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RESEARCH ARTICLE

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

Ed Maunder,[1]* Helen E. Bradley,[2]* Colleen S. Deane,[3,4] Adrian B. Hodgson,[5] Michael Jones,[2] Sophie Joanisse,[6] Alice M. Turner,[7,8] Leigh Breen,[2] Andrew Philp,[9,10] and Gareth A. Wallis[2]

[1]*Sports Performance Research Institute New Zealand, Auckland University of Technology, Auckland, New Zealand;* [2]*School of Sport, Exercise, and Rehabilitation Sciences, University of Birmingham, Birmingham, United Kingdom;* [3]*Department of Sport and Health Sciences, College of Life and Environmental Sciences, University of Exeter, Exeter, United Kingdom;* [4]*Living Systems Institute, University of Exeter, Exeter, United Kingdom;* [5]*Lucozade Ribena Suntory Limited, Uxbridge, United Kingdom;* [6]*Department of Sport and Exercise Sciences, Manchester Metropolitan University, Manchester, United Kingdom;* [7]*Institute for Applied Health Research, College of Medical and Dental Sciences, University of Birmingham, Birmingham, United Kingdom;* [8]*University Hospitals Birmingham National Health Services Foundation Trust, Heartlands Hospital, Birmingham, United Kingdom;* [9]*Healthy Ageing Research Theme, Garvan Institute of Medical Research, Darlinghurst, New South Wales, Australia; and* [10]*St. Vincent's Medical School, University of New South Wales Medicine, University of New South Wales Sydney, Sydney, New South Wales, Australia*

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 wk) 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% $\dot{V}O_{2max}$ on three occasions. Diets contained lower [LOW, 2.40 ± 0.66 g CHO·kg^{-1}·day^{-1}, 21.3 ± 0.5% of energy intake (EI)], moderate (MOD, 4.98 ± 1.31 g CHO·kg^{-1}·day^{-1}, 46.3 ± 0.7% EI), or higher (HIGH, 6.48 ± 1.56 g CHO·kg^{-1}·day^{-1}, 60.5 ± 1.6% EI) CHO. Preexercise muscle glycogen content was lower in LOW [54.3 ± 26.4 mmol·kg^{-1} wet weight (ww)] compared with 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 [respiratory exchange ratio (RER): 0.81 ± 0.01] compared with 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}·day^{-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}·day^{-1}, ~21% EI).

NEW & NOTEWORTHY The data presented here suggest 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}·day^{-1} [46–61% energy intake (EI)] range. In contrast, reduction in short-term dietary CHO intake to ~2.4 g CHO·kg^{-1}·day^{-1} (21% EI) evoked clear changes indicative of increased fat and decreased CHO metabolism during exercise.

diet; exercise; fat metabolism; muscle glycogen; running economy

INTRODUCTION

Glycogen is the storage form of carbohydrate (CHO) energy in animals, primarily located in skeletal muscles and the liver (1, 2). Glycogen availability is sensitive to exercise and nutrition (3–6), and glycogen depletion has been implicated in fatigue during prolonged moderate-to-vigorous intensity exercise (2, 7, 8). Muscle glycogen is also recognized as a potent regulator of acute substrate metabolism during prolonged exercise (9) and is increasingly implicated in the regulation of exercise training adaptation (10–12). 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 wk) dietary interventions that reduce CHO intake and lower muscle glycogen content elicit

* E. Maunder and H. E. Bradley contributed equally to this work.
Correspondence: G. A. Wallis (e-mail: G.A.Wallis@bham.ac.uk).
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increased fatty acid and reduced CHO oxidation during subsequent moderate-intensity exercise (9). 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·kg^{-1}·day^{-1}), moderate (~4–5 g CHO·kg^{-1}·day^{-1}), and/or high (>6.5 g CHO·kg^{-1}·day^{-1}) dietary CHO intake (3, 13, 14). Despite apparent achievement of graded preexercise muscle glycogen availability, subsequent effects on fuel utilization during exercise were inconsistent with similar (13, 14) or augmented (3) CHO oxidation in the high compared with moderate CHO condition. While informative, these early studies were limited by lack of consistent dietary control (3, 14) and/or low statistical power to detect differences in fuel utilization between conditions (13, 14). As such, the effect of short-term graded dietary CHO intakes on preexercise muscle glycogen, whole body, and muscle fuel utilization during exercise requires clarification.

The main aims of the present investigation were to examine the effect of short-term diet-exercise interventions that provided graded dietary CHO intake [i.e., ~21% energy intake (EI) or ~2.4 g CHO·kg^{-1}·day^{-1}, ~46% EI or ~5.0 g CHO·kg^{-1}·day^{-1}, and ~61% EI or ~6.5 g CHO·kg^{-1}·day^{-1}] on preexercise resting muscle glycogen content, muscle, and whole body fuel utilization during prolonged, moderate-intensity exercise. It was hypothesized that preexercise muscle glycogen content and muscle and whole body fuel utilization during subsequent moderate-intensity exercise would be graded in line with the preceding dietary CHO intake. Diet-induced manipulation of fuel utilization during exercise is likely underpinned by altered systemic (15, 16) and/or local (i.e., muscle) (17) fuel availability, supported by altered expression of proteins implicated in up- or downregulating CHO and fatty acid metabolism (18). Thus the expression of selected genes encoding proteins involved in fuel metabolism was quantified to gain further insights into the potential of skeletal muscle to adapt to varying levels of CHO intake.

MATERIALS AND METHODS

Participants

Thirteen recreationally active males took part in the present investigation [age, 26 ± 7 yr; height, 177.3 ± 7.8 cm; mass, 71.4 ± 7.8 kg; and maximum oxygen uptake ($\dot{V}O_{2max}$), 49.7 ± 6.7 ml·kg^{-1}·min^{-1}; data are means ± SD]. Participants were required to be aged 18–45 yr with a body mass index of 20–25 kg·m^{-2} and a $\dot{V}O_{2max}$ of 40–60 ml·kg^{-1}·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 crossover design in which participants completed three 4-day diet-exercise interventions in randomized order, with a 1- to 4-wk intervening washout period (Fig. 1). Following an initial assessment to determine $\dot{V}O_{2max}$ and assess the treadmill speed versus 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-day completion of a weighed diet record (*days −2* and *−1*), 1-day consumption of a standardized diet (*day 0*), and a 4-day exercise-diet manipulation (lower, moderate, or higher carbohydrate) finishing with a 60-min treadmill run at 65% $\dot{V}O_{2max}$ with heart rate measurement, expired gas analysis, and venous blood sampling throughout, with pre- and postexercise muscle biopsies.

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 before an incremental treadmill test to exhaustion. The test started at 7–10 km·h^{-1} against a 1% incline, and

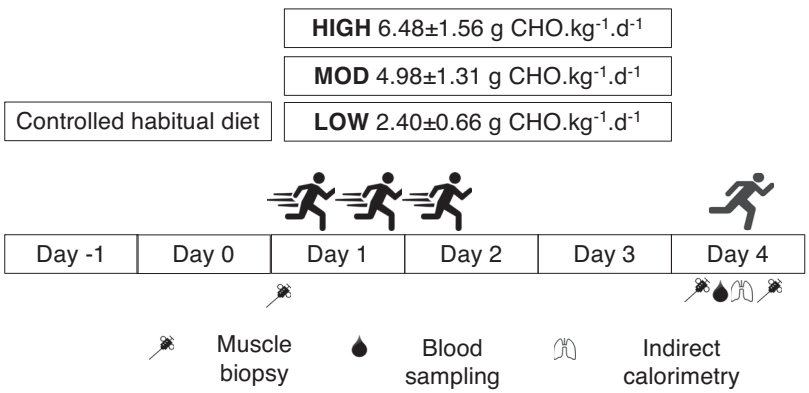

Figure 1. Schematic overview of the experimental design. After 2 days of controlled habitual diet consumption, participants undertook successive bouts of interval running exercise across *days 1* and *2*. Isocaloric diets of lower (LOW), moderate (MOD), and higher (HIGH) carbohydrate (CHO) intakes were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

the speed was increased by 2 km·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 versus $\dot{V}O_2$ relationship for use in the experimental trials. $\dot{V}O_{2max}$ 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 ($\leq$2 mL·kg^{-1}·min^{-1}); *2*) heart rate $\leq$10 beats·min^{-1} of age-predicted maximum (220 beats·min^{-1} minus age in years), and *3*) respiratory exchange ratio >1.1.

Familiarization Procedures

Participants returned to the laboratory ~2–7-days 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. 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 3,427 ± 1,140, 3,146 ± 926, and 2,621 ± 724 kcal, respectively, which was subsequently used to estimate required energy intake on each day during subsequent experimental trials in each volunteer.

Preexperimental Procedures

The experimental trials commenced with a 3-day pretrial phase (*days −2*, *−1*, and *0*). On *days −2* and *−1*, participants recorded a 2-day weighed diet record using digital weighing scales and blank diaries provided. These diaries were analyzed 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 standardized diet provided by the researchers (50% carbohydrate, 35% fat, and 15% protein, with total energy intake equal to estimated daily energy expenditure during familiarization). Participants refrained from exercise on *days −1* and *0*.

Experimental Procedures: Glycogen-Depleting Exercise

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

Following the biopsy, a standardized breakfast was consumed (one-third of daily energy intake: 50% carbohydrate, 35% fat, and 15% protein). Two 50-min supervised high-intensity interval sessions were then performed on a treadmill, separated by 3–4 h. These sessions involved a 10-min period at the speed estimated at 70% $\dot{V}O_{2max}$ followed by 5 × 3-min intervals at 90% $\dot{V}O_{2max}$, with 3-min active recovery between intervals (1.5 min at 25% $\dot{V}O_{2max}$ and 1.5 min at 50% $\dot{V}O_{2max}$). A further 10-min period was then performed at 70% $\dot{V}O_{2max}$. A low-carbohydrate lunch was consumed between sessions (one-sixth of daily energy intake: <25% carbohydrate, >65% fat, and 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*.

Experimental Procedures: Dietary Manipulation

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

Participants collected all urine output on *day 3*, and on the morning of *day 4*, 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 nonmeasured nitrogen sources (e.g., ammonia, urate) (19), and analyzed enzymatically using a semiautomated 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. Postvoid 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

Table 1. *Dietary intervention characteristics*

	Habitual	*Day 0*	LOW	MOD	HIGH
Energy, kcal·day^{-1}	2,250 ± 603	2,736 ± 797	3,080 ± 917	3,084 ± 921	3,145 ± 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}·day^{-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·day^{-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

Values are means ± SD. Lower (LOW; n = 11), moderate (MOD; n = 11), and higher (HIGH; n = 12) conditions are calculated from the averages from *days 1–3*. CHO, carbohydrate.

resting whole body fat and carbohydrate oxidation using stoichiometric equations (20). 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% $\dot{V}O_{2max}$. 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 postexercise 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; and *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 precooled 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 (1,800 *g* for 1 min at 4°C), and the supernatant was analyzed in duplicate for glucose concentration using a semiautomatic analyzer (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 analyzed, with the intra-assay coefficient of variation <10%.

Muscle embedded in OCT compound was cryosectioned and analyzed for type 1 and 2 muscle fiber-specific IMTG (BODIPY D3922, Thermo Fisher Scientific) and cytochrome *c* oxidase 4 (COX4, primary antibody 459600, Thermo Fisher Scientific) content using immunohistochemical approaches as described elsewhere (21, 22). BODIPY immunofluorescence images were captured using a Leica DMIRE2 confocal microscope with a ×40 oil immersion objective (1.25 NA). COX4 images were captured using a Nikon E600 microscope coupled to a SPOT RT KE color three shot CCD camera. Images were analyzed using Image Pro Plus 5.1.

For analysis of basal muscle gene expression, RNA was extracted from 20–40 mg of powdered vastus lateralis tissue using Tri reagent (1 mL, Sigma-Aldrich, T9424) for four samples per participant; baseline, LOW preexercise, MOD preexercise, and HIGH preexercise. Following addition of

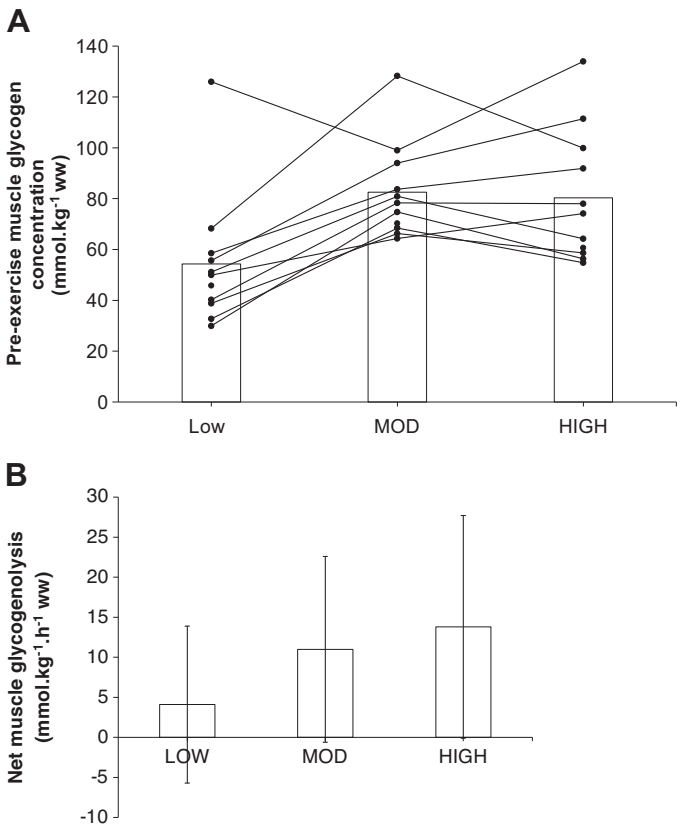

Figure 2. Muscle glycogen concentration (*A*) pre-60-min steady-state treadmill running at 65% $\dot{V}O_{2max}$ (mean and individual concentrations) and net utilization (mean ± 95% confidence interval) (*B*) during 60-min treadmill running at 65% $\dot{V}O_{2max}$ in lower (LOW; n = 11), moderate (MOD; n = 11), and higher (HIGH; n = 12) conditions.

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, 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 (184–400 ng) reverse transcribed to cDNA using the RT2 First Strand kit (Qiagen, 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 (Bio-Rad). Then, 2.8 ng cDNA was added to each well. All primers were commercially available from Qiagen, and Supplemental Table S1 displays the list of genes analyzed alongside their Qiagen catalog number and Refseq# (all Supplemental material is available at 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 $2-\Delta\Delta CT$ method (23). 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 are 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) and centrifuged at 1,006 *g* for 15 min at 4°C. Plasma or serum was then extracted and stored in aliquots at −70°C until analysis. All collected samples were analyzed using enzymatic colorimetric assays for glucose (Glucose Oxidase Kit, Instrumentation Laboratories, Cheshire, UK), nonesterified fatty acids (NEFA; Randox, London, UK), glycerol (Randox, London, UK), and lactate (Randox, London, UK) using a semiautomatic 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%). Epinephrine and norepinephrine were measured pre- and postexercise using a commercially available kit (CatCombi ELISA kit, Human, IBL International, GER; CV: 25.7% and 22.7%, respectively).

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

	Adjusted Mean (SE/95% CI)			Mean Difference (95% CI)		
Glycogen/IMTG (Trial, Contrast, and *P* Value)	**LOW**	**MOD**	**HIGH**	**MOD-LOW**	**HIGH-LOW**	**HIGH-MOD**
Preexercise muscle glycogen, mmol·kg^{-1} wet wt						
Estimate	54.3	82.8	81.6	28.5	27.3	−1.2
(95% CI)	(41.5, 67.1)	(70.0, 95.6)	(68.5, 94.8)	(15.8, 41.2)	(14.3, 40.4)	(−14.2, 11.9)
P value				0.0002	0.0004	0.85
Net muscle glycogen utilization, mmol·kg^{-1} wet wt						
Estimate	4.2	11.3	13.5	7.1	9.3	2.1
(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)
P value				0.15	0.09	0.68
Preexercise type I fiber IMTG, %						
Estimate	11.6	10.7	9.3	−0.9	−2.3	−1.4
(SE/95% CI)	(1.3)	(1.3)	(1.4)	(−4.1, 2.3)	(−5.6, 1.0)	(−4.7, 1.9)
P value				0.55	0.16	0.39
Postexercise type I fiber IMTG, %						
Estimate	8.8	6.6	8.8	−2.1	−0.47	1.7
(SE/95% CI)	(1.0)	(1.0)	(1.0)	(−4.0, −0.2)	(−2.47, 1.54)	(−0.3, 3.6)
P value				0.03	0.63	0.09
Net type I fiber IMTG utilization, %						
Estimate	2.5	3.8	1.6	1.3	−0.9	2.2
(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)
P value				0.24	0.41	0.06
Preexercise type II fiber IMTG, %						
Estimate	5.0	6.4	5.3	1.5	0.3	−1.2
(SE/95% CI)	(0.8)	(0.8)	(0.9)	(−1.0, 3.9)	(−2.6, 2.9)	(−3.7, 1.4)
P value				0.21	0.79	0.35
Postexercise type II fiber IMTG, %						
Estimate	5.3	4.5	4.9	−0.8	−0.41	0.38
(95% CI)	(0.8)	(0.7)	(0.8)	(−2.5, 0.9)	(−2.3, 1.4)	(−1.4, 2.1)
P value				0.34	0.64	0.65
Net type II fiber IMTG utilization, %						
Estimate	−0.3	1.7	0.0	2.0	0.2	−1.8
(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)
P value				0.07	0.84	0.12

CI, confidence interval; IMTG, intramuscular triglyceride; LOW (n = 11), MOD (n = 11), and HIGH (n = 12), lower, moderate, and higher conditions.

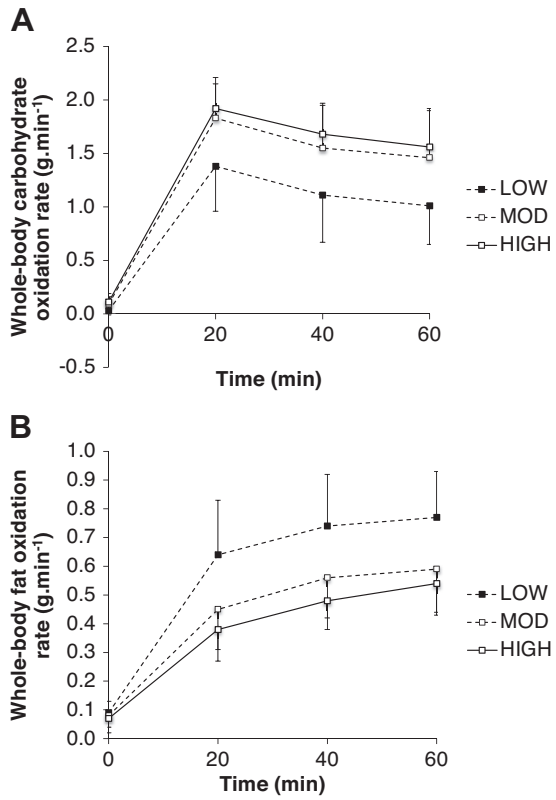

Figure 3. Whole body rates of carbohydrate (*A*) and fat oxidation (*B*) during 60-min steady-state treadmill running at 65% $\dot{V}O_{2max}$ in lower (LOW; $n = 11$), moderate (MOD; $n = 11$), and higher (HIGH; $n = 12$) conditions.

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 (20):

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

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

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

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 preexercise 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 (13). Assuming a two-sided 5% significance level, with the use of a within-subject SD of 20 $mmol \cdot kg^{-1}$ wet wt (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 and 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 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 preexercise muscle glycogen, muscle utilization, and fat oxidation during exercise were explored using Pearson product-moment correlations. For muscle gene expression, 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 Bonferroni adjustment (i.e., 0.05/#comparisons).

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

	Adjusted Mean, $g \cdot min^{-1}$			Mean Difference, $g \cdot min^{-1}$		
Oxidation Rates (Trial, Contrast, and *P* Value)	**LOW**	**MOD**	**HIGH**	**MOD-LOW**	**HIGH-LOW**	**HIGH-MOD**
CHO oxidation, $g \cdot min^{-1}$						
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, $g \cdot min^{-1}$						
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

CI, confidence interval; RER, respiratory exchange ratio; LOW ($n = 11$), MOD ($n = 11$), and HIGH ($n = 12$), lower, moderate, and higher conditions.

RESULTS

Intervention Characteristics

Of the 13 participants who received at least 1 of the dietary interventions, 10 participants completed all 3 periods, 1 completed 2 of the 3 periods (HIGH and LOW), and 2 participants completed 1 period (1 HIGH and 1 MOD). Hence the number of participants completing each of the dietary interventions was LOW = 11, MOD = 11, and 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% $\dot{V}O_{2max}$; $P = 0.84$).

Muscle Substrate Metabolism

Preexercise 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; and HIGH, 31.1 ± 13.2 mean fluorescence intensity per fiber; $P = 0.14$) or type II (LOW, 20.7 ± 10.7; MOD, 24.8 ± 10.1; and HIGH, 23.6 ± 9.6 mean fluorescence intensity per fiber; $P = 0.20$) fibers between trials. Habitual resting muscle glycogen concentration was 75.6 ± 18.8 mmol·kg^{-1} wet wt, consistent with the lower range of expected values of participants of similar overall fitness status (24). Pre- and postexercise muscle glycogen concentrations were significantly lower in LOW vs. MOD and HIGH, but MOD and HIGH were not significantly different (Fig. 2). Net muscle glycogen utilization was not significantly different between trials, although 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 preexercise muscle glycogen concentration ($r = 0.62$; $P = 0.0001$) but not net muscle glycogen utilization ($r = 0.18$; $P = 0.32$).

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

Whole Body Substrate Oxidation Rates

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

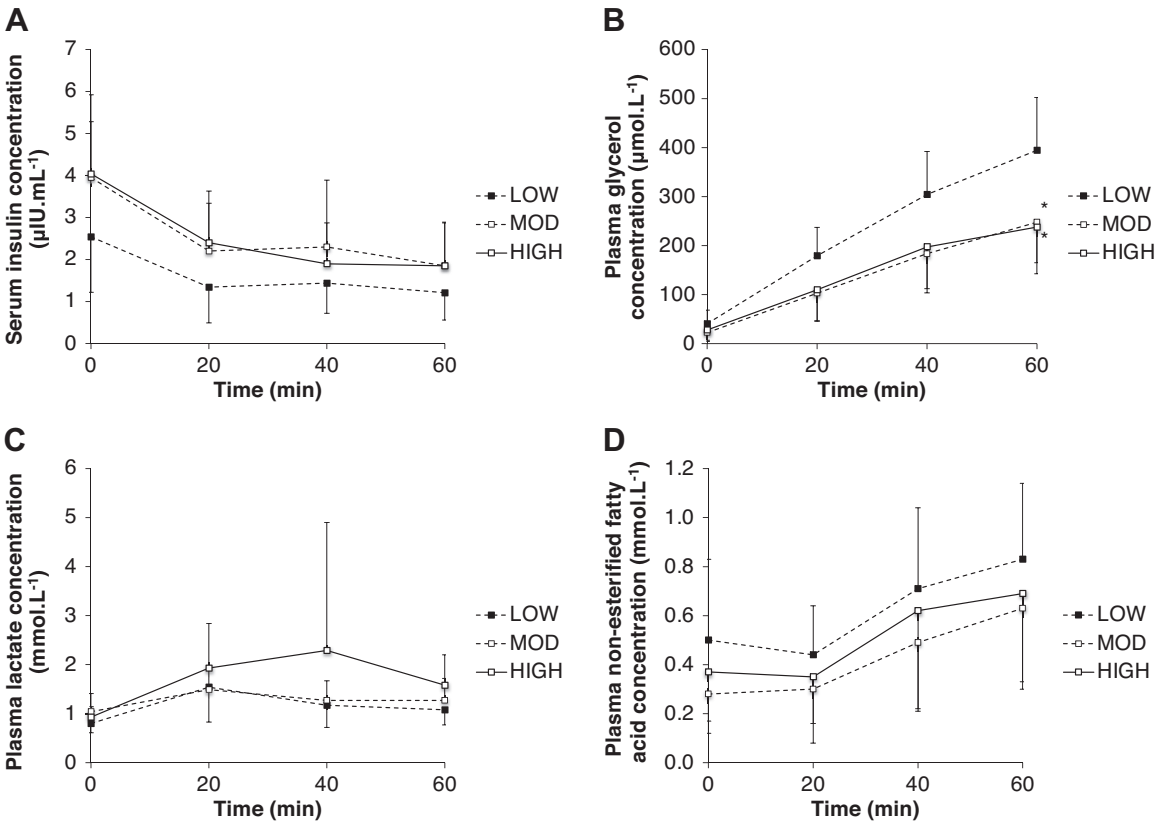

Figure 4. Serum insulin (*A*) and plasma glycerol (*B*), lactate (*C*), and nonesterified fatty acid (*D*) concentrations during 60-min treadmill running at 65% $\dot{V}O_{2MAX}$ in lower (LOW; $n = 11$), moderate (MOD; $n = 11$), and higher (HIGH; $n = 12$) conditions. *Mean exercise area under the curve was different in MOD, HIGH, vs. LOW ($P < 0.05$).

MOD (0.09 ± 0.07 g·min^{-1}; P = 0.12) or MOD and HIGH (P = 0.53; Fig. 3*A*). Preexercise resting whole body fat oxidation rates were not significantly different between trials (LOW, 0.09 ± 0.04 g·min^{-1}; MOD, 0.08 ± 0.04 g·min^{-1}; and HIGH, 0.07 ± 0.05 g·min^{-1}; $P > 0.05$; Fig. 3*B*). Whole body carbohydrate oxidation during exercise was significantly lower in LOW compared with MOD and HIGH, but MOD and HIGH were not significantly different (Fig. 3*A* and Table 3). Whole body fat oxidation during exercise was significantly greater in LOW compared with MOD and HIGH, but MOD and HIGH were not significantly different (Fig. 3*B* and Table 3). Dietary CHO intake during the intervention period was not significantly associated with whole body fat oxidation during exercise (r = −0.26; P = 0.16).

Blood Responses

Blood data are shown in Fig. 4, and statistical comparisons are summarized in Table 4. Preexercise blood variables were not significantly different between trials, other than plasma lactate concentration being significantly lower in MOD versus LOW. Plasma glucose, lactate, NEFA, epinephrine, norepinephrine, and serum insulin concentrations during exercise were not significantly different between trials. Plasma glycerol concentrations during exercise were significantly greater in LOW than MOD and HIGH, whereas MOD and HIGH were not significantly different.

Gene Expression

Preexercise metabolic gene expression for the 34 genes quantified in LOW, MOD, and HIGH is shown in Supplemental Table S1, with between-trial comparisons shown in Supplemental Table S2. Three genes were significantly different between trials following correction for multiple comparisons (n = 102, so $P < 0.0005$). mRNA expression of fatty acid binding protein 3 (FABP3), malonyl-CoA decarboxylase (MLYCD), and uncoupling protein 3 (UCP3) were all significantly lower in MOD and HIGH than LOW (Fig. 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., $P < 0.016$). With the use of this approach, mRNA expression of ACSL1, PDK2, and PNPLA2 was significantly

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

	Median			Median Difference		
Plasma/Serum (Trial, Contrast, and *P* Value)	LOW	MOD	HIGH	MOD-LOW	HIGH-LOW	HIGH-MOD
Insulin, μIU·mL^{-1}						
Median	1.35	2.35	2.28	1.14	1.11	−0.12
(Min, max)	(0.31, 2.65)	(0.95, 4.52)	(0.84, 3.85)			
P value				0.02	0.02	0.57
Glucose, mmol·L^{-1}						
Median	4.92	5.12	5.25	0.02	0.09	0.09
(Min, max)	(4.44, 5.57)	(4.56, 5.39)	(4.48, 5.60)			
P value				0.73	0.55	0.25
Glycerol, μmol·L^{-1}						
Median	254.0	114.0	134.7	−100.8	−98.3	1.8
(Min, max)	(134.8, 298.5)	(81.8, 231.5)	(59.3, 309.0)			
P value				0.008	0.008	0.73
Lactate, mmol·L^{-1}						
Median	1.20	1.34	1.57	0.12	0.17	0.11
(Min, max)	(0.36, 2.08)	(0.65, 1.87)	(0.69, 5.22)			
P value				0.46	0.15	0.31
NEFA, mmol·L^{-1}						
Median	0.67	0.43	0.44	−0.21	−0.23	0.11
(Min, max)	(0.26, 0.92)	(0.16, 0.84)	(0.16, 1.23)			
P value				0.04	0.02	1.00

	Adjusted Mean			Mean Difference		
	LOW	MOD	HIGH	MOD-LOW	HIGH-LOW	HIGH-MOD
Preexercise epinephrine, pg·mL^{-1}						
Estimate	152.5	152.3	150.4	−0.2	−2.2	−1.9
(SE/95% CI)	(13.9)	(15.2)	(15.0)	(−36.8, 36.4)	(−38.8, 34.5)	(−41.3, 37.4)
P value				0.99	0.90	0.92
Postexercise epinephrine, pg·mL^{-1}						
Estimate	278.1	249.6	249.0	−28.5	−29.1	−0.58
(SE/95% CI)	(25.0)	(23.7)	(23.7)	(−77.4, 20.4)	(−78.9, 20.7)	(−48.7, 47.5)
P value				0.23	0.23	0.98
Preexercise norepinephrine, pg·mL^{-1}						
Estimate	316.9	428.4	327.2	111.5	10.3	−101.2
(SE/95% CI)	(253.6)	(74.3)	(73.6)	(−30.7, 253.8)	(−132.0, 152.7)	(−255.8, 53.4)
P value				0.12	0.88	0.18
Postexercise norepinephrine, pg·mL^{-1}						
Estimate	793.4	891.5	860.2	98.1	66.8	−31.3
(SE/95% CI)	(135.1)	(128.8)	(128.9)	(−149.8, 346.1)	(−185.9, 319.5)	(−275.4, 212.8)
P value				0.41	0.58	0.79

CI, confidence interval; LOW (n = 11), MOD (n = 11), and HIGH (n = 12), lower, moderate, and higher conditions.

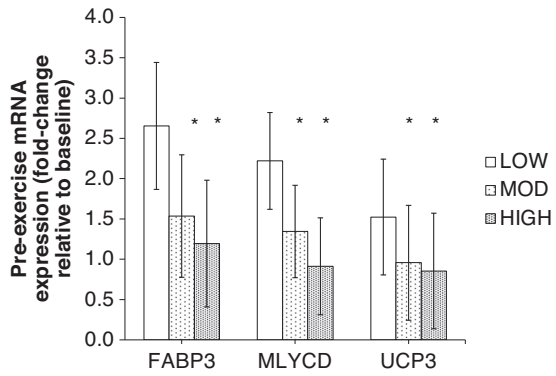

Figure 5. mRNA expression of metabolic genes before 60-min steady-state treadmill running at 65% $\dot{V}O_{2max}$ in lower (LOW; *n* = 11), moderate (MOD; *n* = 11), and higher (HIGH; *n* = 12) conditions, expressed as fold-change relative to baseline (*day 1* of the 1st experimental trial). *Significantly different vs. LOW ($P < 0.0001$). FABP3, fatty acid binding protein 3; MLYCD, malonyl-CoA decarboxylase; UCP3, uncoupling protein 3.

lower in MOD and HIGH than LOW, and mRNA expression of CD36, CPT1B, HADHA, and SLC27A1 was all significantly lower in HIGH than LOW (Supplemental Table S2).

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 preexercise muscle glycogen availability was not observed. Rather, the main findings were as follows: *1*) preexercise 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 (3, 14), HIGH did not produce significantly greater preexercise muscle glycogen concentration than MOD, despite 1.5 g CHO·kg^{-1}·day^{-1} greater CHO ingestion in the preceding 48 h (Fig. 2). Differences in preexercise muscle glycogen concentration between MOD and HIGH could have been observed with greater CHO intake in HIGH. However, Costill and colleagues (13) observed graded preexercise 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 normalize 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 coworkers (25). The absence of differences in preexercise 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 (24). 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 preexercise gene expression (Tables 2–4 and Supplementary Table S2). A relatively modest net muscle glycogen use was seen in the present study (Fig. 2 and 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; Ref. 13), the exercise modality (i.e., net glycogen use in vastus lateralis is lower in level running than cycle ergometry; Refs. 24, 26), and the moderate exercise intensity employed (14). Previous work with similar CHO intakes in the HIGH condition as the present study reported similar respiratory exchange ratio responses to exercise as compared with moderate or mixed CHO intakes; however, in contrast to the present study, this was observed despite elevated muscle glycogen availability in the high CHO conditions (13, 14). With higher rates of CHO ingestion (~8 g CHO·kg^{-1}·day^{-1}), clearer exercise-metabolic differences have been observed (3). Accordingly, the addition of the present data to existing literature suggests increasing short-term CHO intake from ~4.5 to ~6.5 g CHO·kg^{-1}·day^{-1} (~45 to ~70% EI) in recovery from exercise does not discernibly influence metabolic responses to subsequent moderate-intensity exercise (13, 14), and that more aggressive increases in CHO intake may be required to alter fuel metabolism during exercise (3).

In contrast to the largely similar response between MOD and HIGH, consistent metabolic differences were observed in LOW. This included lowered preexercise muscle glycogen availability (Table 2), decreased CHO and increased fatty acid oxidation during exercise (Table 3), elevated plasma glycerol concentrations (Table 4), and upregulation of several genes implicated in substrate metabolism, such as FABP3, MLYCD, and UCP3, with several other genes possibly differentially expressed in LOW (Supplemental Table S2). These data align with previous research reporting decreased CHO and increased fatty acid metabolism during exercise commenced with lowered muscle glycogen (27–31). It is clearly plausible reduced muscle glycogen availability contributed to the altered fuel use per se (9), although it is also possible the additional dietary fat intake resulted in adaptations that augmented fatty acid oxidation in LOW (32). Regardless of the precise mechanism, the present data indicate short-term CHO intakes of ~2.4 g CHO·kg^{-1}·day^{-1} (~21% EI) in recovery from exercise are sufficient to reduce muscle glycogen availability and alter substrate metabolism during subsequent moderate-intensity exercise, consistent with what might be expected from studies of non-ketogenic low-CHO,

high-fat diets (33). Whether a "threshold" dietary CHO intake exists, somewhere between ~2.5 and ~4.5 g CHO·kg^{-1}·day^{-1} (i.e., ~20–45% EI), at which this metabolic shift takes place requires further investigation.

The gene expression data demonstrate LOW induced a coordinated change in basal skeletal muscle gene expression favoring fatty acid utilization, which is consistent with the metabolic data observed during subsequent moderate-intensity exercise (Supplemental Table S2). Given the 48-h recovery following the previous exercise bout in the present investigation, this coordinated change in muscle gene expression can be confidently attributed to the dietary manipulations (34). Our data align with previous work demonstrating increased expression of FABP (35), UCP3 (36), PDK2 (37), CPT1 (26), CD36 (38), and HADHA (35) with low CHO availability. To our knowledge, a prior nutrient-exercise induced regulation of ACSL1, MLYCD, PNPLA2, and SLC27A1 gene expression has not previously been shown in human muscle, but their upregulation with lower CHO intake is consistent with an intracellular environment favoring fatty acid utilization. Altered expression of these genes and/or the proteins they encode for has been observed after a period of endurance exercise training, a stimulus expected to augment the capacity for fatty acid metabolism in skeletal muscle (39–42). Increased UCP3 gene expression in the present investigation is interesting in the context of research showing impaired exercise economy following ingestion of a low CHO-high-fat diet (43–45), given UCP3 is implicated in uncoupling oxidative phosphorylation from ATP synthesis and mitochondrial fatty acid export when supply exceeds oxidation capacity (46–48). While significant between-diet effects on running economy were not seen in the present investigation (data not shown), possibly due to the low exercise intensity (45), the data, albeit at the gene level, provide a plausible mechanism for low-CHO availability-induced impairments in exercise economy observed elsewhere (43–45). Collectively, the gene expression data confirm several previous observations and add new insights into the coordinated mRNA response to diet-induced alterations in CHO availability in humans.

In summary, the present data demonstrate, within a model of short-term 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}·day^{-1} range (46–61% of daily EI). In contrast, more marked reductions in CHO intake (~2.4 g·kg^{-1}·day^{-1}, ~21% EI) lowered resting muscle glycogen concentration, altered resting expression of genes related to fatty acid utilization in skeletal muscle, and ultimately increased whole body fat oxidation during subsequent moderate-intensity exercise. The data presented herein combined with that of previous reports suggest that metabolic responses appear somewhat resistant to short-term dietary CHO change within the 4.5–6.5 g CHO·kg^{-1}·day^{-1} (45–70% EI) range (13, 14) but are affected by more aggressive CHO increases (>6.5 g CHO·kg^{-1}·day^{-1}, >70% EI) or decreases (<2.5 g CHO·kg^{-1}·day^{-1}, <20% EI) (3). Whether a threshold exists between 4.5 and 2.5 g CHO·kg^{-1}·day^{-1} (45%-20% EI) whereby fatty acid metabolism is augmented remains to be tested. These findings help to provide a useful framework for researchers when examining responses to exercise-diet manipulations. Furthermore, for those interested in optimizing fat oxidation, the results provide insights into the range of moderate to higher short-term CHO intakes within which fat oxidation is maintained and highlight the degree of dietary change necessary to induce clear alterations in in fat oxidation during exercise.

SUPPLEMENTAL DATA

Supplemental material is available at https://doi.org/10.25500/edata.bham.00000609.

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DISCLOSURES

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AUTHOR CONTRIBUTIONS

G.A.W., H.E.B., A.B.H., S.J., and A.P. conceived and designed research; G.A.W., H.E.B., C.S.D., M.J., S.J., A.M.T., and L.B. performed experiments; H.E.B., C.S.D., and S.J. analyzed data; E.M., G.A.W., H.E.B., and S.J. interpreted results of experiments; E.M. and G.A.W. prepared figures; E.M. and G.A.W. drafted manuscript; E.M., G.A.W., H.E.B., C.S.D., S.J., and A.P. edited and revised manuscript; E.M., G.A.W., H.E.B., C.S.D., A.B.H., M.J., S.J., A.M.T., L.B., and A.P. approved final version of manuscript.

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