Title: Effects of short-term graded dietary carbohydrate intake on intramuscular and whole-body metabolism during moderate-intensity exercise

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Abstract

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

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

Running head: Exercise metabolism following graded carbohydrate intake

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 $CHO \cdot kg^{-1} \cdot d^{-1}$ (46-61% EI) range. In contrast, reduction in short-term dietary CHO intake to ~2.4 g $CHO \cdot kg^{-1} \cdot d^{-1}$ (21% EI) evoked clear changes indicative of increased fat and decreased CHO metabolism during exercise.

Introduction

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

Short-term (i.e., ≤1 week) dietary interventions that reduce CHO intake and lower muscle glycogen content elicit increased fatty acid and reduced CHO oxidation during subsequent moderate-intensity exercise (18). However, the magnitude of alteration in CHO intake required to elicit distinct effects on muscle glycogen and subsequent metabolic responses to exercise remains poorly understood. Assessing the magnitude of alteration in CHO intake required to elicit distinct effects on muscle glycogen and subsequent metabolic responses to exercise requires several (i.e., >2) experimental conditions. Three classic studies compared muscle and/or whole-body fuel use during exercise performed after short-term low (<2.5 g $CHO \cdot kg^{-1} \cdot day^{-1}$), 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 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

Materials and methods

Participants

Thirteen recreationally-active males took part in the present investigation (age, 26 ± 7 y; height, 177.3 ± 7.8 cm; mass, 71.4 ± 7.8 kg; maximum oxygen uptake [$\dot{V}O_2max$], 49.7 ± 6.7 $ml \cdot kg^{-1} \cdot min^{-1}$; data are mean ± standard deviation [SD]). Participants were required to be aged 18-45 years with a body mass index of 20-25 $kg \cdot m^{-2}$ and a $\dot{V}O_2max$ of 40-60 $ml \cdot kg^{-1} \cdot min^{-1}$. Prospective participants were excluded if they had known or suspected intolerance or hypersensitivity to the planned dietary interventions or were taking medication. All participants provided written informed consent and all procedures were approved by the Health Research Authority of the United Kingdom (15/WM/0452). The study was registered at clinicaltrials.gov as NCT02605291 and conducted in accordance with the Declaration of Helsinki.

Study design

This study adopted a cross-over design in which participants completed three four-day diet-exercise interventions in randomised order, with a 1-4 week intervening washout period (**Figure 1**). Following an initial assessment to determine $\dot{V}O_2max$ and assess the treadmill speed vs. oxygen consumption ($\dot{V}O_2$) relationship, participants completed a familiarization to the experimental protocol. The familiarization period was also used to estimate typical daily energy expenditure within the intervention periods. The experimental procedures consisted of 2-d completion of a weighed diet record (day -2 and -1), 1-d consumption of a standardised diet (day 0), and a four-day exercise-diet manipulation (lower, moderate, or higher carbohydrate) finishing with a

60-min treadmill run at 65% $\dot{V}O_2$max with heart rate measurement, expired gas analysis, and venous blood sampling throughout, with pre- and post-exercise muscle biopsies.

***Insert Figure 1

Initial assessment

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

Familiarization procedures

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

Pre-experimental procedures

The experimental trials commenced with a 3-d pre-trial phase (day -2, -1, and 0). On day -2 and -1, participants recorded a 2-d weighed diet record using digital weighing scales and blank diaries provided. These diaries were analysed for energy and macronutrient intake (Dietplan 6.70.67, Forestfield Software Ltd.) and participants were asked to repeat these diets on day -2 and -1 of subsequent experimental trials. On day 0, participants consumed a standardised diet provided by the researchers (50% carbohydrate, 35% fat, 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, ≤20%; fat, ≥65%; protein, ~15%), moderate (MOD:
247 carbohydrate, ~50%; fat, ~35%, protein, ~15%), or higher (HIGH:
248 carbohydrate, ≥65%; fat, ≤20%; protein, ~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, 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*. Pre- and 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).

Muscle analysis

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

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.

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 µL, 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

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

Plasma and serum analysis

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

Expired gas analysis

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

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

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

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

Statistical analysis

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

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

Results

Intervention characteristics

Of the 13 participants who received at least one of the dietary interventions, 10 participants completed all three periods, one completed two of the three periods (HIGH and LOW) and two participants completed one period (one HIGH, one MOD). Hence the number of participants completing each of the dietary interventions was LOW=11, MOD=11, HIGH=12. Participants who did not contribute any data to a treatment period were not included in that group. The achieved relative exercise intensity for the 60-min treadmill exercise bouts was similar between-trials (LOW, 64.6 ± 2.0; MOD, 64.4 ± 2.4, and HIGH, 64.8 ± 2.5% $\dot{V}O_2$max, P = 0.84).

Muscle substrate metabolism

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

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

***Insert Figure 2

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

***Insert Table 2

Whole-body substrate oxidation rates

Pre-exercise resting whole-body carbohydrate oxidation rate was significantly greater in HIGH (0.11 ± 0.08 g·min^{-1}) compared to LOW (0.03 ± 0.04 g·min^{-1}, $P < 0.05$), whereas no significant differences were observed between LOW 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 ± 0.04 g·min^{-1}; MOD, 0.08 ± 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

***Insert Panel Figure 4

***Insert Table 4

Gene expression

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

***Insert Figure 5

Discussion

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

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

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

Consequently, metabolic responses during exercise were similar between MOD and HIGH, with no clear differences in muscle glycogen use, whole-body substrate oxidation rates, blood variables, or pre-exercise gene expression (Tables 2-4, Supplementary Table 2). A relatively modest net muscle glycogen use was seen in the present study (Figure 2, Table 2) which may be explained by several factors such as the muscle group sampled (i.e., vastus lateralis shows lower net glycogen use than soleus or gastrocnemius during level running; (12)), the exercise modality (i.e., net glycogen use in vastus lateralis is lower in level running than cycle ergometry; (2, 3)) and the 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 $CHO \cdot kg^{-1} \cdot d^{-1}$),
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 $CHO \cdot kg^{-1} \cdot d^{-1}$ (~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
579 CHO intakes of ~2.4 g CHO·kg^{-1}·d^{-1} (~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·kg^{-1}·d^{-1} (i.e., ~20-45% 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 ~5.0-6.5 g CHO·kg^{-1}·d^{-1} range (46-
620 61% of daily EI). In contrast, more marked reductions in CHO intake (~2.4
621 g·kg^{-1}·d^{-1}, ~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 4.5-6.5 g CHO·kg^{-1}·d^{-1}

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

Table 1. Dietary intervention characteristics

Table 2. Statistical summary of muscle glycogen and intramuscular triglycerides concentrations during the 60-min steady-state treadmill running at 65% $\dot{V}O_2$max in LOW, MOD, and HIGH

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

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Figure headings

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% $\dot{V}O_2$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% 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.

Figure 3. Whole-body rates of (a) carbohydrate and (b) fat oxidation during 60-min steady-state treadmill running at 65% $\dot{V}O_2$max in LOW, MOD, and HIGH conditions.

Figure 4. Serum (a) insulin and plasma (b) glycerol, (c) lactate, (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 ($P < 0.05$).

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 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

		Adjusted mean (SE/95% CI)			Mean difference (95% CI)		
	Trial, contrast	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)
	P-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)
	P-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 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	Median	1.35	2.35	2.28	1.14	1.11	-0.12
($\mu IU.mL^{-1}$)	(Min, max)	(0.31, 2.65)	(0.95, 4.52)	(0.84, 3.85)	-	-	-
	P-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
($\mu mol.L^{-1}$)	(Min, max)	(134.8, 298.5)	(81.8, 231.5)	(59.3, 309.0)	-	-	-
	P-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)	-	-	-
	P-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)
($pg.mL^{-1}$)	*P*-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)
($pg.mL^{-1}$)	*P*-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)
($pg.mL^{-1}$)	*P*-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)

($pg.mL^{-1}$)	*P*-value	-	-	-	0.41	0.58	0.79

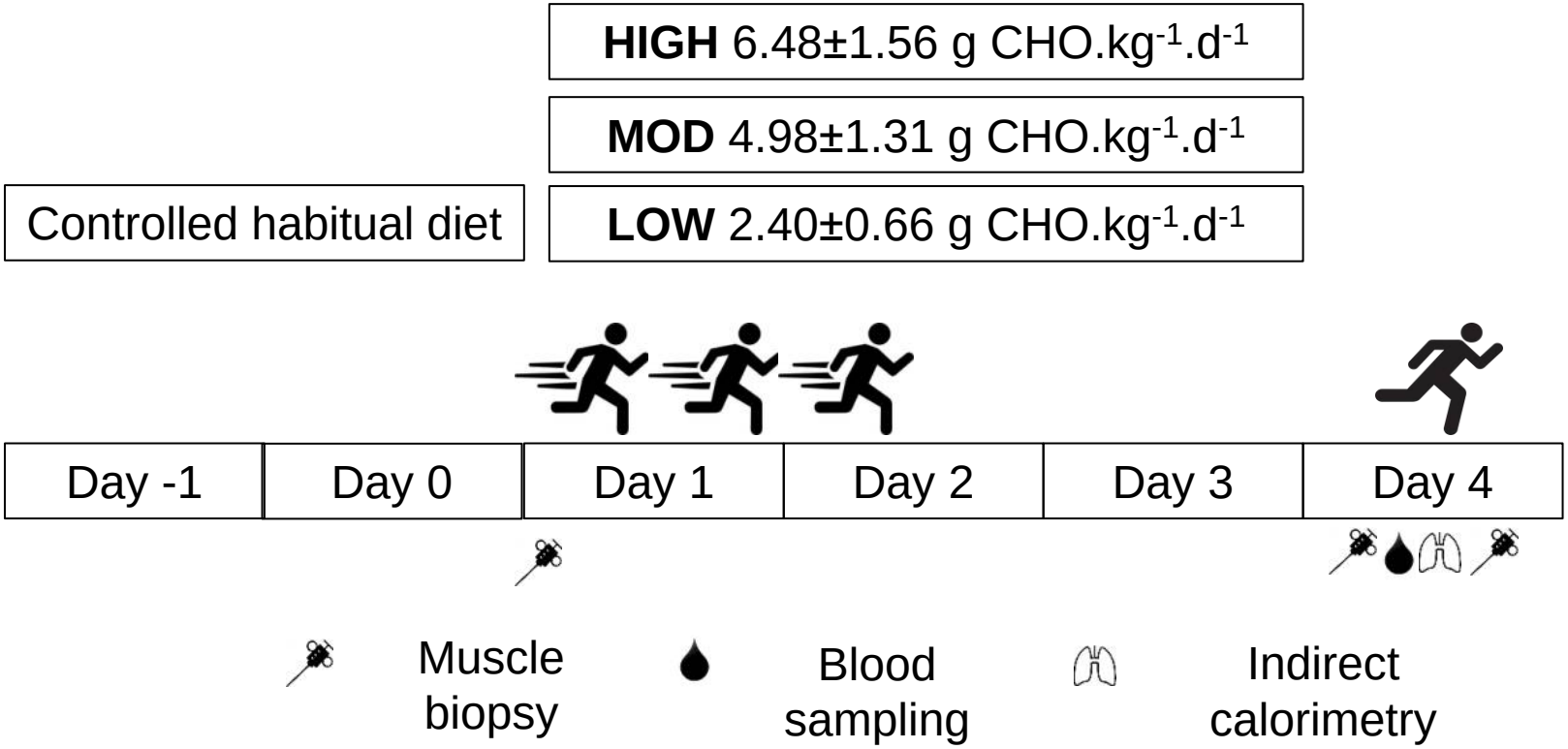


Figure 1. Schematic overview of the experimental design. After two days of controlled habitual diet consumption, participants undertook successive bouts of interval running exercise across Days 1 and 2. Isocaloric diets of lower (LOW), moderate (MOD) and higher (HIGH) carbohydrate (CHO) intakes were provided across Days 1-3. Metabolic responses to 60-min running at ~65% $\dot{V}O_2$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.

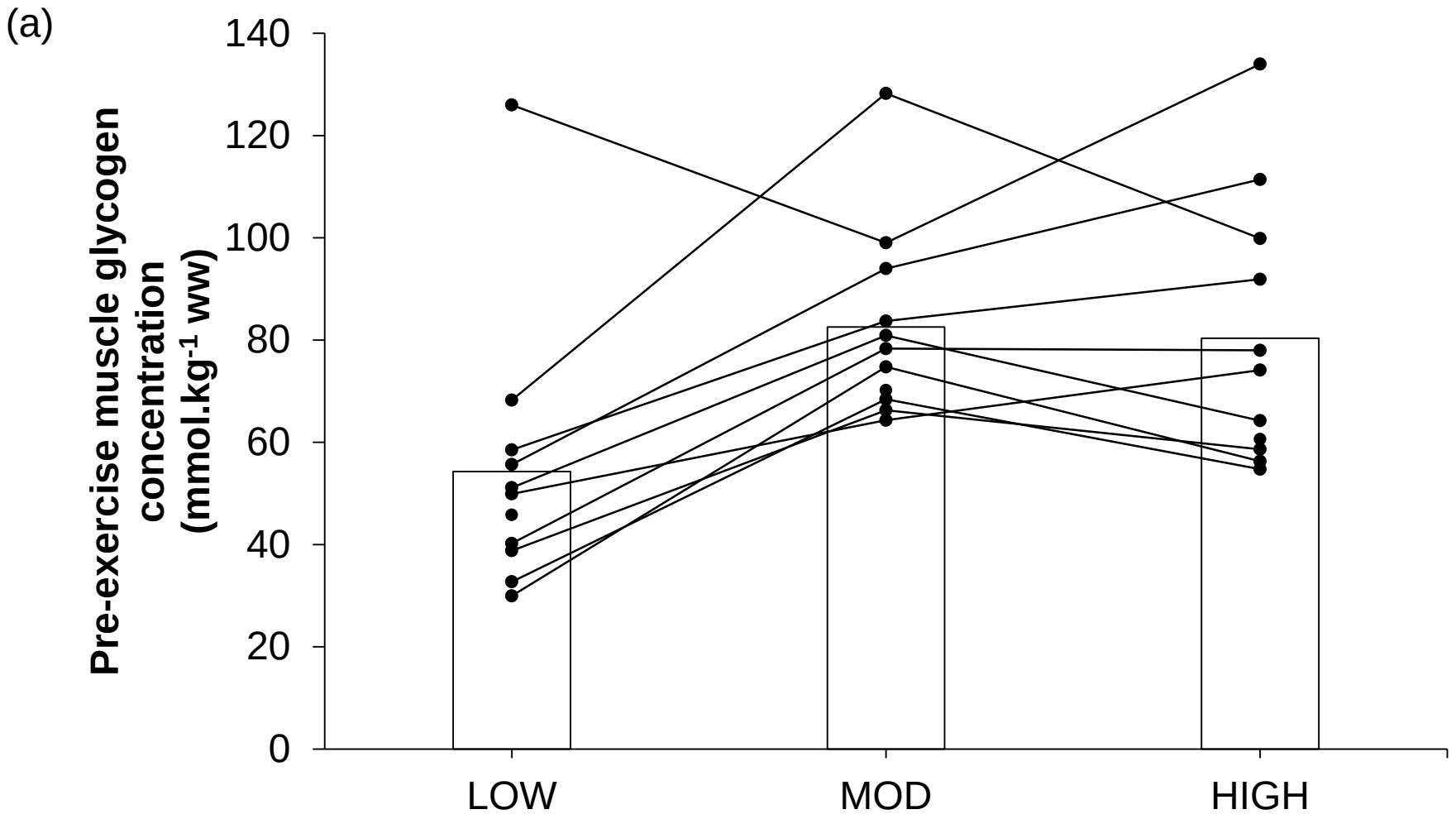
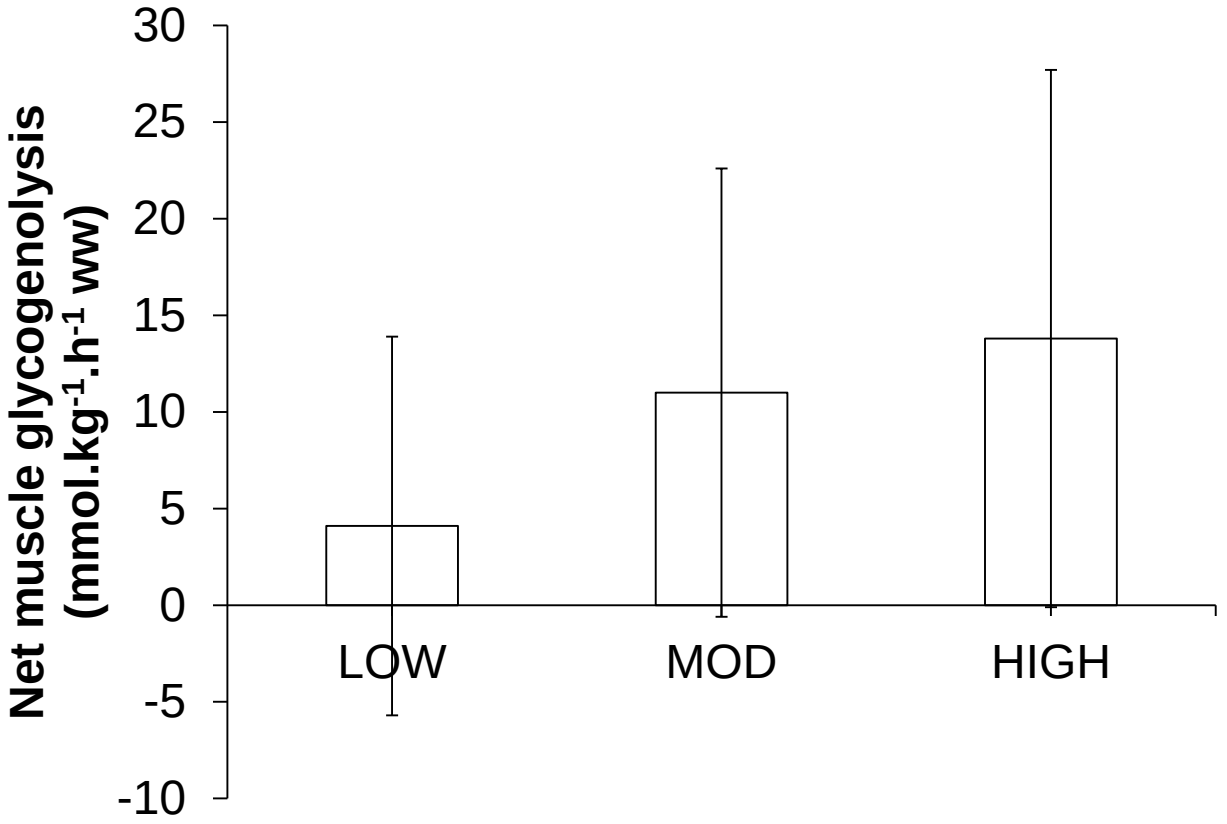


Figure 2. Muscle glycogen (a) concentration pre- 60-min steady-state treadmill running at 65% VO_2max (mean and individual concentrations) and (b) net utilization (mean±95% CI) during 60-min treadmill running at 65% VO_2max in LOW, MOD, and HIGH.

(b)



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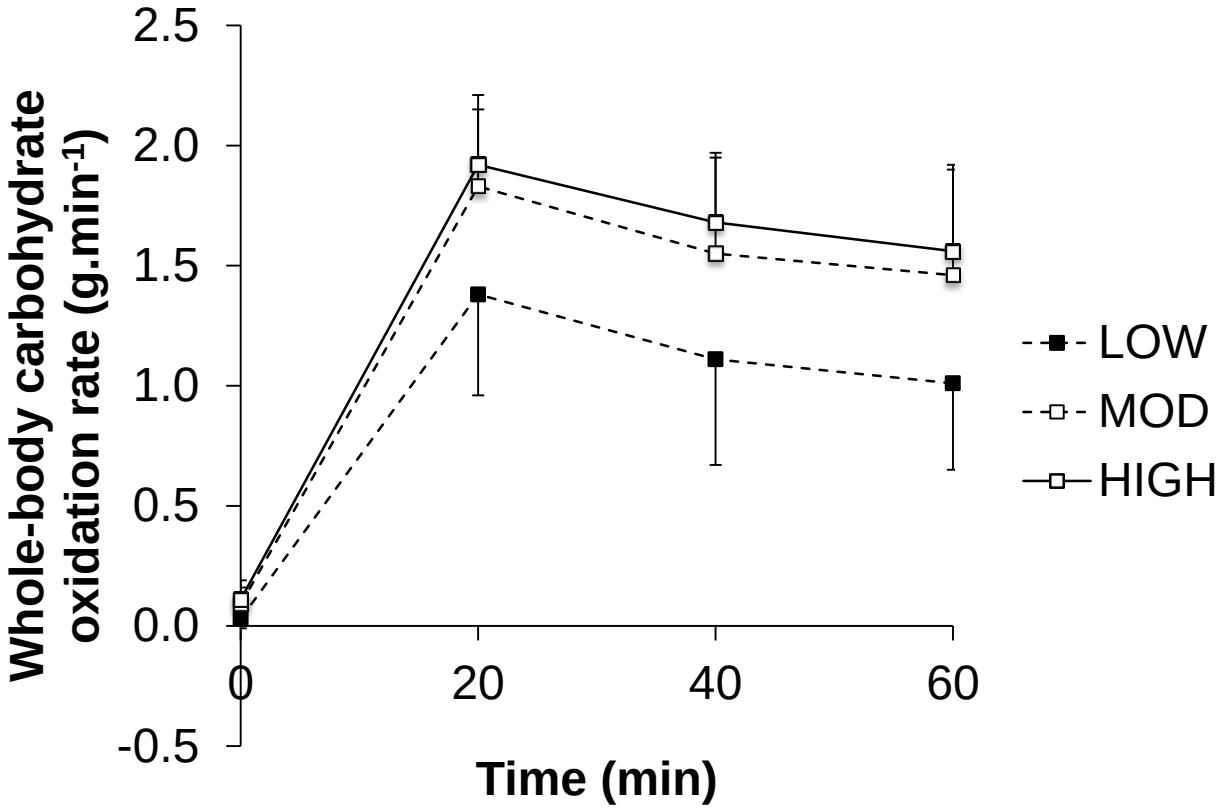
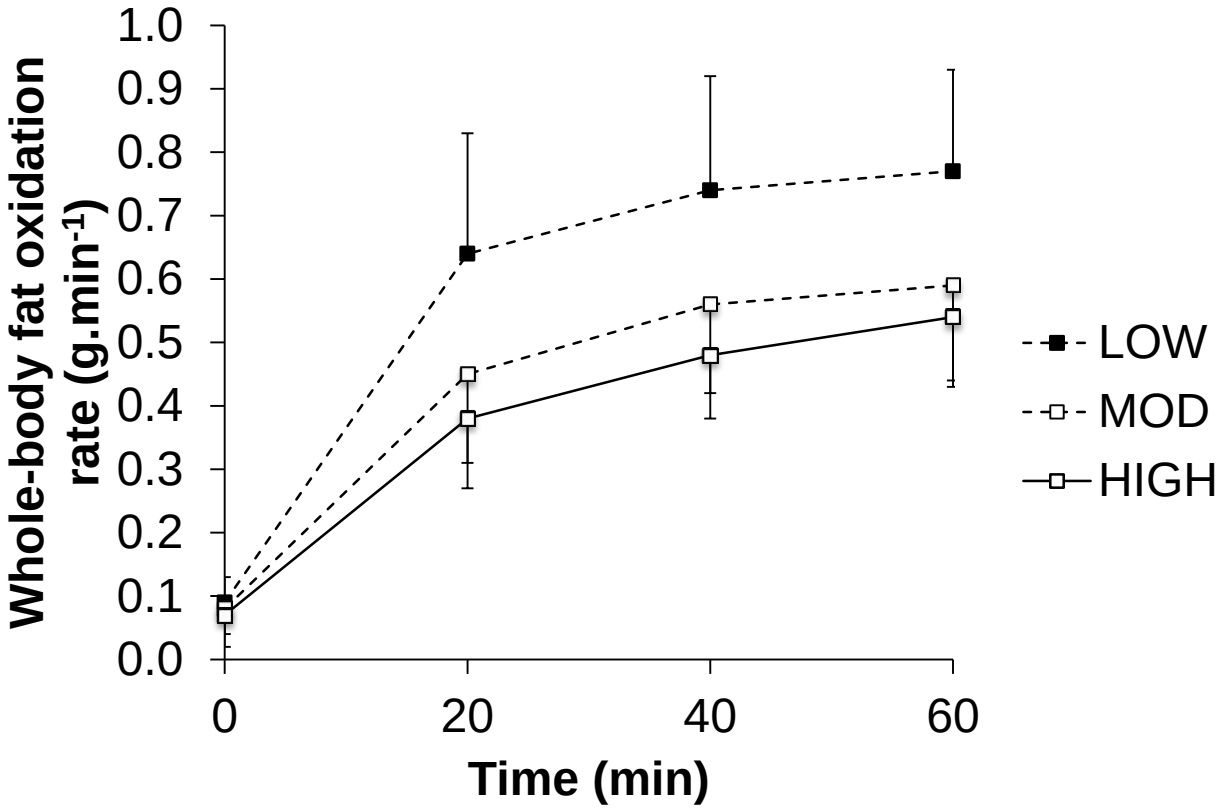


Figure 3. Whole-body rates of (a) carbohydrate and (b) fat oxidation during 60-min treadmill running at 65%VO_{2max} in LOW, MOD, and HIGH conditions.

(b)



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(a)

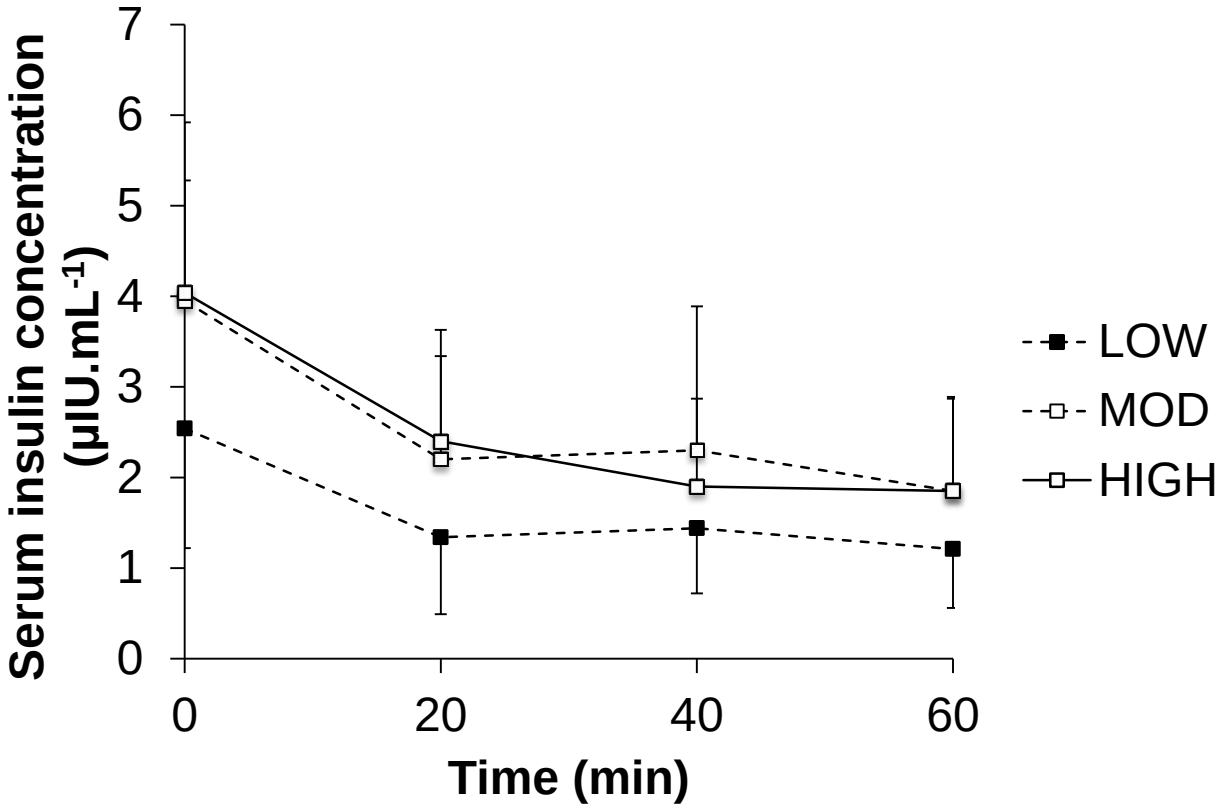
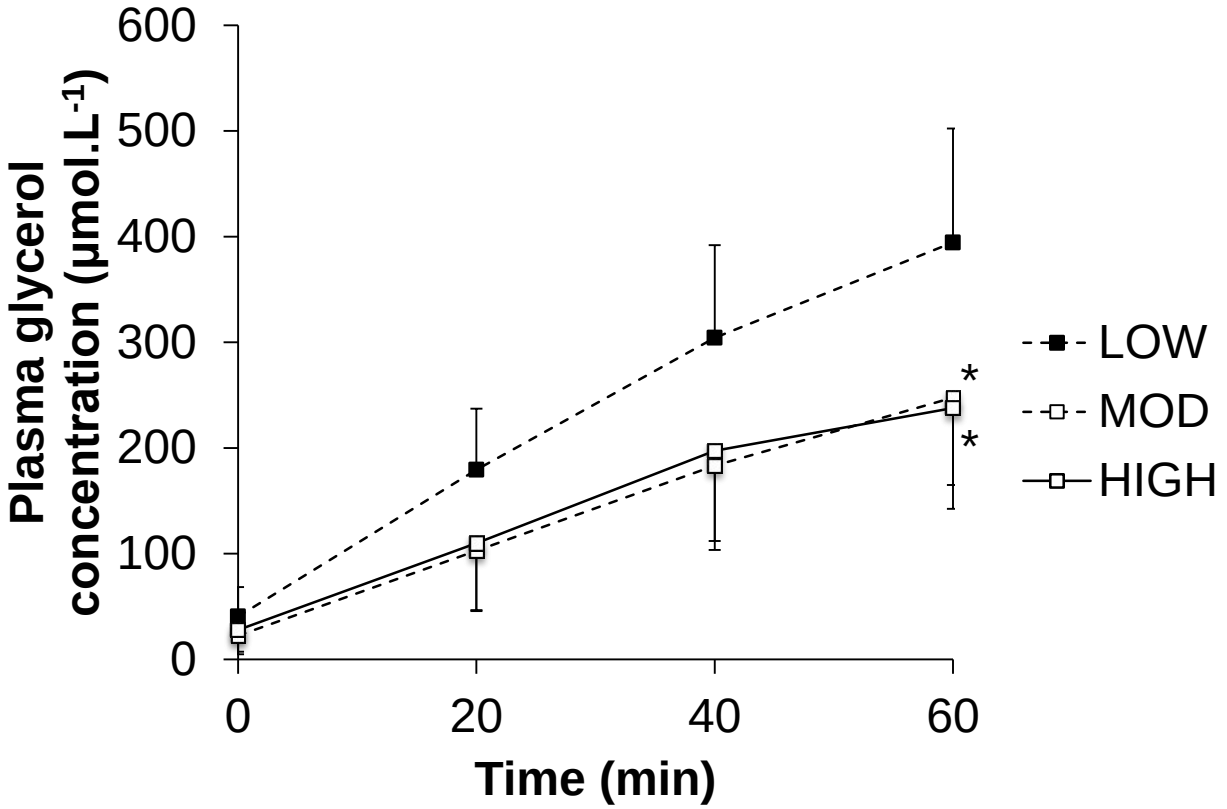


Figure 4. Serum (a) insulin and plasma (b) glycerol, (c) lactate and (d) non-esterified fatty acid concentrations during 60-min treadmill running at 65% $\dot{V}O_2$max in LOW, MOD, and HIGH conditions. '*' denotes mean exercise AUC was different in MOD, HIGH vs. LOW ($P < 0.05$).

(b)



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(c)

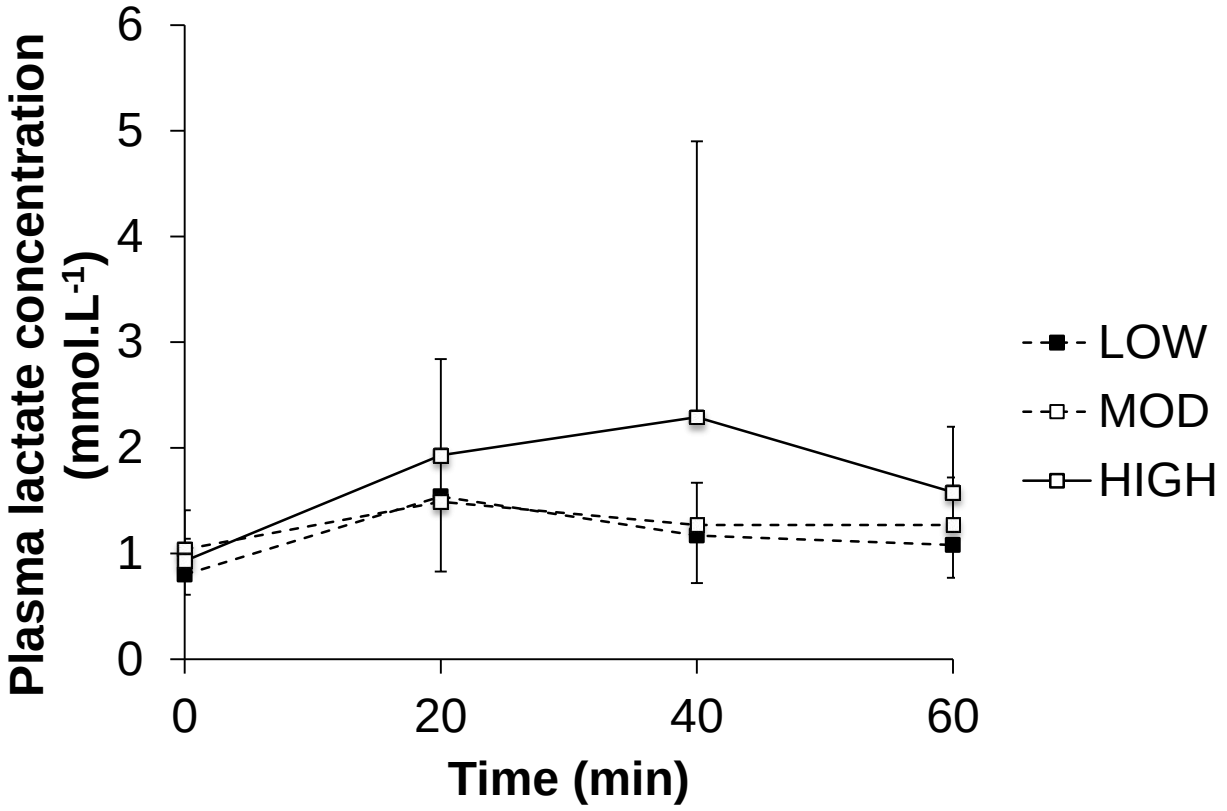

Figure 4. Serum (a) insulin and plasma (b) glycerol, (c) lactate and (d) non-esterified fatty acid concentrations during 60-min treadmill running at 65% $\dot{V}O_2$max in LOW, MOD, and HIGH conditions. '*' denotes mean exercise AUC was different in MOD, HIGH vs. LOW ($P < 0.05$).

(d)

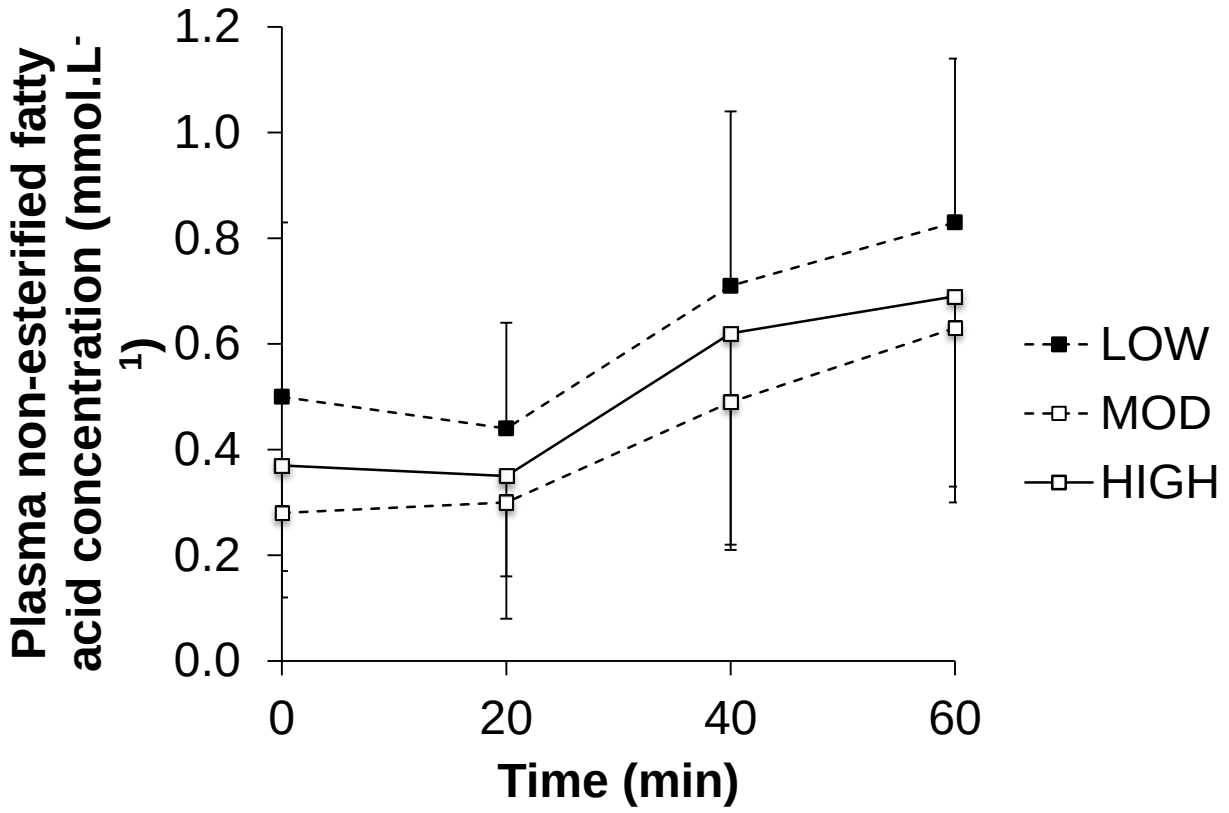

Figure 4. Serum (a) insulin and plasma (b) glycerol, (c) lactate and (d) non-esterified fatty acid concentrations during 60-min treadmill running at 65% $\dot{V}O_2$max in LOW, MOD, and HIGH conditions. '*' denotes mean exercise AUC was different in MOD, HIGH vs. LOW ($P < 0.05$).

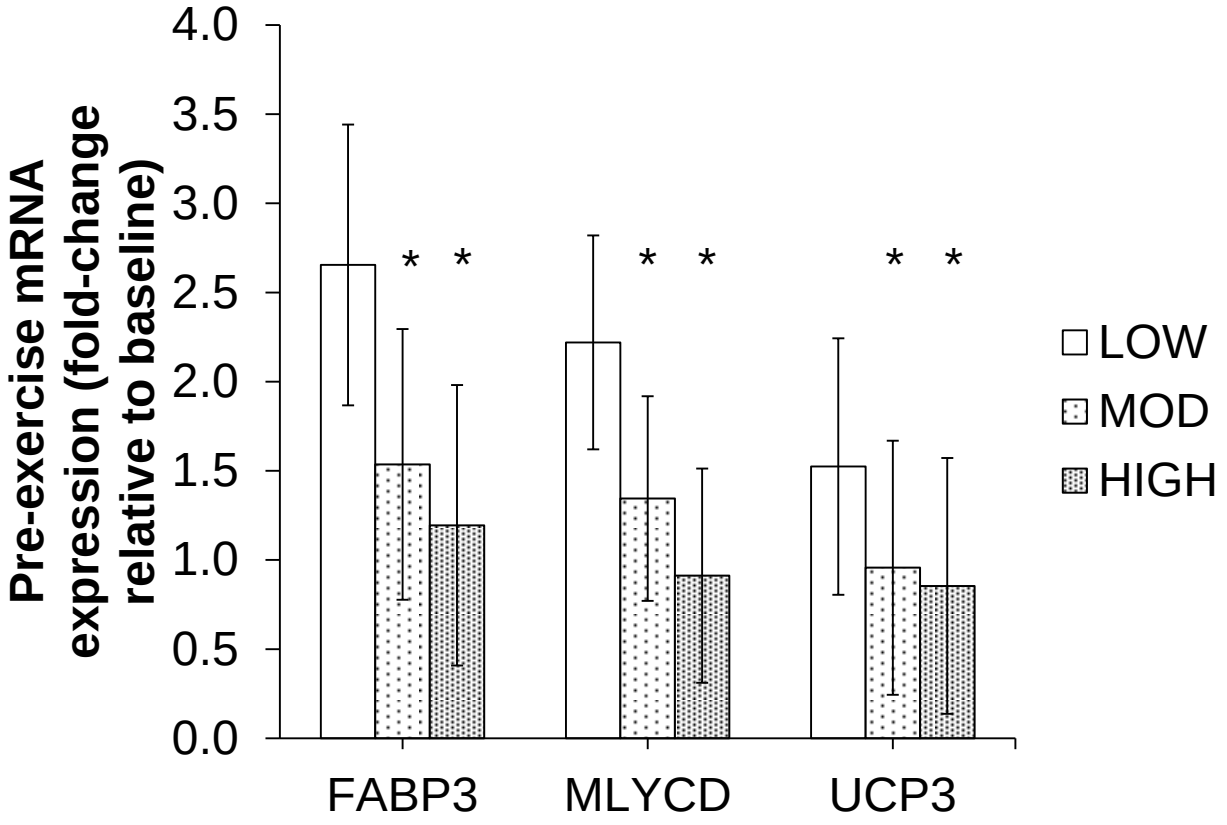

Figure 5. mRNA expression of metabolic genes prior to 60-min steady-state treadmill running at 65%VO_{2max} in LOW, MOD, and HIGH conditions, expressed as fold-change relative to baseline (day 1 of the first experimental trial) and 99.95% confidence intervals. '*' denotes significantly different vs.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