

Effects of exercise followed by a low-carbohydrate diet on peak fat oxidation and mitochondrial coupling control and efficiency in healthy trained men

K. K. Lange¹ , L. Becker² , T. Rømer^{1,3} , M. Hassing¹ , S. T. Høybye¹, N. Smailes¹, P. Pardo¹, B. Danisman¹, P. F. Andersen⁴ , S. Larsen^{1,5}  and J. W. Helge¹ 

¹Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark

²Technical University of Munich, Munich, Germany

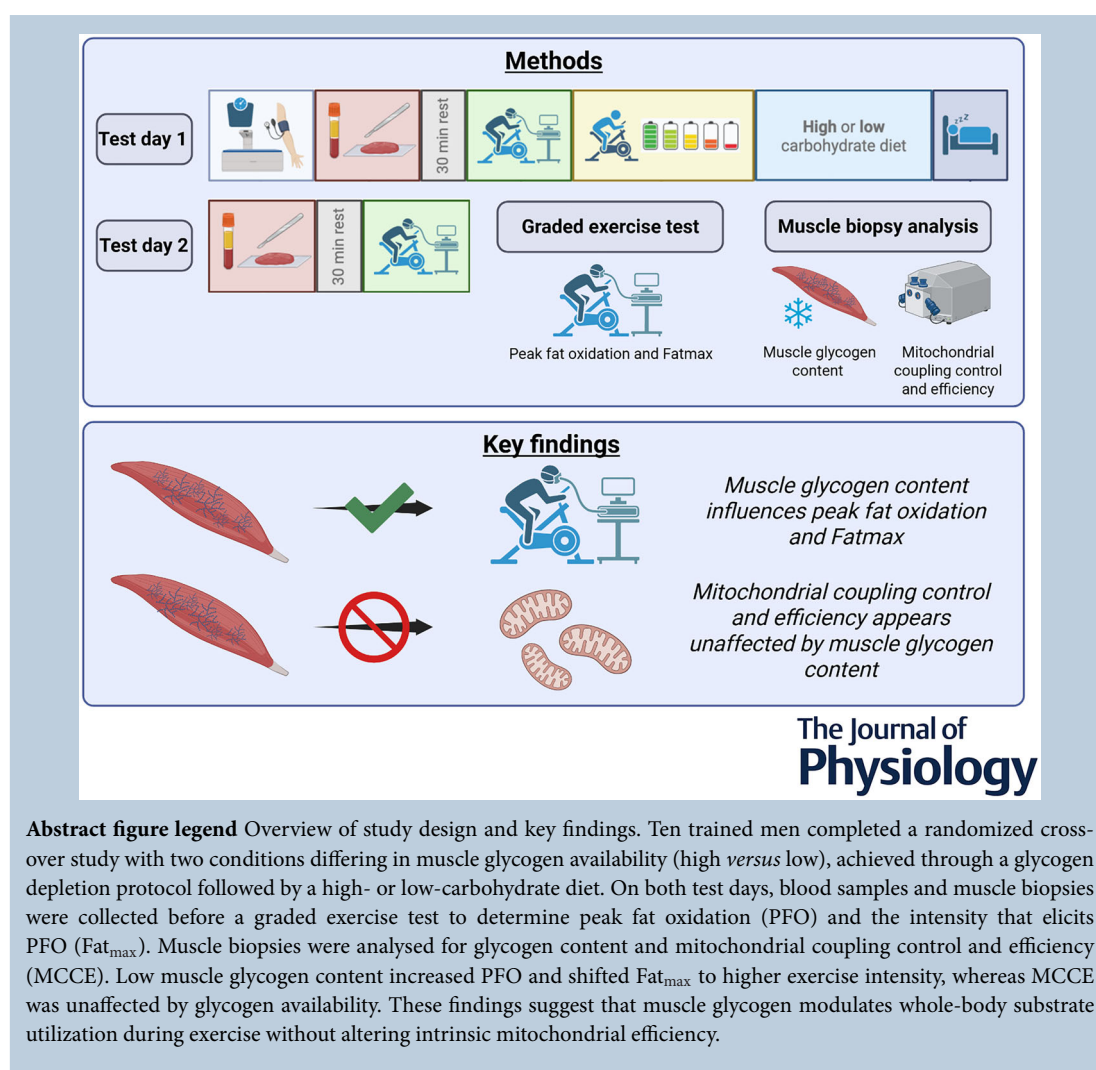
³Team Denmark (Danish Elite Sports Organization), Copenhagen, Denmark

⁴Department of Cellular and Molecular Medicine, University of Copenhagen, Copenhagen, Denmark

⁵Clinical Research Centre, Medical University of Białystok, Białystok, Poland

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Abstract Whole-body fat oxidation correlates with mitochondrial oxidative capacity, emphasizing the role of mitochondrial function in systemic and skeletal muscle substrate utilization during exercise – a relationship that may be modulated by the prevailing fuel availability. Muscle glycogen is an important fuel during exercise due to its effects on substrate metabolism, but its influence on peak fat oxidation (PFO) and mitochondrial coupling control and efficiency (MCCE) remains unclear. The aim of the study was to investigate the influence of muscle glycogen content on PFO, the intensity eliciting PFO (Fat_{max}) and MCCE. Ten trained men participated in a randomized crossover study comprising two interventions conducted over two consecutive days. Day 1 included anthropometric assessments, blood sampling, a muscle biopsy and a graded exercise test followed by a glycogen depletion protocol. The following day blood sampling, muscle biopsy and the graded exercise test were repeated. A diet high or low in carbohydrate was consumed between the days to manipulate glycogen levels. The experiment was repeated after 7–14 days. Muscle samples were analysed for glycogen and triacylglycerol content and MCCE. PFO increased after both interventions and was further enhanced by the combined exercise and low-carbohydrate intervention, where muscle glycogen was low. Fat_{max} increased only following the combined exercise and low-carbohydrate intervention. MCCE was unaffected by the interventions. The study concludes that exercise-induced glycogen depletion together with a low-carbohydrate diet increases PFO and Fat_{max} but does not influence MCCE. These findings have implications towards understanding and optimizing fat oxidation and as a potential read out of metabolic flexibility.

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Corresponding author K. K. Lange: Department of Biomedical Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Copenhagen, Denmark. Email: Kristine.lange@sund.ku.dk

Key points

- Whole-body fat oxidation is linked to mitochondrial capacity, yet the role of endogenous fuel availability in this relationship has not been studied.
- Muscle glycogen is a primary exercise fuel, but its specific influence on peak fat oxidation (PFO), the exercise intensity that elicits PFO (Fat_{max}) and mitochondrial coupling control and efficiency (MCCE) is unclear.
- Ten trained men completed a randomized crossover study using glycogen depletion and dietary intervention to alter glycogen levels, with exercise tests and muscle biopsies performed at baseline and postintervention.
- Exercise-induced glycogen depletion combined with a low-carbohydrate diet increased both PFO and Fat_{max} , whereas there was no effect on MCCE.
- These findings clarify the regulation of PFO and the combined effect of exercise-induced glycogen depletion and low carbohydrate diet, and that this is not regulated through changes in mitochondrial function.

Kristine Kjær Lange is a human biologist and research coordinator at the Department of Biomedical Sciences at University of Copenhagen, Denmark. Her research focuses on muscle metabolism, exercise physiology and metabolic regulation. A key highlight of her career has been the opportunity to present her research at recognized international conferences. She aspires to become a highly respected researcher, contributing to the advancement of basic science while ensuring that her findings are meaningful, applicable and beneficial to the populations they intend to serve.



Introduction

The capacity to oxidize fat during exercise is crucial for endurance performance and metabolic health. Because carbohydrate stores are limited while fat reserves are virtually inexhaustible (Maunder et al., 2018), enhanced fat oxidation provides a performance advantage during endurance exercise and performance, particularly when carbohydrate availability declines (Frandsen et al., 2017). Conversely, low fat oxidation capacity is linked to attenuated performance, as well as fat accumulation, obesity and metabolic diseases (Robinson et al., 2015; Rosenkilde et al., 2010; Zurlo et al., 1990), highlighting its essential role in metabolic health.

Macronutrient intake and endogenous storage strongly influence fat oxidation as well as peak fat oxidation (PFO) through their effect on substrate availability. Low-carbohydrate diets, ketogenic diets and fasting increase whole-body PFO during exercise (Frandsen et al., 2019; Tarry et al., 2025; Volek et al., 2016), whereas carbohydrate feeding suppresses PFO (Achten & Jeukendrup, 2003). Substrate availability and fat and carbohydrate oxidation are also influenced by exercise intensity, and the crossover concept outlines this distribution across the exercise intensity spectrum (Brooks & Mercier, 1994). Two papers by Romijn et al. (1993) and van Loon et al. (2001) outlined the contribution from endogenous and exogenous substrates across the exercise intensity spectrum, as well as the bell-shaped curve of absolute fat oxidation as a function of exercise intensity. In a study of MacArdle patients it was demonstrated that the PFO occurred at 94% of their aerobic capacity, when exercise was sustained without contribution of glycogen as a substrate (Rodriguez-Lopez et al., 2023). In non-pathological condition glycogen breakdown is linked to glycogen storage and exercise intensity (Impey et al., 2018), and thus strongly influences substrate selection and oxidation rates during exercise. However, a knowledge gap exists as to whether and how muscle glycogen modulates PFO and the intensity that elicits PFO (Fat_{max}).

Endurance training increases mitochondrial content and enzyme activity in skeletal muscle (Holloszy & Coyle, 1984), thereby enhancing fat oxidation by upregulating proteins involved in fatty acid transport and oxidative metabolism (Fritzen et al., 2020). Accordingly, whole-body fat oxidation correlates with mitochondrial oxidative capacity (Holloway et al., 2006; Sahlin et al., 2007), emphasizing the role of mitochondrial function in systemic and muscle substrate utilization during exercise – a relationship that may be modulated by the prevailing fuel availability (Demine et al., 2014). Mitochondrial coupling control and efficiency, reflected by the phosphate–oxygen ratio (P/O ratio) and respiratory control ratio (RCR), may also be influenced by substrate

availability. However, this relationship has not been directly investigated, and only indirect evidence exists linking substrate availability to mitochondrial coupling control and efficiency (Fernstrom et al., 2007; Nisr & Affourtit, 2014). Another study investigating the effects of acute exercise on mitochondrial respiration reported no change in mitochondrial efficiency following acute short-term exercise despite likely alterations in substrate availability (Newsom et al., 2021). From an evolutionary perspective, fluctuating energy availability driven by environmental and climatic variation could have shaped mitochondrial coupling control and efficiency. During scarcity, tighter coupling would promote energy conservation by maximizing ATP yield per substrate, whereas uncoupled respiration under energy abundance would facilitate thermogenesis and prevent oxidative stress (Brand, 2000; Wallace et al., 2003). In line with this framework, mitochondria from naturally fasted, winter-acclimatized king penguin chicks exhibit approximately 15% higher coupling efficiency (P/O ratio) compared to experimentally fed chicks, suggesting that energy availability under harsh conditions is associated with a more economical mitochondrial management of resources to secure survival (Monternier et al., 2014). Thus, size, location and variability of endogenous substrate stores likely exert an influence on mitochondrial efficiency and its regulation, although, this has yet to be explored.

To assess these knowledge gaps, this study examined the relationships under experimentally manipulated muscle glycogen conditions in a study of trained male participants. The aim was to investigate the influence of the muscle glycogen content on PFO, Fat_{max} and muscle mitochondrial coupling control and efficiency. We hypothesized that low muscle glycogen concentrations, accompanied by elevated plasma free fatty acid (FFA) concentrations, would increase PFO and Fat_{max} but reduce muscle mitochondrial coupling control and efficiency compared to normal muscle glycogen concentrations.

Methods

Ethical approval

The study was approved by the Science Ethics Committee (H-20023946) and conforms to the standards set by the Declaration of Helsinki. The participants received written and oral information about possible risks associated with the study before they volunteered and signed the informed consent form.

Participants

Ten young healthy males were included in the study. Inclusion criteria were age between 18 and 40 years,

body mass index (BMI) between 18 and 25 kg/m², fitness level >55 ml O₂/min/kg and a regular participation (>3 sessions per week) in endurance-dominated sports such as running, cycling or triathlon. Exclusion criteria were previous or present cardiovascular disease and medication that affect metabolism. The participants were recruited through advertising on Facebook.

Experimental design

The study was a randomized crossover study consisting of two experimental interventions of each two consecutive test days. The difference between the two interventions was the diet intake on day 1, and the order was randomized and separated by 7–14 days (Fig. 1). In the 4 days leading up to intervention 1 the participants were asked to consume a regular mixed non-standardized diet and to write down their dietary intake to replicate this during the 4 days prior intervention 2. Furthermore, daily self-reported physical activity was recorded for the 4 days prior to each intervention. Participants were instructed to abstain from strenuous exercise for 48 h and from any exercise for 24 h prior to test day 1.

Experimental diets

After the experiments on test day 1, participants consumed either a high-carbohydrate (H-CHO) or a low-carbohydrate (L-CHO) diet to maximize or minimize muscle glycogen resynthesis, respectively. The diets were isocaloric and tailored to the individual energy requirements, calculated using the Harris–Benedict equation (Harris & Benedict, 1918). The average energy composition of the H-CHO diet was 72.6 energy percentage (E%) carbohydrate, 15.3 E% fat and 12.1 E% protein, whereas the L-CHO diet contained 9.1 E% carbohydrate, 74.5 E% fat and 16.4 E% protein. Both experimental diets were distributed across four meals. The initial meal was consumed immediately following the glycogen depletion protocol and was prepared by the study project team. The remaining three meals were prepared and consumed by the participants at home. Participants recorded their food intake, including any deviations from the prescribed dietary regimen.

Experimental protocol

At each of the four test days, participants reported to the laboratory at the same time in the morning (08.00 AM) to

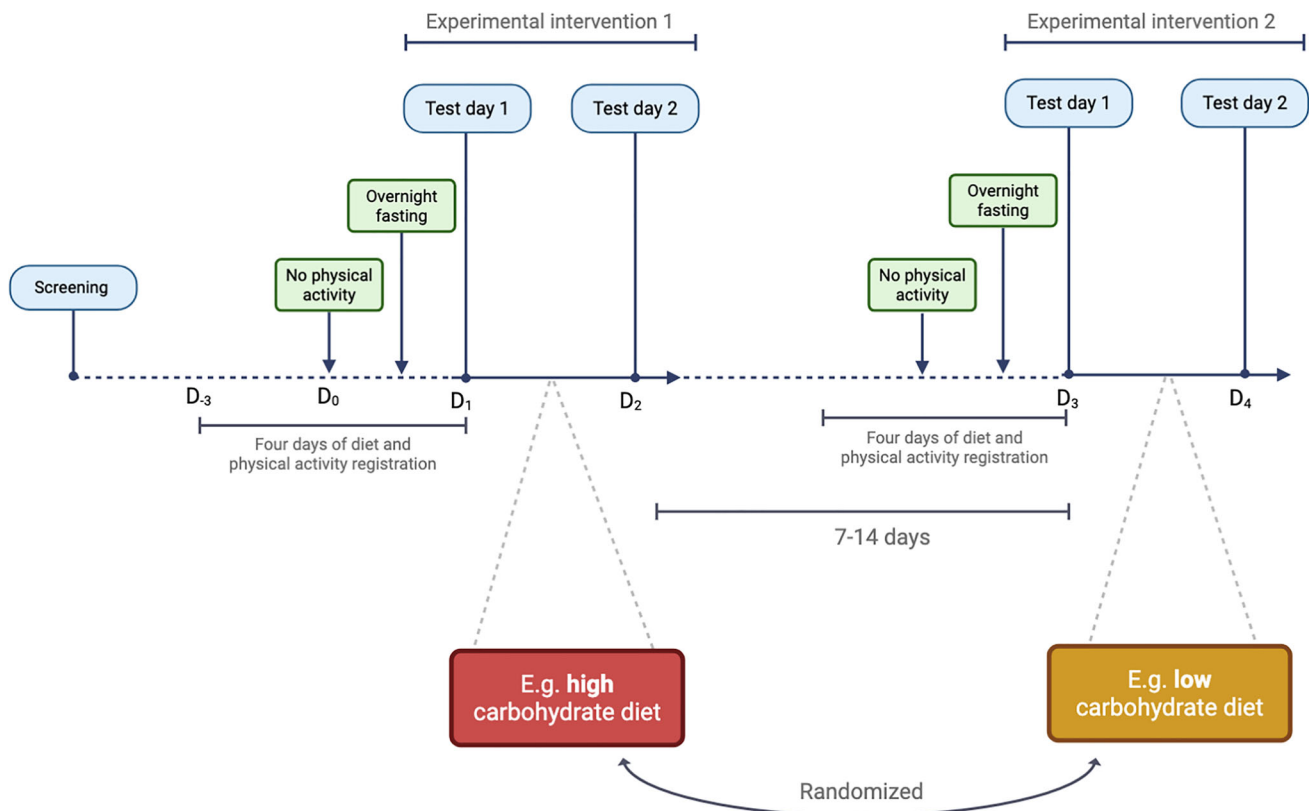


Figure 1. Experimental design and timeline

A total of 10 participants ($n = 10$) were included for the randomized crossover study consisting of two experimental interventions of each two consecutive test days.

standardize testing time and thereby minimize potential confounding effects of diurnal variation between the interventions. Initially on test day 1, measurements of height and weight were performed followed by assessment of body composition using dual-energy X-ray absorptiometry (DXA, Lunar, iDXA, G&E Medical Systems Lunar, Wisconsin, WI, USA, Encore version 14.10.022). The participants were allowed to consume water in the morning and were encouraged to empty their bladder before the DXA scan. After the DXA scan, and with the participants retained in a supine position, blood pressure was measured thrice with 60 s between measurements. After this a venous blood sample was obtained from the antecubital vein and immediately centrifuged (Rotina 380R, Hettich ApS) at 4000 g for 10 min at 4°C and stored at −80°C for later analysis. Subsequently, a muscle biopsy from m. vastus lateralis was obtained under sterile conditions using local anaesthesia (5 mL Xylocaine 20 mg/mL, Aspen Pharma Trading Lt, Ireland) and the Bergström technique. The muscle sample was immediately freed from any visible blood and divided into a part placed in isolation buffer used for mitochondrial analysis and a part immediately frozen in liquid nitrogen and stored at −80°C for the analyses of muscle glycogen and intramuscular triacylglycerol (IMTG) concentrations, as well as oxidative enzyme activity of citrate synthase (CS) and hydroxyacyl-CoA dehydrogenase (HAD). After a 30 min rest the participants performed a graded exercise test on a bike ergometer to determine PFO, Fat_{max} and peak oxygen consumption ($\dot{V}_{\text{O}_{2\text{peak}}}$). Finally, a glycogen depletion protocol was performed as detailed below. After this the participants had their first meal (H-CHO or L-CHO), which completed day 1. The next day (test day 2) the participants revisited the laboratory and repeated the weight measurement, a venous blood sample, a muscle biopsy and the graded exercise test. In the second intervention a muscle biopsy was only obtained on day 2.

Graded exercise test

A graded exercise protocol to determine PFO and Fat_{max} ($\text{Fat}_{\text{oxidation}}$ test) was performed on a bike ergometer (Monark LC7TT, Monark Exercise AB, Vansbro, Sweden), and pulmonary oxygen uptake and carbon dioxide excretion were measured using a mixing chamber and an automated online system (Quark CPET, COSMED, Rome, Italy). After an initial 5 min resting period on the bike ergometer participants began cycling at an initial intensity of 60 W for 5 min. The workload was subsequently increased by 35 W every 3 min until a steady-state respiratory exchange ratio (RER) above 0.95 was reached. Average $\dot{V}_{\text{O}_2}$ and $\dot{V}_{\text{CO}_2}$ from minute 03.30 to 04.30 during the resting period and from minute 01.30 to 02.30 at each

intensity were used to calculate fat oxidation rates using the Frayn equation (Frayn, 1983). After a 5 min break the graded exercise was continued to determine $\dot{V}_{\text{O}_{2\text{peak}}}$ and maximum watts (W_{max}) with an initial workload of 150 W for 1 min, and subsequently the workload increased with 25 W every minute until voluntary exhaustion. Immediately after both the $\text{Fat}_{\text{oxidation}}$ and the $\dot{V}_{\text{O}_{2\text{peak}}}$ test the participants were asked about the rate of perceived exertion (RPE). $\dot{V}_{\text{O}_{2\text{peak}}}$ was determined as the highest oxygen consumption during 30 consecutive seconds. The credibility of the $\dot{V}_{\text{O}_{2\text{peak}}}$ test was investigated by checking for $\dot{V}_{\text{O}_2}$ plateau, defined as a change of <2.0 mL/min/kg with increasing workload. If the primary criteria were not met, two of the following three secondary criteria were needed to be fulfilled for the $\dot{V}_{\text{O}_{2\text{peak}}}$ test to be approved: (1) RER exceeding 1.10, (2) heart rate (HR) within 10 beats per minute (bpm) of age-predicted maximum heart rate (HR_{max}) and (3) RPE exceeding 17 on Borg's 6–20 scale (Borg, 1982). W_{max} was defined as the highest average power output that could be maintained for minimum 10 s at each intensity and was used to calculate the intensities applied in the glycogen depletion protocol.

The data from the graded exercise test were used for calculation of PFO and Fat_{max} to create a second-degree polynomial regression curve fit, with fat oxidation as the dependent variable and relative exercise intensity given in % of $\dot{V}_{\text{O}_{2\text{peak}}}$ as the independent variable.

Glycogen depletion protocol

The participants underwent a four-phase glycogen-depleting cycling exercise protocol adapted from Wojtaszewski et al. (2003) on a bike ergometer (Monark, LC7TT, Monark Exercise AB). The participants performed cycle exercise at 70% of W_{max} for 45 min followed by 45–60 min of high-intensity intervals consisting of 2 min intervals starting at 95% of W_{max} , interspersed with 2 min of active recovery at 40% of W_{max} . When the participants were unable to keep the rpm above 60, the intensity of the interval was decreased with 5% of W_{max} . The interval session continued until the participants were unable to maintain an intensity of 70% of W_{max} . A minimum of 12 and a maximum of 15 intervals were performed before being proceeded to the next phase. The participants then performed 30 min of continuous work on a combined arm crank and regular bike ergometer (Monark 839E, Monark Exercise AB). The arm crank ergometer was set at 50 W, whereas the bike ergometer was set at 50% of W_{max} . Finally, the participants performed a time trial, where they had to work until voluntary exhaustion. The intensity of this phase started at 85% of W_{max} for 1 min and was lowered with 5% every minute until the intensity reached 70% of W_{max} . At 70% of W_{max} the participant had to work for

as long as possible. Time until exhaustion was noted. To minimize heat stress, participants were provided with water *ad libitum* and the option of circulating air. Electrolytes without carbohydrates were provided to minimize dehydration. The glycogen depletion protocol lasted for 2.5–3 h and was well tolerated by all participants.

Analytical procedures

Blood analysis. The blood samples were analysed for concentrations of plasma glucose, insulin, FFA, triacylglycerol (TAG), total cholesterol, low-density lipoprotein (LDL), high-density lipoprotein (HDL), glycerol, ketones, leptin and high-sensitivity C-reactive protein (hsCRP) using an automatic analyser (COBAS e 601 and c 501 module, Roche Diagnostics, Switzerland). Two millilitres of full blood was drawn with a safePico blood gas aspirator syringe (safePico Aspirator, Radiometer, Denmark) to analyse haemoglobin, haematocrit and lactate (ABL800 Flex, radiometer, Denmark) immediately after aspiration. On test day 1 in experimental intervention 1, glycated haemoglobin (HbA1c) was measured and analysed (DCA Vantage Analyzer, Siemens Healthcare Diagnostics, USA). Plasma insulin and glucose concentrations were used to calculate homeostatic model assessment of insulin resistance (HOMA-IR) (Levy et al., 1998).

Muscle analysis. The frozen muscle samples were freeze-dried and dissected free from blood, connective tissue and adipose tissue prior to biochemical analysis. The content of muscle glycogen and IMTG, as well as muscle CS and HAD activity, was determined as previously described (Kiens & Richter, 1996; Larsen et al., 2015).

Mitochondrial respiration analysis. The mitochondrial respiratory measurements were carried out in isolated muscle mitochondria as previously described (Mogensen & Sahlin, 2005). Mitochondrial respiration was measured using high-resolution respirometry (Oxygraph-2k; Oroboros Instruments GmbH, Innsbruck, Austria), and measurements were made in duplicates in a respiration medium (MIR05) at 37°C in a low oxygenated environment (0–200 nmol/mL oxygen). Polarographic oxygen sensors measured the oxygen concentration in the chambers over time, reflecting the respiratory flux. The measurements used two specific protocols with the aim of stimulating both carbohydrate and fatty acid pathways. In short, the carbohydrate protocol started with adding the isolated muscle mitochondria (5 µL). Next, malate (2 mM) and pyruvate (5 mM) were added, followed by adding a non-saturating amount of ADP (0.2 mM). The addition of ADP increased the respiration because of

complex V stimulation and, thereby, ATP production. After restoration of the respiratory flux to baseline, a saturating amount of ADP (5 mM) was added together with magnesium chloride (MgCl₂) (3 mM). Hereafter, glutamate (10 mM) was added to observe maximal respiratory capacity in complex I. Finally, succinate (10 mM) was added to observe maximal respiratory capacity in complex I + II. The fatty acid protocol was similar to the aforementioned protocol, except that pyruvate was replaced with palmitoylcarnitine (10 µM) as the second step, whereas pyruvate was added after the saturating ADP titration. The amount of substrate presented in the parenthesis is the final concentration in the chambers.

Data analysis of the mitochondrial respiratory data was done using DatLab software (DatLab version 7.4.0.4, Oroboros Instruments GmbH, Innsbruck, Austria). The data were analysed for mitochondrial coupling control and efficiency, measured as the P/O ratio and RCR, respectively, and mitochondrial respiratory capacity. The P/O ratio was determined as the amount of ATP produced relative to the oxygen consumed, assuming that all available ADP is converted to ATP (Mogensen et al., 2006). The RCR was calculated as the ratio between maximal respiration in state 3 and leak respiration in state 2. An average oxygen flux rate after each substrate titration was calculated to analyse mitochondrial respiratory capacity.

Statistical analyses. Statistical analyses were performed using RStudio (Posit Software, PBC, version 2024.09.1, Boston, MA, USA). Excel (Microsoft Excel, version 16.89.1, Redmond, Washington) was used to calculate means and SDs of all results presented in the tables. Prism (GraphPad, San Diego, CA, USA) was used to graphically present the data.

Data were analysed for normality using QQ-plot and Shapiro–Wilk tests. If not normally distributed the data were log transformed, and if still not normally distributed non-parametric tests were used. A two-way ANOVA mixed model was used to detect any significant changes during and between the experimental interventions. As a *post hoc* test a Tukey's test or a pairwise *t* test was performed if a statistically significant interaction was observed. A paired *t* test was used to investigate statistical significance between the diets. A one-way ANOVA was performed to investigate changes in mitochondrial coupling control and efficiency, the content of muscle glycogen and IMTG and CS and HAD activity from baseline to H-CHO day 2 and L-CHO day 2. The analysis of changes in mitochondrial respiratory capacity between the test days was performed using a linear mixed model with repeated measures. A repeated-measures correlation analysis was performed on selected outcome variables of

Table 1. Calculated mean daily intake of energy and nutrients in the experimental diets.

Dietary intake <i>n</i>	H-CHO diet 10	L-CHO diet 10	<i>t</i> test <i>P</i> -value
Energy (kJ)	14 028 ± 1868	15 205 ± 3835	<i>P</i> = 0.239
Carbohydrate (E%)	72.2 ± 1.6	8.9 ± 1.0	<i>P</i> < 0.0001
Carbohydrate (g/day)	580 ± 69	66 ± 9	<i>P</i> < 0.0001
Carbohydrate (g/kg)	7.4 ± 1.3	0.8 ± 0.2	<i>P</i> < 0.0001
Fat (E%)	15.3 ± 1.9	74.5 ± 0.5	<i>P</i> < 0.0001
Fat (g/day)	60 ± 14	306 ± 75	<i>P</i> < 0.0001
Fat (g/kg)	0.8 ± 0.2	3.9 ± 1.2	<i>P</i> < 0.0001
Protein (E%)	12.1 ± 1.0	16.4 ± 1.4	<i>P</i> < 0.0001
Protein (g/day)	97 ± 10	149 ± 51	<i>P</i> = 0.006
Protein (g/kg)	1.2 ± 0.2	1.9 ± 0.8	<i>P</i> = 0.008

Note: Data are presented for 10 participants for both the H-CHO and L-CHO diet. Values are presented as means ± SD. The intake on the experimental diets was calculated from dietary diaries and presented in E%, g/day and g/kg.

the experimental trial that were hypothesized to exhibit significant interrelationships.

Data are presented as means ± SD. Selected data are presented graphically as individual values with means ± 95% confidence limits. For all analysis significance was set at the 0.05 level of confidence.

Results

Dietary energy intake was similar across the interventions (Table 1). The subjects adhered well to the prescribed meals on day 1 in the interventions, resulting in a significantly different consumption of carbohydrate and fat between the diets (Table 1). The protein intake in E% (*P* < 0.0001), g/day (*P* = 0.006) and g/kg (0.008) was significantly higher in the L-CHO diet compared to the H-CHO diet (Table 1).

Participants characteristic

BMI, lean body mass (LBM), fat percentage, visceral fat and systolic and diastolic blood pressure remained unchanged during the experimental trial (Table 2). Body weight was reduced (*P* < 0.05) similarly from day 1 to day 2 (0.9 ± 0.7 kg and 1.1 ± 0.9 kg) in the H-CHO and L-CHO intervention, respectively (Table 2).

Muscular characteristics

Muscle glycogen content was reduced (*P* = 0.029) by 30.5% from baseline to H-CHO day 2 (from 566 ± 155 to 393 ± 115 nmol/mg dry weight) and reduced (*P* < 0.001) by 69.3% from baseline to L-CHO day 2 (from 566 ± 155 to 174 ± 27 nmol/mg dry weight). Muscle glycogen content was 55.9% lower (*P* = 0.0006) on L-CHO day 2 compared to H-CHO day 2 (Fig. 2A). IMTG content and

CS and HAD activity were not influenced by the interventions (Table 3B).

Fat oxidation

PFO increased by 24.3% (*P* = 0.021) from day 1 to day 2 in the H-CHO intervention (from 0.37 ± 0.09 to 0.46 ± 0.09 g/min) and by 89.7% (*P* < 0.0001) in the L-CHO intervention (from 0.39 ± 0.07 to 0.74 ± 0.11 g/min). PFO was 60.9% higher (*P* < 0.0001) on L-CHO day 2 compared to H-CHO day 2 (Fig. 2B). A repeated-measures correlation revealed a strong negative association between muscle glycogen content and PFO (repeated-measures correlation coefficient (*r_{rm}*) = −0.81, 95% confidence interval (CI) = −0.92 to −0.59, degrees of freedom (df) = 19, *P* < 0.001) (Fig. 2C). Fat_{max} increased (*P* = 0.035) by 36.6% from day 1 to day 2 in the L-CHO intervention (from 41 ± 5% of $\dot{V}_{O_{2peak}}$ to 56 ± 7% of $\dot{V}_{O_{2peak}}$) but remained unchanged (*P* = 0.343) in the H-CHO intervention (Table 2).

Exercise capacity ($\dot{V}_{O_{2peak}}$, RER, RPE and *W_{max}*)

A $\dot{V}_{O_2}$ plateau was observed in 22 of 40 $\dot{V}_{O_{2peak}}$ tests. In the remaining 18 tests, at least two of the three secondary criteria were met.

$\dot{V}_{O_{2peak}}$ remained unchanged during the H-CHO (*P* > 0.999) and L-CHO (*P* = 0.645) intervention (Table 2). RER decreased at the last step of the $\dot{V}_{O_{2peak}}$ test from day 1 to day 2 by 3.3% (*P* = 0.011) in the H-CHO intervention (from 1.21 ± 0.07 to 1.17 ± 0.04) and by 16.4% (*P* < 0.0001) in the L-CHO intervention (from 1.22 ± 0.04 to 1.02 ± 0.05). Additionally, a markedly lower (*P* < 0.0001) RER was observed on L-CHO day 2 compared to H-CHO day 2 (Table 2). From the Fat_{oxidation} test the RPE was rated 14.3% (*P* < 0.05) and 21.3%

Table 2. Characteristics of the participants throughout the experimental trial

Participant characteristics <i>n</i>	H-CHO day 1 10	H-CHO day 2 10	L-CHO day 1 10	L-CHO day 2 10
Anthropometrics				
Age (years)	26.1 ± 3.0	-	-	-
Body weight (kg)	79.6 ± 9.2	78.7 ± 9.2 ^a	79.5 ± 9.5	78.4 ± 9.2 ^a
BMI (kg/m ²)	23.3 ± 1.6	23.0 ± 1.7	23.3 ± 1.7	22.9 ± 1.6
Lean body mass (kg)	68.5 ± 5.3	-	67.7 ± 6.2	-
Fat percentage (%)	14.7 ± 3.5	-	14.6 ± 3.0	-
Visceral fat (g)	275 ± 112	-	223 ± 131	-
Systolic BP (mmHg)	123 ± 7	-	122 ± 8	-
Diastolic BP (mmHg)	70 ± 6	-	69 ± 5	-
Performance				
$\dot{V}_{O_{2peak}}$ (mL O ₂ /min)	4718 ± 352	4710 ± 273	4685 ± 455	4586 ± 384
Fitness level (mL O ₂ /min/kg)	59.8 ± 5.7	60.4 ± 5.7	59.3 ± 5.9	58.8 ± 4.4
Fat _{max} (% of $\dot{V}_{O_{2peak}}$)	41 ± 5	44 ± 4	41 ± 5	56 ± 7 ^{ab}
W_{max}	375 ± 35	375 ± 27	375 ± 29	350 ± 24 ^a
Maximum HR (bpm)	188 ± 7	186 ± 8	185 ± 10	181 ± 11 ^b
RPE (Fat oxidation test)	12.6 ± 2.3	14.4 ± 0.8 ^a	13.6 ± 0.8	16.5 ± 1.1 ^{ab}
RPE ($\dot{V}_{O_{2peak}}$ test)	19.2 ± 0.8	19.6 ± 0.7 ^a	19.2 ± 0.8	19.2 ± 0.6 ^b
RER ($\dot{V}_{O_{2peak}}$ test)	1.21 ± 0.07	1.17 ± 0.04 ^a	1.22 ± 0.04	1.02 ± 0.05 ^{ab}

Note: Data are presented for 10 participants at each test day. Values are presented as means ± SD.

Abbreviations: BMI, body mass index; BP, blood pressure; $\dot{V}_{O_{2peak}}$, peak oxygen consumption; Fat_{max}, intensity (% of $\dot{V}_{O_{2peak}}$) that elicits PFO; W_{max} , maximum watt; bpm, beats per minute; RPE, rate of perceived exertion; RER, respiratory exchange ratio.

^a $P < 0.05$ within the same intervention.

^b $P < 0.05$ between L-CHO day 2 and H-CHO day 2.

($P < 0.05$) higher from day 1 to day 2 in the H-CHO (from 12.6 ± 2.3 to 14.4 ± 0.8) and L-CHO intervention (from 13.6 ± 0.8 to 16.5 ± 1.1), respectively. Additionally, RPE from the Fat_{oxidation} test was rated 14.6% higher on L-CHO day 2 compared to H-CHO day 2. W_{max} decreased ($P = 0.008$) by 6.7% from day 1 to day 2 in the L-CHO intervention (from 375 ± 28 to 350 ± 24 W) but remained unchanged ($P = 0.614$) in the H-CHO intervention. A trend ($P = 0.063$) towards a lower W_{max} on L-CHO day 2 compared to H-CHO day 2 was observed (Table 2).

Concentration of blood parameters at rest

The plasma concentration of FFA increased ($P < 0.001$) from day 1 to day 2 with 114.7% in the H-CHO intervention (from 272 ± 87 to 584 ± 187 $\mu\text{mol/L}$) and with 141.6% in the L-CHO intervention (from 298 ± 139 to 720 ± 218 $\mu\text{mol/L}$) (Table 3). Moreover, a repeated-measures correlation revealed a positive association between plasma FFA and PFO ($r_{tm} = 0.79$, 95% CI = 0.61–0.89, $df = 29$, $P < 0.001$) (Fig. 2D). Plasma glucose concentrations decreased ($P \leq 0.0001$) by 5.8% from day 1 to day 2 in the L-CHO intervention (from 5.2 ± 0.3 to 4.9 ± 0.3 mmol/L) but remained unchanged

($P > 0.9999$) in the H-CHO intervention (Table 3). Plasma insulin was not influenced by the interventions, but a 36% lower ($P = 0.001$) plasma insulin was observed on L-CHO day 2 compared to H-CHO day 2 (Table 3). Plasma glycerol concentration increased ($P < 0.0001$) with 93.8% and 126.7% from day 1 to day 2 in the H-CHO (42.2 ± 11.8 to 81.8 ± 32.0 $\mu\text{mol/L}$) and L-CHO intervention (47.6 ± 16.1 to 107.9 ± 53.3 $\mu\text{mol/L}$), respectively. The plasma concentration of glycerol was higher ($P = 0.006$) on L-CHO day 2 compared to H-CHO day 2 (Table 3).

Haemoglobin and haematocrit decreased by 5.1% (from 15.8 ± 1.1 to 15.0 ± 1.2 g/dL, $P = 0.0007$) and 5.0% ($48.3 \pm 3.3\%$ to $45.9 \pm 3.4\%$, $P = 0.002$), respectively, from day 1 to day 2 in the L-CHO intervention, whereas no changes ($P = 0.118$ and $P = 0.082$, respectively) were observed in the H-CHO intervention (Table 3).

TAG decreased ($P = 0.002$) by 44.7% from day 1 to day 2 in the L-CHO intervention (from 0.94 ± 0.34 to 0.52 ± 0.10 mmol/L) and was 40.2% lower ($P = 0.010$) on day 2 in the L-CHO intervention compared to day 2 in the H-CHO intervention. TAG did not change during the H-CHO intervention (Table 3). Total cholesterol decreased ($P = 0.011$) by 8.2% from day 1 to day 2 in the L-CHO intervention (4.25 ± 0.57 to 3.90 ± 0.60 mmol/L)

Table 3. (A) Resting values of haemoglobin, haematocrit and HbA1c in blood, glucose, insulin, lactate, FFA, TAG, total cholesterol, LDL, HDL, glycerol, ketones, leptin and hsCRP in plasma and calculated insulin resistance index (HOMA-IR) at H-CHO and L-CHO day 1 and day 2. Data are presented for 10 participants at each test day. (B) Resting muscle values of IMTG and CS and HAD activity at baseline, H-CHO day 2 and L-CHO day 2.

(A) Blood characteristics	H-CHO day 1	H-CHO day 2	L-CHO day 1	L-CHO day 2
N	10	10	10	10
Haemoglobin (g/dL)	15.6 ± 0.8	15.3 ± 0.7	15.8 ± 1.1	15.0 ± 1.2 ^a
Haematocrit (%)	47.9 ± 2.7	46.8 ± 2.3	48.3 ± 3.3	45.9 ± 3.4 ^a
HbA1c (%)	5.5 ± 0.3	-	-	-
Glucose (mmol/L)	5.3 ± 0.3	5.3 ± 0.2	5.2 ± 0.3	4.9 ± 0.3 ^{ab}
Insulin (pmol/L)	43.7 ± 21.8	47.3 ± 18.6	37.9 ± 14.1	30.3 ± 10.0 ^b
HOMA-IR	1.5 ± 0.8	1.6 ± 0.6	1.3 ± 0.5	1.0 ± 0.4 ^{ab}
Lactate (mmol/L)	0.80 ± 0.25	0.83 ± 0.30	0.83 ± 0.28	0.79 ± 0.16
FFA (μmol/L)	272 ± 87	584 ± 187 ^a	298 ± 139	720 ± 218 ^{ab}
Triacylglycerol (mmol/L)	1.02 ± 0.42	0.87 ± 0.29	0.94 ± 0.34	0.52 ± 0.10 ^{ab}
Total cholesterol (mmol/L)	4.17 ± 0.72	3.98 ± 0.72	4.25 ± 0.57	3.90 ± 0.60 ^a
LDL (mmol/L)	2.52 ± 0.54	2.36 ± 0.59	2.58 ± 0.47	2.36 ± 0.49
HDL (mmol/L)	1.46 ± 0.46	1.47 ± 0.40	1.51 ± 0.36	1.53 ± 0.34
Glycerol (μmol/L)	42.2 ± 11.8	81.8 ± 32.0 ^a	47.6 ± 16.1	107.9 ± 53.3 ^{ab}
Ketones (mmol/L)	0.04 ± 0.01	0.09 ± 0.06	0.05 ± 0.02	0.37 ± 0.11 ^{ab}
Leptin (pg/mL)	1801 ± 1226	1315 ± 756	1761 ± 1052	1055 ± 373 ^a
hsCRP (mg/L)	0.60 ± 0.78	1.94 ± 1.14 ^a	0.45 ± 0.41	2.19 ± 1.35 ^a

(B) Muscle characteristics	Baseline	H-CHO day 2	L-CHO day 2
N	9	10	10
IMTG (nmol/mg)	58.1 ± 37.3	38.2 ± 14.4	76.9 ± 56.2

N	10	10	10
CS activity (μmol/g*min)	210.6 ± 48.0	227.8 ± 43.6	243.3 ± 43.7
HAD activity (μmol/g*min)	111.0 ± 32.0	121.9 ± 24.9	125.0 ± 32.5

Note: Data are presented for 10 participants at each test day besides IMTG at baseline where only data from 9 participants are presented. Values are presented as means ± SD.

Abbreviations: CS, citrate synthase; FFA, free fatty acid; HAD, 3-hydroxyacyl-CoA dehydrogenase; HbA1c, glycated haemoglobin; HDL, high-density lipoprotein; HOMA-IR, homeostatic model assessment of insulin resistance; hsCRP, high-sensitivity C-reactive protein; IMTG, intramuscular triacylglycerol; LDL, low-density lipoprotein; TAG, triacylglycerol.

^a $P < 0.05$ versus day 1 within the experimental intervention.

^b $P < 0.05$ L-CHO day 2 versus H-CHO day 2.

(Table 3). No changes were observed in LDL or HDL (Table 3). Plasma concentration of leptin decreased ($P = 0.017$) by 40.1% from day 1 to day 2 in the L-CHO intervention (from 1761 ± 1052 to 1055 ± 373 pg/mL), whereas no change ($P = 0.146$) was observed in the H-CHO intervention (Table 3).

HOMA-IR decreased ($P = 0.041$) by 23.1% in the L-CHO intervention (from 1.3 ± 0.5 to 1.0 ± 0.4), remained unchanged ($P = 0.883$) in the H-CHO intervention and was 37.5% lower ($P = 0.0007$) on day 2 in the L-CHO intervention compared to day 2 in the H-CHO intervention (Table 3). The plasma concentration of hsCRP increased from day 1 to day 2 by 223% ($P = 0.002$) in the H-CHO (from 0.60 ± 0.78 to 1.94 ± 1.14 mg/L)

and by 387% ($P < 0.0001$) in the L-CHO intervention (0.45 ± 0.41 to 2.19 ± 1.35 mg) (Table 3).

Mitochondrial coupling control and efficiency and respiratory capacity

The P/O ratio was unchanged from baseline to day 2 in the H-CHO (with carbohydrate substrates: $P = 0.449$; with fat substrates: $P = 0.952$) and L-CHO (with carbohydrate substrates: $P = 0.353$; with fat substrates: $P = 0.849$) interventions (Fig. 3A and B). No significant difference in the P/O ratio was observed between the H-CHO and L-CHO interventions with carbohydrate substrates (mean

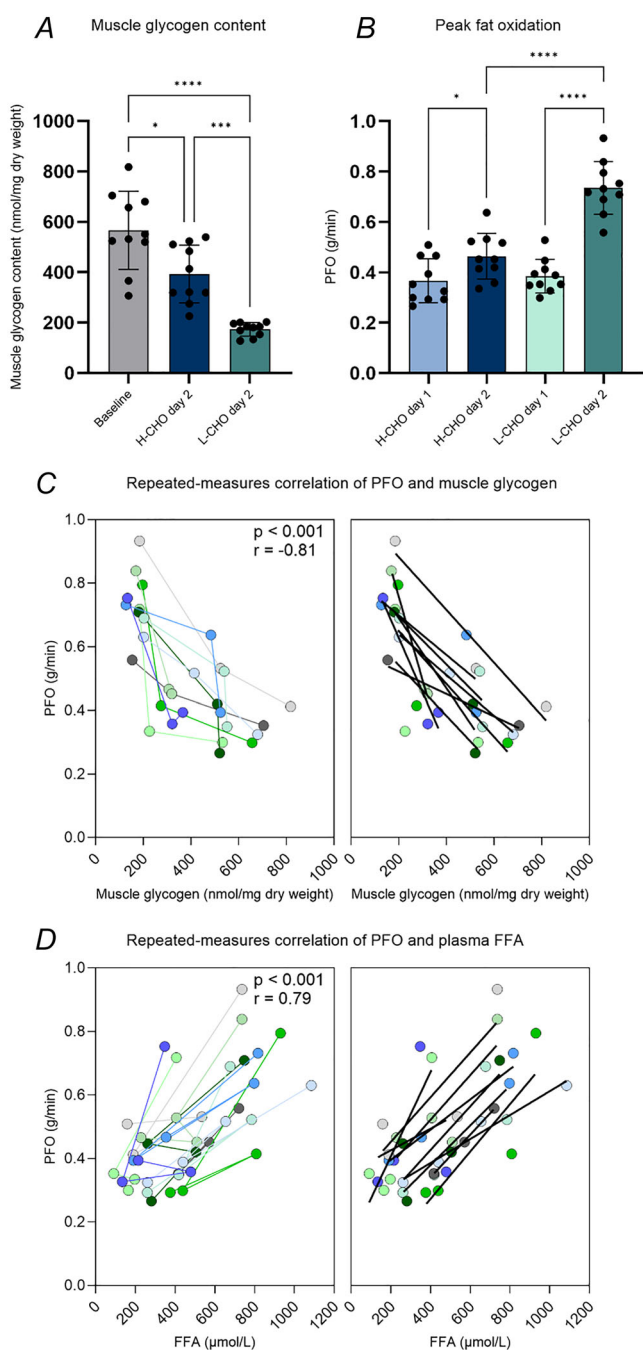


Figure 2. Association between peak fat oxidation and substrate availability of muscle glycogen and plasma free fatty acids

A, resting muscle glycogen content measured at baseline, H-CHO day 2 and L-CHO day 2. **B**, PFO measured during exercise on H-CHO day 1 and day 2 and L-CHO day 1 and day 2. **C**, repeated-measures correlation between muscle glycogen content and PFO. Muscle glycogen content is plotted against PFO across experimental conditions in all participants. In the left-hand-side graph, each point represents an individual measurement (one colour for each participant), and each line represents within-subject variation across conditions. In the right-hand-side graph, the black line indicates the overall linear association for each participant estimated using repeated measures. **D**, repeated-measures correlation between

plasma FFA and PFO. Plasma FFA concentration is plotted against PFO across experimental conditions in all participants. In the left-hand-side graph, each line represents within-subject variation across conditions, and each point represents an individual measurement (one colour for each participant). In the right-hand-side graph, the black lines indicate the overall linear association for each participant, estimated using repeated-measures correlation. Data are presented for 10 participants ($n = 10$). Values are presented as means $\pm$ SD. * $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$. PFO, peak fat oxidation; FFA, free fatty acid.

difference = 0.02 ATP per O_2 molecule, Cohen's $d_z = 0.04$, 95% CI = -0.30 to 0.34). No significant difference in the P/O ratio with fatty acid substrates was observed between H-CHO and L-CHO interventions (mean difference = 0.09 ATP per O_2 molecule, Cohen's $d_z = 0.33$, 95% CI = -0.10 to 0.28), indicating a small-to-moderate effect size with considerable uncertainty.

The RCR did not change from baseline to day 2 in the H-CHO (with carbohydrate substrates: $P = 0.709$; with fat substrates: $P = 0.896$) or L-CHO (with carbohydrate substrates: $P = 0.999$; with fat substrates: $P = 0.920$) interventions (Fig. 3C and D). Furthermore RCR did not differ between H-CHO and L-CHO under either carbohydrate- or fatty acid-supported respiration protocols (carbohydrate: mean difference = -0.54 , $d_z = -0.08$, 95% CI = -5.04 to 3.97 ; fat: mean difference = -0.53 , Cohen's $d_z = -0.08$, 95% CI = -4.78 to 3.71).

The mitochondrial respiratory capacity normalized to CS activity did not change significantly during the interventions (Table 4). However, a trend towards a higher respiratory capacity in complex I + II with fatty acid substrates was observed from baseline to day 2 in the L-CHO intervention ($P = 0.092$). No changes in respiratory capacity were observed between the interventions in the carbohydrate substrate protocol (Table 4). No meaningful differences in carbohydrate-supported mitochondrial respiratory capacity were observed between the H-CHO and L-CHO interventions (mean difference = 5.39 , Cohen's $d_z = 0.17$, 95% CI = -17.12 to 27.90). Similarly, fatty acid-supported mitochondrial respiratory capacity showed no significant difference between the H-CHO and L-CHO conditions (mean difference = 4.26 , Cohen's $d_z = 0.14$, 95% CI = -17.78 to 26.31).

Discussion

The key novel finding of this study was that muscle glycogen content influences PFO and Fat_{max} . As hypothesized, we found increases in both PFO and Fat_{max} in conditions with low compared to high muscle glycogen concentrations.

In the present study the participants went through a glycogen-depleting cycling exercise protocol followed

by a four-meal diet for 8–9 h, which, combined with the overnight fast, provides an overall effect on substrate availability and oxidation. The inability to achieve full muscle glycogen resynthesis by H-CHO day 2, despite a carbohydrate rich diet, was most likely a consequence of the short dietary intervention. Depending

on the initial depletion level, pre-exercise muscle glycogen content, the type and timing of carbohydrate intake and exercise within the preceding 24 h several reviews indicate that a minimum of 24 h of a carbohydrate-rich diet is necessary to reach pre-exercise muscle glycogen values (Burke et al., 2017; Murray & Rosenbloom, 2018).

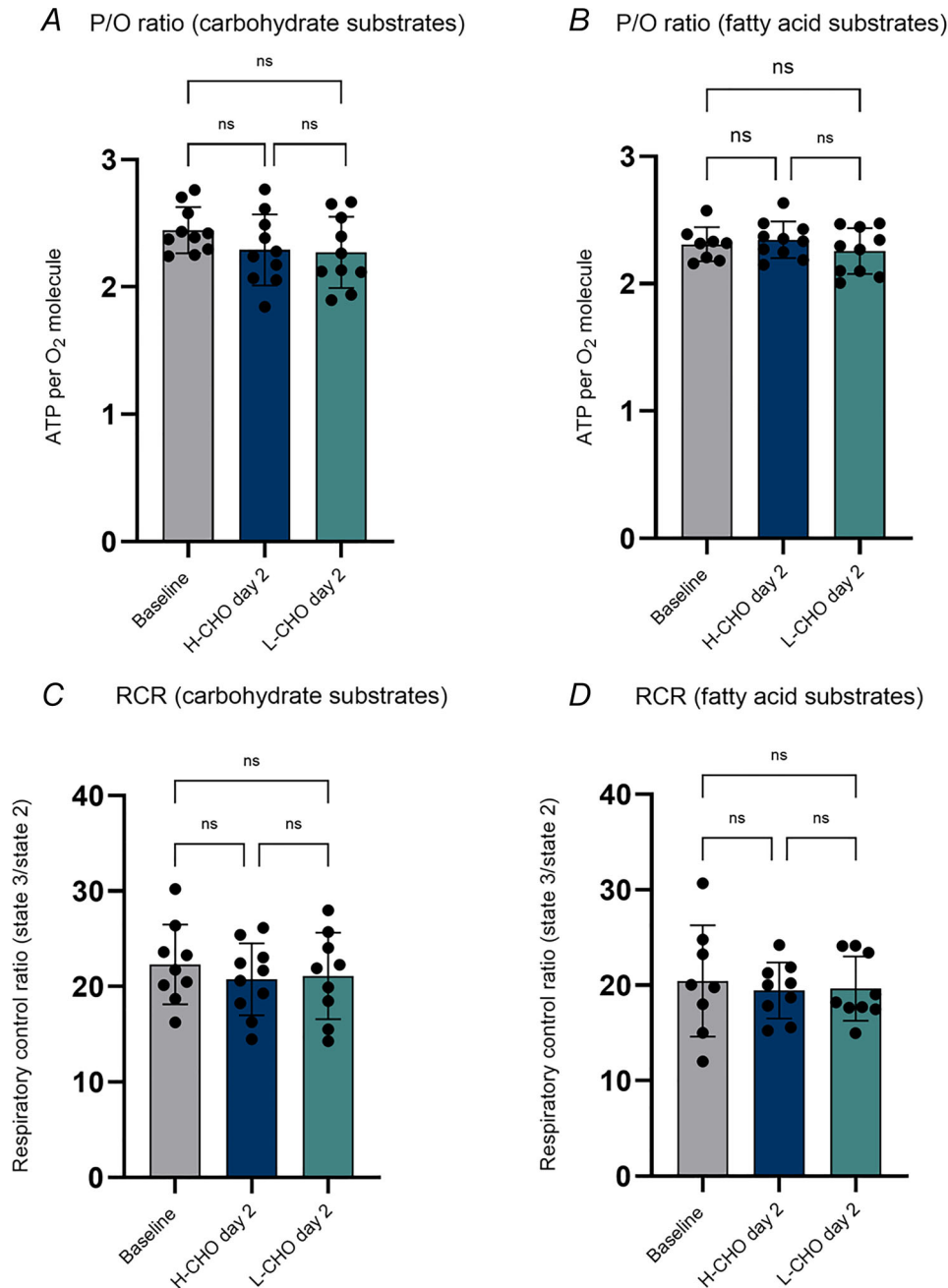


Figure 3. Mitochondrial coupling control and efficiency across experimental conditions

Effect of high or low muscle glycogen content on mitochondrial efficiency measured at baseline, H-CHO day 2 and L-CHO day 2 as (A) the P/O ratio with carbohydrate substrates, (B) the P/O ratio with fatty acid substrates, (C) RCR with carbohydrate substrates and (D) RCR with fatty acid substrates. Data are presented for 10 participants ($n = 10$). Values are presented as means $\pm$ SD. P/O, phosphate/oxygen; RCR, respiratory control ratio; ns, not significant.

Table 4. Mitochondrial respiratory capacity at baseline, H-CHO day 2 and L-CHO day 2

Respiratory capacity (carbohydrate substrates) N	Baseline 9	H-CHO day 2 10	L-CHO day 2 9
Malate + pyruvate (pmol/s*mL/CS activity (μmol/min))	1.7 ± 0.4	2.1 ± 0.7	2.1 ± 0.9
ADP _{saturated} (pmol/s*mL/CS activity (μmol/min))	22.8 ± 3.4	28.9 ± 9.5	29.0 ± 13.6
Complex I (pmol/s*mL/CS activity (μmol/min))	25.1 ± 3.4	31.0 ± 10.4	31.2 ± 14.3
Complex I + II (pmol/s*mL/CS activity (μmol/min))	36.9 ± 5.7	44.0 ± 15.0	42.9 ± 20.9
Respiratory capacity (fatty acid substrates) N	Baseline 8	H-CHO day 2 10	L-CHO day 2 9
Malate + PalmC (pmol/s*mL/CS activity (μmol/min))	1.8 ± 0.4	2.1 ± 0.7	2.2 ± 1.1
ADP _{saturated} (pmol/s*mL/CS activity (μmol/min))	6.5 ± 3.5	9.7 ± 7.6	7.3 ± 4.4
Complex I (pmol/s*mL/CS activity (μmol/min))	20.6 ± 4.2	26.7 ± 9.8	26.2 ± 11.3
Complex I + II (pmol/s*mL/CS activity (μmol/min))	34.2 ± 6.0	41.3 ± 13.7	41.1 ± 17.8 ^a

Note: Mitochondrial respiratory capacity was measured as oxygen flux per volume normalized to CS activity with carbohydrate and fatty acid substrates. Data are presented at baseline for 9 and 8 participants with carbohydrate and fatty acid substrates, respectively. Data are presented at H-CHO day 2 for 10 participants for both substrate protocols. Data are presented at L-CHO day 2 for 9 participants in both substrate protocols. Values are presented as means ± SD.

Abbreviations: CS, citrate synthase; PalmC, palmitoylcarnitine.

^a $P < 0.10$ versus baseline.

Consequently, the study design successfully induced substantial differences in muscle glycogen content between baseline, H-CHO day 2 and L-CHO day 2. This allowed for the investigation of mitochondrial coupling control and efficiency and PFO and Fat_{max} across varying glycogen levels, providing the foundation for exploring potential underlying mechanisms.

The effect of muscle glycogen on PFO

PFO increased when muscle glycogen content was low, supporting the study's first hypothesis. This was accompanied by elevated plasma FFA, known to promote fat oxidation (Frandsen et al., 2019; Helge et al., 2001). These results extend the findings of Frandsen et al. (2019), who demonstrated that PFO during graded exercise increased in a dose-dependent manner with plasma FFA, particularly in the fasted state compared to a carbohydrate-fed state. Although muscle glycogen content was not measured in that study, it was speculated to influence the observed differences in PFO and plasma FFA. Similarly, both Pilegaard et al. (2002) and Krstrup et al. (2006) showed that low muscle glycogen elevates plasma FFA and glycerol during exercise, further linking glycogen availability to substrate oxidation. However, because the current study only assessed resting plasma levels this interpretation remains speculative and requires further investigation.

An observed between-subject-within-time point variation in both muscle glycogen and plasma FFA concentration at a given PFO reflects that parameters

other than muscle glycogen and plasma FFA influence PFO on a single occasion. As documented by the resting values of plasma TAG, ketones, glucose and insulin during the interventions the overall substrate milieu also differs between the test days. Consequently, the observed changes in PFO between test days and interventions cannot be unequivocally attributed to differences in muscle glycogen content alone. However, we do not have blood values during exercise, and it is therefore difficult to predict their exact contribution to the resulting PFO.

Both short-term (Støa et al., 2016; Tarry et al., 2025) and long-term (Helge et al., 2001) modifications in dietary macronutrient composition are known to alter the availability of endogenous and exogenous substrates, which, in turn, influence substrate selection during exercise. By design, the difference in dietary carbohydrate, fat and protein composition between the experimental diets clearly affected endogenous and exogenous substrate availability and, thus, an interplay of several contributions, and not only glycogen, may have affected the observed changes in PFO. Furthermore, the between-subject variation may be influenced by differences in muscle fibre type composition, fibre recruitment patterns and subcellular glycogen utilization (Vigh-Larsen et al., 2022), which exhibit substantial heterogeneity even at the single-fibre level and may therefore differ between participants, potentially contributing to the absence of association observed on a single occasion. Overall, we acknowledge that muscle glycogen content alone cannot solely be the explanation for the changes in PFO.

However, when examining within-subject associations between PFO and muscle glycogen (Fig. 2C), as well as

between PFO and plasma FFA concentrations (Fig. 2D), a strong negative association with muscle glycogen and a strong positive association with plasma FFA are observed. This indicates that on an individual level, concentrations of muscle glycogen and plasma FFA, to some extent, do influence PFO.

Although muscle glycogen content and PFO appear closely linked, the underlying mechanism is not fully explained by the present data. The findings suggest that substrate availability – particularly plasma FFA and muscle glycogen – plays a role in regulating fat oxidation consisting with prior studies (Frandsen et al., 2019; Helge et al., 2001; Romijn et al., 1995). Interestingly, intracellular long-chain fatty acids (LCFA) have been demonstrated to rise with increasing intensity, even as fat oxidation declines in favour of carbohydrate use (Kiens et al., 1999), implying that substrate availability alone does not determine oxidation rates at very high intensities. Low muscle glycogen content may trigger molecular adaptations that shift metabolism towards a higher fat oxidation. For example, pyruvate dehydrogenase kinase 4 (PDK4) expression increases during exercise when muscle glycogen is low (Pilegaard et al., 2002), potentially inhibiting glucose oxidation. Similar increases in PDK activity were seen after just 1 day of low-carbohydrate diet (Peters et al., 2001). Although these adaptations remain speculative due to lack of protein expression data in the current study they highlight the potential role of glycogen content in modulating metabolic pathways during exercise.

The effect of muscle glycogen on Fat_{max}

In the present study Fat_{max} increased alongside elevated plasma FFA on L-CHO day 2, whereas no increase in Fat_{max} was observed on H-CHO day 2 despite elevated PFO and plasma FFA. Although the exact determinants of interindividual variability in Fat_{max} remain unclear, these findings align with Jeukendrup and Achten (2001), proposing that Fat_{max} occurs near the glycolytic threshold, possibly linking glucose metabolism to Fat_{max} regulation. Notably, plasma glucose decreased significantly on L-CHO day 2 but remained unaltered on H-CHO day 2. The reduction in plasma glucose availability may attenuate the glycolytic flux, thereby promoting lipid utilization at higher exercise intensities contributing to the observed increase in Fat_{max} .

The availability of free carnitine has been proposed as a potential mechanism behind a shift towards greater lipid oxidation at higher intensities (Stephens et al., 2007; van Loon et al., 2001). At higher glycolytic rates, excess acetyl-CoA accumulation can deplete free carnitine by forming acetylcarnitine, thereby limiting transport of LCFA into the mitochondria. In contrast,

the lower glycolytic flux associated with reduced plasma glucose may preserve free carnitine availability, enhancing mitochondrial uptake and oxidation of fatty acids. This may explain, at least in part, the augmented Fat_{max} observed on day 2 in the L-CHO intervention, contrasting with the absence of such an increase in the H-CHO intervention.

The influence of muscle glycogen on mitochondrial coupling control and efficiency

Contrary to our hypothesis, mitochondrial coupling control and efficiency remained unchanged during the intervention despite altered muscle glycogen and plasma FFA levels. This contrast with Fernström et al. (2007), who reported reduced mitochondrial efficiency immediately after 24 h of ultra-endurance exercise. Although muscle glycogen was not directly measured in their study, the prolonged exercise, a fourfold increase in plasma FFA and elevated mitochondrial fat oxidation strongly suggest substantial glycogen depletion and, thereby, maybe a link between substrate availability and mitochondrial efficiency. However, despite similar participant characteristics and experimental protocols differences in exercise duration, timing of biopsies and acute oxygen demand may explain the discrepancy. These findings suggest that postexercise recovery time may play a critical role in normalizing mitochondrial efficiency following glycogen-depleting exercise. Notably, Fernström et al. (2007) also reported a sustained reduction in mitochondrial efficiency 28 h postexercise despite likely partial glycogen resynthesis and normalize FFA levels. This suggests that neither glycogen content nor plasma FFA concentration alone directly modulates mitochondrial efficiency during recovery, aligning with the present study's findings.

Insulin has been shown to enhance mitochondrial coupling control and efficiency in human muscle cells, an effect lost with palmitate exposure (Nisr & Affourtit, 2014). This suggests higher mitochondrial efficiency in anabolic states and no changes in a catabolic state. In contrast, mitochondrial coupling control remained unchanged across test days in the present study despite elevated plasma FFA, reduced plasma glucose and insulin concentrations and low muscle glycogen on L-CHO day 2, indicating that substrate availability did not influence mitochondrial coupling control during these conditions. These conflicting findings could suggest a limited association between muscle glycogen availability and mitochondrial coupling control and efficiency. However, it could also reflect distinct methodological differences between the studies. Specifically, Nisr and Affourtit (2014) measured mitochondrial coupling control in intact myoblasts using a Seahorse extracellular metabolic flux

analyser. In contrast, the present study evaluated isolated mitochondria utilizing high-resolution respirometry with Oxygraphs. Ultimately, these variations in tissue preparation, cellular integrity and instrument sensitivity are highly likely to account for the discrepancies observed between the two studies.

The trivial standardized effect size and wide CI observed for the P/O ratio measured under carbohydrate-based substrates suggest that any effect of glycogen manipulation on mitochondrial P/O ratio is likely small. This indicates that carbohydrate availability may not meaningfully influence intrinsic mitochondrial efficiency under the present experimental conditions when assessed using a carbohydrate-substrate protocol. Interestingly although not statistically significant, the P/O ratio assessed under the fatty acid substrate protocol demonstrated a small-to-moderate standardized effect size ($d_z = 0.33$), suggesting a potential but uncertain improvement in mitochondrial efficiency following the H-CHO compared to the L-CHO intervention. However, the CIs included zero, indicating that the true effect may range from slightly negative to moderately positive.

The divergence between substrate conditions may reflect that substrate availability is more clearly manifested as alterations in fat-linked, rather than carbohydrate-linked, mitochondrial respiratory efficiency. This suggests that carbohydrate manipulation may primarily influence metabolic flexibility and substrate partitioning rather than intrinsic mitochondrial coupling efficiency.

No differences were observed in RCR between H-CHO and L-CHO day 2 in neither of the substrate protocols. Both conditions showed a small effect size and wide CIs, which could indicate high interindividual variability and limited precision of the estimates. This suggests that glycogen manipulation does not alter mitochondrial coupling control as assessed by RCR under the present experimental conditions. Across both carbohydrate-supported and fatty acid-supported protocols assessing mitochondrial respiratory capacity effect sizes were small to trivial, and the CIs consistently included zero. This indicates substantial uncertainty in the estimates and provides no clear evidence of a directional effect of the interventions on mitochondrial respiratory capacity. Interestingly, we observed a trend towards higher complex I + II respiration with fatty acid substrates in the L-CHO intervention where the glycogen concentration was significantly lowered compared to baseline. It is likely that this is a consequence of the depletion protocol stimulating a trend towards a higher fatty acid oxidation capacity. However, we did not observe a difference in respiratory capacity between the different glycogen conditions with carbohydrate substrates.

Methodological considerations

The randomized crossover design employed in this study is a notable strength, by enabling within-subject comparisons and minimizing interindividual variability, thereby increasing the internal validity of the findings. The protocol allowed the evaluation of three distinct muscle glycogen levels and their influence on key metabolic outcomes.

Limitations of the study include reliance on self-reported diet and activity, introducing potential variability in predepletion glycogen levels. Given the sensitivity of muscle glycogen content to recent carbohydrate intake this may have influenced the degree of glycogen depletion achieved. Potential fatigue effects on day 2 of each intervention may confound interpretation of changes from baseline. Modestly elevated hsCRP levels observed on day 2 may suggest an exercise-induced inflammatory response. However, corroborating evidence, including additional physiological markers of fatigue, is required to support this interpretation. Lastly, the use of only one baseline biopsy, rather than obtaining separate pre-exercise muscle biopsies in both interventions, is a limitation that could have influenced the results by failing to account for potential temporal changes in muscle characteristics or residual effects between experimental conditions. However, the 7–14-day washout period was considered sufficient to allow participants to return to their physiological baseline before commencing the second intervention. This assumption is supported by the absence of significant differences in the baseline measurements obtained on day 1 of each intervention.

It remains unclear whether energy availability *per se* directly influences mitochondrial processes. Although the present study suggests that mitochondrial coupling control and efficiency are not influenced by energy availability, several limitations preclude a definitive conclusion. Uncertainty remains as to whether the design, for example the exercise-induced glycogen depletion, employed in this study, was adequate to detect effects of substrate availability on mitochondrial coupling control and efficiency. Previous studies report conflicting effects of acute exercise on proteins involved in mitochondrial regulation. Although Pilegaard et al. (2000) observed increased transcriptional activity of uncoupler protein 3 (UCP3) after acute exercise, Fernström and colleagues (2004) reported no such effect. Accordingly, the acute exercise performed by participants on test day 1 may have influenced mitochondrial uncoupling and masked potential changes in mitochondrial efficiency on test day 2.

From a speculative evolutionary perspective, mitochondrial coupling control and efficiency may

have been shaped by size, distribution and variability of endogenous substrate stores, favouring energy conservation during scarcity and increased uncoupling during energy abundance in favour of thermogenesis and limitation of oxidative stress. Whether such evolutionary demands translate into detectable, short-term changes in mitochondrial efficiency remains uncertain, and the present model may not be sufficiently sensitive to capture these potentially subtle adaptations.

The lack of *a priori* power calculation, combined with the relatively small sample size ($n = 8$ – 10) and the substantial interindividual variability observed in mitochondrial respiration and CS activity, likely resulted in limited statistical power. Consequently, the study may have been underpowered to detect small-to-moderate intervention-induced changes and increase the risk of type II error despite the crossover design.

Conclusions

In summary, this study found that exercise-induced muscle glycogen depletion and a low-carbohydrate diet influence PFO and Fat_{max} but do not affect mitochondrial coupling control and efficiency. The experimental design effectively manipulated muscle glycogen levels through exercise and dietary interventions, thereby permitting the investigation of the role of this manipulation on PFO, Fat_{max} and mitochondrial coupling control and efficiency. Building on our findings, the changes in PFO associated with divergent muscle glycogen content were likely driven by differences in plasma FFA availability. Notably, these alterations in PFO may also stem from underlying molecular adaptations – an area that warrants further investigation. Although mitochondrial coupling control and efficiency appeared unaffected by muscle glycogen content, the findings emphasize that the observed shifts in substrate oxidation are likely orchestrated at the level of substrate availability and upstream regulatory processes that govern mitochondrial fuel supply.

Perspectives

Future research should delve deeper into the molecular underpinnings of the glycogen-dependent adaptations in PFO and Fat_{max} . Examining the protein expression of key enzymes involved in oxidative pathways within skeletal muscle will be crucial to fully unravel the mechanism by which muscle glycogen depots exert such a significant influence as seen in this study. Additionally, future research should replicate this study design across diverse populations and experimental conditions. Specifically, investigating the impact of altered energy status – such as inducing an energy-deficient state in participants

– would further broaden our understanding of these metabolic mechanisms. Considering the broader health implications, investigating these effects in untrained individuals with and without metabolic challenges presents another interesting aspect for future research, as it allows for the evaluation of these mechanisms without the confounding effects of exercise-induced adaptations.

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Additional information

Data availability statement

Data are available from authors upon reasonable request.

Competing interests

The authors declare no conflicts of interest.

Author contributions

T.R., S.L. and J.W.H. designed the study. K.K.L., L.B., M.H., S.T.H., N.S., P.P., B.D., P.F.A., S.L. and J.W.H. contributed to data acquisition. K.K.L. did the statistical analysis. K.K.L., S.L. and J.W.H. contributed to the interpretation of data. K.K.L. wrote the first manuscript draft. All authors revised and approved the final version of the manuscript. All authors agreed to be accountable for all aspects of the work ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

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Supporting information

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