g CHO \cdot kg $^{-1}\cdot$ d $^{-1}$ (46% to 61% EI), while muscle glycogen, gene
72 expression and metabolic responses were sensitive to more marked
73 reductions in CHO intake (~ 2.4 g CHO \cdot kg $^{-1}\cdot$ d $^{-1}$, $\sim 21\%$ EI).

74

75 **Keywords:** Muscle glycogen, fat metabolism, diet, running economy, exercise

76

77 **Running head:** Exercise metabolism following graded carbohydrate intake

78

79 **New and noteworthy**

80 The data presented here suggests that metabolic responses to steady-state

81 aerobic exercise are somewhat resistant to short-term changes in dietary

82 carbohydrate (CHO) intake within the 5-6.5 g CHO·kg⁻¹·d⁻¹ (46-61% EI) range.

83 In contrast, reduction in short-term dietary CHO intake to ~2.4 g CHO·kg⁻¹·d⁻¹

84 (21% EI) evoked clear changes indicative of increased fat and decreased

85 CHO metabolism during exercise.

86 **Introduction**

87 Glycogen is the storage form of carbohydrate (CHO) energy in animals,
88 primarily located in skeletal muscles and the liver (17, 34). Glycogen
89 availability is sensitive to exercise and nutrition (4, 29, 35, 45), and glycogen
90 depletion has been implicated in fatigue during prolonged moderate-to-
91 vigorous intensity exercise (31, 33, 34). Muscle glycogen is also recognised
92 as a potent regulator of acute substrate metabolism during prolonged exercise
93 (18), and is increasingly implicated in the regulation of exercise training
94 adaptation (6, 20, 22). Therefore, understanding the precise relationship
95 between nutrition, muscle glycogen availability, and metabolic responses to
96 exercise has relevance for exercise performance, training adaptation, and
97 health.

98
99 Short-term (i.e., ≤ 1 week) dietary interventions that reduce CHO intake and
100 lower muscle glycogen content elicit increased fatty acid and reduced CHO
101 oxidation during subsequent moderate-intensity exercise (18). However, the
102 magnitude of alteration in CHO intake required to elicit distinct effects on
103 muscle glycogen and subsequent metabolic responses to exercise remains
104 poorly understood. Assessing the magnitude of alteration in CHO intake
105 required to elicit distinct effects on muscle glycogen and subsequent
106 metabolic responses to exercise requires several (i.e., >2) experimental
107 conditions. Three classic studies compared muscle and/or whole-body fuel
108 use during exercise performed after short-term low (<2.5 g CHO \cdot kg $^{-1}\cdot$ day $^{-1}$),
109 moderate (~ 4 -5 g CHO \cdot kg $^{-1}\cdot$ day $^{-1}$) and/or high (>6.5 g CHO \cdot kg $^{-1}\cdot$ day $^{-1}$) diet
110 CHO intake (4, 12, 16). Despite apparent achievement of graded pre-exercise

111 muscle glycogen availability, subsequent effects on fuel utilization during
112 exercise were inconsistent with similar (12, 16) or augmented (4) CHO
113 oxidation in the high compared to moderate CHO condition. Whilst
114 informative, these early studies were limited by lack of consistent dietary
115 control (4, 16) and/or low statistical power to detect differences in fuel
116 utilization between-conditions (12, 16). As such, the effect of short-term
117 graded dietary CHO intakes on pre-exercise muscle glycogen, whole-body
118 and muscle fuel utilization during exercise requires clarification.

119

120 The main aims of the present investigation were to examine the effect of
121 short-term diet-exercise interventions that provided graded dietary CHO
122 intake (i.e., ~21% energy intake [EI] or ~2.4 g.kg CHO·kg⁻¹·d⁻¹, ~46% EI or
123 ~5.0 g.kg CHO·kg⁻¹·d⁻¹, and ~61% EI or ~6.5 g.kg CHO⁻¹·d⁻¹) on pre-exercise
124 resting muscle glycogen content, muscle and whole-body fuel utilization
125 during prolonged, moderate-intensity exercise. It was hypothesised that pre-
126 exercise muscle glycogen content and muscle and whole-body fuel utilization
127 during subsequent moderate-intensity exercise would be graded in line with
128 the preceding dietary CHO intake. Diet-induced manipulation of fuel utilization
129 during exercise is likely underpinned by altered systemic (14, 48) and/or local
130 (i.e. muscle) (42) fuel availability, supported by altered expression of proteins
131 implicated in up- or down-regulating CHO and fatty acid metabolism (43).
132 Thus, the expression of selected genes encoding proteins involved in fuel
133 metabolism was quantified in order to gain further insights into the potential of
134 skeletal muscle to adapt to varying levels of CHO intake.

135

136 **Materials and methods**

137 *Participants*

138 Thirteen recreationally-active males took part in the present investigation
139 (age, 26 ± 7 y; height, 177.3 ± 7.8 cm; mass, 71.4 ± 7.8 kg; maximum oxygen
140 uptake [$\dot{V}O_{2\max}$], 49.7 ± 6.7 ml·kg⁻¹·min⁻¹; data are mean \pm standard deviation
141 [SD]). Participants were required to be aged 18-45 years with a body mass
142 index of 20-25 kg·m⁻² and a $\dot{V}O_{2\max}$ of 40-60 ml·kg⁻¹·min⁻¹. Prospective
143 participants were excluded if they had known or suspected intolerance or
144 hypersensitivity to the planned dietary interventions or were taking
145 medication. All participants provided written informed consent and all
146 procedures were approved by the Health Research Authority of the United
147 Kingdom (15/WM/0452). The study was registered at clinicaltrials.gov as
148 NCT02605291 and conducted in accordance with the Declaration of Helsinki.

149

150 *Study design*

151 This study adopted a cross-over design in which participants completed three
152 four-day diet-exercise interventions in randomised order, with a 1-4 week
153 intervening washout period (**Figure 1**). Following an initial assessment to
154 determine $\dot{V}O_{2\max}$ and assess the treadmill speed vs. oxygen consumption
155 ($\dot{V}O_2$) relationship, participants completed a familiarization to the experimental
156 protocol. The familiarization period was also used to estimate typical daily
157 energy expenditure within the intervention periods. The experimental
158 procedures consisted of 2-d completion of a weighed diet record (day -2 and -
159 1), 1-d consumption of a standardised diet (day 0), and a four-day exercise-
160 diet manipulation (lower, moderate, or higher carbohydrate) finishing with a

161 60-min treadmill run at 65% $\dot{V}O_{2\max}$ with heart rate measurement, expired
162 gas analysis, and venous blood sampling throughout, with pre- and post-
163 exercise muscle biopsies.

164

165 ***Insert Figure 1

166

167 *Initial assessment*

168 Participants arrived for the initial assessment in the morning after an overnight
169 fast, having refrained from exercise and alcohol consumption for 24 h. Height
170 and body mass was recorded prior to an incremental treadmill test to
171 exhaustion. The test started at 7-10 $\text{km}\cdot\text{h}^{-1}$ against a 1% incline, and the
172 speed was increased by 2 $\text{km}\cdot\text{h}^{-1}$ every 4 min for four continuous stages.
173 Subsequently, the treadmill gradient was increased by 1% every minute until
174 volitional exhaustion. Breath-by-breath measurements of $\dot{V}O_2$ (Oxycon Pro,
175 Jaeger, Wuerzberg, Germany) and heart rate (Polar FT-2, Finland) were
176 obtained throughout. $\dot{V}O_2$ was averaged over the last minute of each 4-min
177 stage, and linear regression was used to estimate the speed vs. $\dot{V}O_2$
178 relationship for use in the experimental trials. $\dot{V}O_{2\max}$ was calculated as the
179 highest rolling 60-s average and considered maximal if two of the following
180 conditions were met: (1) a plateau in $\dot{V}O_2$ despite further increasing workload
181 ($\leq 2 \text{ ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$); (2) heart rate $\leq 10 \text{ beats}\cdot\text{min}^{-1}$ of age-predicted maximum
182 ($220 \text{ beats}\cdot\text{min}^{-1}$ minus age in years), and (3) respiratory exchange ratio > 1.1 .

183

184 *Familiarization procedures*

185 Participants returned to the laboratory ~2-7-d following the initial assessment
186 to begin the familiarization trial. A full familiarization to the experimental
187 procedures was completed, with the exception of dietary manipulation, muscle
188 biopsies, and venous blood sampling. In order to estimate free-living energy
189 expenditure, participants were fitted with an Actiheart (CamNtech Ltd,
190 Cambridge, UK) at the start of day 1 of familiarization and it remained in place
191 until the start of day 4 of familiarization. Free-living energy expenditure was
192 calculated according to manufacturer configurations as the sum of resting
193 energy expenditure, activity energy expenditure and dietary induced
194 thermogenesis (estimated as 10% of total energy expenditure). The estimated
195 EE for Day 1, Day 2, and Day 3 was 3427 ± 1140 , 3146 ± 926 , and $2621 \pm$
196 724 kcal, respectively, which was subsequently used to estimate required
197 energy intake on each day during subsequent experimental trials in each
198 volunteer.

199

200 *Pre-experimental procedures*

201 The experimental trials commenced with a 3-d pre-trial phase (day -2, -1, and
202 0). On day -2 and -1, participants recorded a 2-d weighed diet record using
203 digital weighing scales and blank diaries provided. These diaries were
204 analysed for energy and macronutrient intake (Dietplan 6.70.67, Forestfield
205 Software Ltd.) and participants were asked to repeat these diets on day -2
206 and -1 of subsequent experimental trials. On day 0, participants consumed a
207 standardised diet provided by the researchers (50% carbohydrate, 35% fat,
208 and 15% protein, with total energy intake equal to estimated daily energy

209 expenditure during familiarization). Participants refrained from exercise on day
210 -1 and 0.

211

212 *Experimental procedures: Glycogen-depleting exercise*

213 On day 1, participants reported to the laboratory after an overnight fast and a
214 muscle biopsy was obtained from the lateral portion of the *vastus lateralis*,
215 ~10-15 cm above the patella. Briefly, local anaesthetic was applied to the skin
216 and fascia, and a 5-mm Bergström needle (6G) was used with suction to
217 sample ~50-150 mg of muscle tissue through a small incision. On collection,
218 muscle samples were quickly rinsed with saline, blotted dry, dissected free of
219 visible fat and connective tissue, separated into 3-4 ~25 mg pieces
220 (dependent on yield) with some pieces immediately frozen in liquid nitrogen
221 (for glycogen and gene expression analysis) and one of the pieces embedded
222 in specialist medium (Tissue Tek® O.C.T.™ Compound, Sakura Finetek
223 Europe, NET) prior to freezing in liquid nitrogen cooled isopentane (for
224 intramuscular triglyceride [IMTG] analysis). All muscle was stored at -70°C
225 until further analysis. Muscle biopsies were only obtained on day 1 in the first
226 experimental trial to ascertain habitual resting skeletal muscle glycogen and
227 IMTG content and baseline gene expression.

228

229 Following the biopsy, a standardized breakfast was consumed (one-third of
230 daily energy intake, 50% carbohydrate, 35% fat, 15% protein). Two 50-min
231 supervised high-intensity interval sessions were then performed on a
232 treadmill, separated by 3-4 hours. These sessions involved a 10-min period at
233 the speed estimated at 70% $\dot{V}O_{2\max}$ followed by 5 x 3-min intervals at 90%

234 $\dot{V}O_{2\max}$, with 3-min active recovery between-intervals (1.5 min at 25%
235 $\dot{V}O_{2\max}$ and 1.5 min at 50% $\dot{V}O_{2\max}$). A further 10-min period was then
236 performed at 70% $\dot{V}O_{2\max}$. A low carbohydrate lunch was consumed
237 between-sessions (one-sixth of daily energy intake, <25% carbohydrate,
238 >65% fat, 15% protein), and then again following the second interval training
239 session. A further single interval training session was repeated after an
240 overnight fast on the morning of day 2.

241

242 *Experimental procedures: Dietary manipulation*

243 Participants were randomly allocated to one of three experimental diets, which
244 were consumed following the second interval session of day 1 and on day 2
245 and 3 of the protocol. These diets were constructed to be lower (LOW:
246 carbohydrate, $\leq 20\%$; fat, $\geq 65\%$; protein, $\sim 15\%$), moderate (MOD:
247 carbohydrate, $\sim 50\%$; fat, $\sim 35\%$, protein, $\sim 15\%$), or higher (HIGH:
248 carbohydrate, $\geq 65\%$; fat, $\leq 20\%$; protein, $\sim 15\%$) in carbohydrate. Diets were
249 isocaloric and total energy intake was equal to estimated daily energy
250 expenditure determined using heart-rate accelerometry during familiarization.
251 All diets were prepared by the researchers, and participants were given
252 written instructions regarding their consumption. The final consumed dietary
253 intervention characteristics are shown in **Table 1**.

254

255 ***Insert Table 1

256

257 Participants collected all urine output on day 3, and on the morning of day 4,
258 which was subsequently used to correct substrate oxidation rates (resting

only) estimated via gas exchange for urinary nitrogen excretion. Urinary nitrogen content was estimated by correcting urinary urea and creatinine by 1.11 to account for non-measured nitrogen sources (e.g. ammonia, urate) (5), and analyzed enzymatically using a semi-automated analyzer (ILab 650, Instrumentation Laboratory, Bedford, MA) and commercially available kits (IL Test urea, IL Test creatinine, Instrumentation Laboratories, Cheshire, UK).

Experimental procedures: Metabolic assessment

On day 4, participants returned to the laboratory after an overnight fast. Post-void body mass was measured and a resting metabolic assessment was undertaken. Participants lay supine under a ventilated hood connected to an indirect calorimeter (GEM, GEM Nutrition Ltd, Cheshire, UK) that enabled the collection of expired gases for estimation of resting whole-body fat and carbohydrate oxidation using stoichiometric equations (15). An antecubital venous cannula was then inserted and a 10 mL resting blood sample was collected. A muscle sample was then obtained from the *vastus lateralis* according to the procedures described above.

Following the resting muscle biopsy, participants ran on a treadmill for 60-min at 65% $\dot{V}O_{2\max}$. Venous blood (10 mL) and 4-min expired gas samples (Oxycon Pro, Jaeger, Wuerzburg, Germany) were collected every 20 min during exercise, and heart rate (Polar Electro Oy, Kemple, Finland) was collected continuously and recorded every 10 min. Immediately following exercise, a further muscle sample was obtained from the *vastus lateralis*. Pre- and post-exercise biopsies were collected from the same leg within each trial,

284 with samples taken at least 2 cm proximal from previous biopsy sites to
285 minimize the impact of local inflammation from previous sampling. For
286 consistency, the sampled leg order was standardized between participants
287 (familiarization – right leg, trial 1 – left leg, trial 2 – right leg, trial 3 – left leg).

288

289 *Muscle analysis*

290 For determination of muscle glycogen concentration, duplicate samples of 10-
291 15 mg of frozen muscle were powdered using a pestle and mortar pre-cooled
292 on dry ice. Thereafter, samples were hydrolyzed by adding 500 μL of 2 $\text{mol}\cdot\text{L}^{-1}$
293 HCl and incubated for 2 h at 95°C. After cooling to room temperature,
294 samples were neutralized with 500 μL of 2 $\text{mol}\cdot\text{L}^{-1}$ NaOH. Samples were then
295 vortexed, centrifuged (1800 g for 1 minute at 4°C) and the supernatant
296 analysed in duplicate for glucose concentration using a semi-automatic
297 analyser (ILab 650, Instrumentation Laboratory, Bedford, MA) and
298 commercially-available kit (Glucose Oxidase kit, Instrumentation Laboratories,
299 Cheshire, UK). Muscle glycogen content was taken as the average of the
300 duplicate muscle samples analysed, with the intra-assay coefficient of
301 variation <10%.

302

303 Muscle embedded in OCT compound was cryosectioned and analyzed for
304 Type 1 and 2 muscle fibre specific IMTG (BODIPY D3922, Thermofisher
305 Scientific, USA) and cytochrome c oxidase 4 (COX4, primary antibody
306 459600, Thermofisher Scientific, USA) content using immunohistochemical
307 approaches as described elsewhere (13, 40). BODIPY immunofluorescence
308 images were captured using a Leica DMIRE2 confocal microscope with a 40x

oil immersion objective (1,25 NA). COX4 images were captured using a Nikon E600 microscope coupled to a SPOT RT KE colour three shot CCD camera. Images were analysed using Image Pro Plus 5.1.

For analysis of basal muscle gene expression, RNA was extracted from 20-40 mg powdered vastus lateralis tissue using Tri reagent (1 mL, Sigma Aldrich, UK, T9424) for four samples per participant; baseline, LOW pre-exercise, MOD pre-exercise and HIGH pre-exercise. Following addition of chloroform (200 μ L, Acros organics 268320025), tubes were mixed vigorously, incubated at room temperature for 5 min and centrifuged for 10 min at 4°C at 12 000 g. The RNA phase was mixed with an equal volume of ice cold 70 % ethanol and RNA was purified on Reliaprep spin columns (Promega, USA, Z6111) according to the manufacturer's instructions. The LVis function of the FLUOstar Omega microplate reader was used to measure RNA concentration to ensure all samples for each participant had the same amount of RNA (184 ng - 400 ng) reverse transcribed to cDNA using the RT2 First Strand kit (Qiagen, UK, 330401). Quantitative RT-PCR analysis was performed using custom designed 384-well RT2 PCR Profiler Arrays (Qiagen) and RT2 SYBR Green Mastermix (Qiagen) on a CFX384 Real-Time PCR Detection system (BioRad). 2.8 ng cDNA was added to each well. All primers were commercially available from Qiagen and **Supplementary Table 1** displays the list of genes analysed alongside their Qiagen catalogue number and Refseq# (all supplemental material can be found at DOI: <https://doi.org/10.25500/edata.bham.00000609>). The absence of genomic DNA, the efficiency of reverse-transcription and the efficiency of the PCR

334 assay were assessed for each sample and conformed to the manufacturer's
335 limits in each case. Relative mRNA expression was determined using the 2-
336 $\Delta\Delta CT$ method (28). The C(t) values for housekeeper genes beta actin
337 (Refseq# NM_001101), ribosomal protein lateral stalk subunit P0 (Refseq#
338 NM_001002) and beta-2-microglobulin (Refseq# NM_004048) showed no
339 statistical differences between-groups. Therefore the mean C(t) of these three
340 housekeeper genes was used as an internal control. Data for LOW, MOD and
341 HIGH is presented as a fold-change from the baseline sample.

342

343 *Plasma and serum analysis*

344 Venous blood samples were placed into ethylenediaminetetraacetic acid-
345 containing, lithium-heparin-containing, or serum tubes (BD, New Jersey, USA)
346 and centrifuged at 1006 g for 15 minutes at 4°C. Plasma or serum was then
347 extracted and stored in aliquots at -70°C until analysis. All collected samples
348 were analysed using enzymatic colorimetric assays for glucose (GLU,
349 Glucose Oxidase kit, Instrumentation Laboratories, Cheshire, UK), non-
350 esterified fatty acids (NEFA, Randox, London, UK), glycerol (GLY, Randox,
351 London, UK), and lactate (LAC, Randox, London, UK) using a semi-automatic
352 analyzer (ILab 650, Instrumentation Laboratory, Bedford, MA). Intra-assay
353 coefficient of variation (CV) was <2.0% for all metabolite assays. Insulin was
354 analyzed by enzyme-linked immunoassays using a commercially available kit
355 (Ultra-sensitive Insulin ELISA kit, Human, DRG Diagnostics, Marburg, GER;
356 CV – 13.5%). Adrenaline and noradrenaline were measured pre- and post-
357 exercise using a commercially available kit (CatCombi ELISA kit, Human, IBL
358 International, GER; CV – 25.7% and 22.7%, respectively).

359

360 *Expired gas analysis*

361 $\dot{V}O_2$ and $\dot{V}CO_2$ were used to estimate whole-body rates of carbohydrate and
362 fat oxidation throughout exercise using the following equations (15):

363

364 Whole-body carbohydrate oxidation ($g \cdot min^{-1}$) = $(4.55 \times \dot{V}CO_2) - (3.21 \times$
365 $\dot{V}O_2)$

366

367 Whole-body fat oxidation ($g \cdot min^{-1}$) = $(1.67 \times \dot{V}O_2) - (1.67 \times \dot{V}CO_2)$

368

369 Where $\dot{V}O_2$ and $\dot{V}CO_2$ are in $L \cdot min^{-1}$.

370

371 *Statistical analysis*

372 Statistical procedures were conducted using commercially available software
373 (SAS Version 9.4, SAS Institute, Cary, NC). Sample size determinations were
374 made using the pre-exercise muscle glycogen content on Day 4 as the
375 primary outcome. Based on prior work reporting on glycogen
376 depletion/repletion patterns with similar exercise-dietary interventions, resting
377 glycogen was predicted to be highest in HIGH, and $\leq 70\%$ and $\leq 40\%$ of that
378 seen in HIGH with MOD and LOW, respectively (12). Assuming a two-sided
379 5% significance level, with the use of a within-subject SD of $20 \text{ mmol} \cdot \text{kg}^{-1} \text{ ww}$
380 (estimated from published studies reporting resting glycogen content), a
381 sample size of 12 was required to provide 95% power to detect the
382 differences predicted (i.e., HIGH vs. MOD, MOD vs. LOW).

383

384 Data are presented as raw means \pm SD, with statistical summaries where
385 appropriate presented in tabular form. Muscle-related outcomes, substrate
386 oxidation and plasma catecholamines were assessed using linear mixed
387 models, with intervention group (LOW, MOD, or HIGH) as fixed effects, and
388 subject as a random effect. For muscle glycogen and IMTG related-outcomes,
389 baseline concentration/content recorded on Day 1 of the first intervention
390 period was included as a covariate. From the models, adjusted means with
391 95% CIs or standard error (SE) were calculated. In addition, pairwise
392 differences (HIGH vs. MOD, MOD vs. LOW, and HIGH vs. LOW) were
393 calculated and presented as 95% CIs and associated unadjusted *P*-values.
394 Associations between dietary CHO intake and pre-exercise muscle glycogen,
395 muscle utilization and fat oxidation during exercise were explored using
396 Pearson product-moment correlations. For muscle gene expression, in order
397 to account for multiplicity, adjusted means with 99.95% CIs were calculated
398 for fold changes relative to baseline, with pairwise differences between
399 interventions periods calculated and presented as means \pm 99.95% CIs and
400 associated unadjusted *P*-values. Plasma metabolites and insulin data were
401 not normally distributed, and these data are presented as means \pm SD as
402 profiles across time with pairwise differences and associated unadjusted *P*-
403 values determined from Wilcoxon sign rank tests performed on time-averaged
404 AUC data. Statistical significance was only inferred when unadjusted *P*-values
405 met the threshold for significance after Bonferonni adjustment (i.e.,
406 0.05/#comparisons).

407

408 **Results**

409 *Intervention characteristics*

410 Of the 13 participants who received at least one of the dietary interventions,
411 10 participants completed all three periods, one completed two of the three
412 periods (HIGH and LOW) and two participants completed one period (one
413 HIGH, one MOD). Hence the number of participants completing each of the
414 dietary interventions was LOW=11, MOD=11, HIGH=12. Participants who did
415 not contribute any data to a treatment period were not included in that group.
416 The achieved relative exercise intensity for the 60-min treadmill exercise
417 bouts was similar between-trials (LOW, 64.6 ± 2.0 ; MOD, 64.4 ± 2.4 , and
418 HIGH, $64.8 \pm 2.5\%$ $\dot{V}O_{2\max}$, $P = 0.84$).

419

420 *Muscle substrate metabolism*

421 Pre-exercise COX4 protein content, as a marker of mitochondrial density, did
422 not differ in type I (LOW, 26.6 ± 12.9 ; MOD, 32.7 ± 13.3 ; HIGH, 31.1 ± 13.2
423 mean fluorescence intensity per fibre, $P = 0.14$) or type II (LOW, 20.7 ± 10.7 ;
424 MOD, 24.8 ± 10.1 ; HIGH, 23.6 ± 9.6 mean fluorescence intensity per fibre, P
425 = 0.20) fibres between-trials. Habitual resting muscle glycogen concentration
426 was 75.6 ± 18.8 mmol·kg⁻¹ ww, consistent with the lower range of expected
427 values of participants of similar overall fitness status (2). Pre- and post-
428 exercise muscle glycogen concentrations were significantly lower in LOW vs.
429 MOD and HIGH, but MOD and HIGH were not significantly different (**Figure**
430 **2**). Net muscle glycogen utilization was not significantly different between-
431 trials, though 95% confidence intervals suggest there was net utilization in
432 MOD and HIGH but not LOW (**Table 2**). Dietary CHO during the intervention

433 period was positively associated with pre-exercise muscle glycogen
434 concentration ($r = 0.62$, $P = 0.0001$) but not net muscle glycogen utilization (r
435 $= 0.18$, $P = 0.32$).

436

437 ***Insert Figure 2

438

439 Habitual resting IMTG content was 7.33 ± 4.80 and 3.77 ± 2.25 % area lipid
440 staining for Type 1 and 2 fibres, respectively. Pre-exercise IMTG content
441 were not significantly different between-interventions in type I or type II fibres.
442 Post-exercise IMTG content in type I fibres was significantly greater in LOW
443 than MOD, but not HIGH, and MOD and HIGH were not significantly different.
444 Post-exercise IMTG concentration in type II fibres was not significantly
445 different between-interventions. Net IMTG utilization was not significantly
446 different between-interventions in type I or type II fibres, however, 95%
447 confidence intervals suggest there was net IMTG utilization in type I fibres in
448 LOW and MOD but not HIGH, and in type II fibres in MOD but not LOW or
449 HIGH (**Table 2**).

450

451 ***Insert Table 2

452

453 *Whole-body substrate oxidation rates*

454 Pre-exercise resting whole-body carbohydrate oxidation rate was significantly
455 greater in HIGH (0.11 ± 0.08 g·min⁻¹) compared to LOW (0.03 ± 0.04 g·min⁻¹,
456 $P < 0.05$), whereas no significant differences were observed between LOW
457 and MOD (0.09 ± 0.07 g·min⁻¹, $P = 0.12$) or MOD and HIGH ($P = 0.53$, **Figure**

458 **3a**). Pre-exercise resting whole-body fat oxidation rates were not significantly
459 different between-trials (LOW, $0.09 \pm 0.04 \text{ g}\cdot\text{min}^{-1}$; MOD, $0.08 \pm 0.04 \text{ g}\cdot\text{min}^{-1}$;
460 HIGH, $0.07 \pm 0.05 \text{ g}\cdot\text{min}^{-1}$; $P > 0.05$, **Figure 3b**). Whole-body carbohydrate
461 oxidation during exercise was significantly lower in LOW compared to MOD
462 and HIGH, but MOD and HIGH were not significantly different (**Figure 3a**,
463 **Table 3**). Whole-body fat oxidation during exercise was significantly greater in
464 LOW compared to MOD and HIGH, but MOD and HIGH were not significantly
465 different (**Figure 3b**, **Table 3**). Dietary CHO intake during the intervention
466 period was not significantly associated with whole-body fat oxidation during
467 exercise ($r = -0.26$, $P = 0.16$).

468

469 ***Insert Panel Figure 3

470

471 ***Insert Table 3

472

473 *Blood responses*

474 Blood data is shown in **Figure 4** and statistical comparisons are summarized
475 in **Table 4**. Pre-exercise blood variables were not significantly different
476 between-trials, other than plasma lactate concentration being significantly
477 lower in MOD vs. LOW. Plasma glucose, lactate, NEFA, adrenaline,
478 noradrenaline and serum insulin concentrations during exercise were not
479 significantly different between-trials. Plasma glycerol concentrations during
480 exercise were significantly greater in LOW than MOD and HIGH, whereas
481 MOD and HIGH were not significantly different.

482

483 ***Insert Panel Figure 4

484

485 ***Insert Table 4

486

487 *Gene expression*

488 Pre-exercise metabolic gene expression for the 34 genes quantified in LOW,
489 MOD, and HIGH is shown in **Supplementary Table 1**, with between-trial
490 comparisons shown in Supplementary Table 2. Three genes were significantly
491 different between-trials following correction for multiple comparisons ($N = 102$,
492 so $P < 0.0005$). mRNA expression of FABP3, MLYCD, and UCP3 were all
493 significantly lower in MOD and HIGH than LOW (**Figure 5**). With a less
494 conservative statistical approach, correction for multiple comparisons within
495 each gene ($N = 3$), a further seven genes were differentially expressed
496 between-trials (i.e. $P < 0.016$). Using this approach, mRNA expression of
497 ACSL1, PDK2, and PNPLA2 was significantly lower in MOD and HIGH than
498 LOW, and mRNA expression of CD36, CPT1B, HADHA, and SLC27A1 were
499 all significantly lower in HIGH than LOW (**Supplementary Table 2**).

500

501 ***Insert Figure 5

502 **Discussion**

503 The aim of the present investigation was to assess muscle glycogen
504 availability and muscle and whole-body metabolic responses to moderate-
505 intensity exercise following short-term lower, moderate or higher dietary CHO
506 intake. Contrary to our hypothesis, graded pre-exercise muscle glycogen
507 availability was not observed. Rather, the main findings were: 1) pre-exercise
508 muscle glycogen content was not different between MOD and HIGH; 2) MOD
509 and HIGH produced broadly similar metabolic responses before and during
510 subsequent moderate-intensity exercise, and; 3) metabolic responses were
511 uniquely sensitive to lowered dietary CHO intake. That is, the LOW condition
512 showed reduced resting muscle glycogen, elevated whole-body fat oxidation
513 rates and plasma glycerol concentrations during exercise, and increased
514 skeletal muscle expression of several genes encoding proteins implicated in
515 fat utilization.

516

517 As stated, and in contrast to our hypothesis (4, 16), HIGH did not produce
518 significantly greater pre-exercise muscle glycogen concentration than MOD,
519 despite $1.5 \text{ g CHO} \cdot \text{kg} \cdot \text{day}^{-1}$ greater CHO ingestion in the preceding 48 h
520 (**Figure 2**). Differences in pre-exercise muscle glycogen concentration
521 between MOD and HIGH could have been observed with greater CHO intake
522 in HIGH. However, Costill and colleagues (12) observed graded pre-exercise
523 muscle glycogen using similar CHO intakes as the present study, but applied
524 over a 24-h recovery period. It is plausible the recovery duration following
525 glycogen-depleting exercise is influential in the grading of muscle glycogen
526 concentration to CHO ingestion; the 48 h used in the presented study may

527 have been sufficient for muscle glycogen to normalise between MOD and
528 HIGH at the CHO intakes provided. This contention may be further
529 substantiated by the graded muscle glycogen observed ~15-16 h following
530 glycogen-depleting exercise with 0, 3.6, and 7.6 g.kg⁻¹ CHO ingestion in a
531 more recent study by Hearn and co-workers (21). The absence of
532 differences in pre-exercise muscle glycogen content could also be attributable
533 to the aerobic fitness status of the study cohort, given that those with a higher
534 fitness status have greater capacity for muscle glycogen storage (2). The fate
535 of the additional CHO provided in HIGH is not readily apparent from the
536 present data, although oxidation and/or storage as liver glycogen are
537 possibilities. Regardless, the present data show that when the recovery
538 duration after successive bouts of high intensity interval exercise is 48 h,
539 increasing dietary CHO intake from ~5.0 to ~6.5 g CHO.kg⁻¹.day⁻¹ confers no
540 additional benefit to muscle glycogen storage.

541

542 Consequently, metabolic responses during exercise were similar between
543 MOD and HIGH, with no clear differences in muscle glycogen use, whole-
544 body substrate oxidation rates, blood variables, or pre-exercise gene
545 expression (Tables 2-4, Supplementary Table 2). A relatively modest net
546 muscle glycogen use was seen in the present study (Figure 2, Table 2) which
547 may be explained by several factors such as the muscle group sampled (i.e.,
548 vastus lateralis shows lower net glycogen use than soleus or gastrocnemius
549 during level running; (12)), the exercise modality (i.e., net glycogen use in
550 vastus lateralis is lower in level running than cycle ergometry; (2, 3)) and the
551 moderate exercise intensity employed (16). Previous work with similar CHO

552 intakes in the HIGH condition as the present study reported similar respiratory
553 exchange ratio (RER) responses to exercise as compared to moderate or
554 mixed CHO intakes; however, in contrast to the present study, this was
555 observed despite elevated muscle glycogen availability in the high CHO
556 conditions (12, 16). With higher rates of CHO ingestion ($\sim 8 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$),
557 clearer exercise-metabolic differences have been observed (4). Accordingly,
558 the addition of the present data to existing literature suggests increasing
559 short-term CHO intake from ~ 4.5 to $\sim 6.5 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (~ 45 to $\sim 70\%$ EI) in
560 recovery from exercise does not discernibly influence metabolic responses to
561 subsequent moderate-intensity exercise (12, 16), and that more aggressive
562 increases in CHO intake may be required to alter fuel metabolism during
563 exercise (4).

564

565 In contrast to the largely similar response between MOD and HIGH,
566 consistent metabolic differences were observed in LOW. This included
567 lowered pre-exercise muscle glycogen availability (**Table 2**), decreased CHO
568 and increased fatty acid oxidation during exercise (**Table 3**), elevated plasma
569 glycerol concentrations (**Table 4**), and up-regulation of several genes
570 implicated in substrate metabolism, such as FABP3, MLYCD, and UCP3, with
571 several other genes possibly differentially expressed in LOW
572 (**Supplementary Table 2**). These data align with previous research reporting
573 decreased CHO and increased fatty acid metabolism during exercise
574 commenced with lowered muscle glycogen (19, 26, 32, 46, 47). It is clearly
575 plausible reduced muscle glycogen availability contributed to the altered fuel
576 use *per se* (18), although it is also possible the additional dietary fat intake

577 resulted in adaptations that augmented fatty acid oxidation in LOW (27).
578 Regardless of the precise mechanism, the present data indicate short-term
579 CHO intakes of ~ 2.4 g CHO \cdot kg $^{-1}\cdot$ d $^{-1}$ ($\sim 21\%$ EI) in recovery from exercise are
580 sufficient to reduce muscle glycogen availability and alter substrate
581 metabolism during subsequent moderate-intensity exercise, consistent with
582 what might be expected from studies of non-ketogenic low-CHO, high-fat diets
583 (7). Whether a 'threshold' dietary CHO intake exists, somewhere between
584 ~ 2.5 and ~ 4.5 g CHO \cdot kg $^{-1}\cdot$ d $^{-1}$ (i.e., $\sim 20\text{--}45\%$ EI), at which this metabolic shift
585 takes place requires further investigation.

586

587 The gene expression data demonstrate LOW induced a coordinated change
588 in basal skeletal muscle gene expression favouring fatty acid utilization, which
589 is consistent with the metabolic data observed during subsequent moderate-
590 intensity exercise (**Supplementary Table 2**). Given the 48-h recovery
591 following the previous exercise bout in the present investigation, this
592 coordinated change in muscle gene expression can be confidently attributed
593 to the dietary manipulations (49). Our data align with previous work
594 demonstrating increased expression of FABP (30), UCP3 (39), PDK2 (11),
595 CPT1 (3), CD36 (10), and HADHA (30) with low CHO availability. To our
596 knowledge, a prior nutrient-exercise induced regulation of ACSL1, MLYCD,
597 PNPLA2, and SLC27A1 gene expression has not previously been shown in
598 human muscle, but their up-regulation with lower CHO intake is consistent
599 with an intracellular environment favouring fatty acid utilization. Altered
600 expression of these genes and/or the proteins they encode for has been
601 observed after a period of endurance exercise training, a stimulus expected to

602 augment the capacity for fatty acid metabolism in skeletal muscle (1, 23, 25,
603 44). Increased UCP3 gene expression in the present investigation is
604 interesting in the context of research showing impaired exercise economy
605 following ingestion of a low CHO-high fat diet (8, 9, 41), given UCP3 is
606 implicated in uncoupling oxidative phosphorylation from ATP synthesis, and
607 mitochondrial fatty acid export when supply exceeds oxidation capacity (36–
608 38). Whilst significant between-diet effects on running economy were not seen
609 in the present investigation (data not shown), possibly due to the low exercise
610 intensity (41), the data, albeit at the gene level, provide a plausible
611 mechanism for low-CHO availability-induced impairments in exercise
612 economy observed elsewhere (8, 9, 41). Collectively, the gene expression
613 data confirms several previous observations and adds new insights into the
614 coordinated mRNA response to diet-induced alterations in CHO availability in
615 humans.

616

617 In summary, the present data demonstrate, within a model of short-term
618 exercise-diet manipulation, graded metabolic responses to altering dietary
619 CHO intake do not appear present in the $\sim 5.0\text{--}6.5\text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ range (46–
620 61% of daily EI). In contrast, more marked reductions in CHO intake (~ 2.4
621 $\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, $\sim 21\%$ EI) lowered resting muscle glycogen concentration, altered
622 resting expression of genes related to fatty acid utilization in skeletal muscle,
623 and ultimately increased whole-body fat oxidation during subsequent
624 moderate-intensity exercise. The data presented herein combined with that of
625 previous reports suggests that metabolic responses appear somewhat
626 resistant to short-term dietary CHO change within the $4.5\text{--}6.5\text{ g CHO}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$

627 (45-70% EI) range (12, 16), but are affected by more aggressive CHO
628 increases ($>6.5 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, $>70\% \text{ EI}$) or decreases ($<2.5 \text{ g CHO} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$,
629 $<20\% \text{ EI}$) (4). Whether a threshold exists between 4.5 and 2.5 $\text{g} \cdot \text{CHO} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$
630 (45%-20% EI) whereby fatty acid metabolism is augmented remains to be
631 tested. These findings help to provide a useful framework for researchers
632 when examining responses to exercise-diet manipulations. Furthermore, for
633 those interested in optimizing fat oxidation, the results provide insights into the
634 range of moderate to higher short-term CHO intakes within which fat oxidation
635 is maintained, and highlight the degree of dietary change necessary to induce
636 clear alterations in in fat oxidation during exercise.

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- 820
- 821

822 **Table headings**

823

824 Table 1. Dietary intervention characteristics

825

826 Table 2. Statistical summary of muscle glycogen and intramuscular
827 triglycerides concentrations during the 60-min steady-state treadmill running
828 at 65% $\dot{V}O_{2\max}$ in LOW, MOD, and HIGH

829

830 Table 3. Statistical summary of whole-body substrate oxidation rates during
831 the 60-min steady-state treadmill running at 65% $\dot{V}O_{2\max}$ in LOW, MOD, and
832 HIGH

833

834 Table 4. Statistical summary of plasma and serum concentrations at rest (R)
835 and during the 60-min steady-state treadmill running at 65% $\dot{V}O_{2\max}$ (Ex) in
836 LOW, MOD, and HIGH

837 **Figure headings**

838

839 Figure 1. Schematic overview of the experimental design. After two days of
840 controlled habitual diet consumption, participants undertook successive bouts
841 of interval running exercise across Days 1 and 2. Isocaloric diets of lower
842 (LOW), moderate (MOD) and higher (HIGH) carbohydrate (CHO) intakes
843 were provided across Days 1-3. Metabolic responses to 60-min running at
844 $\sim 65\% \dot{V}O_{2\max}$ was assessed on the morning of Day 4, in the overnight fasted
845 state, and ~ 48 h after the last exercise bout. A pre-exercise muscle biopsy on
846 Day 1 was taken on only one occasion. CHO intakes expressed as grams of
847 CHO per kilogram body mass.

848

849 Figure 2. Muscle glycogen (a) concentration pre- 60-min steady-state
850 treadmill running at $65\% \dot{V}O_{2\max}$ (mean and individual concentrations) and
851 (b) net utilization (mean \pm 95% CI) during 60-min treadmill running at 65%
852 $\dot{V}O_{2\max}$ in LOW, MOD, and HIGH.

853

854 Figure 3. Whole-body rates of (a) carbohydrate and (b) fat oxidation during
855 60-min steady-state treadmill running at $65\% \dot{V}O_{2\max}$ in LOW, MOD, and
856 HIGH conditions.

857

858 Figure 4. Serum (a) insulin and plasma (b) glycerol, (c) lactate, (d) non-
859 esterified fatty acid concentrations during 60-min treadmill running at 65%
860 $\dot{V}O_{2\max}$ in LOW, MOD, and HIGH conditions. ‘*’ denotes mean exercise AUC
861 was different in MOD, HIGH vs. LOW ($P < 0.05$).

862

863 Figure 5. mRNA expression of metabolic genes prior to 60-min steady-state
864 treadmill running at $65\% \dot{V}O_{2\max}$ in LOW, MOD, and HIGH conditions,
865 expressed as fold-change relative to baseline (day 1 of the first experimental
866 trial). ‘*’ denotes significantly different vs. LOW ($P < 0.0001$).

867

Table 1. Dietary intervention characteristics

| | Habitual | Day 0 | LOW | MOD | HIGH |
|--|------------|-------------|-------------|-------------|-------------|
| Energy (kcal.d ⁻¹) | 2250 ± 603 | 2736 ± 797 | 3080 ± 917 | 3084 ± 921 | 3145 ± 913 |
| Contribution to energy intake (%) | | | | | |
| CHO | 46.9 ± 8.4 | 48.4 ± 4.0 | 21.3 ± 0.8 | 46.3 ± 0.7 | 60.5 ± 1.6 |
| Fat | 33.3 ± 6.6 | 33.6 ± 2.8 | 63.2 ± 1.2 | 38.3 ± 0.7 | 24.3 ± 1.8 |
| Protein | 18.4 ± 5.9 | 14.8 ± 0.7 | 15.0 ± 0.7 | 14.8 ± 0.6 | 14.2 ± 0.8 |
| Total (g.kg ⁻¹ .d ⁻¹) | | | | | |
| CHO | | 4.66 ± 1.20 | 2.40 ± 0.66 | 4.98 ± 1.31 | 6.48 ± 1.56 |
| Fat | | 1.44 ± 0.40 | 3.07 ± 0.80 | 1.89 ± 0.54 | 1.25 ± 0.36 |
| Protein | | 1.42 ± 0.34 | 1.65 ± 0.45 | 1.60 ± 0.42 | 1.56 ± 0.40 |
| Total (g.d ⁻¹) | | | | | |
| CHO | 267 ± 96 | 328 ± 93 | 169 ± 53 | 322 ± 141 | 460 ± 124 |
| Fat | 81 ± 21 | 101 ± 30 | 215 ± 63 | 133 ± 42 | 89 ± 30 |
| Protein | 103 ± 40 | 100 ± 26 | 115 ± 35 | 113 ± 33 | 111 ± 31 |

LOW, MOD and HIGH are calculated from the averages from Day 1-3. Mean ± SD.

Table 2. Statistical summary of muscle glycogen and intramuscular triglycerides concentrations during the 60-min steady-state treadmill running at 65%VO_{2max} in LOW, MOD, and HIGH

| | Trial, contrast | Adjusted mean (SE/95% CI) | | | Mean difference (95% CI) | | |
|--|----------------------|---------------------------|-------------------|-------------------|--------------------------|---------------------|--------------------|
| | | LOW | MOD | HIGH | MOD-LOW | HIGH-LOW | HIGH-MOD |
| Pre-exercise muscle glycogen | Estimate (95% CI) | 54.3 (41.5, 67.1) | 82.8 (70.0, 95.6) | 81.6 (68.5, 94.8) | 28.5 (15.8, 41.2) | 27.3 (14.3, 40.4) | -1.2 (-14.2, 11.9) |
| (mmol.kg ⁻¹ ww) | P-value | - | - | - | 0.0002 | 0.0004 | 0.85 |
| Net muscle glycogen utilization | Estimate (95% CI) | 4.2 (-3.7, 12.1) | 11.3 (3.5, 19.2) | 13.5 (4.8, 22.1) | 7.1 (-2.9, 17.2) | 9.3 (-1.5, 20.1) | 2.1 (-8.7, 12.9) |
| (mmol.kg ⁻¹ ww) | P-value | - | - | - | 0.15 | 0.09 | 0.68 |
| Pre-exercise type I fibre IMTG (%) | Estimate (SE/95% CI) | 11.6 (1.3) | 10.7 (1.3) | 9.3 (1.4) | -0.9 (-4.1, 2.3) | -2.3 (-5.6, 1.0) | -1.4 (-4.7, 1.9) |
| | P-value | - | - | - | 0.55 | 0.16 | 0.39 |
| Post-exercise type I fibre IMTG (%) | Estimate (SE/95% CI) | 8.8 (1.0) | 6.6 (1.0) | 8.8 (1.0) | -2.1 (-4.0, -0.2) | -0.47 (-2.47, 1.54) | 1.7 (-0.3, 3.6) |
| | P-value | - | - | - | 0.03 | 0.63 | 0.09 |
| Net type I fibre IMTG utilization (%) | Estimate (95% CI) | 2.5 (0.7, 4.4) | 3.8 (2.0, 5.5) | 1.6 (-0.3, 3.5) | 1.3 (-0.9, 3.4) | -0.9 (-3.3, 1.4) | -2.2 (-4.5, 0.1) |
| | P-value | - | - | - | 0.24 | 0.41 | 0.06 |
| Pre-exercise type II fibre IMTG (%) | Estimate (SE/95% CI) | 5.0 (0.8) | 6.4 (0.8) | 5.3 (0.9) | 1.5 (-1.0, 3.9) | 0.3 (-2.6, 2.9) | -1.2 (-3.7, 1.4) |
| | P-value | - | - | - | 0.21 | 0.79 | 0.35 |
| Post-exercise type II fibre IMTG (%) | Estimate (95% CI) | 5.3 (0.8) | 4.5 (0.7) | 4.9 (0.8) | -0.8 (-2.5, 0.9) | -0.41 (-2.3, 1.4) | 0.38 (-1.4, 2.1) |
| | P-value | - | - | - | 0.34 | 0.64 | 0.65 |
| Net type II fibre IMTG utilization (%) | Estimate (95% CI) | -0.3 (-2.0, 1.4) | 1.7 (0.1, 3.2) | 0.0 (-1.8, 1.7) | 2.0 (-0.2, 4.2) | 0.2 (-2.2, 2.7) | -1.8 (-4.1, 0.5) |
| | P-value | - | - | - | 0.07 | 0.84 | 0.12 |

Table 3. Statistical summary of whole-body substrate oxidation rates during the 60-min steady-state treadmill running at 65%VO_{2max} in LOW, MOD, and HIGH

| | | Adjusted mean (g.min ⁻¹) | | | Mean difference (g.min ⁻¹) | | |
|---|-----------------|--------------------------------------|--------|--------|--|----------------|---------------|
| | Trial, contrast | LOW | MOD | HIGH | MOD-LOW | HIGH-LOW | HIGH-MOD |
| CHO oxidation (g.min ⁻¹) | Estimate | 1.16 | 1.60 | 1.72 | 0.44 | 0.57 | 0.12 |
| | (SE/95% CI) | (0.12) | (0.12) | (0.12) | (0.13, 0.75) | (0.24, 0.89) | (-0.20, 0.44) |
| | <i>P</i> -value | - | - | - | 0.008 | 0.002 | 0.43 |
| Fat oxidation (g.min ⁻¹) | Estimate | 0.72 | 0.54 | 0.47 | -0.18 | -0.25 | -0.07 |
| | (SE/95% CI) | (0.04) | (0.04) | (0.05) | (-0.28, -0.08) | (-0.35, -0.15) | (-0.17, 0.03) |
| | <i>P</i> -value | - | - | - | 0.001 | <0.0001 | 0.17 |
| RER | Estimate | 0.81 | 0.86 | 0.88 | 0.05 | 0.07 | 0.02 |
| | (SE/95% CI) | (0.01) | (0.01) | (0.01) | (0.02, 0.07) | (0.04, 0.09) | (-0.01, 0.04) |
| | <i>P</i> -value | - | - | - | 0.0005 | <0.0001 | 0.14 |

Table 4. Statistical summary of plasma concentrations during the 60-min steady-state treadmill running at 65%VO_{2max} in LOW, MOD, and HIGH

| | | Median | | | Median difference | | |
|---|-----------------|----------------------|---------------|---------------|--------------------------|-----------------|-----------------|
| | Trial, contrast | LOW | MOD | HIGH | MOD-LOW | HIGH-LOW | HIGH-MOD |
| Insulin ($\mu\text{IU}\cdot\text{mL}^{-1}$) | Median | 1.35 | 2.35 | 2.28 | 1.14 | 1.11 | -0.12 |
| | (Min, max) | (0.31, 2.65) | (0.95, 4.52) | (0.84, 3.85) | - | - | - |
| | <i>P</i> -value | - | - | - | 0.02 | 0.02 | 0.57 |
| Glucose ($\text{mmol}\cdot\text{L}^{-1}$) | Median | 4.92 | 5.12 | 5.25 | 0.02 | 0.09 | 0.09 |
| | (Min, max) | (4.44, 5.57) | (4.56, 5.39) | (4.48, 5.60) | - | - | - |
| | <i>P</i> -value | - | - | - | 0.73 | 0.55 | 0.25 |
| Glycerol ($\mu\text{mol}\cdot\text{L}^{-1}$) | Median | 254.0 | 114.0 | 134.7 | -100.8 | -98.3 | 1.8 |
| | (Min, max) | (134.8, 298.5) | (81.8, 231.5) | (59.3, 309.0) | - | - | - |
| | <i>P</i> -value | - | - | - | 0.008 | 0.008 | 0.73 |
| Lactate ($\text{mmol}\cdot\text{L}^{-1}$) | Median | 1.20 | 1.34 | 1.57 | 0.12 | 0.17 | 0.11 |
| | (Min, max) | (0.36, 2.08) | (0.65, 1.87) | (0.69, 5.22) | - | - | - |
| | <i>P</i> -value | - | - | - | 0.46 | 0.15 | 0.31 |
| NEFA ($\text{mmol}\cdot\text{L}^{-1}$) | Median | 0.67 | 0.43 | 0.44 | -0.21 | -0.23 | 0.11 |
| | (Min, max) | (0.26, 0.92) | (0.16, 0.84) | (0.16, 1.23) | - | - | - |
| | <i>P</i> -value | - | - | - | 0.04 | 0.02 | 1.00 |
| | | Adjusted mean | | | Mean difference | | |
| | | LOW | MOD | HIGH | MOD-LOW | HIGH-LOW | HIGH-MOD |
| Pre-exercise adrenaline ($\text{pg}\cdot\text{mL}^{-1}$) | Estimate | 152.5 | 152.3 | 150.4 | -0.2 | -2.2 | -1.9 |
| | (SE/95% CI) | (13.9) | (15.2) | (15.0) | (-36.8, 36.4) | (-38.8, 34.5) | (-41.3, 37.4) |
| | <i>P</i> -value | - | - | - | 0.99 | 0.90 | 0.92 |
| Post-exercise adrenaline ($\text{pg}\cdot\text{mL}^{-1}$) | Estimate | 278.1 | 249.6 | 249.0 | -28.5 | -29.1 | -0.58 |
| | (SE/95% CI) | (25.0) | (23.7) | (23.7) | (-77.4, 20.4) | (-78.9, 20.7) | (-48.7, 47.5) |
| | <i>P</i> -value | - | - | - | 0.23 | 0.23 | 0.98 |
| Pre-exercise noradrenaline ($\text{pg}\cdot\text{mL}^{-1}$) | Estimate | 316.9 | 428.4 | 327.2 | 111.5 | 10.3 | -101.2 |
| | (SE/95% CI) | (253.6) | (74.3) | (73.6) | (-30.7, 253.8) | (-132.0, 152.7) | (-255.8, 53.4) |
| | <i>P</i> -value | - | - | - | 0.12 | 0.88 | 0.18 |
| Post-exercise noradrenaline | Estimate | 793.4 | 891.5 | 860.2 | 98.1 | 66.8 | -31.3 |
| | (SE/95% CI) | (135.1) | (128.8) | (128.9) | (-149.8, 346.1) | (-185.9, 319.5) | (-275.4, 212.8) |
| | <i>P</i> -value | - | - | - | - | - | - |

| | | | | | | | |
|------------------------|-----------------|---|---|---|------|------|------|
| (pg.mL ⁻¹) | <i>P</i> -value | - | - | - | 0.41 | 0.58 | 0.79 |
|------------------------|-----------------|---|---|---|------|------|------|

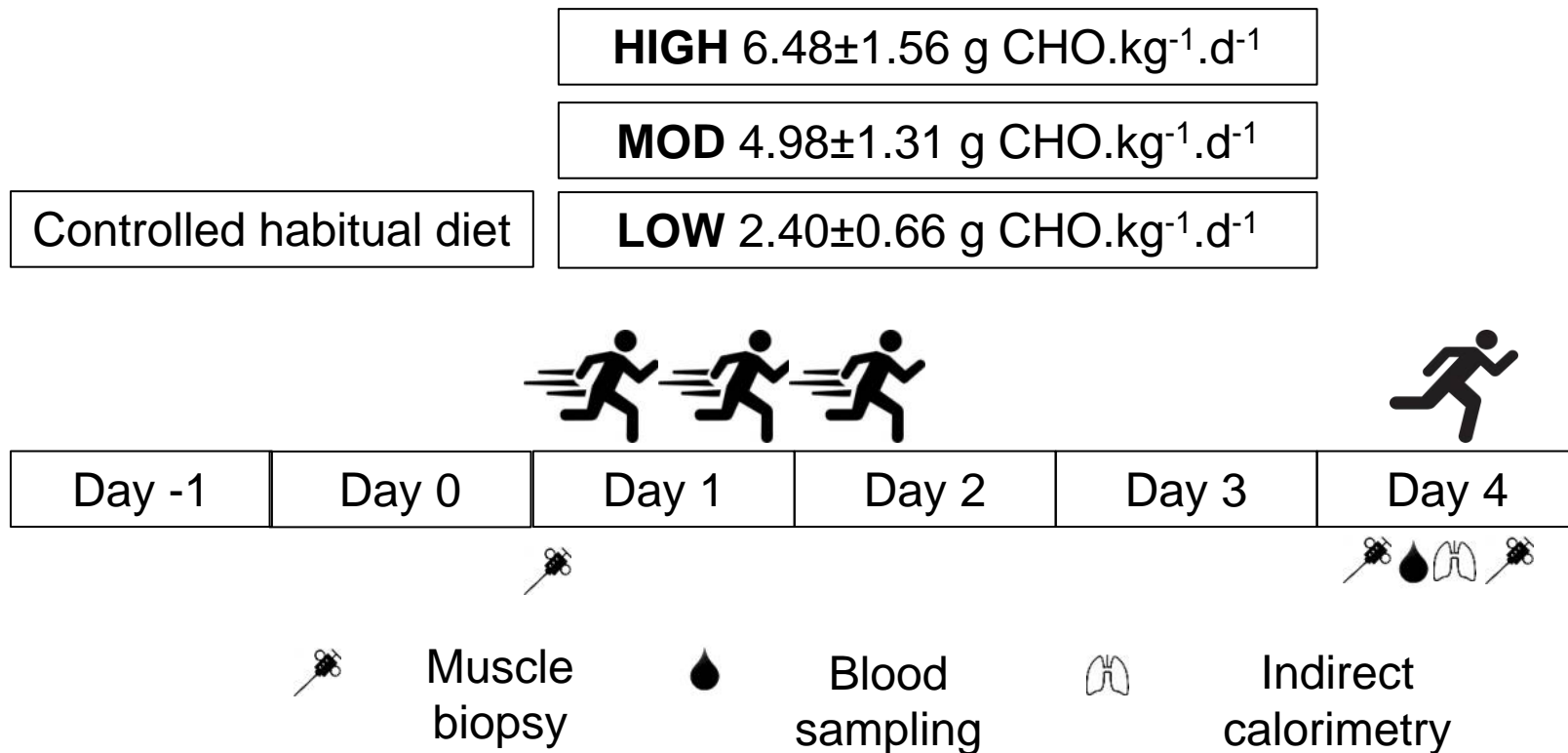


Figure 1. Schematic overview of the experimental design. After two days of controlled habitual diet consumption, participants undertook successive bouts of interval running exercise across Days 1 and 2. Isocaloric diets of lower (LOW), moderate (MOD) and higher (HIGH) carbohydrate (CHO) intakes were provided across Days 1-3. Metabolic responses to 60-min running at $\sim 65\% \dot{V}O_{2\max}$ was assessed on the morning of Day 4, in the overnight fasted state, and $\sim 48 \text{ h}$ after the last exercise bout. A pre-exercise muscle biopsy on Day 1 was taken on only one occasion. CHO intakes expressed as grams of CHO per kilogram body mass.

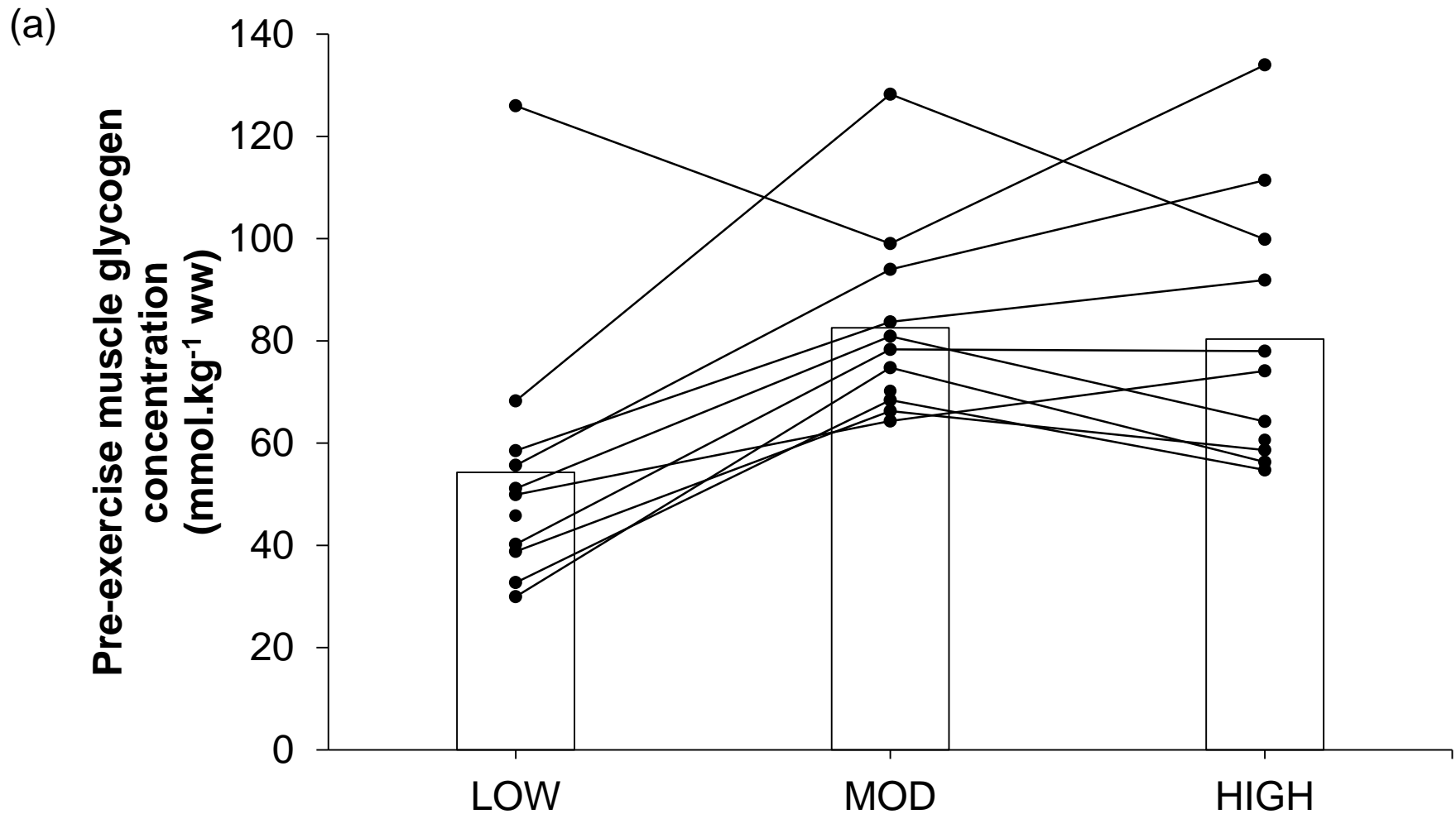


Figure 2. Muscle glycogen (a) concentration pre- 60-min steady-state treadmill running at 65% VO_2max (mean and individual concentrations) and (b) net utilization (mean \pm 95% CI) during 60-min treadmill running at 65% VO_2max in LOW, MOD, and HIGH.

(b)

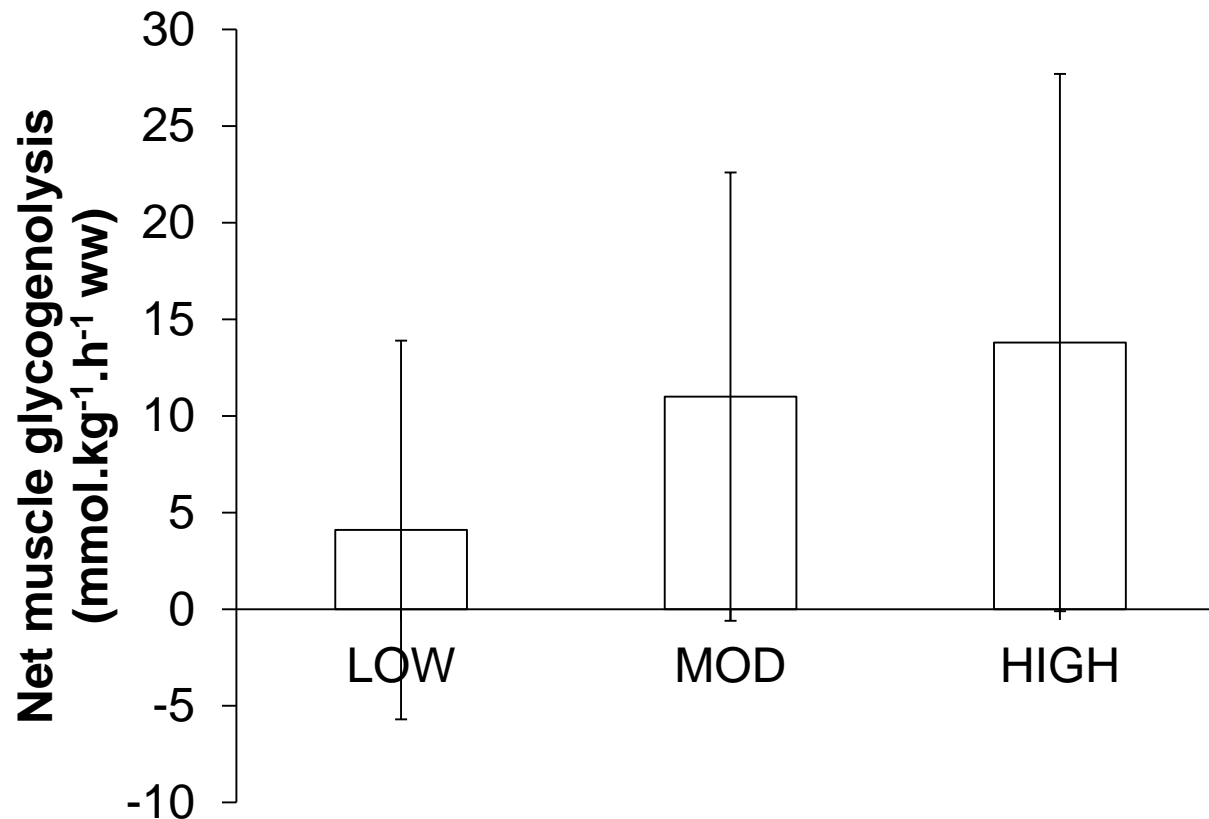


Figure 2. Muscle glycogen (a) concentration pre- 60-min steady-state treadmill running at 65% VO_2max (mean and individual concentrations) and (b) net utilization (mean \pm 95% CI) during 60-min treadmill running at 65% VO_2max in LOW, MOD, and HIGH.

(a)

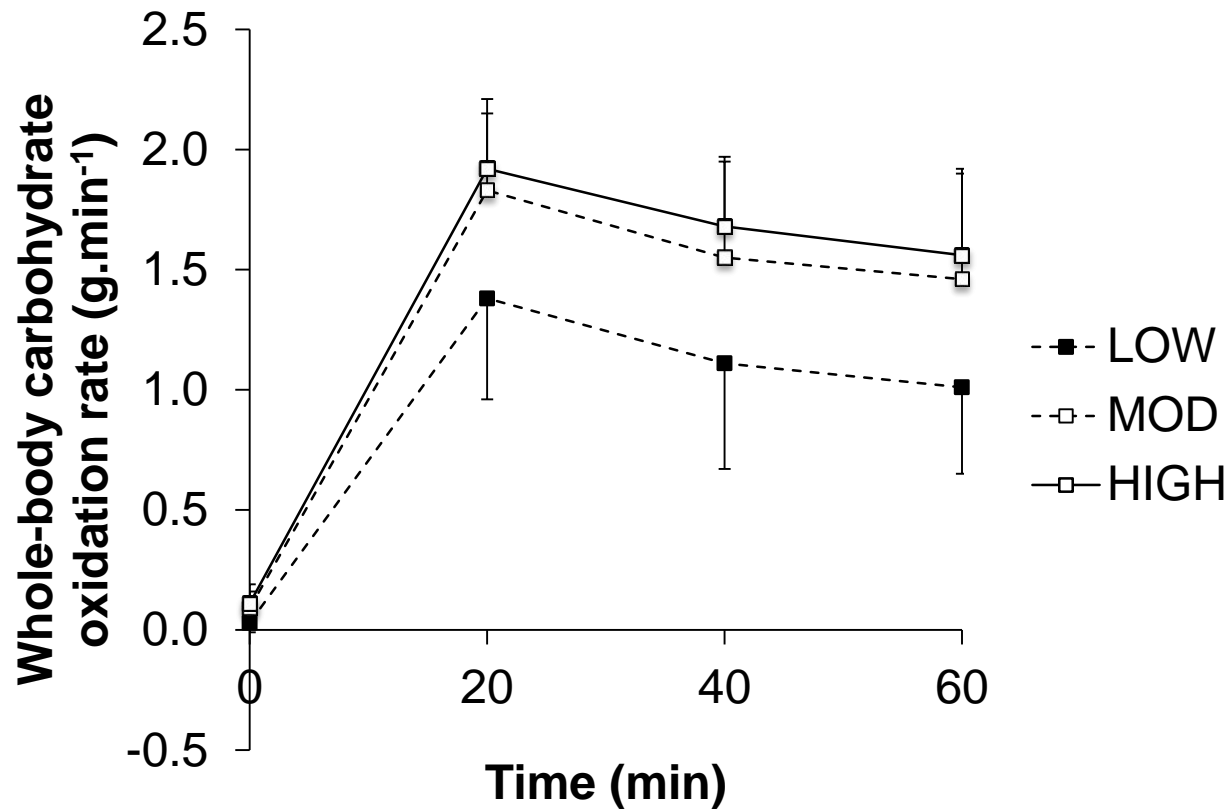


Figure 3. Whole-body rates of (a) carbohydrate and (b) fat oxidation during 60-min treadmill running at 65% $\text{VO}_{2\text{max}}$ in LOW, MOD, and HIGH conditions.

(b)

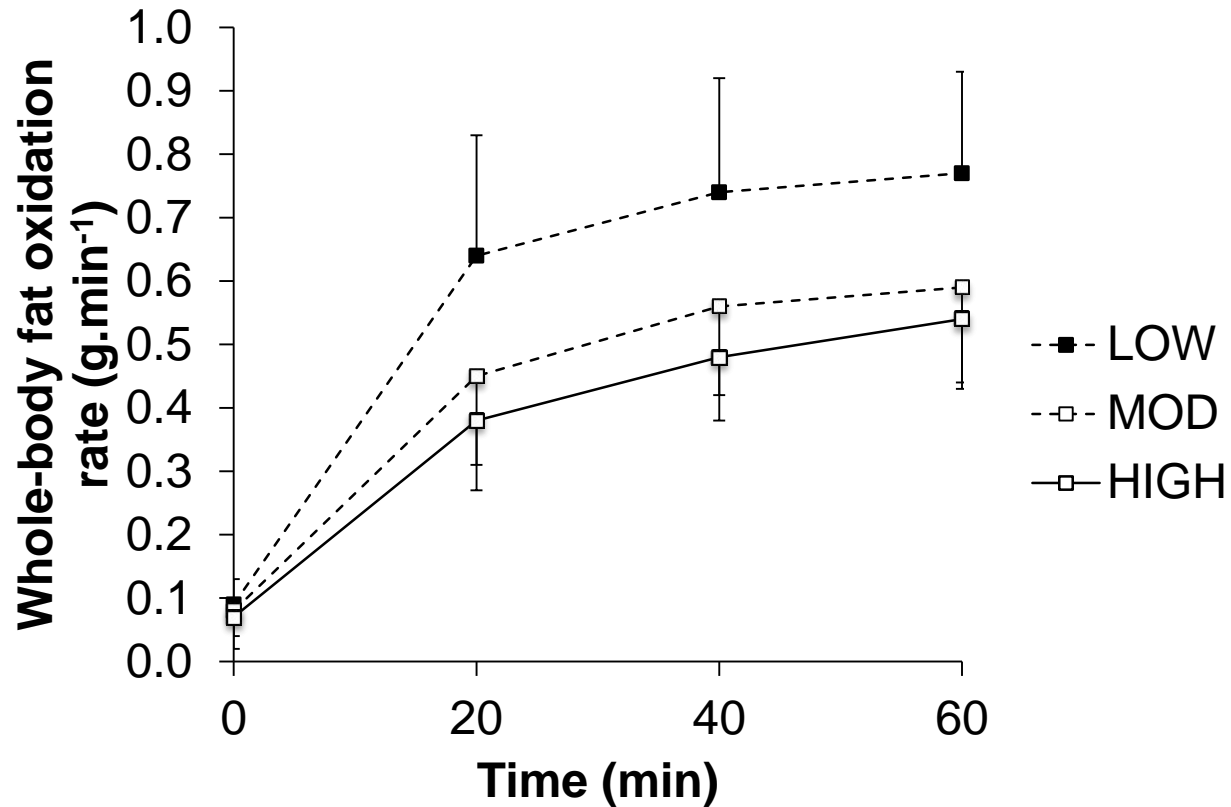


Figure 3. Whole-body rates of (a) carbohydrate and (b) fat oxidation during 60-min treadmill running at 65% $\text{VO}_{2\text{max}}$ in LOW, MOD, and HIGH conditions.

(a)

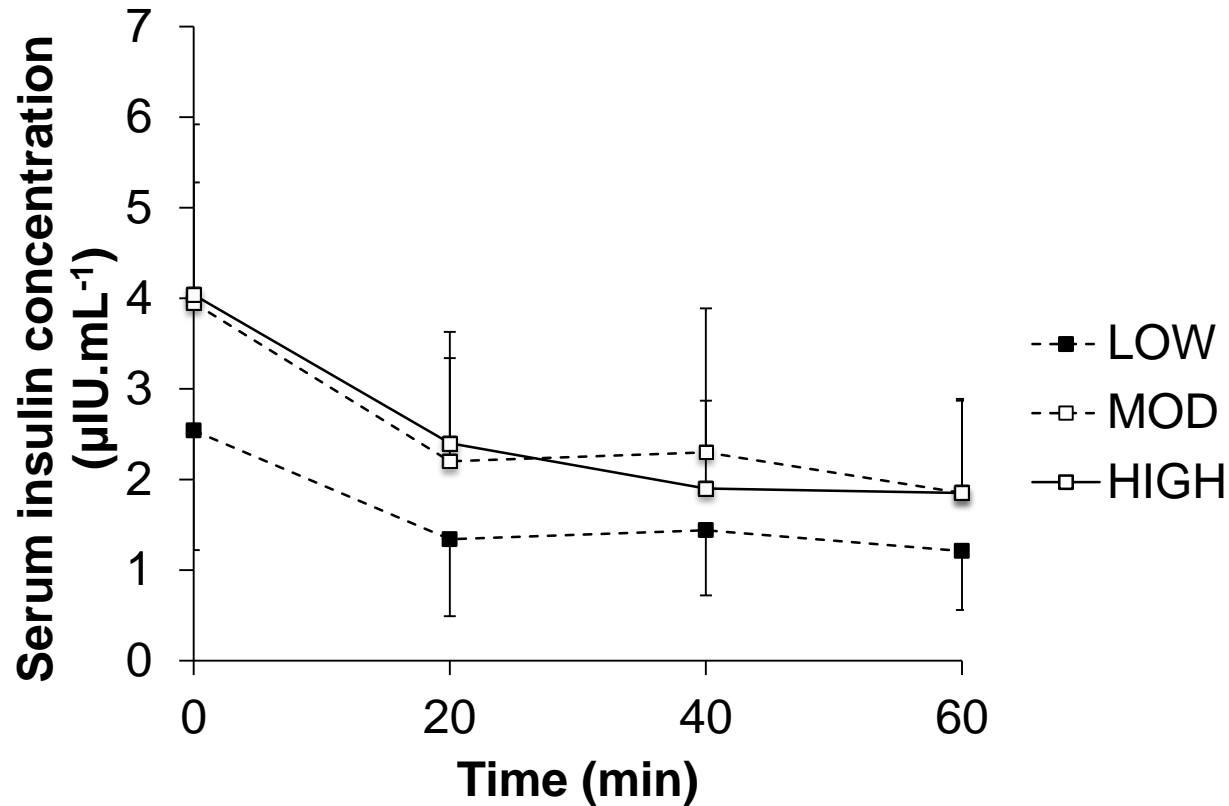


Figure 4. Serum (a) insulin and plasma (b) glycerol, (c) lactate and (d) non-esterified fatty acid concentrations during 60-min treadmill running at 65% $\dot{V}O_2\text{max}$ in LOW, MOD, and HIGH conditions. “*” denotes mean exercise AUC was different in MOD, HIGH vs. LOW ($P < 0.05$).

(b)

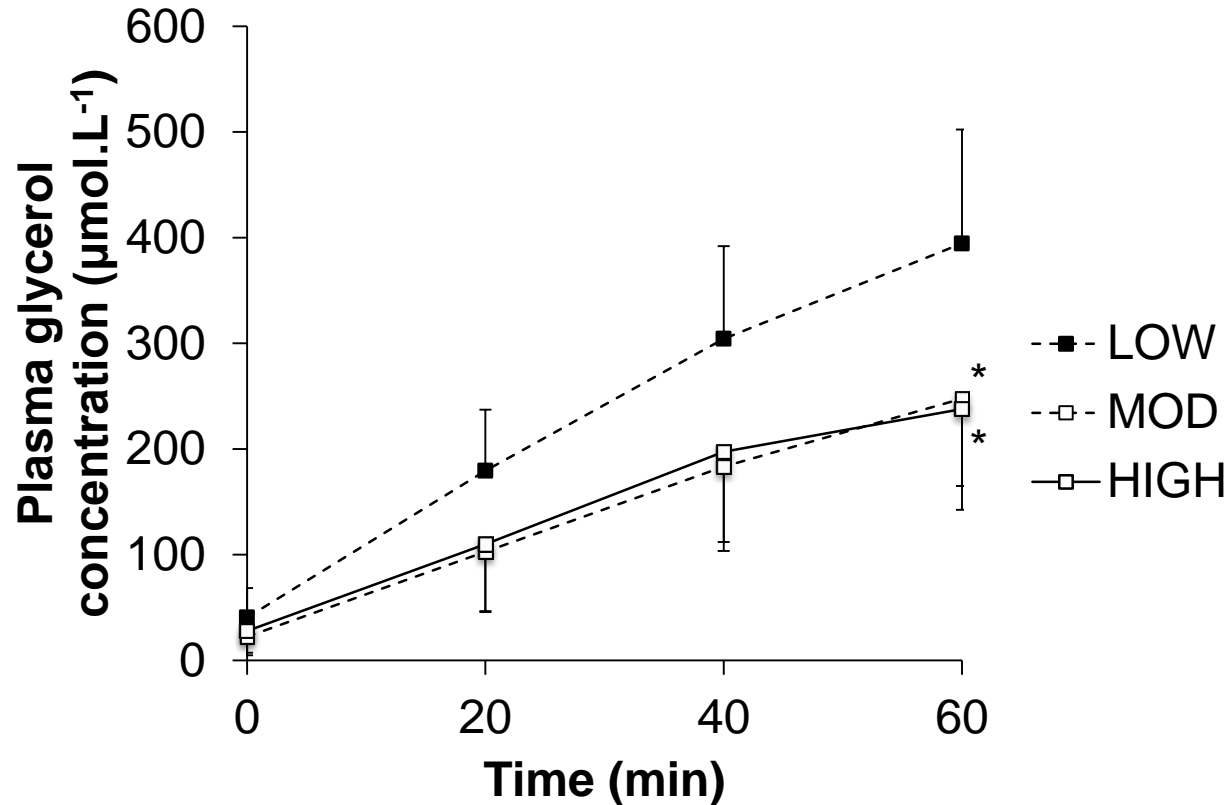


Figure 4. Serum (a) insulin and plasma (b) glycerol, (c) lactate and (d) non-esterified fatty acid concentrations during 60-min treadmill running at 65% $\dot{V}O_2$ max in LOW, MOD, and HIGH conditions. “*” denotes mean exercise AUC was different in MOD, HIGH vs. LOW ($P < 0.05$).

(c)

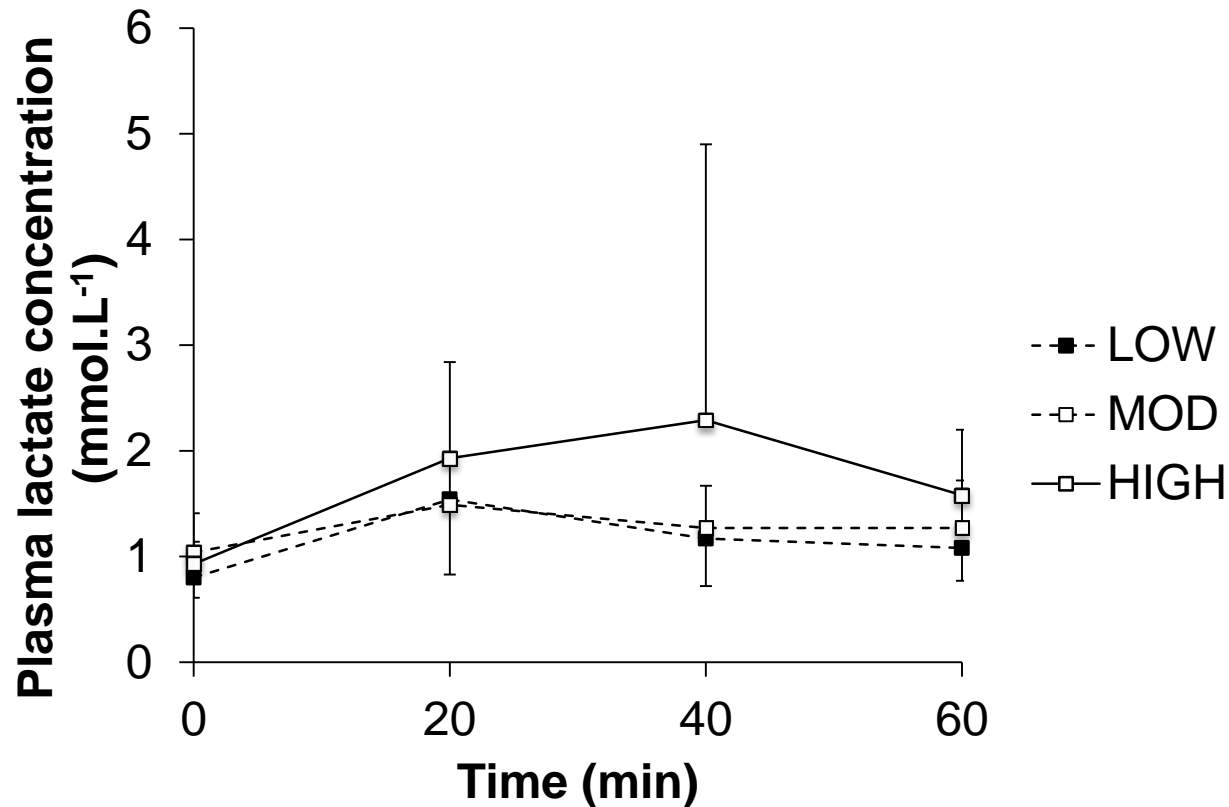


Figure 4. Serum (a) insulin and plasma (b) glycerol, (c) lactate and (d) non-esterified fatty acid concentrations during 60-min treadmill running at 65% $\dot{V}O_2\text{max}$ in LOW, MOD, and HIGH conditions. “*” denotes mean exercise AUC was different in MOD, HIGH vs. LOW ($P < 0.05$).

(d)

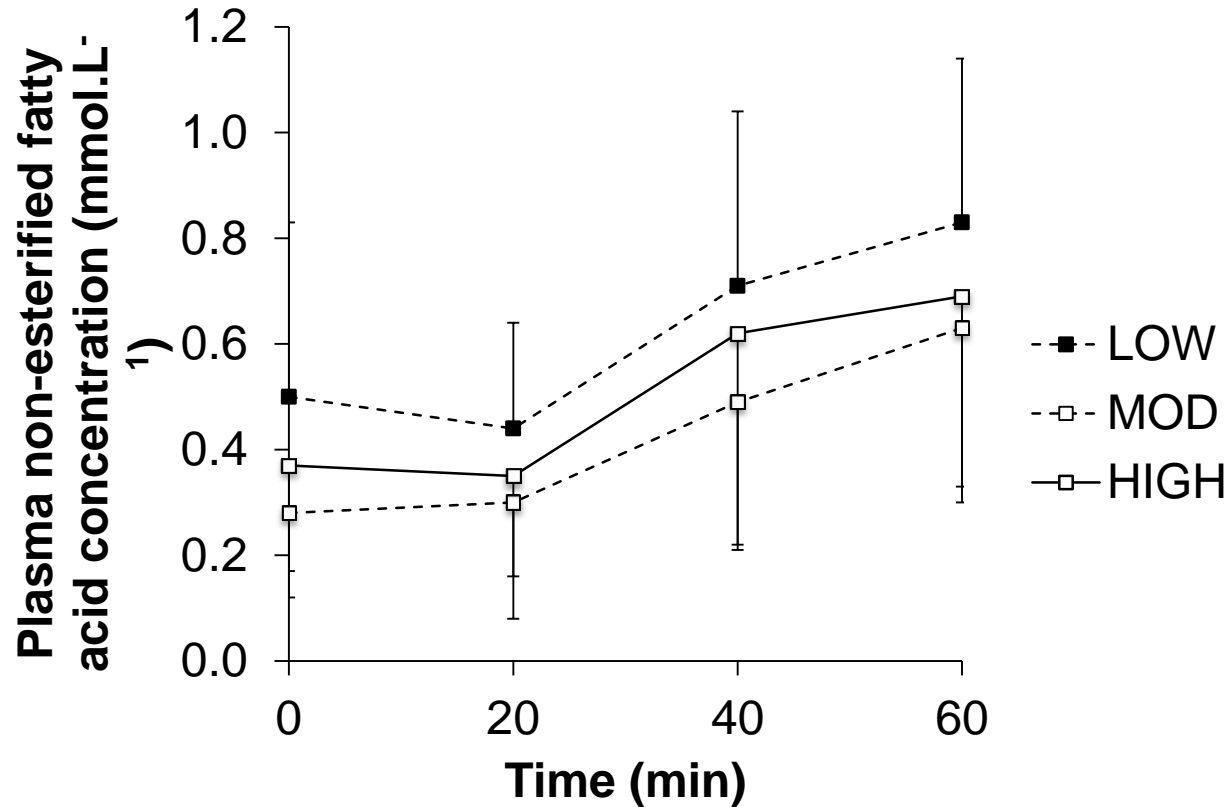


Figure 4. Serum (a) insulin and plasma (b) glycerol, (c) lactate and (d) non-esterified fatty acid concentrations during 60-min treadmill running at 65% $\dot{V}O_2\text{max}$ in LOW, MOD, and HIGH conditions. “*” denotes mean exercise AUC was different in MOD, HIGH vs. LOW ($P < 0.05$).

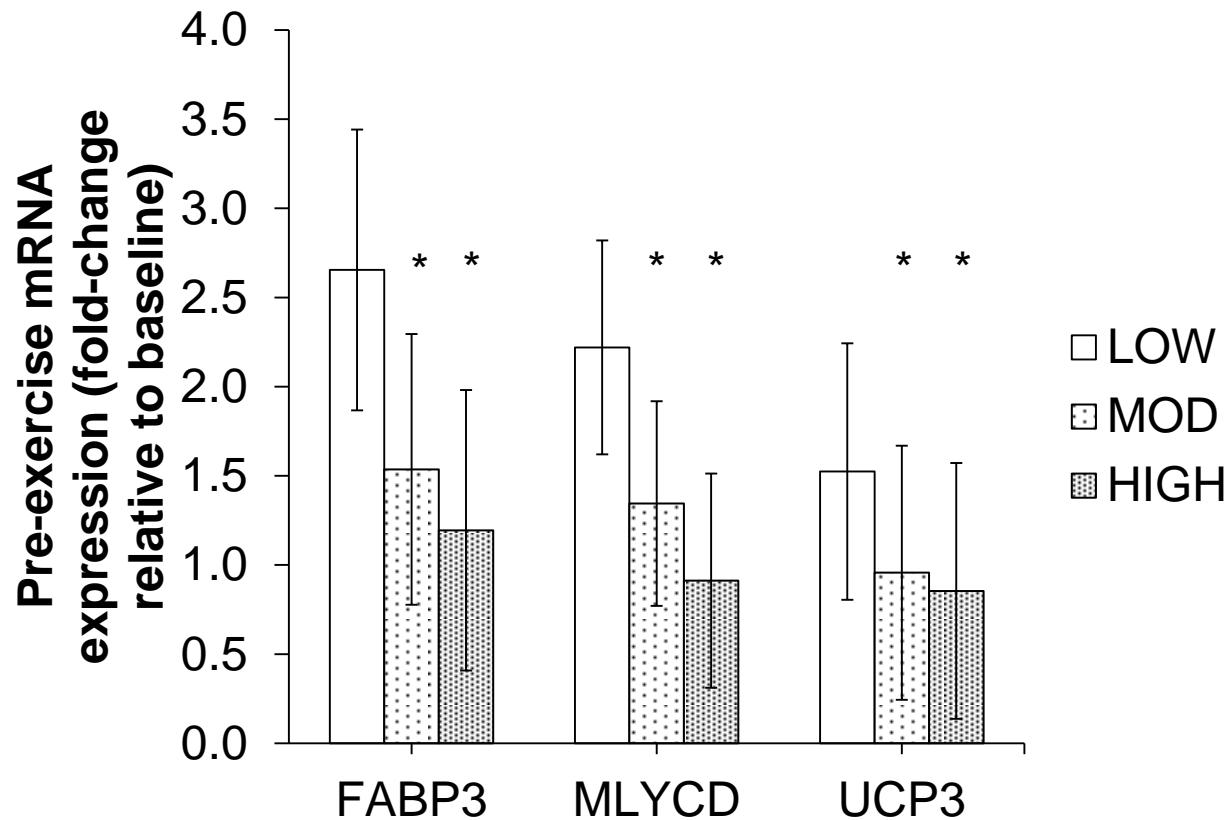


Figure 5. mRNA expression of metabolic genes prior to 60-min steady-state treadmill running at 65% $\text{VO}_{2\text{max}}$ in LOW, MOD, and HIGH conditions, expressed as fold-change relative to baseline (day 1 of the first experimental trial) and 99.95% confidence intervals. ‘*’ denotes significantly different vs. LOW ($P < 0.0001$).