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JTG, KDT, DLH, EJS, JAB and DT designed the research; RME, JTG AH, HS, RLT and J-PW conducted the research, RME, JTG, AH, HS, FK, and GAW analyzed the data, RME and JTG performed the statistical analysis, RME and JTG primarily wrote the paper, and all authors contributed to earlier versions of the manuscript and read and approved the final version of the manuscript.

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Running Head: Pre-exercise feeding and postprandial glucose flux

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ABSTRACT

Aims: To characterize postprandial glucose flux after exercise in the fed *versus* overnight fasted-state and to investigate potential underlying mechanisms. **Methods:** In a randomized order, twelve men underwent breakfast-rest (BR; 3 h semi-recumbent), breakfast-exercise (BE; 2 h semi-recumbent before 60-min of cycling (50% peak power output) and overnight fasted-exercise (FE; as per BE omitting breakfast) trials. An oral glucose tolerance test (OGTT) was completed post-exercise (post-rest on BR). Dual stable isotope tracers ([U-^{13}C] glucose ingestion and [6,6-$^{2}H_2$] glucose infusion) and muscle biopsies were combined to assess postprandial plasma glucose kinetics and intramuscular signaling, respectively. Plasma intestinal fatty acid binding (I-FABP) concentrations were determined as a marker of intestinal damage. **Results:** Breakfast before exercise increased post-exercise plasma glucose disposal rates during the OGTT, from 44 g·120 min^{-1} in FE [35 to 53 g·120 min^{-1}] (mean [normalized 95% CI]) to 73 g·120 min^{-1} in BE [55 to 90 g·120 min^{-1}; p = 0.01]. This higher plasma glucose disposal rate was, however, offset by increased plasma glucose appearance rates (principally OGTT-derived), resulting in a glycemic response that did not differ between BE and FE (p = 0.11). Plasma I-FABP concentrations during exercise were 264 pg·mL^{-1} [196 to 332 pg·mL^{-1}] lower in BE *versus* FE (p = 0.01). **Conclusion:** Breakfast before exercise increases post-exercise postprandial plasma glucose disposal, which is offset (primarily) by increased appearance rates of orally-ingested glucose. Therefore, metabolic responses to fed-state exercise cannot be readily inferred from studies conducted in a fasted state.

52 **Key words:** Breakfast; Exercise; Insulin sensitivity; Glycemia; Metabolism

INTRODUCTION

Postprandial glycemia is a strong predictor of future mortality and morbidity. Even in people without diabetes, those with greater blood glucose excursions after feeding are at an increased risk of cardiovascular disease (47, 48). This glycemic response to food ingestion is dictated by blood glucose kinetics (i.e. the balance between the rates of glucose appearance into blood and glucose disposal from blood into peripheral tissues). Exercise potently increases glucose disposal from the blood into skeletal muscle (52), and regular exercise is therefore recommended as a lifestyle strategy to improve glycemic control.

Habitual responses to exercise and nutrition are however, the culmination of not only chronic adaptations, but also the acute effects of each exposure to these daily behaviors (5, 6, 22). For example, each bout of exercise potently stimulates post-exercise insulin sensitivity and muscle glucose uptake (52). However, despite increases in blood glucose disposal rates, endurance-type exercise does not always reduce postprandial glucose excursions in the post-exercise period (20, 54). The finding that postprandial blood glucose concentrations are not lowered post-exercise is because when exercise is performed (at least in the fasted state), the increase in postprandial blood glucose disposal after exercise can be offset - *and even superseded* - by increases in both endogenous and meal-derived blood glucose appearance rates (34, 54).

Whilst fasting prior to laboratory trials is common in order to control for baseline metabolic status, these conditions may preclude the application of findings to situations most representative of daily living. For example, most people living in developed countries spend the majority of a typical day in the postprandial state (13, 55). Therefore, most eating occasions and exercise sessions will take place in the context of this postprandial situation (23). It has previously been shown by others that plasma glucose fluxes *during* exercise (16), and by us that plasma glucose *concentrations* after exercise (24), are elevated by pre-exercise feeding. However, the effect of prior feeding on *post-exercise* plasma glucose flux has never been assessed. Therefore, there is a distinct lack of understanding regarding postprandial glucose kinetics under scenarios that are most representative of daily living, and it may not be valid to generalize existing observations of exercise in the fasted state. Moreover, an understanding of the underlying mechanisms responsible for any differences in postprandial glucose flux post-exercise, with prior feeding *versus* fasting, is still required.

This study therefore aimed to characterize postprandial plasma glucose kinetics after: 1) breakfast and rest; 2) breakfast and exercise and 3) overnight fasted-state exercise, while also exploring potential mechanisms (intramuscular signaling and markers of intestinal damage) to explain any differences in glucose flux between these conditions.

MATERIALS AND METHODS

Ethical Approval

All trials were undertaken at the University of Bath (Bath, UK) in accordance with the Declaration of Helsinki. The study was approved by the National Health Service South-West Research Ethics Committee (reference: 15/SW/0006) and registered at clinicaltrials.gov as NCT02258399. Written, informed consent was obtained from all participants prior to their participation.

Study Design

This study was a randomized cross-over design (randomization performed by JTG with Research Randomizer version 3.0, http://www.randomizer.org/). Preliminary testing was followed by three trials (separated by > 7 d), namely, breakfast-rest (BR), breakfast-exercise (BE) and overnight fasted-exercise (FE). A schematic for the study protocol is shown in **Figure 1**. For all trials participants arrived at the laboratory after a 12 to 14-h overnight fast. In BR, a porridge breakfast was consumed, followed by 3 h of rest, and then a 2-h oral glucose tolerance test (OGTT). In BE, the same breakfast was consumed, before 2 h rest and 60 min of cycling, prior to the OGTT. In FE, breakfast was omitted but the trial otherwise replicated BE. By necessity of design (food intake/exercise) the intervention was open label. Within-lab testing conditions were not different across the trials ([mean ± SD] ambient temperature [23.7 ± 0.5 °C on BR, 23.7 ± 0.6 °C on BE, 23.6 ± 0.7 °C on FE) and barometric pressure [734 ± 5 mmHg on BR, 736 ± 6 mmHg on BE, 736 ± 5 mmHg on FE]; all $p > 0.05$).

121

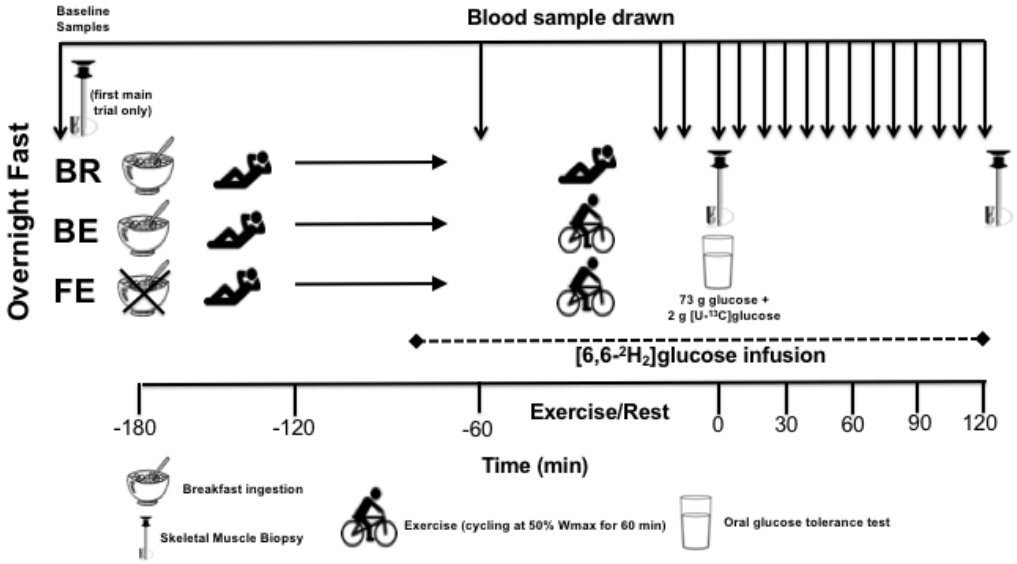

Figure 1. Protocol schematic. An oral glucose tolerance test was conducted after breakfast followed by rest (BR), breakfast followed by exercise (BE), or extended overnight fasting followed by exercise (FE). Dual stable isotope tracers ([U-^{13}C] glucose ingestion and [6,6-$^{2}H_2$] glucose infusion) and muscle biopsies were combined to assess postprandial plasma glucose kinetics and intramuscular signaling, respectively.

122

123 **Participants**

124 Twelve healthy and physically active men (self-reported as regular exercisers
125 engaging in at least 30 min of exercise a minimum of 3 times per week) were
126 recruited from Bath and North East Somerset, between May and November
127 2015. Participant characteristics are shown in **Table 1**. Exclusion criteria
128 included any history of metabolic disease, or condition that may have posed
129 undue personal risk to the participant or introduced bias to the study.

130

131 **Preliminary Testing**

Participants were asked to refrain from strenuous physical activity for 24 h prior to preliminary testing, but were asked to otherwise maintain their normal physical activity behaviors. They abstained from alcoholic and caffeinated drinks for 24 h prior to this visit. Food intake ceased at 8 pm on the evening before testing and participants fasted overnight (minimum 12 h), consuming only water (*ad libitum*) during this period. In addition, they were asked to consume 568 mL of water at least 1 h prior to testing, and to void immediately prior to arriving at the laboratory. Upon arrival, the participant's stature was measured (Frankfurt plane) to the nearest 0.1 cm using a stadiometer (Seca Ltd, Birmingham, UK). Body mass was recorded to the nearest 0.1 kg (only light clothing permitted) using electronic weighing scales (BC543 Monitor, Tanita, Tokyo, Japan). A whole-body dual energy x-ray absorptiometry scan was completed to quantify fat and fat-free mass (DEXA; Discovery, Hologic, Bedford, UK).

Participants then performed an incremental cycling exercise test at a self-selected cadence on an electronically-braked ergometer (Excalibur Sport, Lode Lode® Groningen, Netherlands). They were permitted to adjust the saddle and handlebar heights to their preferred position, which were replicated for cycling during the exercise trials. The initial exercise intensity was 50 W and this was increased by 50 W every four min, for four stages. Thereafter, the intensity was increased by 20 W every min until volitional exhaustion. Heart rate (Polar Electro Oy, Kempele, Finland) and continuous breath-by-breath measurements were recorded throughout (TrueOne2400, ParvoMedics, Sandy, USA). Volume

and gas analyzers were calibrated with a 3 L calibration syringe (Hans Rudolph, Kansas City, USA) and a calibration gas (balance nitrogen mix; 16.04% O_2, 5.06% CO_2; BOC Industrial Gases, Linde AG, Munich, Germany), respectively. Peak power output (PPO) was calculated as the work rate of the last completed stage, plus the fraction of time spent in the final non-completed stage, multiplied by the work rate increment. Peak oxygen uptake ($\dot{V}O_2$ peak) was calculated as the highest average $\dot{V}O_2$ over a rolling 30-s period.

Main Trials

Participants refrained from strenuous physical activity, alcohol and caffeine for 24 h before all trials. They recorded the composition of their evening meal on the day before the first main trial and replicated this meal for subsequent trials, in accordance with procedures for standardizing postprandial glucose tolerance testing (10). This pre-trial standardisation protocol has been previously shown to be effective at producing overnight-fasted muscle glycogen concentrations, liver glycogen concentrations and intramyocellular lipid that are standardised across multiple trial days, in a similar population (21). To help ensure physical activity standardization, participants completed a physical activity diary and wore a physical activity monitor (Actiheart^{TM}; Cambridge Neurotechnology, Papworth, UK) for 24 h before all trials (pre-trial 24-h physical activity energy expenditure [(mean ± standard deviation) 988 ± 500 kcal on BR, 1022 ± 521 kcal on BE, 992 ± 313 kcal on FE; all $p > 0.05$; $n = 9$].

Participants arrived at the laboratory at 0800 ± 1 h following a 12 to 14-h overnight fast and this arrival time was replicated for the subsequent trials. They were asked to void and all further urine samples were collected for the remainder of the trial to allow for urinary nitrogen excretion to be estimated from urine urea concentrations. Participants then placed their dominant hand into a heated-air box (Mass Spectrometry Facility; The University of Vermont & University of Vermont Medical Center, Burlington, USA) set to 55 °C. After 20 min of rest, an intravenous catheter (BD Venflon Pro, BD, Helsingborg, Sweden) was fitted into a heated dorsal hand vein (retrograde) and a 10-mL baseline blood sample was drawn, before a 5-min expired gas sample was collected. On the first main trial for each participant (see **Figure 1**), a baseline muscle sample was taken from the *vastus lateralis* to allow for an assessment of the pathways involved in exercise and insulin signaling in muscle [5' AMP-activated protein kinase (AMPK), acetyl-CoA carboxylase (ACC), protein kinase B (herein refered to as Akt2), and Akt substrate of 160 kDa (AS160).

In BE and BR, a porridge breakfast was then consumed within 10 min (3 h pre-OGTT) and in FE participants were allowed water only. The breakfast was 72 g of instant refined oats (Oatso Simple Golden Syrup, Quaker Oats) and 360 mL of semi-skimmed milk (Tesco), providing 431 kcal of energy ([1803 kJ]; 65 g CHO, 11 g FAT, 19 g PRO). The breakfast was high-carbohydrate (57% of energy intake) and high glycemic-index [oatmeal, made from mix (Quaker Oats) has a glycemic index of 69 (17)], as is commonly consumed in developed countries. Due to the co-ingestion of milk, this breakfast would produce a high

203 insulinemic response (38, 46). Breakfast consumption (or omission on FE) was
204 followed by 2 h of rest, where participants remained in a semi-recumbent
205 position, completing resting activities (e.g. watching television), with expired gas
206 samples collected every 60 min. After 1 h 40 min of rest, (1 h 20 min pre-OGTT)
207 a catheter was inserted into an antecubital vein (the contralateral arm to the one
208 used for blood sampling). A primed infusion of [6,6-$^{2}H_{2}$]glucose was initiated
209 and continued for the remaining within-lab component of the trial (Cambridge
210 Isotope Laboratories, MA, USA; prime: 13.5 μmol.kg^{-1}; infusion: 0.35
211 μmol.kg^{-1}.min^{-1}). After 20 min (60 min pre-OGTT), and on BE and FE only,
212 participants began 60 min of cycling at 50% PPO on an ergometer (Lode
213 Corival, Lode B.V, Groningen, Netherlands). The cadence was self-selected
214 (replicated for both exercise trials) and the power output was monitored via a
215 computerized system. In BR, participants remained rested in the semi-
216 recumbent position during this period. Expired gas samples were collected
217 every 15 min and blood samples were collected at 40 and 50 min of exercise
218 (20 and 10 min pre-OGTT). Immediately post-exercise (or post-rest in BR) a
219 muscle sample was taken from the *vastus lateralis*. Then a 2-h OGTT was
220 completed, with arterialized blood sampled at 10-min intervals and expired gas
221 sampled every 60 min. The OGTT was 73 g of glucose (81 g of dextrose
222 monohydrate when corrected for water content; Myprotein, Northwich, UK) and
223 2 g of [U-^{13}C]glucose (99%; Cambridge Isotope Laboratories, MA, USA), to
224 allow the rate of appearance of the orally ingested glucose (Ra_{OGTT}) to be
225 assessed. A final muscle sample was taken post-OGTT (OGTT 120 min).
226

Tracer approach, blood sampling and analysis

A dual-tracer approach was employed, where the tracer infusion rate was doubled during exercise (on BE and FE) to account for an expected increase in endogenous glucose production (1) and reduced to 80% of baseline at OGTT 20 min (all trials) to account for an expected suppression of endogenous glucose production after oral glucose ingestion (9). This approach reduces changes in the tracer-to-tracee ratio, thereby permitting more accurate estimations of glucose kinetics(4). Arterialized blood was sampled from a heated dorsal hand vein at baseline, at 60-min intervals during the initial 2-h rest period, at 40 and 50 min of the exercise period (or post-rest in BR) and at 10-min intervals during the OGTT. Whole blood was dispensed into ethylenediaminetetraacetic acid-coated tubes (BD, Oxford, UK) which were first centrifuged (4 °C and 3500 *g*) for 10-min (Heraeus Biofuge Primo R, Kendro Laboratory Products Plc., UK) to obtain plasma. The plasma was then dispensed into 0.5 mL aliquots and immediately frozen at -20 °C, before longer-term storage at -80 °C.

Plasma glucose (intra-assay coefficient of variation [CV], 3.2%; inter-assay CV 3.8%), lactate (intra-assay CV, 1.0%; inter-assay CV 4.8%), and triglyceride (intra-assay CV, 1.4%; inter-assay CV 4.0%) concentrations were measured using an automated analyzer (Daytona; Randox Lab, Crumlin, UK) as per the manufacturer's instructions. Plasma insulin concentrations were measured using a commercially available ELISA kit (Mercodia AB, Uppsala, Sweden; intra-assay CV, 5.7%; inter-assay CV 9.9%). Plasma intestinal fatty acid binding

protein (I-FABP) concentrations were measured as a marker of intestinal cell damage using a commercially available ELISA kit (Hycult Biotech; intra-assay CV, 6.0%). Plasma NEFA concentrations were determined using an enzymatic colorimetric assay (WAKO Diagnostics; intra-assay CV, 8.9%; inter-assay CV 10.4%). For all of these analyses, all plasma samples were analyzed in batch after all sample collection was completed, and for a given participant all samples (from the three trials) were run on the same plate.

Plasma [U-^{13}C]glucose and [$^{2}H_2$]glucose enrichments were determined by gas chromatography-mass spectrometry (GC-MS: GC, Agilent 6890N; MS, Agilent 5973N; Agilent Technologies, Stockport, UK). Plasma glucose was extracted using methanol-chloroform and hydrochloric acid, dried under nitrogen gas, and then derivatised using the heptofluorobuyric acid method as previously described(30). The glucose derivative was acquired by selected ion monitoring at mass-to-charge ratios (*m*/*z*) 519, 521 and 525 for [^{12}C], [6,6-$^{2}H_2$]- and [U-^{13}C]-glucose, respectively. Glucose enrichments of [^{13}C] and [$^{2}H_2$] in plasma were determined using standard curves for [^{13}C] and [$^{2}H_2$] glucose, and enrichments were expressed relative to those at 519 (M+0). The baseline sample was used for every trial to account for background isotopic plasma enrichments. To reduce any impact of analytical variability on calculations of glucose kinetics, glucose and enrichment data were curve fitted as previously described (63).

Muscle sampling and analysis

Muscle samples were collected from the *vastus lateralis* under local anesthesia (~5 mL of 1% lidocaine, Hameln Pharmaceuticals Ltd., Brockworth, UK). Samples were taken from a 3-5-mm incision at the anterior aspect of the thigh with a 5-mm Bergstrom biopsy needle technique adapted for suction(57). Samples were immediately extracted from the needle and frozen in liquid nitrogen, before longer-term storage at -80 °C. The order of dominant or non-dominant leg was counterbalanced across trials for the OGTT 0 min and the 120 min samples. Samples were taken from separate skin incision sites, with these > 2 cm proximal to any previous incision on the same leg (59). For the OGTT 0 min sample (post exercise [or post-rest in BR]) the incision was made prior to cycling (BE and FE) and closed with Steristrips, to allow for an immediate sample to be taken post-exercise.

Frozen wet tissue (20-30 mg) was freeze-dried, powdered, and dissected free of visible blood and connective tissue and added to ice cold lysis buffer [50 mM Tris (pH 7.4), 150 mM NaCl, 0.5% Sodium deoxycholate; 0.1% SDS and 0.1% NP-40] with a protease (Thermo Scientific) and phosphatase inhibitor cocktail (Millipore). Samples were homogenized with a dounce homogenizer (~ 40 passes), incubated for 60 min at 4 °C with rotation, and centrifuged for 10 min (4 °C and 20,000 *g*). The protein content of the resultant supernatant was measured via a bicinchoninic acid (BCA) protein assay kit (Thermo Scientific). For Western blots, an equal amount of protein (40 µg) was loaded per lane for each sample and separated using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on Tris-glycine SDS–polyacrylamide gels (7% for

298 p-AMPK^{Thr172}, total AMPK, p-ACC^{Ser79} and total ACC, 10% for p-Akt^{Ser473} and
299 total Akt2 and 8% for p-Akt^{Thr308}, p-AS160^{Thr642} and total AS160). Gels were
300 electro-blotted using a semi-dry transfer onto a nitrocellulose membrane.
301 Membranes were washed in Tris-buffered saline (0.09% NaCl, 100 mM Tris–
302 HCl pH 7.4) with 0.1% Tween 20 (TBS-T) and incubated for 30-min in a
303 blocking solution (5% non-fat dry milk in TBS-T; Marvel, Premier International
304 Foods Ltd, UK). The membranes were incubated overnight at 4 °C with primary
305 antibodies against p-AMPK^{Thr172}, p-ACC^{Ser79}, p-Akt^{Ser473}, p-Akt^{Thr308} and p-
306 AS160^{Thr642} (Cell Signaling Technologies, USA). In the morning, membranes
307 were washed in TBS-T and incubated with a 1:4000 dilution of anti-species IgG
308 horseradish peroxidase-conjugated secondary antibodies made up in the
309 aforementioned blocking solution. After further washes, membranes were
310 incubated in an enhanced chemiluminescence (ECL) reagent and visualized
311 using a chemiluminescent imager (EpiChemi II Darkroom, UVP, Upland, USA).
312 Nitrocellulose membranes were then incubated for 30-min at 50 °C in a
313 stripping solution [62.5 mM Tris pH 6.7, 2%SDS, 100 mM 2-mercaptoethanol],
314 before re-blotting for total AMPK, total ACC, total Akt2 (Cell Signaling
315 Technologies, USA) and total AS160 (Merck-Milliopore, UK). For these
316 analyses, all samples from each participant (all three trials) were run on the
317 same gel. Band densities were quantified using VisionWorksLS Image
318 Acquisition and Analysis Software for Windows (UVP, Upland, USA). For all of
319 the signalling molecules reported in this experiment a ratio of phosphorylated to
320 total protein was calculated and the results were expressed relative to the
321 baseline sample.

322

323 **Substrate utilization**

324 Expired gas samples were collected at baseline, during the initial 2-h rest period
325 and the OGTT at 60-min intervals (for 5 min), and at 15-min intervals (for 1 min)
326 during the exercise period (or rest in BR). For all samples, participants were
327 provided with the mouthpiece 1 min before gas collections for a stabilization
328 period. Samples were collected in 200 L Douglas bags (Hans Rudolph, Kansas
329 City, USA) through falconia tubing (Baxter, Woodhouse and Taylor Ltd,
330 Macclesfield, UK). Concurrent measures of inspired air were made to correct for
331 changes in ambient O_2 and CO_2 concentrations(7). Expired O_2 and CO_2
332 concentrations were measured in a known volume of each sample, using
333 paramagnetic and infrared transducers respectively (Mini HF 5200, Servomex
334 Group Ltd., Crowborough, East Sussex, UK). The sensor was calibrated using
335 concentrations of low (99.998% Nitrogen, 0% O_2 and CO_2) and high (balance
336 nitrogen mix, 16.04% O_2, 5.06% CO_2) calibration gases (both BOC Industrial
337 Gases, Linde AG, Munich, Germany). Urinary nitrogen excretion was estimated
338 from urine urea concentrations, which were measured on an automated
339 analyzer (Daytona; Randox Lab, Crumlin, UK), to allow for protein oxidation to
340 be accounted for in calculations of substrate utilization rates.

341

342 **Calculations and statistical analysis**

343 A sample size estimation was completed *a priori* with the total rate of plasma
344 glucose appearance as the primary outcome measure. Rose *et al. (54)* reported
345 a difference in the total plasma glucose appearance of (mean ± SD) 1600 ±

346 1300 μmol·kg^{-1} during an OGTT after rest *versus* after fasted exercise. Using
347 this effect size, and an alpha level of 0.05, we calculated that 12 participants
348 were required for an 80% probability of statistically detecting an effect in the
349 Ra_{TOTAL} using a crossover design with three trials and a two-tailed, one-way
350 ANOVA.
351
352 The total and incremental area underneath the concentration-time curve (AUC
353 or iAUC, respectively) for each plasma metabolites or hormones was calculated
354 using the trapezoid rule. The AUC or iAUC for each plasma metabolite or
355 hormone was then divided by either the duration of the total within-lab period
356 (300-min) or the OGTT observation period (120-min), as appropriate, to provide
357 a time-averaged value (mmol·L^{-1}), which are used as summary measures.
358 Plasma glucose and insulin concentrations during the OGTT were used to
359 estimate insulin sensitivity (ISI) according to the equation of Matsuda (41): [FPG
360 and FPI are fasting plasma glucose and insulin concentrations, and MPG and
361 MPI are mean plasma glucose and insulin concentrations in the OGTT (41)]:
362

$$ISI_{MATSUDA}(au) = \frac{10{,}000}{\sqrt{FPG(mgdL^{-1}) \bullet FPI(mIUmL^{-1}) \bullet MPG(mgdL^{-1}) \bullet MPI(mIUmL^{-1})}}$$

363
364 Plasma glucose kinetics were determined using Radziuk's two-compartment
365 non-steady state model (50, 51) and SAMM II software (SAAM II v2.3, The
366 Epilson Group, Charlottesville, VA, USA). This model reduces errors in
367 estimations of glucose kinetics that are apparent when using Steele's (56) one

368 compartment model (53). The total rate of plasma glucose appearance
369 (Ra_{TOTAL}) and glucose disappearance (Rd) were calculated as follows:
370
371 ***Equation 1:***

$$Ra_{TOTAL}(t) = \frac{F}{E_1(t)} - \frac{V_1 \cdot G(t)}{E_1(t)} \cdot \dot{E}_1(t) + k_{12}\left(\frac{q_2^{iv}(t)}{E_1(t)} - Q_2(t)\right)$$

372 ***Equation 2:***

$$Rd\ (t) = Ra_{TOTAL} - V_1 \cdot \dot{G}(t) - k_{21} \cdot V_1 \cdot G(t) + k_{12} \cdot Q_2(t)$$

373
374 Where F is the [6,6-$^2H_2$] infusion, V_1 is the glucose volume of distribution [4% of
375 body mass (kg)], $E_1(t)$ the [$^2H_2$] plasma glucose enrichment (mole percent
376 excess) at time *t*, $\dot{E}_1(t)$ the change in E over time [derivate of E], G*(t)* the
377 plasma glucose concentrations at time t, $\dot{G}(t)$ the change in G over time
378 [derivate of G], k_{12} and k_{21} are fixed rate constants between the peripheral and
379 the accessible compartments (0.05 min^{-1} and 0.07 min^{-1} respectively) and q_2^{iv}
380 and Q_2 are the amounts of the tracer [$^2H_2$] and tracee in the peripheral
381 compartment respectively, evaluated by integrating the two-compartment
382 model.
383
384 The [U-^{13}C] enrichment of the orally ingested glucose and the Ra_{TOTAL} (from
385 *Equation 1*) were used to calculate the plasma rate of appearance of glucose
386 from the OGTT (Ra_{OGTT}). In these equations, r_1 is the ratio of the infusion [$^2H_2$]
387 and oral [U-^{13}C] glucose tracer concentrations in plasma, $\dot{r}(t)$ is the change in r
388 over time [derivate of r], g is the [U-^{13}C] glucose tracer in plasma, q_o^{iv} is the

389 amount of the [U-^{13}C] tracer in the peripheral compartment (by integrating the
390 two-compartment model), and E_{OGTT} is the [U-^{13}C] enrichment of the OGTT.
391
392 ***Equation 3:***

$$ra^{O}(t) = Ra_{TOTAL}(t)\frac{F}{r_1(t)} - \frac{V_1 \cdot g(t)}{r_1(t)} \cdot \dot{r}_1(t) + k_{12}\left[\frac{q_2^{iv}(t)}{r_1(t)} - q_2^0(t)\right]$$

393 ***Equation 4:***

$$Ra_{OGTT}(t) = ra^{O}(t)\left[\frac{1}{E_{OGTT}}\right]$$

394 The metabolic clearance rate was calculated as the Rd divided by the plasma
395 glucose concentrations for a given time point (G_1)
396
397 ***Equation5:***

$$\text{Metabolic Clearance Rate} = Rd(t) / G_1$$

398 Rates of whole-body fat and carbohydrate utilization were calculated using the
399 expired gas samples and stoichiometric equations (31). Adjustments were
400 made to account for the contribution made by the oxidation of protein
401 (estimated via urinary urea nitrogen). Plasma glucose utilization was assumed
402 to be equivalent to the plasma glucose rate of disappearance (Rd) as has been
403 confirmed previously(32). Muscle glycogen utilization during exercise (BE and
404 FE only) was calculated as total carbohydrate utilization during exercise minus
405 plasma glucose utilization during exercise. Due to these methods, this estimate
406 of muscle glycogen utilization will include the utilization of other non-glucose
407 carbohydrates (e.g. lactate). Both the production and utilization of ketone

408 bodies can influence the respiratory exchange ratio and therefore theoretically
409 complicate the estimates of carbohydrate oxidation during exercise. However,
410 during short-duration, moderate intensity exercise this effect is negligible (31).
411 Within-lab, energy expenditure was determined assuming that lipids, glucose
412 and glycogen give 40.81, 15.64 and 17.36 $kJ \cdot g^{-1}$ respectively (31).
413
414 One-way, repeated measures ANOVA were used to assess differences
415 between trials at baseline and for summary measures (e.g. AUCs). If multiple
416 comparisons were necessary, two-way repeated measures ANOVAs (time x
417 trial) was used to identify differences between trials. Degrees of freedom for *F*
418 values were Greenhouse-Geisser corrected for epsilon < 0.75, with Huynh-Feldt
419 corrections used for less severe asphericity. If time x trial interaction effects
420 were identified, multiple paired *t*-tests were used to locate variance, with Holm-
421 Bonferroni step-wise adjustments to control for inflated type I errors. Pearson *r*
422 and Spearman *R* were used to explore correlations between variables display
423 normal and non-normal distribution, respectively. Unless otherwise stated, data
424 in text, figures and tables are means ± 95% confidence intervals, which were
425 normalized by removing between-subject variance (presented as 95% nCI)(39).
426 All statistical analyses were completed using IBM SPSS statistics version 22 for
427 windows (IBM, New York, USA), with the exception of the Holm-Bonferroni
428 step-wise adjustments and the calculation of normalized confidence intervals
429 which were completed using Microsoft Excel [2013]. Graph Pad Prism 7
430 software (La Jolla, CA, USA) was used for preparation of the manuscript
431 figures. A complete set of muscle samples was only obtained from nine

participants. Due to cannulation difficulties, for one participant's BR trial the last blood sample obtained was at OGTT 70 min and for a different participant's BE trial the last sample was at OGTT 60 min. For these trials (2 of 36) the group average was used for the missing data. Sensitivity analysis was completed for all measures involving blood samples and including/excluding these two participants did not influence any of the primary outcome measures. For clarity, the *n* is presented in all figure and table legends.

RESULTS

Plasma glucose kinetics

The plasma glucose disappearance rate (Rd) displayed a time x trial interaction (F = 3.123, p = 0.05), whereby plasma glucose Rd was higher during exercise *versus* rest (**Figure 2A**). Compared to extended overnight fasting, breakfast ingestion prior to exercise further increased the plasma glucose Rd during and after exercise (i.e. during the OGTT; **Figure 2A**). A main effect of trial was detected for the plasma glucose Rd during the OGTT (F = 7.079, p = 0.01), whereby the Rd was 45 g·120 min^{-1} in BR [95% nCI: 36 to 62 g·120 min^{-1}] *versus* 73 g·120 min^{-1} in BE ([95% nCI: 55 to 90 g·120 min$^{-1;}$ p = 0.09 *versus* BR) and 44 g·120 min^{-1} in FE [95% nCI: 35 to 53 g·120 min^{-1}]; p = 0.01 *versus* BE). Metabolic clearance rates showed a main effect of trial, with the highest rates also apparent in BE (**Figure 2B**; F = 7.849, p < 0.01 *versus* BR and FE).

A main effect of trial was detected for Ra_{TOTAL} during the OGTT ([g·120 min^{-1}]; F = 3.915, p = 0.05) which was highest in BE (**Figure 2C)**. However, after post-hoc adjustment the difference between trials was less apparent (p = 0.19 for BE *versus* BR and p = 0.09 for BE *versus* FE). A similar pattern was observed for the rate of appearance of glucose from the OGTT in plasma (Ra_{OGTT}) and a trial x time interaction was detected (**Figure 2D**; F = 3.134, p = 0.04). A main effect of trial was detected for the total Ra_{OGTT} (F = 5.915, p = 0.02), which was 49 g·120 min^{-1} in BE [95% nCI: 44 to 53 g·120 min^{-1}] (65% of the OGTT [59 to 71%]) *versus* 42 g·120 min^{-1} in BR [95% nCI: 36 to 46 g·120 min^{-1}] (56% of the OGTT [50 to 62%]; p = 0.11 *versus* BE) and 41 g·120 min^{-1} in FE [95% nCI: 35

464 to 47 g] (55% of the OGTT [49 to 61%] *p* = 0.06 *versus* BE). The plasma
465 enrichment of [$^{2}H_{2}$] - and [^{13}C]- glucose are shown in **Figures 2E** and **2F**,
466 respectively.
467

468 **Plasma glucose concentrations**

469 No difference between trials was detected for plasma glucose concentrations at
470 baseline (Figure 3A; p > 0.05). Thereafter, a trial x time interaction was
471 apparent (F = 2.957, p = 0.01). During the exercise period (rest in BR), plasma
472 glucose concentrations were higher in BR *versus* BE at 40 min, and in BR
473 *versus* FE at 50 min (both p < 0.05). At OGTT 0 min, glucose concentrations
474 were higher in BR *versus* BE, and during the OGTT they were initially higher in
475 BR *versus* BE and in BR *versus* FE (all p < 0.05), but no further differences
476 were then detected (Figure 3A). Peak plasma glucose concentrations were
477 higher in BR *versus* BE (p = 0.03), but not different in BE *versus* FE (Table 2; p
478 > 0.05). A main effect of trial was detected for the within-lab (300-min) glucose
479 AUC which was higher in BR *versus* BE (Table 2; p = 0.05). However, no main
480 effect of trial was detected for the OGTT (120-min) iAUC (Figure 4A; F = 2.524,
481 p = 0.11).
482

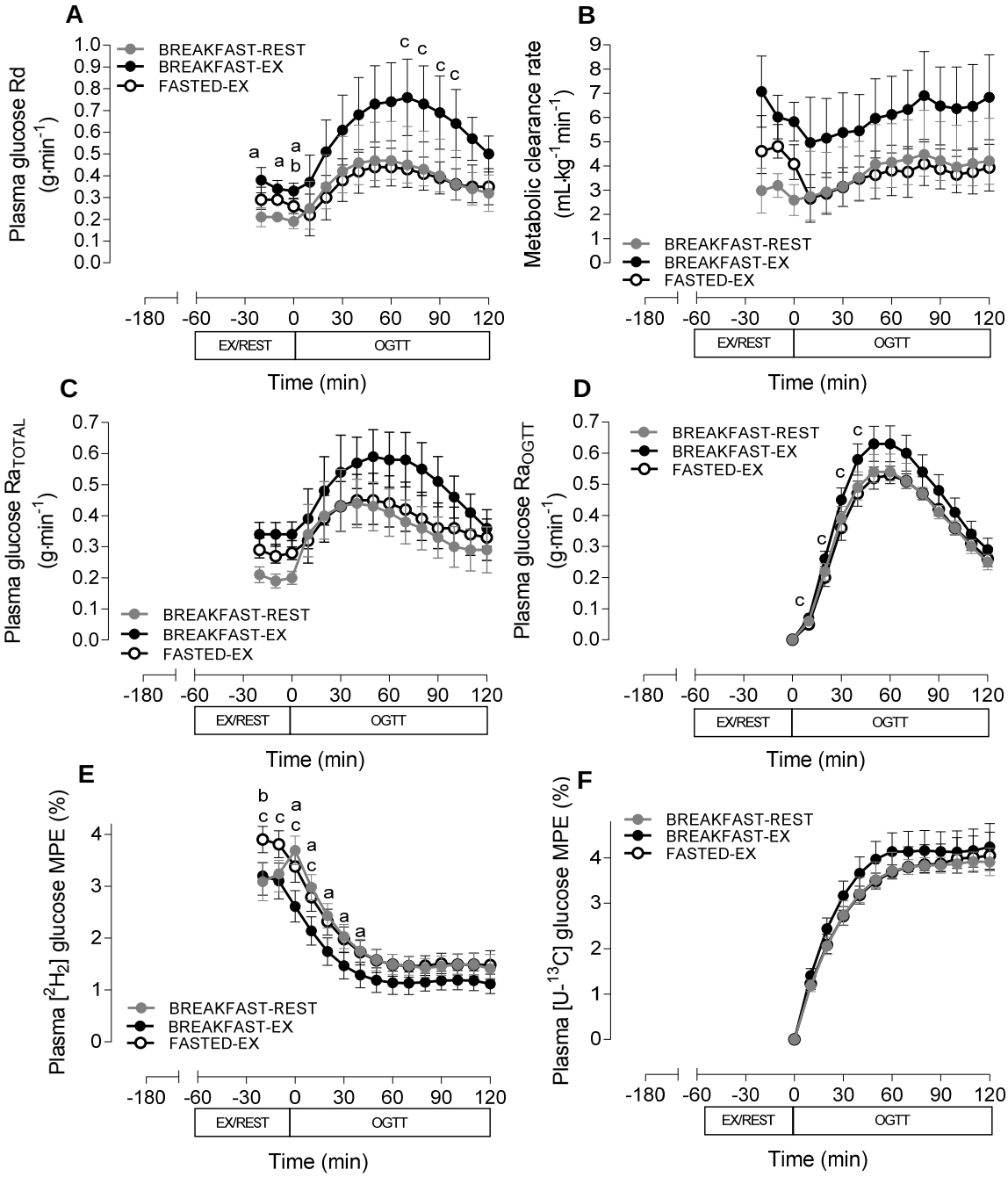

Figure 2. The plasma glucose disposal rate (Rd) (**A**), metabolic clearance rate (**B**), the total rate of plasma glucose appearance (Ra_{TOTAL}) (**C**), the rate of appearance of glucose in plasma from the oral glucose tolerance test (Ra_{OGTT}) (**D**), and the plasma enrichments of [$^2H_2$]-glucose (**E**) and [^{13}C]-glucose (**F**) before and during an oral glucose tolerance test that was conducted after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Data are means ± normalized 95% confidence intervals. *n* = 12 healthy men. a = difference between breakfast rest *versus* breakfast exercise; b = breakfast rest *versus* fasted exercise and c = breakfast exercise *versus* fasted exercise with $p < 0.05$.

485

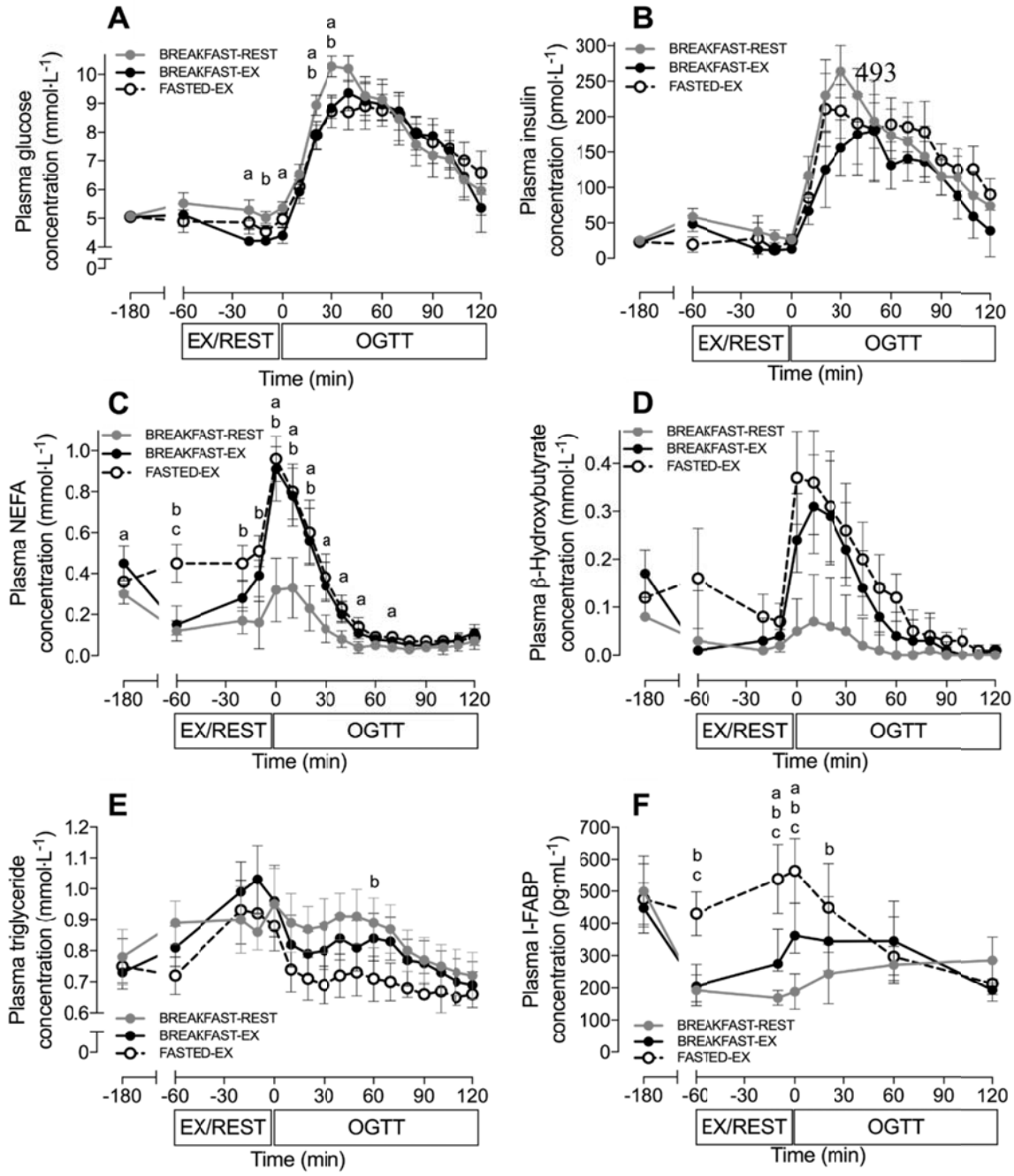

Figure 3. Plasma glucose (**A**), plasma insulin (**B**), plasma non-esterified fatty acids (NEFA; **C**), plasma β-hydroxybuturate (**D**) plasma triglyceride (**E**), and plasma intestinal fatty acid binding protein (IFAB-P; **F**) concentrations before and during an oral glucose tolerance test that was conducted after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Data are means ± normalized 95% confidence intervals. *n* = 12 healthy men. a = difference between breakfast rest *versus* breakfast exercise; b = breakfast rest *versus* fasted exercise and c = breakfast exercise *versus* fasted exercise with $p < 0.05$.

486

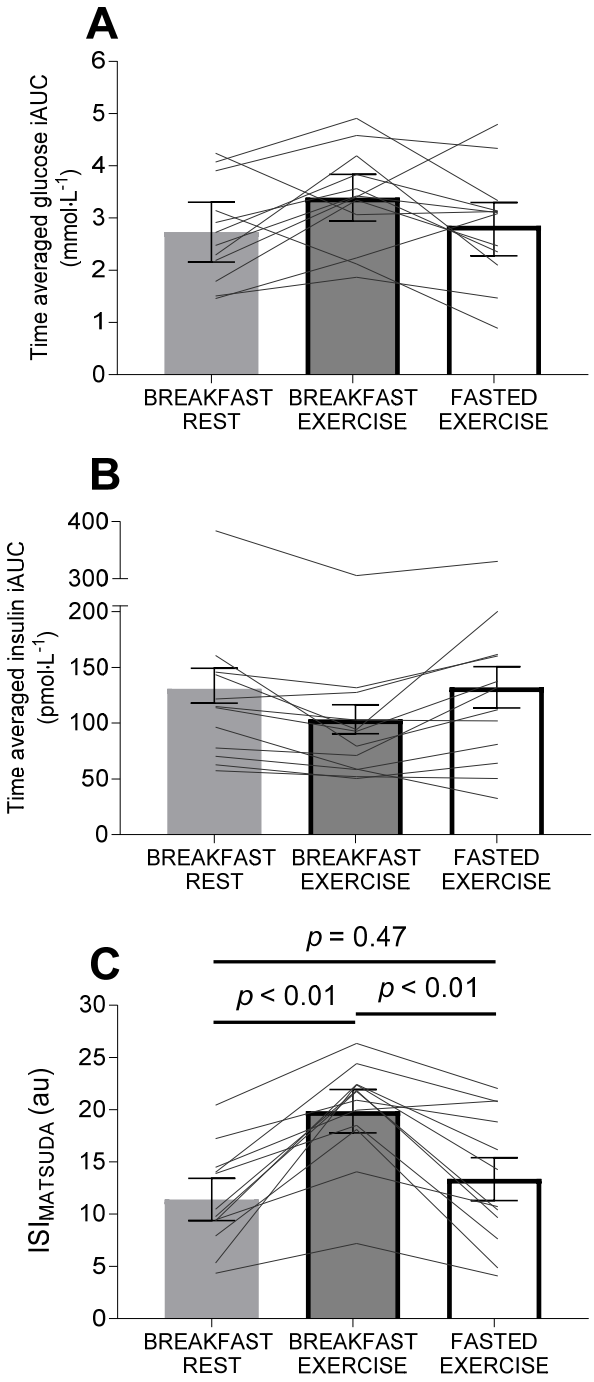

Figure 4. The time-averaged (120-min) plasma glucose (**A**) and plasma insulin (**B**) incremental area under the curves (iAUC) and the Matsuda insulin sensitivity index (**C**; $ISI_{MATSUDA}$) for an oral glucose tolerance test conducted after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Data are means ± normalized 95% confidence intervals, with individual data shown as grey lines. *n* = 12 healthy men.

487 **Plasma insulin concentrations**

488 At baseline, there was no difference between trials for plasma insulin
489 concentrations (**Figure 3B**; $p > 0.05$). Main effects of time ($F = 4.351$, $p < 0.01$)
490 and trial ($F = 7.986$, $p < 0.01$) were detected, but there was no trial x time
491 interaction effect ($F = 2.395$, $p = 0.07$). Peak (and time to peak) plasma insulin
492 concentrations are shown in **Table 2**. A main effect of trial was detected for the
493 within-lab (300-min) insulin AUC which was higher in BR *versus* BE ($p < 0.01$),
494 but not different in BE *versus* FE (**Table 2**; $p = 0.10$). A main effect of trial was
495 apparent for the insulin OGTT iAUC [**Figure 4B**; (120-min) $F = 5.132$, $p = 0.02$]
496 which was lower in BE *versus* BR [by 27.34 pmol·L^{-1} (95% nCI: 12.10 to 45.80
497 pmol·L^{-1}); $p = 0.02$] and lower in BE *versus* FE [by 28.67 pmol·L^{-1} (95% nCI:
498 10.21 to 47.12 pmol·L^{-1}); $p = 0.04$]. There was a main effect of trial for the
499 $ISI_{MATSUDA}$ insulin sensitivity index (**Figure 4C**; $F = 22.790$, $p < 0.01$), which was
500 higher in BE *versus* BR [by 8.45 au, (95% nCI: 6.42 to 10.47 au); $p < 0.01$] and
501 in BE *versus* FE [by 6.49 au (95% nCI: 2.93 to 8.51 au); $p < 0.01$].
502
503 **Plasma non-esterified fatty acid (NEFA) concentrations**
504 A main effect of trial ($F = 4.314$, $p = 0.04$) was detected for plasma NEFA at
505 baseline, with concentrations of 0.30 mmol·L^{-1} in BR (95% nCI: 0.25 to 0.35
506 mmol·L^{-1}), 0.45 mmol·L^{-1} in BE (95% nCI: 0.36 to 0.53 mmol·L^{-1}; $p = 0.03$ BE
507 *versus* BR) and 0.36 mmol·L^{-1} in FE (95% nCI: 0.31 to 0.44 mmol·L^{-1}; $p = 0.12$
508 FE *versus* BR and BE). Thereafter, a time x trial interaction effect was apparent
509 (**Figure 3C**; $F = 11.438$, $p < 0.01$), where plasma NEFA concentrations were
510 lowered by breakfast consumption in BR and BE, and remained lower during

511 the exercise in BE *versus* FE, before increasing during the initial OGTT period
512 in BE and FE *versus* BR. A main effect of trial was detected for the total within-
513 lab plasma NEFA (300-min) AUC and the NEFA OGTT (120-min) AUC which
514 in both instances was lower in BR *versus* BE and FE (**Table 2**; all $p < 0.01$).
515
516 **Other plasma metabolites**
517 No differences were detected between trials at baseline for plasma β-
518 hydroxybuturate concentrations (**Figure 3D;** $p > 0.05$). Thereafter, a time x trial
519 interaction effect was apparent ($F = 6.310$, $p < 0.01$) where concentrations were
520 lowered by breakfast in BR and BE. Plasma β-hydroxybuturate concentrations
521 remained lower during exercise in BE *versus* FE, but increased during the
522 OGTT with BE and FE *versus* BR. However, with post-hoc adjustment, the
523 differences between trials for plasma β-hydroxybuturate concentrations became
524 less clear (all $p > 0.05$). The within-lab (300-min) β-hydroxybuturate AUC was
525 lower with BR *versus* BE ($p = 0.03$), but did not differ in BE and FE (**Table 2**; p
526 $= 0.35$). No baseline differences were detected for triglyceride concentrations
527 (**Figure 3E**, $p > 0.05$) but a time x trial interaction effect was apparent ($F =$
528 3.994, $p < 0.01$). There was an effect of trial for the within-lab (300-min) and
529 OGTT (120-min) triglyceride AUC, which tended to be lower in FE *versus* BE,
530 but with post-hoc adjustment this difference between trials was less clear
531 (**Table 2;** $p > 0.05$). Plasma lactate concentrations at baseline were not different
532 across trials ($p > 0.05$) but were lower in BR *versus* BE and FE in the exercise
533 period (rest in BR) and at OGTT 0 min, but were then higher in BR *versus* BE
534 during the OGTT (time x trial; $F = 20.305$, $p < 0.01$). No effect of trial was

535 detected for the total within-lab (300-min) lactate AUC, but a main effect of trial
536 was detected for the lactate OGTT (120-min) AUC, which higher in BR *versus*
537 BE (**Table 2;** $p < 0.01$).
538
539 **Plasma intestinal fatty acid binding protein (I-FABP) concentrations**
540 There was no difference between trials at baseline for plasma I-FABP
541 concentrations (**Figure 3F;** $p > 0.05$), but these were lower after breakfast (time
542 x trial interaction effect; $F = 6.844$, $p < 0.01$) in BR and BE *versus* FE (both $p <$
543 0.05). During and post-exercise (or rest in BR), I-FABP concentrations were
544 lower in BR and BE *versus* FE and remained lower in BR *versus* FE until OGTT
545 20 min (all $p < 0.05$). The within-lab (300-min) I-FABP AUC was lower in BR
546 and BE *versus* FE (**Table 2**; $p = 0.01$ and $p = 0.05$ respectively).
547
548 **Activation of exercise and insulin signaling pathways in skeletal muscle**
549 Time x trial interaction effects were apparent for $AMPK^{Thr172}$ (ratio p-AMPK to
550 total-AMPK) and ACC^{Ser79} (ratio pACC to total ACC) phosphorylation, if
551 normalized to the baseline muscle sample (**Figure 5A**; $F = 5.154$, $p = 0.04$ and
552 **Figure 5B**, $F = 5.881$, $p = 0.02$, respectively). Compared to the breakfast-rest
553 trial (BR), skeletal muscle $AMPK^{Thr172}$ phosphorylation was higher post-exercise
554 (or post-rest in BR) in the breakfast and exercise (BE) trial [by 1.9 fold (95%
555 nCI: 0.9 to 2.8 fold); $p = 0.04$] and was also higher in BE *versus* the fasted-
556 exercise (FE) trial [by 1.0 fold (95% nCI: 0.2 to 2.0 fold); $p = 0.01$]. A similar
557 pattern was apparent for ACC phosphorylation, which was higher post-exercise
558 (or post-rest in BR) in BE *versus* BR [by 6.7 fold (95% nCI: 5.4 to 8.0 fold); $p =$

559 0.03] but did not differ between BE and FE (p = 0.09). By OGTT 120 min,
560 ACC^{Ser79} and AMPK^{Thr172} phosphorylation had returned to baseline levels in all
561 three trials (all p > 0.05). No time x trial interaction (F = 2.110, p = 0.16) nor a
562 main effect of trial (F = 0.098, p = 0.83) was detected for Akt^{Ser473} (ratio p-
563 Akt^{Ser473} to total-Akt2) phosphorylation (**Figure 6A**). A main effect of time (F =
564 9.907, p = 0.01) was observed, where Akt^{Ser473} phosphorylation was elevated at
565 OGTT 120 min in all trials. Similarly no time x trial interaction (F = 1.533, p =
566 0.25) nor a main effect of trial (F = 0.484, p = 0.56) was detected for Akt^{Thr308}
567 (ratio p-Akt^{Thr308} to total-Akt2) phosphorylation (**Figure 6B**). A main effect of
568 time (F = 10.598, p = 0.01) was also detected for this phosphorylation site,
569 whereby Akt^{Thr308} phosphorylation was elevated at OGTT 120 min in all trials.
570 For AS160^{Thr642} phosphorylation (ratio p-AS160^{Thr642} to total-AS160), a time x
571 trial interaction was detected (**Figure 6C**; F = 4.430, p = 0.03), whereby the
572 AS160^{Thr642} phosphorylation was not different between BR and BE at any time,
573 was higher pre-OGTT in BE compared to FE (p = 0.04), but was not different
574 between BE and FE at 120-min post-OGTT (p = 0.69).

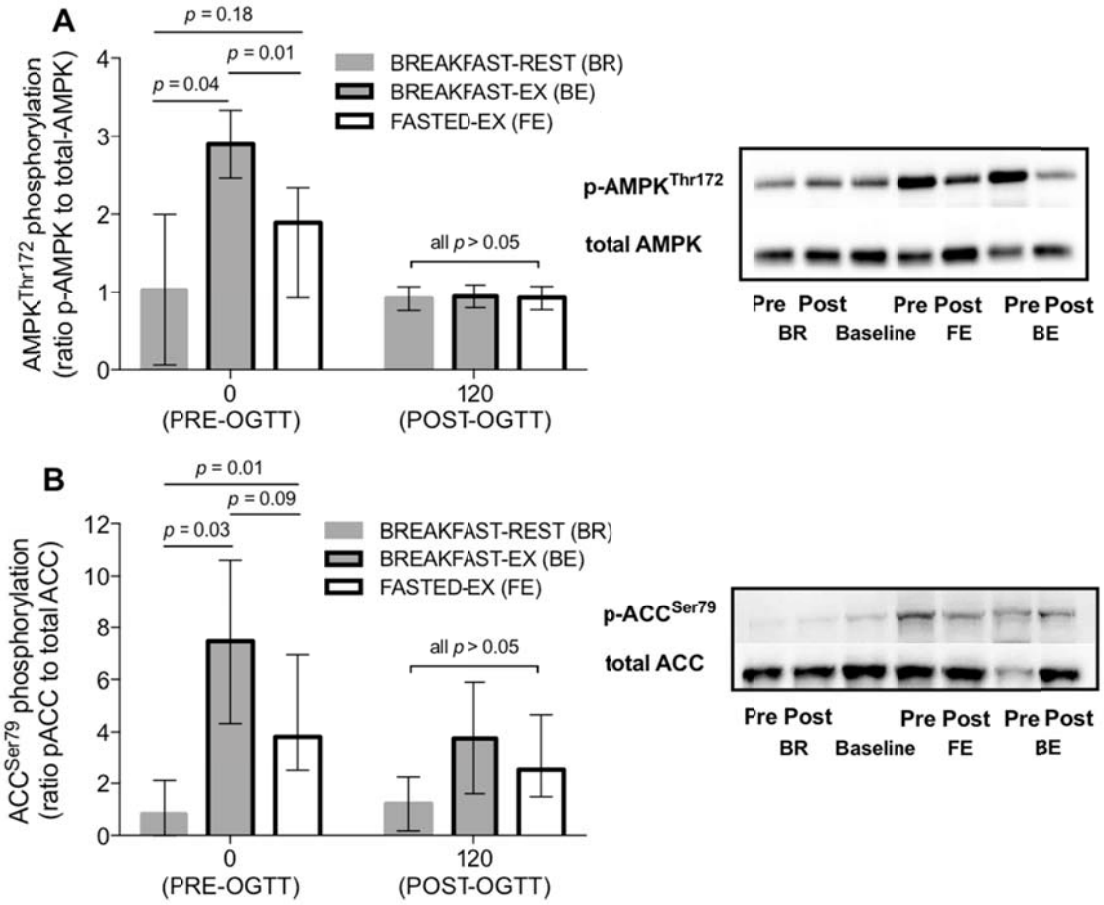

Figure 5. The phosphorylation of 5' AMP-activated protein kinase (**A**; phospho AMPK^{Thr172}, ratio p-AMPK to total-AMPK) and the phosphorylation of acetyl-CoA carboxylase (**B**; phospho ACC^{Ser79}, ratio p-ACC to total-ACC) before (PRE-OGTT) and after (POST-OGTT) an oral glucose tolerance test that was conducted after breakfast followed by rest (BREAKFAST-REST [BR]), breakfast followed by exercise (BREAKFAST-EX [BE]), or extended overnight fasting followed by exercise (FASTED-EX [FE]). All samples were taken from the *vastus lateralis*. Samples were normalized to the baseline muscle sample for each participant (collected in the resting fasted state on the first main trial for each participant). Data are means ± normalized 95% confidence intervals. *n* = 9 healthy men.

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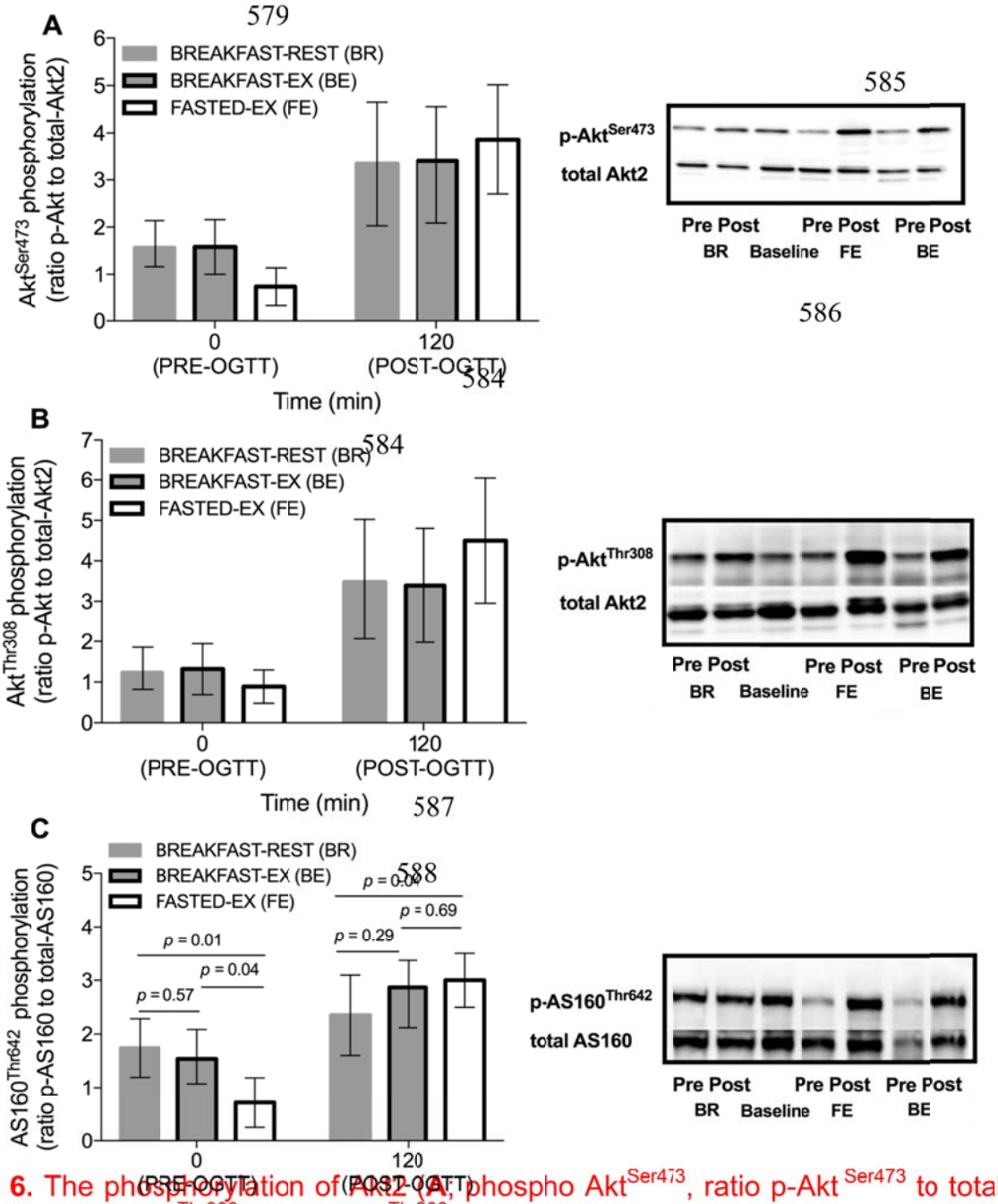

Figure 6. The phosphorylation of Akt2 (**A**; phospho Akt^{Ser473}, ratio p-Akt^{Ser473} to total-Akt2 and **B**; phosphor Akt^{Thr308}, ratio p-Akt^{Thr308} to total-Akt2) and the phosphorylation of AS160 (**C**; AS160^{Thr642}, ratio p-AS160^{Thr642} to total-AS160) before (PRE-OGTT) and after (POST-OGTT) an oral glucose tolerance test that was conducted after breakfast followed by rest (BREAKFAST-REST [BR]), breakfast followed by exercise (BREAKFAST-EX [BE]), or extended overnight fasting followed by exercise (FASTED-EX [FE]). All samples were taken from the *vastus lateralis*. Samples were normalized to the baseline muscle sample for each participant (collected in the resting fasted state on the first main trial for each participant). Data are means ± normalized 95% confidence intervals. *n* = 9 healthy men.

586 **Substrate utilization**

587 Across the duration of the trial carbohydrate utilization was higher in BE *versus*
588 BR (**Figure 7**; by 514 kcal, [95% nCI: 452 to 576 kcal] and higher in BE *versus*
589 FE (by 124 kcal, [95% nCI: 18 to 230 kcal], both $p < 0.01$). This difference in
590 carbohydrate utilization between BE and FE was derived from a higher
591 utilization of plasma glucose *and* other carbohydrate sources (i.e. primarily
592 muscle glycogen, but also plasma lactate) in BE ($p = 0.02$ and $p = 0.04$
593 respectively). Within-lab fat utilization did not differ between BR and BE ($p =$
594 0.25), but was higher in FE *versus* BE (by 138 kcal, [95% nCI: -6 to 224 kcal], p
595 = 0.03). Muscle glycogen utilization during exercise (g·kg body mass^{-1}) was
596 positively correlated ($R = 0.64$, $p < 0.01$) with skeletal muscle ACC^{Ser79}
597 phosphorylation (ratio p-ACC to total-ACC) after exercise conducted following
598 breakfast consumption (BE) or extended overnight fasting (FE).
599

601

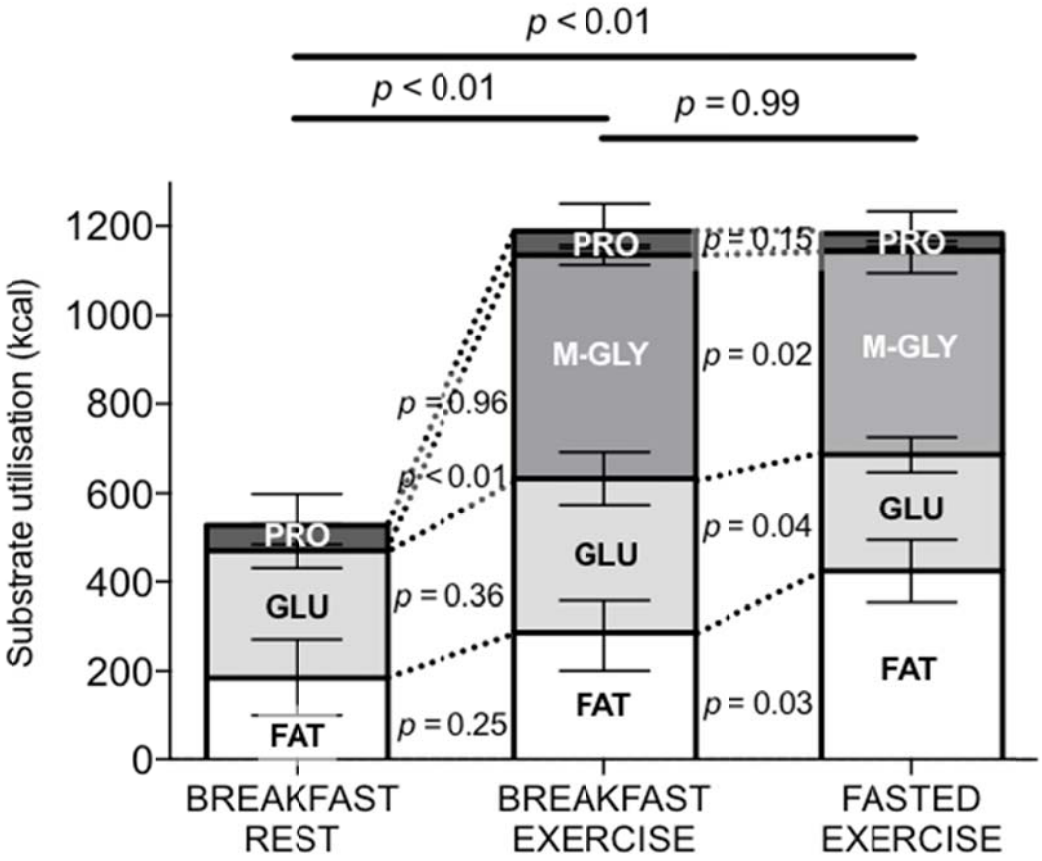

Figure 7. Substrate utilization after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Within-lab substrate utilization was calculated assuming that the plasma glucose disappearance rate was equivalent to plasma glucose utilization. Glycogen utilization during exercise (on breakfast exercise and fasted exercise only) was calculated as total carbohydrate utilization (from indirect calorimetry) minus plasma glucose utilization during exercise. This estimate of muscle glycogen utilization will therefore include the utilization of other non-glucose (e.g. lactate) carbohydrates. GLU = glucose; M-GLY = muscle glycogen; PRO = protein. Data are presented as means ± normalized 95% confidence intervals. *n* = 12 healthy men.

602

DISCUSSION

This is the first study to assess the effect of pre-exercise feeding on postprandial plasma glucose kinetics after exercise. Our data demonstrate that pre-exercise feeding increases plasma glucose disposal during meals consumed after exercise, despite lower insulinemia in this condition. Characterizing glucose flux at meals is important because this determines postprandial glycemia; a predictor of cardiovascular disease risk (47, 48). Previous work has only studied postprandial glucose flux after fasted-state exercise. As most people consume food and perform exercise while still in a postprandial period from a prior meal (13, 23, 55) our results describe the physiological responses to feeding that are more readily applicable to scenarios that are representative of normal daily living. Moreover, our novel data demonstrate that metabolic responses to exercise conducted in an overnight fasted state and to meals that are consumed post-exercise cannot be easily extrapolated to conditions where breakfast has been consumed.

The disposal of plasma glucose (the disappearance rate) into skeletal muscle is elevated after exercise, via insulin-dependent and -independent pathways (25). We observed a higher postprandial plasma glucose disposal rate with breakfast *versus* fasting before exercise. The higher postprandial plasma glucose rate of disposal with pre-exercise breakfast ingestion was apparent despite lower insulinemia in the breakfast-exercise trial. At rest, breakfast consumption improves glucose tolerance and insulin sensitivity at subsequent meals [known as the “second-meal effect” (5, 20, 26)]. Mechanisms likely relate to delayed

626 gastric emptying (19), a potentiation of early phase insulinemia at the second
627 meal (37) and enhanced glucose uptake into muscle due to increased GLUT4
628 trafficking (18). Our findings show that breakfast ingestion (*versus* fasting) prior
629 to exercise enhances subsequent glucose disposal at post-exercise meals in
630 the presence of lower insulinemia, suggesting that the second-meal effect is
631 maintained even if exercise is performed between meals. Whilst the effects of
632 pre-exercise feeding on the metabolic responses during subsequent exercise is
633 well characterised (15, 43), our data therefore provide new insights regarding
634 postprandial glucose metabolism after exercise in the fed *versus* fasted state.
635
636 Molecular insulin-signaling pathways are instrumental mediators of glucose
637 disposal in response to exercise and/or nutrition (11). We therefore determined
638 the activation status of key proteins involved in glucose uptake in skeletal
639 muscle [the primary site of postprandial glucose disposal (14)]. Akt activation
640 (Thr^{308} and Ser^{473} phosphorylation) two hours after OGTT began, was
641 unaffected by prior exercise or prior breakfast ingestion. However, the timing of
642 muscle sampling could be responsible for this result. It is possible that
643 differences in Akt activation between trials may have been apparent earlier in
644 the postprandial period, as peak Akt phosphorylation can be variable, occurring
645 as early as 30 min after an OGTT (35). Thus, despite the lack of a measurable
646 difference, we cannot rule out a role for insulin signaling in the glucose disposal
647 responses we observed. Distal proteins within the insulin signalling pathway can
648 be activated after exercise, without detectable differences in Akt
649 phosphorylation (61). We therefore measured $AS160^{Thr642}$ activation as this

650 phosphorylation site has been previously shown to be activated by both insulin
651 *and* exercise stimulation (58). However, our data show that AS160^{Thr642}
652 phosphorylation was not different two hours post-OGTT with breakfast *versus*
653 fasting before exercise, providing further evidence that differences in early
654 stages of the activation of the insulin signalling pathway were not responsible
655 for the higher plasma glucose disposal rate we observed when breakfast was
656 ingested before exercise.
657
658 AMPK activity also plays a key role in muscle glucose uptake and stimulate
659 GLUT4 translocation (27). The greater post-exercise skeletal muscle AMPK
660 activation with breakfast prior to exercise may have contributed to the higher
661 glucose disposal rate in that trial. This AMPK response seems to be specific to
662 skeletal muscle, as we have previously shown that post-exercise adipose tissue
663 AMPK content is unaffected by pre-exercise feeding (12). The increase in
664 skeletal muscle AMPK activity with pre-exercise feeding that we report in the
665 present study may seem surprising given that the ingestion of large amounts (>
666 200 g) of carbohydrate before *and* during exercise can blunt AMPK signaling in
667 muscle (3, 29). This blunting is partly because low muscle glycogen
668 concentrations stimulate AMPK activity (42). The *modest amount* (65 g) of
669 carbohydrate ingested by participants in our study may explain why we did not
670 observe an elevated AMPK response in our fasted-exercise trial. For example,
671 when smaller carbohydrate doses are ingested before and/or during exercise (~
672 120 g or less) the exercise induced increase in the phosphorylation of AMPK
673 and ACC is not always suppressed compared to when a placebo is ingested (2,

674 36, 58), although in one study this result was apparent despite a suppression of
675 α2-AMPK activity when carbohydrate was ingested (2).
676
677 The heightened AMPK response observed in the current study with breakfast
678 before exercise may be explained by the *type* of carbohydrate ingested before
679 exercise (high-glycemic index) in the breakfast-exercise trial, as this can
680 stimulate muscle glycogen use during exercise, especially when no
681 carbohydrate is ingested during the activity (62). Thus, the high-glycemic index
682 breakfast with a modest carbohydrate content in the present study, may have
683 stimulated muscle glycogen utilization during exercise without supplying
684 sufficient carbohydrate to replace additional glycogen utilization, resulting in
685 lower post-exercise muscle glycogen concentrations with breakfast *versus*
686 fasting before exercise. This could explain the enhanced exercise-induced
687 AMPK response if breakfast was consumed prior to exercise. However, we had
688 insufficient tissue to measure muscle glycogen *concentrations* in all participants,
689 and our data only provide rates of muscle glycogen *utilization* (which was higher
690 with feeding *versus* fasting before exercise). Nevertheless, in the two
691 participants for whom we had sufficient tissue to perform glycogen analyses,
692 these data were in agreement with the tracer-derived calculations of muscle
693 glycogen utilization (data not shown) and AMPK signaling assessed by western
694 blotting. Consistent with this, the present data demonstrated that post-exercise
695 ACC^{Ser79} phosphorylation – as a marker of activation of the AMPK pathway -
696 positively correlated with muscle glycogen utilization during exercise. Thus,
697 taken together our results suggest that the increased post-exercise plasma

698 glucose disposal rates with pre-exercise feeding *versus* fasting may have been
699 mediated through enhanced AMPK signaling, which is itself a consequence of
700 altered fuel use during exercise. AMPK signaling and resulting muscle glycogen
701 concentrations following exercise after breakfast should now be explored.
702

703 The higher plasma glucose disposal rate (despite lower plasma insulin
704 concentrations), that we observed in the breakfast-exercise trial, may be due to
705 differences in GLUT4 trafficking downstream of the signalling proteins we
706 measured. Insulin-stimulated GLUT4 translocation and insulin sensitivity of
707 skeletal muscle are increased *in vitro*, if muscle is pre-treated with insulin,
708 without differences in Akt^{Ser473} or Akt^{Thr308} activation, or the transport activity of
709 GLUT4 (18). This suggests priming of skeletal muscle by prior insulin exposure
710 enhances subsequent insulin action. Pre-treatment with insulin *and* exercise
711 augments this response, possibly because insulin (33) and exercise (28)
712 stimulate GLUT4 translocation from different intracellular stores (8, 49). As
713 such, in the current work, prior breakfast *and* exercise (the multiple stimuli in
714 BE), may have enhanced skeletal muscle GLUT4 translocation during the
715 OGTT. Although technically challenging future work should now quantify GLUT4
716 trafficking with fed *versus* fasted state exercise to confirm this. It is also possible
717 that hepatic glucose disposal accounts for some of the increase in glucose
718 disposal following BE *versus* FE. Similarly to skeletal muscle, insulin-stimulated
719 hepatic glucose uptake is enhanced by prior exposure to insulin in dogs (44,
720 45). Thus, assuming that this response persists in humans and after exercise,

721 priming of the liver by prior breakfast could also contribute to greater glucose
722 disposal during meals consumed post-exercise.

723

724 The increases in post-exercise glucose disposal with prior breakfast
725 consumption were, however, offset by alterations in plasma glucose
726 appearance. As such, postprandial glucose *concentrations* did not differ
727 between the two exercise trials. Postprandial plasma glucose appearance rates
728 are determined by three main factors: 1) the appearance of glucose from the
729 meal; 2) residual appearance of glucose from previous meals; and/or 3) liver
730 glucose output (glycogenolysis or gluconeogenesis). We showed that
731 alterations in the postprandial plasma glucose appearance rate (Ra_{TOTAL}) after
732 exercise with prior breakfast was mostly driven by increased appearance of
733 glucose from the post-exercise OGTT. These alterations in the Ra_{TOTAL} suggest
734 that differences in gut function (i.e. increased intestinal damage or absorptive
735 capacity) and/or splanchnic blood flow altered the Ra_{TOTAL}. Thus, breakfast prior
736 to exercise may alter postprandial glycemia via factors related to intestinal
737 absorption and splanchnic handling of glucose, rather than just glucose
738 metabolism by skeletal muscle.

739

740 There are several potential mechanisms which may explain the differences in
741 glucose appearance rates due to intestinal absorption and/or splanchnic
742 handling of glucose. Plasma I-FABP concentrations are used as a marker of
743 damage to intestinal epithelial cells (60). We noted lower plasma I-FABP
744 concentrations in the breakfast-exercise *versus* fasted-exercise trial, despite

745 increased plasma glucose appearance rates of the orally-ingested glucose
746 post-exercise. It is therefore unlikely that increased intestinal damage was
747 responsible for the higher plasma glucose appearance rates we observed with
748 feeding *versus* fasting before exercise. The better maintenance of splanchnic
749 perfusion during exercise with prior feeding is a likely explanation for this
750 response (15). If splanchnic perfusion was better maintained during exercise
751 with prior feeding, this may have also *directly* facilitated higher OGTT-derived
752 glucose appearance rates in that trial *versus* the fasted-exercise trial. It should
753 be acknowledged however, that this intestinal damage response may be
754 specific to cycling, and could differ with other exercise modalities (e.g. running),
755 within the context of pre-exercise feeding. An alternative mechanism for higher
756 appearance rate of orally-ingested glucose with feeding before exercise could
757 also be that apical glucose transporters were primed by the prior breakfast
758 ingestion (40). Although further underlying mechanism(s) remain unclear, and
759 should therefore be investigated with future work, we showed that a major
760 determinant of post-exercise glycemia (plasma glucose appearance rates) are
761 altered by pre-exercise feeding and that this is unlikely to be explained by
762 increases in intestinal damage.
763
764 Our results show that the metabolic and intramuscular signaling responses to
765 exercise conducted in a fed state cannot be readily inferred from responses
766 observed with exercise in a fasted state. As well as a continual investigation of
767 the mechanisms responsible for differences in postprandial glucose metabolism
768 with altered pre-exercise feeding, future work should study whether the results

769 we observed are apparent with different post-exercise meals (including the co-
770 ingestion of fat and protein). Moreover, if the acute alterations in postprandial
771 metabolism translate into longer-term differences in insulin sensitivity with
772 repeated bouts of exercise in the fed *versus* fasted state, and in overweight and
773 obese populations, should now be a focus for future work.
774
775 To conclude, eating breakfast (*versus* fasting) before exercise increases post-
776 exercise plasma glucose disposal rates but this is offset by increases in
777 appearance rates of (mainly) orally-ingested glucose, a result that does not
778 appear to be explained by a greater intestinal damage response to the exercise.
779 We showed that pre-exercise breakfast consumption lowers insulinema at
780 meals that are consumed post-exercise, providing new evidence that the
781 second meal effect is maintained even when exercise is performed between
782 eating occasions.
783

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789

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995 **TABLES**

Table 1. Participant Characteristics

Age (y)	23 (3)
Stature (cm)	179.8 (4.4)
Body mass (kg)	75.3 (7.9)
Body Mass Index (kg·m^{-2})	23.3 (2.3)
Fat mass (kg)[1]	10.6 (3.7)
Fat Mass Index (kg·m^{-2})[1]	3.26 (1.12)
Body fat (%)[1]	14 (4)
Fat free mass (kg)[1]	65.5 (6.4)
$\dot{V}O_2$peak (L·min^{-1}) *	4.00 (0.72)
$\dot{V}O_2$peak (mL·kg·min^{-1}) *	53 (10)
Peak Power Output (W)	317 (67)
HR_{MAX}, (beats·min^{-1})	189 (10)

Data are presented as means and (standard deviation). $\dot{V}O_2$peak = peak oxygen uptake. *n* = 12. **n* = 11, due to technical difficulties with the breath-by-breath analysis during one participant's preliminary testing. [1] = derived by dual-energy x-ray absorptiometry (DEXA).

Table 2. Peak plasma concentrations, time to peak concentrations, and the time-averaged area under the curve (AUC) for various metabolites and hormones measured during the total within-lab period (300-min) and/or during an oral glucose tolerance test observation (120-min) that was conducted after breakfast followed by rest (Breakfast-Rest), breakfast followed by exercise (Breakfast-Exercise), or extended overnight fasting followed by exercise (Fasted-Exercise).

	Breakfast-Rest	**Breakfast-Exercise**	**Fasted-Exercise**	
Peak glucose conc. (mmol·L^{-1})	10.62 (9.98, 11.25)	9.65 (9.27, 10.03) [a]	9.22 (8.42, 10.45)	F = 4.895, p = 0.03
Time to peak glucose (min)	***36 (24, 47)***	***49 (36, 62)***	***49 (36, 61)***	***F = 2.588, p = 0.12***
Glucose AUC_{TOTAL} (mmol·L^{-1})	6.41 (6.21, 6.60)	6.05 (5.90, 6.15) [a]	6.07 (5.93, 6.27)	F = 6.126, p = 0.01

Peak insulin conc. (pmol·L^{-1})	***286 (231, 341)***	***209 (148, 269)***	***282 (222, 337)***	***F = 3.900, p = 0.07***
Time to peak insulin (min)	38 (26, 49)	56 (47, 65) [a]	43 (33, 54)	F = 4.579, *p* = 0.03
Insulin AUC$_{TOTAL}$ (pmol·L^{-1})	***88 (79, 97)***	***62 (54, 71) [aa]***	***76 (67, 85)***	***F = 9.28, p < 0.01***
NEFA AUC$_{TOTAL}$(mmol·L^{-1})	0.16 (0.12, 0.19)	0.28 (0.24, 0.33)[aa]	0.37 (0.31,0.42)[bb]	F = 24.98, p < 0.01
NEFA AUC$_{OGTT}$ (mmol·L^{-1})	***0.10 (0.06, 0.14)***	***0.24 (0.20, 20.29) [aa]***	***0.26 (0.22, 0.31) [bb]***	***F = 15.51, p < 0.01***
Triglyceride AUC$_{TOTAL}$ (mmol·L^{-1})	0.85 (0.79, 0.91)	0.81 (0.75, 0.86)	0.75 (0.70, 0.79)	F = 4.319, p = 0.03
Triglyceride AUC$_{OGTT}$ (mmol·L^{-1})	***0.84 (0.77, 0.92)***	***0.79 (0.72, 0.86)***	***0.70 (0.64, 0.77)***	***F = 4.677, p = 0.02***
Lactate AUC$_{TOTAL}$ (mmol·L^{-1})	1.05 (0.96, 1.13)	1.19 (1.08, 1.30)	1.16 (1.07, 1.27)	F = 3.645, p = 0.06
Lactate AUC$_{OGTT}$ (mmol·L^{-1})	***1.12 (1.04, 1.20)***	***0.97 (0.89, 1.04) [aa]***	***1.03 (0.96, 1.11)***	***F = 6.479, p = 0.02***
β-hydroxybuturate AUC$_{TOTAL}$ (mmol·L^{-1})	0.03 (0.00, 0.06)	0.08 (0.06, 0.13) [a]	0.14 (0.09, 0.19) [b]	F = 5.936, p = 0.03
I-FABP AUC$_{TOTAL}$ (pg·mL^{-1})	***279 (242, 317)***	***304 (267, 366)***	***415 (353, 476) [b cc]***	***F = 10.87, p < 0.01***

Data are means and (normalized 95% confidence intervals). conc. = concentration; AUC$_{TOTAL}$ = the time-averaged area underneath the concentration-time curve for the total within-lab period (300-min); AUC$_{OGTT}$ = the time-averaged area underneath the concentration-time curve for the oral glucose tolerance test (120-min); I-FABP = intestinal fatty acid binding protein. *n* = 12 healthy men [a] represents a difference between breakfast rest and breakfast exercise, [b] a difference between breakfast rest and fasted exercise and [c] a difference between breakfast exercise and fasted exercise with $p < 0.05$. [aa], [bb] or [cc] is the same difference between trials but with $p < 0.01$.

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LEGENDS TO FIGURES

Figure 1. Protocol schematic. An oral glucose tolerance test was conducted after breakfast followed by rest (BR), breakfast followed by exercise (BE), or extended overnight fasting followed by exercise (FE). Dual stable isotope tracers ([U-^{13}C] glucose ingestion and [6,6-$^{2}H_2$] glucose infusion) and muscle biopsies were combined to assess postprandial plasma glucose kinetics and intramuscular signaling, respectively.

Figure 2. The plasma glucose disposal rate (Rd) (**A**), metabolic clearance rate (**B**), the total rate of plasma glucose appearance (Ra_{TOTAL}) (**C**), the rate of appearance of glucose in plasma from the oral glucose tolerance test (Ra_{OGTT}) (**D**), and the plasma enrichments of [$^{2}H_2$]-glucose (**E**) and [^{13}C]-glucose (**F**) before and during an oral glucose tolerance test that was conducted after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Data are means ± normalized 95% confidence intervals. *n* = 12 healthy men. a = difference between breakfast rest *versus* breakfast exercise; b = breakfast rest *versus* fasted exercise and c = breakfast exercise *versus* fasted exercise with $p < 0.05$.

Figure 3. Plasma glucose (**A**), plasma insulin (**B**), plasma non-esterified fatty acids (NEFA; **C**), plasma β-hydroxybuturate (**D**) plasma triglyceride (**E**), and plasma intestinal fatty acid binding protein (IFAB-P; **F**) concentrations before and during an oral glucose tolerance test that was conducted after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Data are means ± normalized 95% confidence intervals. *n* = 12 healthy men. a = difference between breakfast rest *versus* breakfast exercise; b = breakfast rest *versus* fasted exercise and c = breakfast exercise *versus* fasted exercise with $p < 0.05$.

Figure 4. The time-averaged (120-min) plasma glucose (**A**) and plasma insulin (**B**) incremental area under the curves (iAUC) and the Matsuda insulin sensitivity index (**C**; $ISI_{MATSUDA}$) for an oral glucose tolerance test conducted after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Data are means ± normalized 95% confidence intervals, with individual data shown as grey lines. *n* = 12 healthy men.

Figure 5. The phosphorylation of 5' AMP-activated protein kinase (**A**; phospho AMPK^{Thr172}, ratio p-AMPK to total-AMPK) and the phosphorylation of acetyl-CoA carboxylase (**B**; phospho ACC^{Ser79}, ratio p-ACC to total-ACC) before (PRE-OGTT) and after (POST-OGTT) an oral glucose tolerance test that was

conducted after breakfast followed by rest (BREAKFAST-REST [BR]), breakfast followed by exercise (BREAKFAST-EX [BE]), or extended overnight fasting followed by exercise (FASTED-EX [FE]). All samples were taken from the *vastus lateralis*. Samples were normalized to the baseline muscle sample for each participant (collected in the resting fasted state on the first main trial for each participant). Data are means ± normalized 95% confidence intervals. *n* = 9 healthy men.

Figure 6. The phosphorylation of Akt2 (**A**; phospho Akt^{Ser473}, ratio p-Akt^{Ser473} to total-Akt2 and **B**; phosphor Akt^{Thr308}, ratio p-Akt^{Thr308} to total-Akt2) and the phosphorylation of AS160 (**C**; $AS160^{Thr642}$, ratio p-$AS160^{Thr642}$ to total-AS160) before (PRE-OGTT) and after (POST-OGTT) an oral glucose tolerance test that was conducted after breakfast followed by rest (BREAKFAST-REST [BR]), breakfast followed by exercise (BREAKFAST-EX [BE]), or extended overnight fasting followed by exercise (FASTED-EX [FE]). All samples were taken from the *vastus lateralis*. Samples were normalized to the baseline muscle sample for each participant (collected in the resting fasted state on the first main trial for each participant). Data are means ± normalized 95% confidence intervals. *n* = 9 healthy men.

Figure 7. Within-lab (300-min) substrate utilization after breakfast followed by rest (BREAKFAST-REST), breakfast followed by exercise (BREAKFAST-EX), or extended overnight fasting followed by exercise (FASTED-EX). Within-lab substrate utilization was calculated assuming that the plasma glucose disappearance rate was equivalent to plasma glucose utilization. Glycogen utilization during exercise (on breakfast exercise and fasted exercise only) was calculated as total carbohydrate utilization (from indirect calorimetry) minus plasma glucose utilization during exercise. This estimate of muscle glycogen utilization will therefore include the utilization of other non-glucose (e.g. lactate) carbohydrates. GLU = glucose; M-GLY = muscle glycogen; PRO = protein; CHO = carbohydrate. Data are presented as means ± 95% confidence intervals. *n* = 12 healthy men.