**Read­\_me\_file for: *Dataset for "Lipid metabolism links nutrient-exercise timing to insulin sensitivity in overweight men"*** *This document provides additional information regarding values in the excel data file relating to the two studies. References are also made to the methods in the manuscript.*

**STUDY OVERVIEWS**

**Trial Registrations:** Both studies were registered at <https://clinicaltrials.gov>

(NCT02397304 for the **Acute Study** and NCT02744183 for the **Training Study**).

As detailed in the manuscript, this study consisted of two separate experiments. In the **Acute Study (Tab 1 in the excel data file)**, 12 sedentary, overweight or obese men were recruited from the Birmingham region of the UK. Participants performed two trials in a randomized crossover design, comprising 60 min of cycling at 65 % V̇O2peak, followed by a 3 h recovery period. In one trial, participants consumed breakfast (25 % estimated daily energy requirements; 65 % carbohydrate, 20 % fat and 15 % protein) 90 min before exercise. In the other trial (separated by 7-14 d), they performed the exercise after an overnight fast (breakfast eaten post-exercise). Substrate utilization was assessed during exercise and venous blood was sampled pre-, during- and post-exercise. In a subset of participants (*n*=8) *vastus lateralis* muscle was sampled pre- and post-exercise to assess intramuscular triglyceride and glycogen utilization. A third muscle sample (3 h post-exercise) assessed intramuscular gene expression responses (*n*=7).

In the **Training Study** **(Tab 2 in the excel data file)** 30, overweight or obese, sedentary men (non-exercisers) were recruited from the Bath region of the UK. This was a single-blind, randomized, controlled trial, with participants allocated to a no-exercise control group (CON; *n*=9) a breakfast before exercise group (BR-EX; *n*=12) or an exercise before breakfast group (EX-BR; *n*=9) for 6-weeks. Exercise training was supervised moderate-intensity cycling (50 % peak power output [PPO] in weeks 1-3 and 55 % PPO for weeks 4-6) and progressed from 30 min (week 1) to 40 min (week 2) to 50-min (weeks 3-6) three times per week. For all participants, breakfast was provided (1.3 g carbohydrate per kg), but consumed 2 h before exercise in BR-EX, 2 h after exercise in EX-BR, or at rest in CON. Pre- and post-intervention, an oral glucose tolerance test, a *vastus lateralis* muscle sample (fasting & rested) and an exercise test were undertaken. Substrate utilization rates were assessed during every exercise training session.

**MEASURES IN BOTH STUDIES (EXCEL FILE TABS 1 & 2)**

HEIGHT

Height was measured as the distance from the floor (feet against the wall, barefoot, ankles together) to the top of the head (against the wall, looking ahead) after maximal inspiration and straight legs (nearest 0.1 cm) using a stadiometer (Holtain Ltd., UK).

BODY MASS

The weight of each participant was measured to the nearest 0.1 kg using electronic scales (TANITA Inner Scan Body Composition Monitor-BC453, Tokyo) whilst they wore minimal clothing (lightweight shorts and t-shirt only).

BODY MASS INDEX

Body mass index was body mass (kilograms) divided by height (m) squared.

WAIST AND HIP CIRCUMFERENCES

Waist & hip circumferences were measured to the nearest 0.1 cm. Waist circumference was taken at the midpoint between the lower margin of the last palpable rib and the top of the iliac crest and hip circumference was measured at the widest part of the buttocks.

INCREMENTAL EXERCISE TEST

In both studies, participants completed incremental exercise tests on an electronically-braked ergometer. In the **Acute Study**, the starting intensity was 35 W and increased by 35 W every 3 min until volitional exhaustion. In the **Training Study**, the starting intensity was 50 W which was increased by 25 W every 3 min. Continuous breath-by-breath measurements were recorded (**Acute Study**: Oxycon Pro, Jeager, Wurzburg, Germany; **Training Study**: TrueOne2400, ParvoMedics, Sandy, USA). Volume and gas analyzers were calibrated using a 3-L calibration syringe and a known calibration gas (16.04% O2, 5.06% CO2; BOC Industrial Gases, Linde AG, Germany).

PEAK POWER OUTPUT (PPO)

Peak power output was the work rate of the last completed stage, plus the fraction of time in the final non-completed stage, multiplied by the work rate increment.

VO2 PEAK

Peak oxygen uptake (VO2 peak) was taken as the highest average recorded oxygen uptake value over a rolling 30 second period.

**ADDITIONAL MEASURES FOR ACUTE STUDY (EXCEL FILE: TAB 1)**

PLASMA GLUCOSE CONCENTRATIONS

At each time point (as specified in the manuscript and excel data file) 10 mL blood was sampled from an antecubital forearm vein and 6 mL of the sample was dispensed into ethylenediaminetetraacetic acid-coated tubes and centrifuged (4°C at 3500 rpm) for 15 min. Resultant plasma was dispensed into 0.5 mL aliquots and frozen at -20°C, before storage at -80°C. Plasma glucose concentrations were measured on an ILAB 650 Clinical Chemistry Analyzer (Instrumentation Laboratory, Warrington, UK).

SERUM INSULIN CONCENTRATIONS

At each time point (as specified in the manuscript and excel data file) 10 mL blood was sampled from an antecubital forearm vein and 4 mL was allowed to clot in a plain vacutainer for 10 min. The tube was then centrifuged (4°C at 3500 rpm) for 15 min. Resultant serum was dispensed into 0.5 mL aliquots and frozen at -20°C, before storage at -80°C. Serum insulin concentrations were measured using an ELISA kit (Invitrogen; Cat#KAQ1251) and Biotek ELx800 analyzer (Biotek Instruments, Vermont, USA).

PLASMA NON-ESTERIFIED FATTY ACID (NEFA) CONCENTRATIONS

At each time point (as specified in the manuscript and excel data file) 10 mL blood was sampled from an antecubital forearm vein and 6 mL of the sample was dispensed into ethylenediaminetetraacetic acid-coated tubes and centrifuged (4°C at 3500 rpm) for 15 min. Resultant plasma was dispensed into 0.5 mL aliquots and frozen at -20°C, before storage at -80°C. Plasma NEFA concentrations were measured on an ILAB 650 Clinical Chemistry Analyzer (Instrumentation Laboratory, Warrington, UK).

PLASMA GLYCEROL CONCENTRATIONS

At each time point (as specified in the manuscript and excel data file) 10 mL blood was sampled from an antecubital forearm vein and 6 mL of the sample was dispensed into ethylenediaminetetraacetic acid-coated tubes and centrifuged (4°C at 3500 rpm) for 15 min. Resultant plasma was dispensed into 0.5 mL aliquots and frozen at -20°C, before storage at -80°C. Plasma glycerol concentrations were measured on an ILAB 650 Clinical Chemistry Analyzer (Instrumentation Laboratory, Warrington, UK).

WHOLE-BODY CARBOHYDRATE AND FAT OXIDATION

Rates of whole-body carbohydrate and fat oxidation were estimated from V̇O2 and V̇CO2 values (assessed using indirect calorimetry from expired air samples which were collected at 25-30 and 55-60 min of exercise) and via stoichiometric equations using research of Frayn <https://www.physiology.org/doi/abs/10.1152/jappl.1983.55.2.628>.

EXERCISE-INDUCED CHANGE IN LIPID DROPLET/FIBRE AREA (%)

*Vastus lateralis* samples were collected under local anaesthesia (~ 5 mL 1 % lidocaine, Hameln Pharmaceuticals Ltd., Brockworth, UK), from a 3-6-mm incision at the anterior aspect of the thigh with a 5-mm Bergstrom biopsy needle technique adapted for suction. To enable the analysis of the intramuscular (IMTG) lipid content ~ 15-20 mg of each sample was embedded in Tissue-Tek OCT (Sigma Aldrich, Dorset, UK) on cork disc and frozen in cooled isopentane, before being transferred into an aluminium cryotube and stored at -80°C (*the* *methods used to measure the IMTG content of the muscle samples is detailed within the manuscript*). Quantification of the lipid droplets was performed using Image J software and the intramuscular lipid content of each sample was calculated as the percent area of bodipy staining of the total fibre area ([bodipy stained area [um2] / area of muscle [um2]\*100). The pre- and post-exercise samples were used to calculate the exercise-induced change in the lipid droplet of the samples.

EXERCISE-INDUCED CHANGE IN MUSCLE GLYCOGEN CONCENTRATION

*Vastus lateralis* samples were collected under local anaesthesia (~5 mL 1 % lidocaine, Hameln Pharmaceuticals Ltd., Brockworth, UK), from a 3-6-mm incision at the anterior aspect of the thigh with a 5-mm Bergstrom biopsy needle technique adapted for suction. Briefly, 10-15 mg of frozen tissue was powdered and transferred into a glass tube pre-cooled on dry ice. Thereafter, the samples were hydrolyzed by adding a 500 µl of 2M HCL and then incubated for 2 h at 95 ºC. After cooling to room temperature, 500µl 2M NaOH was added. Samples were centrifuged and the supernatant was analyzed for glucose concentrations via an ILAB 650 Clinical Chemistry Analyzer (Instrumentation Laboratory, Warrington, UK). The pre- and post-exercise muscle samples were used to calculate the exercise-induced change in the muscle glycogen concentration of samples.

EXERCISE-INDUCED CHANGE IN MRNA GENE EXPRESSION (fold-change).

*Vastus lateralis* samples were collected under local anaesthesia (~5 mL 1 % lidocaine, Hameln Pharmaceuticals Ltd., Brockworth, UK), from a 3-6-mm incision at the anterior aspect of the thigh with a 5-mm Bergstrom biopsy needle technique adapted for suction. The mRNA expression of 34 genes was analyzed using a custom RT2 Profiler PCR Array (*the* *methods used to measure the IMTG content of the muscle samples is detailed within the manuscript*). First, RNA was extracted from 20-40 mg of powdered muscle tissue using Tri reagent. After addition of chloroform, tubes were mixed, incubated and centrifuged for 10 min at 4 oC at 12 000 g. The RNA phase was mixed with an equal volume of 70 % ethanol and RNA was purified. The LVis function of the FLUOstar Omega microplate reader was used to measure RNA to ensure all samples for each participant had the same amount of RNA, reverse transcribed to cDNA using a RT2 First Strand kit. Quantitative RT-PCR analysis was performed using custom designed 384-well RT2 PCR Profiler Arrays and RT2 SYBR Green Mastermix on a CFX384 Real-Time PCR Detection. All primers are commercially available. Relative mRNA expression was determined via the 2-∆∆CT method. The fold-change in mRNA expression was calculated using a 3 h post-exercise *versus* a baseline muscle sample.

**ADDITIONAL MEASURES FOR TRAINING STUDY (EXCEL FILE: TAB 2)**

PEAK FAT UTILISATION

Peak fat utilisation rates were calculated from the incremental exercise test (as detailed previously) Whole-body oxygen uptake (V̇O2) and carbon dioxide production (V̇CO2) were averaged over the last min of each stage. Peak fat utilisation rates were calculated from those averaged values via stoichiometric equations using the research of Frayn <https://www.physiology.org/doi/abs/10.1152/jappl.1983.55.2.628>.

LIPID AND CARBOHYDRATE UTILISATION (EXERCISE SESSIONS)

Participants in the BR-EX and EX-BR groups performed cycling exercise on an ergometer three times weekly for 6-weeks. All training sessions were completed at the University of Bath and were supervised. During exercise, 1-min expired air samples were collected every 10 min. Expired air samples were collected in 200 L Douglas bags with a mouthpiece connected to a two-way, T shaped non-rebreathing valve via falconia tubing and measurements of ambient O2 and CO2 concentrations were recorded. Expired O2 and CO2 concentrations were measured in a known volume of each sample for two min using paramagnetic and infrared transducers. The volume and temperature of expired air samples were measured on a dry gas meter and a digital thermistor. The Haldane transformation was applied to calculate inspired gas volumes and to determine V̇O2 and V̇CO2. Lipid and carbohydrate utilisation rates were then calculated using research by Frayn <https://www.physiology.org/doi/abs/10.1152/jappl.1983.55.2.628>.

ENERGY EXPENDITURE (EXERCISE SESSIONS)

Energy expenditure was calculated assuming that lipids, glucose and glycogen yield 9.75 kcal·g-1, 3.74 kcal·g-1 and 4.15 kcal·g-1 of energy, respectively. It was assumed that there was an equal contribution of glucose and glycogen to carbohydrate utilisation at 40-50 % V̇O2 peak, but with a 20 % and an 80 % contribution at > 50 % V̇O2 peak.

FASTING PLASMA TRIGLYCERIDE CONCENTRATIONS

Prior to any blood sampling participants placed their dominant hand into a heated-air box set to 55°C. After 15 min of rest a catheter was placed (retrograde) into a dorsal hand vein and 10-mL of arterialized blood was drawn for a baseline sample. Plasma was obtained by dispensing blood into ethylenediaminetetraacetic acid-coated tubes (BD Oxford, UK) which were centrifuged for 10 min at 4 °C and 3500 *g* (Heraeus Biofuge Primo R, Kendro Laboratory Products Plc., UK). Plasma was dispensed into 0.5 mL aliquots and frozen at -20°C, before longer-term storage at -80°C. Plasma triglyceride concentrations were measured using an automated analyser (Daytona, Randox). Plasma glycerol concentrations were measured on the analyser and subtracted from the measured triglyceride concentration to provide the final (glycerol-corrected) triglyceride concentration, as reported. *The corrected triglyceride concentrations are reported in the excel data file as well as the measured glycerol concentrations.*

FASTING PLASMA HDL CONCENTRATIONS

Prior to any blood sampling participants placed their dominant hand into a heated-air box set to 55°C. After 15 min of rest a catheter was placed (retrograde) into a dorsal hand vein and 10-mL of arterialized blood was drawn for a baseline sample. Plasma was obtained by dispensing blood into ethylenediaminetetraacetic acid-coated tubes (BD Oxford, UK) which were centrifuged for 10 min at 4 °C and 3500 *g* (Heraeus Biofuge Primo R, Kendro Laboratory Products Plc., UK). Plasma was dispensed into 0.5 mL aliquots and frozen at -20°C, before longer-term storage at -80°C. Plasma HDL concentrations were measured using an automated analyser (Daytona, Randox).

FASTING PLASMA LDL CONCENTRATIONS

Prior to any blood sampling participants placed their dominant hand into a heated-air box set to 55°C. After 15 min of rest a catheter was placed (retrograde) into a dorsal hand vein and 10-mL of arterialized blood was drawn for a baseline sample. Plasma was obtained by dispensing blood into ethylenediaminetetraacetic acid-coated tubes (BD Oxford, UK) which were centrifuged for 10 min at 4 °C and 3500 *g* (Heraeus Biofuge Primo R, Kendro Laboratory Products Plc., UK). Plasma was dispensed into 0.5 mL aliquots and frozen at -20°C, before longer-term storage at -80°C. Plasma LDL concentrations were measured using an automated analyser (Daytona, Randox).

FASTING AND POSTPRANDIAL PLASMA GLUCOSE CONCENTRATIONS

Prior to any blood sampling participants placed their dominant hand into a heated-air box set to 55°C. After 15 min of rest a catheter was placed (retrograde) into a dorsal hand vein and 10-mL of arterialized blood was drawn for a baseline sample. Plasma was obtained by dispensing blood into ethylenediaminetetraacetic acid-coated tubes (BD Oxford, UK) which were centrifuged for 10 min at 4 °C and 3500 *g* (Heraeus Biofuge Primo R, Kendro Laboratory Products Plc., UK). Plasma was dispensed into 0.5 mL aliquots and frozen at -20°C, before storage at -80°C. After obtaining a baseline sample, participants ingested 75 grams of glucose (with 300 mL of water) in an oral glucose tolerance test (OGTT). Plasma glucose concentrations were measured using an automated analyser (Daytona, Randox).

FASTING AND POSTPRANDIAL PLASMA INSULIN CONCENTRATIONS

Prior to any blood sampling participants placed their dominant hand into a heated-air box set to 55°C. After 15 min of rest a catheter was placed (retrograde) into a dorsal hand vein and 10-mL of arterialized blood was drawn for a baseline sample. Plasma was obtained by dispensing blood into ethylenediaminetetraacetic acid-coated tubes (BD Oxford, UK) which were centrifuged for 10 min at 4 °C and 3500 *g* (Heraeus Biofuge Primo R, Kendro Laboratory Products Plc., UK). Plasma was dispensed into 0.5 mL aliquots and frozen at -20°C, before storage at -80°C. After obtaining a baseline sample, participants ingested 75 grams of glucose (with 300 mL of water) in an oral glucose tolerance test (OGTT). Plasma insulin concentrations were measured using a commercially available ELISA (Mercodia AB; Cat#10-1113-01)

TIME-AVERAGED AREA UNDER THE CURVE

The time-averaged area underneath the concentration-time curve (AUC) for the plasma glucose and insulin responses were calculated via the trapezoid rule. This was divided by the time over which samples were collected to provide a time-averaged AUC.

ORAL GLUCOSE INSULIN SENSITIVITY INDEX

Plasma glucose and insulin concentrations were used to calculate oral glucose insulin sensitivity (OGIS) as per the instructions provided at; <http://webmet.pd.cnr.it/ogis/>

FASTING AND POSTPRANDIAL PLASMA NEFA CONCENTRATIONS

Prior to any blood sampling participants placed their dominant hand into a heated-air box set to 55°C. After 15 min of rest a catheter was placed (retrograde) into a dorsal hand vein and 10-mL of arterialized blood was drawn for a baseline sample. Plasma was obtained by dispensing blood into ethylenediaminetetraacetic acid-coated tubes (BD Oxford, UK) which were centrifuged for 10 min at 4 °C and 3500 *g* (Heraeus Biofuge Primo R, Kendro Laboratory Products Plc., UK). Plasma was dispensed into 0.5 mL aliquots and frozen at -20°C, before storage at -80°C. After obtaining a baseline sample, participants ingested 75 grams of glucose (with 300 mL of water) in an oral glucose tolerance test (OGTT). Plasma NEFA concentrations were measured using an enzymatic colorimetric assay (WAKO Diagnostics; Cat#999-34691 & Cat#991-34891)

FASTING AND POSTPRANDIAL PLASMA C-PEPTIDE CONCENTRATIONS

Prior to any blood sampling participants placed their dominant hand into a heated-air box set to 55°C. After 15 min of rest a catheter was placed (retrograde) into a dorsal hand vein and 10-mL of arterialized blood was drawn for a baseline sample. Plasma was obtained by dispensing blood into ethylenediaminetetraacetic acid-coated tubes (BD Oxford, UK) which were centrifuged for 10 min at 4 °C and 3500 *g* (Heraeus Biofuge Primo R, Kendro Laboratory Products Plc., UK). Plasma was dispensed into 0.5 mL aliquots and frozen at -20°C, before storage at -80°C. After obtaining a baseline sample, participants ingested 75 grams of glucose (with 300 mL of water) in an oral glucose tolerance test (OGTT) Plasma C-peptide concentrations were measured using a commercially available ELISA (Sigma Aldrich; Cat#EZHCP-20K).

SELF-REPORTED ENERGY INTAKE

Participants were also asked to keep a written record of their food and fluid intake for 4 days over a typical 7-day period pre- and during the last week of the intervention. Weighing scales were provided to increase the accuracy of records. Records were analyzed using Nutritics software (Nutritics Ltd., Dublin, Ireland). The macronutrient composition of each food was taken from the manufacturer’s labels, but where this was not possible foods were analyzed via the software database or comparable brands.

TOTAL DAILY ENERGY EXPENDITURE

Average total daily energy expenditure was calculated as the sum of the resting metabolic rate (RMR), diet-induced thermogenesis (10 % of the self-reported daily energy intake from baseline or follow-up) and physical activity energy expenditure (PAEE). To assess RMR (at baseline and during the last week of the intervention), participants rested in a semi-supine position (for 15 min) before 4 x 5-min expired air samples were collected. Lipid and carbohydrate utilisation rates were calculated from expired air samples via stoichiometric equations based on research of Frayn (*as stated earlier in this file*; <https://www.physiology.org/doi/abs/10.1152/jappl.1983.55.2.628>). To measure free-living PAEE, participants wore an ActiheartTM monitor over 7 days at baseline and during the last week of the intervention (Cambridge Neurotechnology, Papworth, UK). Energy expenditure and heart rate values from rest and exercise were entered in the ActiheartTM software for an individually calibrated model

WESTERN BLOTTING OF MUSCLE SAMPLES

*Vastus lateralis* muscle samples were collected under local anaesthesia (~ 5 mL 1 % lidocaine, Hameln Pharmaceuticals Ltd., Brockworth, UK), from a 3-6-mm incision at the anterior aspect of the thigh using a 5-mm Bergstrom biopsy needle technique adapted for suction. Samples were collected pre- and post-intervention with participants in a fasted, resting state (both from the dominant leg). Muscle was extracted from the needle and frozen in liquid nitrogen, before storage at -80 °C. Western blotting was performed on the muscle samples (*the* *methods used to measure perform the western blotting of the muscle samples is detailed within the manuscript*). The membranes were incubated in an enhanced chemiluminescence reagent and visualized on an imager. Band densities were quantified using Image Studio Lite software and for each sample, bands were normalized to a loading control, before the pre- to post-intervention change was calculated. Pre- and post-intervention samples were included on the same gel.

CITRATE SYNTHASE ACTIVITY

*Vastus lateralis* muscle samples were collected under local anaesthesia (~ 5 mL 1 % lidocaine, Hameln Pharmaceuticals Ltd., Brockworth, UK), from a 3-6-mm incision at the anterior aspect of the thigh using a 5-mm Bergstrom biopsy needle technique adapted for suction. Samples were collected pre- and post-intervention with participants in a fasted, resting state (both from the dominant leg). The activity of citrate synthase was measured using a commercially available assay (Abcam; ab119692).

PHOSPHOLIPID COMPOSITION OF MUSCLE SAMPLES

*Vastus lateralis* muscle samples were collected under local anaesthesia (~ 5 mL 1 % lidocaine, Hameln Pharmaceuticals Ltd., Brockworth, UK), from a 3-6-mm incision at the anterior aspect of the thigh using a 5-mm Bergstrom biopsy needle technique adapted for suction. Samples were collected pre- and post-intervention with participants in a fasted, resting state (both from the dominant leg). Muscle was extracted from the needle and frozen in liquid nitrogen, before storage at -80 °C. The phospholipid composition of the muscle samples was determined (*the* *methods used to measure perform the phospholipid composition of the muscle samples is detailed within the manuscript*). The content of phospholipids was estimated as the sum of the particular fatty acid species and expressed in nanomoles per milligram of dry mass (*these raw data are included in the excel document*). The content of each specific lipid species was normalised to the total content of the phospholipid pool for each sample before the pre- to post-intervention change was calculated (*as reported in the manuscript*). To calculate the sum of changes of phospholipid species, the change was score was added together for each participant as an index of global remodelling (*as reported in the manuscript*).