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1 2 3	Title:	Effects of short-term graded dietary carbohydrate intake on intramuscular and whole-body metabolism during moderate-intensity exercise
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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% VO₂max on three occasions. Diets contained lower (LOW, 58 2.40 ± 0.66 g CHO·kg⁻¹·d⁻¹, 21.3 \pm 0.5% of energy intake [EI]), moderate (MOD, 4.98 ± 1.31 g CHO·kg⁻¹·d⁻¹, $46.3 \pm 0.7\%$ EI), or higher (HIGH, $6.48 \pm$ 59 60 1.56 g CHO kg⁻¹ d⁻¹, 60.5 \pm 1.6% EI) CHO. Pre-exercise muscle glycogen content was lower in LOW (54.3 ± 26.4 mmol·kg⁻¹ wet weight [ww]) compared 61 to MOD (82.6 \pm 18.8 mmol kg⁻¹ ww) and HIGH (80.4 \pm 26.0 mmol kg⁻¹ ww, 62 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 \pm 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.kg⁻¹.d⁻¹ (46% to 61% EI), while muscle glycogen, gene 72 expression and metabolic responses were sensitive to more marked reductions in CHO intake (~2.4 g CHO.kg⁻¹.d⁻¹, ~21% EI). 73

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

The data presented here suggests that metabolic responses to steady-state aerobic exercise are somewhat resistant to short-term changes in dietary carbohydrate (CHO) intake within the 5-6.5 g CHO[·]kg^{-1·}d⁻¹ (46-61% EI) range. In contrast, reduction in short-term dietary CHO intake to ~2.4 g CHO[·]kg^{-1·}d⁻¹ (21% EI) evoked clear changes indicative of increased fat and decreased 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 kg⁻¹ day⁻¹), moderate (~4-5 g CHOkg⁻¹ day⁻¹) and/or high (>6.5 g CHOkg⁻¹ day⁻¹) diet 109 110 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 ~5.0 g.kg CHO·kg⁻¹·d⁻¹, and ~61% EI or ~6.5 g.kg CHO⁻¹.d⁻¹) on pre-exercise 123 124 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 uptake [$\dot{V}O_2$ max], 49.7 ± 6.7 ml·kg⁻¹·min⁻¹; data are mean ± standard deviation 140 141 [SD]). Participants were required to be aged 18-45 years with a body mass index of 20-25 kg·m⁻² and a VO₂max of 40-60 ml·kg⁻¹·min⁻¹. Prospective 142 143 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 VO₂max 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 km h⁻¹ against a 1% incline, and the speed was increased by 2 km·h⁻¹ every 4 min for four continuous stages. 172 173 Subsequently, the treadmill gradient was increased by 1% every minute until 174 volitional exhaustion. Breath-by-breath measurements of VO₂ (Oxycon Pro, 175 Jaeger, Wuerzberg, Germany) and heart rate (Polar FT-2, Finland) were 176 obtained throughout. VO_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 ($\leq 2 \text{ ml·kg}^{-1} \cdot \text{min}^{-1}$); (2) heart rate $\leq 10 \text{ beats min}^{-1}$ of age-predicted maximum 181 182 (220 beats 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 ± 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

Following the biopsy, a standardized breakfast was consumed (one-third of daily energy intake, 50% carbohydrate, 35% fat, 15% protein). Two 50-min supervised high-intensity interval sessions were then performed on a treadmill, separated by 3-4 hours. These sessions involved a 10-min period at the speed estimated at 70% $\dot{V}O_2$ max followed by 5 x 3-min intervals at 90%

 \dot{VO}_2 max, with 3-min active recovery between-intervals (1.5 min at 25% \dot{VO}_2 max and 1.5 min at 50% \dot{VO}_2 max). A further 10-min period was then performed at 70% \dot{VO}_2 max. A low carbohydrate lunch was consumed between-sessions (one-sixth of daily energy intake, <25% carbohydrate, >65% fat, 15% protein), and then again following the second interval training session. A further single interval training session was repeated after an 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, ~50%; fat, ~35%, protein, ~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).

265

266 Experimental procedures: Metabolic assessment

267 On day 4, participants returned to the laboratory after an overnight fast. Post-268 void body mass was measured and a resting metabolic assessment was 269 undertaken. Participants lay supine under a ventilated hood connected to an 270 indirect calorimeter (GEM, GEM Nutrition Ltd, Cheshire, UK) that enabled the 271 collection of expired gases for estimation of resting whole-body fat and 272 carbohydrate oxidation using stoichiometric equations (15). An antecubital 273 venous cannula was then inserted and a 10 mL resting blood sample was 274 collected. A muscle sample was then obtained from the vastus lateralis 275 according to the procedures described above.

276

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, Wuerzberg, 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*. Preand post-exercise biopsies were collected from the same leg within each trial,

with samples taken at least 2 cm proximal from previous biopsy sites to minimize the impact of local inflammation from previous sampling. For consistency, the sampled leg order was standardized between participants (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 μ L of 2 mol.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 mol·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

Muscle embedded in OCT compound was cryosectioned and analyzed for Type 1 and 2 muscle fibre specific IMTG (BODIPY D3922, Thermofisher Scientific, USA) and cytochrome c oxidase 4 (COX4, primary antibody 459600, Thermofisher Scientific, USA) content using immunohistochemical approaches as described elsewhere (13, 40). BODIPY immunofluorescence 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.

312

313 For analysis of basal muscle gene expression, RNA was extracted from 20-40 314 mg powdered vastus lateralis tissue using Tri reagent (1 mL, Sigma Aldrich, 315 UK, T9424) for four samples per participant; baseline, LOW pre-exercise, 316 MOD pre-exercise and HIGH pre-exercise. Following addition of chloroform 317 (200 µL, Acros organics 268320025), tubes were mixed vigorously, incubated 318 at room temperature for 5 min and centrifuged for 10 min at 4°C at 12 000 g. 319 The RNA phase was mixed with an equal volume of ice cold 70 % ethanol 320 and RNA was purified on Reliaprep spin columns (Promega, USA, Z6111) 321 according to the manufacturer's instructions. The LVis function of the 322 FLUOstar Omega microplate reader was used to measure RNA concentration 323 to ensure all samples for each participant had the same amount of RNA (184 324 ng - 400 ng) reverse transcribed to cDNA using the RT2 First Strand kit 325 (Qiagen, UK, 330401). Quantitative RT-PCR analysis was performed using 326 custom designed 384-well RT2 PCR Profiler Arrays (Qiagen) and RT2 SYBR 327 Green Mastermix (Qiagen) on a CFX384 Real-Time PCR Detection system 328 (BioRad). 2.8 ng cDNA was added to each well. All primers were 329 commercially available from Qiagen and **Supplementary Table 1** displays the 330 list of genes analysed alongside their Qiagen catalogue number and Refseg# 331 found DOI: (all supplemental material be at can 332 https://doi.org/10.25500/edata.bham.00000609). The absence of genomic 333 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 (Refseg# 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 $(g \cdot min^{-1}) = (4.55 \times VCO_2) - (3.21 \times VCO_2)$ 365 ^{VO}₂) 366 Whole-body fat oxidation $(q \cdot min^{-1}) = (1.67 \text{ x } \dot{V}O_2) - (1.67 \text{ x } \dot{V}CO_2)$ 367 368 369 Where $\dot{V}O_2$ and $\dot{V}CO_2$ are in L·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 mmol kg⁻¹ 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 ± 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% Cls 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 ± 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 ± 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% VO₂max, 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 \pm 10.1; HIGH, 23.6 \pm 9.6 mean fluorescence intensity per fibre, P 425 = 0.20) fibres between-trials. Habitual resting muscle glycogen concentration 426 was 75.6 \pm 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 g·min ⁻¹ ; MOD, 0.08 \pm 0.04 g·min ⁻¹ ;
460	HIGH, 0.07 ± 0.05 g·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 g CHO kg 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 Hearris 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 \sim 5.0 to \sim 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 (~8 g CHO kg⁻¹ 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 ~6.5 g CHO kg⁻¹ d⁻¹ (~45 to ~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 CHO intakes of ~2.4 g CHO kg⁻¹ d⁻¹ (~21% EI) in recovery from exercise are 579 580 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 ~2.5 and ~4.5 g CHO kg⁻¹ d⁻¹ (i.e., ~20-45% EI), at which this metabolic shift 584 585 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), CPT1 (3), CD36 (10), and HADHA (30) with low CHO availability. To our 595 596 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 CHO intake do not appear present in the ~5.0-6.5 g CHO·kg⁻¹·d⁻¹ range (46-619 620 61% of daily EI). In contrast, more marked reductions in CHO intake (~2.4 $g kg^{-1} d^{-1}$, ~21% EI) lowered resting muscle glycogen concentration, altered 621 622 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 resistant to short-term dietary CHO change within the 4.5-6.5 g CHO·kg⁻¹·d⁻¹ 626

627 (45-70% EI) range (12, 16), but are affected by more aggressive CHO increases (>6.5 g CHO·kg⁻¹·d⁻¹, >70% EI) or decreases (<2.5 g CHO·kg⁻¹·d⁻¹, 628 <20% EI) (4). Whether a threshold exists between 4.5 and 2.5 g·CHO·kg⁻¹.d⁻¹ 629 630 (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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821		

822 Table headings

- 823
- 824 Table 1. Dietary intervention characteristics

825

Table 2. Statistical summary of muscle glycogen and intramuscular
triglycerides concentrations during the 60-min steady-state treadmill running
at 65% VO₂max in LOW, MOD, and HIGH

829

Table 3. Statistical summary of whole-body substrate oxidation rates during the 60-min steady-state treadmill running at 65% $\dot{V}O_2$ max in LOW, MOD, and HIGH

- 834 Table 4. Statistical summary of plasma and serum concentrations at rest (R)
- and during the 60-min steady-state treadmill running at $65\%VO_{2max}$ (Ex) in 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% VO₂max 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

Figure 2. Muscle glycogen (a) concentration pre- 60-min steady-state
treadmill running at 65% VO2max (mean and individual concentrations) and
(b) net utilization (mean±95% CI) during 60-min treadmill running at 65%
VO2max in LOW, MOD, and HIGH.

853

Figure 3. Whole-body rates of (a) carbohydrate and (b) fat oxidation during 60-min steady-state treadmill running at 65% $\dot{V}O_2max$ in LOW, MOD, and HIGH conditions.

857

Figure 4. Serum (a) insulin and plasma (b) glycerol, (c) lactate, (d) nonesterified fatty acid concentrations during 60-min treadmill running at 65% \dot{VO}_2 max in LOW, MOD, and HIGH conditions. '*' denotes mean exercise AUC was different in MOD, HIGH vs. LOW (*P* < 0.05).

862

Figure 5. mRNA expression of metabolic genes prior to 60-min steady-state treadmill running at 65% $\dot{V}O_2$ max in LOW, MOD, and HIGH conditions, expressed as fold-change relative to baseline (day 1 of the first experimental trial). '*' denotes significantly different vs. LOW (*P* < 0.0001).

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 ene	rgy intake (%)				
СНО	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)					
СНО		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)					
СНО	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 \pm SD.

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

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

		Adj	usted mean (g.m	nin ⁻¹)	Mean difference (g.min ⁻¹)			
	Trial, contrast	LOW	MOD	HIGH	MOD-LOW	HIGH-LOW	HIGH-MOD	
CHO oxidation	Estimate	1.16	1.60	1.72	0.44	0.57	0.12	
(g.min⁻¹)	(SE/95% CI)	(0.12)	(0.12)	(0.12)	(0.13, 0.75)	(0.24, 0.89)	(-0.20, 0.44)	
	P-value	-	-	-	0.008	0.002	0.43	
Fat oxidation	Estimate	0.72	0.54	0.47	-0.18	-0.25	-0.07	
(g.min⁻¹)	(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)	
	P-value	-	-	-	0.0005	<0.0001	0.14	

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

		Median			Median difference			
	Trial, contrast	LOW	MOD	HIGH	MOD-LOW	HIGH-LOW	HIGH-MOD	
Insulin	Median	1.35	2.35	2.28	1.14	1.11	-0.12	
(µIU.mL ⁻¹)	(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	Median	4.92	5.12	5.25	0.02	0.09	0.09	
(mmol.L ⁻¹)	(Min, max)	(4.44, 5.57)	(4.56, 5.39)	(4.48, 5.60)	-	-	-	
	P-value	-	-	-	0.73	0.55	0.25	
Glycerol	Median	254.0	114.0	134.7	-100.8	-98.3	1.8	
(µmol.L ⁻¹)	(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	Median	1.20	1.34	1.57	0.12	0.17	0.11	
(mmol.L ⁻¹)	(Min, max)	(0.36, 2.08)	(0.65, 1.87)	(0.69, 5.22)	-	-	-	
	P-value	-	-	-	0.46	0.15	0.31	
NEFA	Median	0.67	0.43	0.44	-0.21	-0.23	0.11	
(mmol.L ⁻¹)	(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	Estimate	152.5	152.3	150.4	-0.2	-2.2	-1.9	
adrenaline	(SE/95% CI)	(13.9)	(15.2)	(15.0)	(-36.8, 36.4)	(-38.8, 34.5)	(-41.3, 37.4)	
(pg.mL ⁻¹)	<i>P</i> -value	-	-	-	0.99	0.90	0.92	
Post-exercise	Estimate	278.1	249.6	249.0	-28.5	-29.1	-0.58	
adrenaline	(SE/95% CI)	(25.0)	(23.7)	(23.7)	(-77.4, 20.4)	(-78.9, 20.7)	(-48.7, 47.5)	
(pg.mL ⁻¹)	<i>P</i> -value	-	-	-	0.23	0.23	0.98	
Pre-exercise	Estimate	316.9	428.4	327.2	111.5	10.3	-101.2	
noradrenaline	(SE/95% CI)	(253.6)	(74.3)	(73.6)	(-30.7, 253.8)	(-132.0, 152.7)	(-255.8, 53.4)	
(pg.mL ⁻¹)	<i>P</i> -value	-	-	-	0.12	0.88	0.18	
Post-exercise	Estimate	793.4	891.5	860.2	98.1	66.8	-31.3	
noradrenaline	(SE/95% CI)	(135.1)	(128.8)	(128.9)	(-149.8, 346.1)	(-185.9, 319.5)	(-275.4, 212.8)	

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

	(pg.mL ⁻¹)	<i>P</i> -value	-	-	-	0.41	0.58	0.79
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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 ~65% \dot{VO}_2 max was assessed on the morning of Day 4, in the overnight fasted state, and ~48 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±95% CI) during 60-min treadmill running at 65% VO_2max in LOW, MOD, and HIGH.

(a)

(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±95% CI) during 60-min treadmill running at 65% VO_2max in LOW, MOD, and HIGH.



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





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



Figure 4. Serum (a) insulin and plasma (b) glycerol, (c) lactate and (d) nonesterified fatty acid concentrations during 60-min treadmill running at 65% \dot{VO}_2 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) nonesterified fatty acid concentrations during 60-min treadmill running at 65% \dot{VO}_2 max in LOW, MOD, and HIGH conditions. '*' denotes mean exercise AUC was different in MOD, HIGH vs. LOW (*P* < 0.05).



Figure 4. Serum (a) insulin and plasma (b) glycerol, (c) lactate and (d) nonesterified fatty acid concentrations during 60-min treadmill running at 65% \dot{VO}_2 max in LOW, MOD, and HIGH conditions. '*' denotes mean exercise AUC was different in MOD, HIGH vs. LOW (*P* < 0.05).



0.2

0.0

0



Time (min)

40

20

- LOW

---- MOD

---HIGH

60

(d)



Figure 5. mRNA expression of metabolic genes prior to 60-min steady-state treadmill running at $65\%VO_{2max}$ 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).