Post-exercise skeletal muscle signaling responses to moderate- to high-intensity steady-state exercise in the fed or fasted state

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Running Head: Skeletal muscle signaling following fed and fasted exercise

Abstract:

Exercise performed in the fasted state acutely increases fatty acid availability and utilization. Furthermore, activation of energy-sensing pathways and fatty acid metabolic genes can be augmented by fasting and fasted exercise. However, whether a similar effect occurs at higher exercise intensities remains poorly understood. This study aimed to assess the effect of fed and fasted exercise upon post-exercise signaling and mRNA responses during moderate- to high-intensity steady-state exercise. Eight male participants (age: 25 ± 2 y, $\dot{V}O_{2peak}$: 47.9 ± 3.8 $mL \cdot kg^{-1} \cdot min^{-1}$) performed one hour of cycling at 70% W_{max} in the fasted (FAST) state or two hours following ingestion of a carbohydrate-rich mixed-macronutrient breakfast (FED). Muscle biopsies were collected pre-, immediately and three hours post-exercise from the *medial vastus lateralis*, whilst venous blood samples were collected throughout the trial. Plasma NEFA and glycerol concentrations were elevated during FAST compared to FED, although substrate utilization during exercise was similar. $AMPK^{Thr172}$ phosphorylation was ~2.5-fold elevated post-exercise in both trials and was significantly augmented by ~30% during FAST. $CREB^{Ser133}$ phosphorylation was elevated ~2-fold during FAST, although $CREB^{Ser133}$ phosphorylation acutely decreased by ~50% immediately post-exercise. mRNA expression of *PDK4* was ~3-4-fold augmented by exercise and ~2-fold elevated throughout FAST, whilst expression of *PPARGC1A* mRNA was similarly activated (~10-fold) by exercise in both FED and FAST. In summary, performing moderate- to high-intensity steady-state exercise in the fasted state increases systemic lipid availability, elevates phosphorylation of $AMPK^{Thr172}$ and $CREB^{Ser133}$, and augments *PDK4* mRNA expression without corresponding increases in whole body fat oxidation and the mRNA expression of *PPARGC1A*.

Key words:

AMPK, PGC1α, substrate utilization, metabolism, breakfast.

Introduction

During endurance exercise, transient perturbations in the cellular and extracellular milieu activate a number of intracellular signaling cascades thought to drive the initial adaptive response to exercise (22). Of note, the exercise-induced activation of AMP-activated protein kinase (AMPK), p38 mitogen activated protein kinase (p38 MAPK) and calcium/calmodulin dependent kinase II (CAMKII) are thought to be central to this response given they activate downstream transcription factors and transcriptional co-activators to initiate mitochondrial biogenesis (4, 5, 13, 21, 30, 62). With regularly repeated exercise bouts, transient upregulation of mRNA and subsequent protein translation manifest as cellular adaptations such as mitochondrial biogenesis (42). Thus the optimization of the acute post-exercise adaptive response within skeletal muscle holds therapeutic potential.

Performing exercise in the fasted state has the potential to produce favorable metabolic adaptations over and above fed exercise training (53). For example, moderate-intensity exercise, performed in the fasted state, increases fatty acid availability and oxidation compared to exercise combined with carbohydrate ingestion (1, 2, 10, 11, 16). At the cellular level, this response may be mediated in part via an AMPK-dependent mechanism, as skeletal muscle AMPKα2 activity is enhanced during moderate-intensity fasted exercise compared to glucose ingestion (2). Despite this, the optimal integration of nutrition and exercise to augment adaptive signaling responses remains elusive. For example, AMPK and other exercise-sensitive signaling molecules can be further activated at more vigorous exercise intensities (e.g. ~80% $\dot{V}O_{2peak}$) (21). However, it remains unclear whether this represents a maximal signaling response to moderate- to high-intensity steady-state exercise or whether

superimposing fasting-induced nutritional stress can further enhance this. Furthermore, fasting *per se* activates additional exercise-sensitive signaling pathways within skeletal muscle including p38 MAPK (26), although this is not apparent in human skeletal muscle (19), and CREB (57), which remain to be studied in a fed versus fasted exercise model in humans. Thus the potential additive or synergistic effect of combining fasting with exercise on signaling pathways within human skeletal muscle remains incompletely understood. Importantly, assessing the acute activation of signaling processes within skeletal muscle does provide pertinent information regarding the adaptive protein and mitochondrial biogenic response (7, 42). However, acute signaling responses do not always predict chronic adaptations (12, 25).

The purpose of this study was to compare the effect of performing moderate- to high-intensity steady-state exercise in the fasted state or following the consumption of a mixed-macronutrient breakfast on post-exercise signaling and mRNA expression in skeletal muscle. It was hypothesized that fasted exercise would result in an elevated *PPARGC1A* and *PDK4* mRNA response associated with augmented AMPK^{Thr172} and CREB^{Ser133} phosphorylation.

Materials and Methods

Participants

Eight recreationally active males (mean ± SD: age, 25 ± 2 years; body mass, 74.6 ± 5.2 kg; peak oxygen uptake ($\dot{V}O_{2peak}$), 47.9 ± 3.8 mL·kg^{-1}·min^{-1}; maximal aerobic power (W_{max}), 272 ± 33 W) were recruited to participate. Participants were fully informed of the study procedures and their right to withdraw before providing written

consent to participate. The study was pre-approved by the National Health Service Research Ethics Committee, Black Country, West Midlands, UK.

Pre-testing

After measuring height (Seca 220, Seca, Birmingham, UK) and body mass (Champ II, OHAUS, Griefensee, Switzerland) participants performed a graded exercise test to exhaustion on a cycle ergometer (Lode Excalibur, Groningen, Netherlands). The test began with a five-minute warm-up at 100 W with power increasing by 35 W every three minutes thereafter. Respiratory variables were measured continuously during exercise using a breath-by-breath metabolic cart (Oxycon Pro, Jaeger, CareFusion, Germany), heart rate was monitored throughout (RCX5, Polar Electro Oy, Kempele, Finland) and ratings of perceived exertion (RPE) were determined using a 6-20 Borg scale during the final 15 seconds of each 3-minute stage (6). $\dot{V}O_{2peak}$ was determined as the highest rolling 30-second average and was stated as being achieved if the following criteria were met: i) heart rate within 10 beats/min of age-predicted maximum and ii) respiratory exchange ratio (RER) > 1.1, or iii) plateau of oxygen consumption despite increasing work-rate. W_{max} was determined as work rate at the last completed stage plus the fraction of time spent in the final non-completed stage multiplied by the increment in work rate (35W).

Experimental trials

Participants performed two experimental trials in a randomized, counter-balanced, crossover design. By necessity of the design (i.e. food intake) it was not possible to blind participants or experimenters. Participants refrained from alcohol for 72 h, caffeine for 24 h and exercise for 48 h prior to each experimental trial. Prior to each

experimental trial, participants were provided with a pre-prepared standardized three-day diet (energy contribution: 61% carbohydrate, 18% fat and 21% protein) matched to individual energy intake (mean ± SD: 2688 ± 450 kcal·day^{-1}) determined by a three-day weighed food diary. While this approach allowed for energy intake to be tailored to the individual, energy intake was likely to be under-reported (36) and therefore under-prescribed.

Participants arrived at the laboratory at ~8 am following an ~12-hour overnight fast. Upon arrival, participants rested in the supine position for approximately ten minutes before a cannula was inserted into an antecubital forearm vein and a baseline venous blood sample was collected. Participants were then provided with a mixed-macronutrient breakfast (FED) (0.9 g·kg^{-1} body mass (BM) of corn flakes cereal, 3.9 mL·kg^{-1} BM of semi-skimmed milk, 1.1 g·kg^{-1} BM of toasted wholemeal bread, 0.3 g·kg^{-1} BM of strawberry jam and 3.2 mL·kg^{-1} BM of orange juice (Sainsburys, UK); energy intake: 710 ± 49 kcal; macronutrients: 1.75 g·kg^{-1} BM carbohydrate (of which 0.66 g·kg^{-1} BM is sugar), 0.1 g·kg^{-1} BM fat and 0.35 g·kg^{-1} BM protein; energy contribution: 75% carbohydrate, 10% fat and 15% protein), which they consumed within the first 15 minutes of the trial, or remained in the fasted state (FAST). The macronutrient composition of the breakfast reflected population trends for relatively greater consumptions of carbohydrates, and thus lowers fat and protein ingestion, at breakfast time (3, 59). Participants rested for two hours prior to providing a pre-exercise skeletal muscle biopsy from the *medial vastus lateralis*. Participants then cycled for one-hour at 70% W_{max} before a second skeletal muscle biopsy was taken immediately post-exercise (completed within two minutes of exercise cessation). Participants then rested in a supine position prior to a third skeletal muscle biopsy

153 being obtained three-hours post-exercise. In order to minimize discomfort for the
154 participant the first two skeletal muscle biopsies were taken from one leg with the
155 third biopsy taken from the contralateral leg. Biopsies were taken from the opposite
156 leg in the second trial. The leg receiving the initial biopsy was randomized and
157 counterbalanced within the study. A new incision was made for each biopsy at least 2
158 cm from the previous site. Venous blood was collected throughout rest periods and
159 during exercise. Respiratory variables were measured pre-exercise and at 15-minute
160 intervals throughout exercise, heart rate was monitored continuously throughout
161 exercise and RPE was determined at 15-minute intervals throughout exercise.
162 Carbohydrate and fat oxidation were calculated from $\dot{V}O_2$ and $\dot{V}CO_2$ using the
163 moderate-high exercise intensities equation of Jeukendrup and Wallis (31) during
164 exercise and Frayn (23) at rest. Participants were allowed to drink water *ad libitum*
165 during rest and exercise periods of trial one, with water intake matched during trial
166 two to that consumed during each period of trial one (fluid intake during exercise: 618
167 ± 341 mL; post-exercise fluid intake: 635 ± 343 mL).
168
169 *Muscle biopsies*
170 Muscle biopsies were obtained from separate incision sites on the *medial vastus*
171 *lateralis* under local anaesthesia (1% lidocaine; B. Braun, Melsungen, Germany) by a
172 Bergström needle adapted with suction. Muscle was rapidly blotted to remove excess
173 blood and flash frozen in liquid nitrogen. Muscle was powdered using a Cellcrusher
174 tissue pulverizer on dry ice and stored at -80°C prior to analysis.
175
176 *Immunoblotting*

Tissue was homogenized in a 10-fold mass excess of ice-cold sucrose lysis buffer (50 mM Tris, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM $Na_4P_2O_7$-$10H_2O$, 270 mM sucrose, 1 M Triton-X, 25 mM β-glycerophosphate, 1 μM Trichostatin A, 10 mM Nicatinamide, 1mM 1,4-Dithiothreitol, 1% Phosphatase Inhibitor Cocktail 2; Sigma, 1% Sigma Phosphatase Inhibitor Cocktail 2; Sigma, 4.8% cOmplete Mini Protease Inhibitor Cocktail; Roche) by shaking in a FastPrep 24 5G (MP Biomedicals) at 6.0 $m \cdot s^{-1}$ for 80 s and centrifuging at 4°C and 8000 g for 10 minutes to remove insoluble material. Protein concentrations were determined by the DC protein assay (Bio-Rad, Hercules, California, USA). Samples were boiled at 97°C for 5 min in laemmli sample buffer and an equal volume of protein (20-50 μg) was separated by SDS-PAGE on 8 - 12.5% gels at a constant current of 23 mA per gel. Proteins were transferred on to BioTrace NT nitrocellulose membranes (Pall Life Sciences, Pensacola, Florida, USA) via wet transfer at 100 V for one hour. Membranes were then stained with Ponceau S (Sigma-Aldrich, Gillingham, UK) and imaged to check for even loading. Membranes were blocked in 3% dry-milk in tris-buffered saline with tween (TBST) for one hour before being incubated in primary antibody overnight at 4°C. Membranes were washed in TBST three times prior to incubation in appropriate horse radish peroxidase (HRP)-conjugated secondary antibody at room temperature for one hour. Membranes were then washed in TBST three times prior to antibody detection via enhanced chemiluminescence HRP substrate detection kit (Millipore, Watford, UK). Imaging and band quantification were undertaken using a G:Box Chemi-XR5 (Syngene, Cambridge, UK).

Antibodies

All primary antibodies were used at a concentration of 1:1000 in TBST unless otherwise stated. Antibodies: AMPKα (2603), p-AMPK^{Thr172} (2535), p-ACC^{Ser79} (3661), CAMKII (3362), p-CAMKII^{Thr268} (12716), CREB (1:500; 9197), p-CREB^{Ser133} (1:500; 9191), GAPDH (1:5000; 2118), p38 (9212), p-p38$^{Thr180/Tyr182}$ (4511) were purchased from Cell Signaling Technology; ACC (05-1098) was purchased from Merck Millipore. Secondary antibodies were used at a concentration of 1:10000 in TBST. Anti-rabbit (7074) and anti-mouse (7076) antibodies were from Cell Signaling Technology

Real-time RT-qPCR

RNA was extracted from ~20 mg of muscle by homogenising in 1 mL of Tri reagent (Sigma Aldrich, Gillingham, UK) using an IKA T10 basic ULTRA-TURRAX homogeniser (IKA, Oxford, UK). Phase separation was achieved by addition of 200 µL of chloroform and centrifugation at 12000 g for 15 minutes. The RNA-containing supernatant was removed and mixed with an equal volume of 2-propanol. RNA was purified on Reliaprep spin columns (Promega, Madison, Wisconsin, USA) using the manufacturers instructions, which includes a DNase treatment step. RNA concentrations were determined using the LVis function of the FLUOstar Omega microplate reader (BMG Labtech, Aylesbury, UK). RNA was diluted to 30 ng/µL and reverse transcribed to cDNA in 20 uL volumes using the nanoScript 2 RT kit and oligo(dT) primers (Primerdesign, Southampton, UK) as per the manufacturers instructions. RT-qPCR analysis of mRNA content was performed in triplicate by using Primerdesign custom designed primers for *PPARGC1A* (Accession number: NM_002612.3; Forward primer: 5'-TTGCTAAACGACTCCGAGAAC-3'; Reverse primer: 5'-GACCCAAACATCATACCCCAAT-3'), *PDK4* (Accession number:

NM_013261; Forward primer: 5'-GAGGGACACTCAGGACACTTTAC-3'; Reverse primer: 5'-TGGAGGAAACAAGGGTTCACAC-3') and commercially available *GAPDH* (Primerdesign) and Precision plus qPCR Mastermix with low ROX and SYBR (Primerdesign) on a QuantStudio3 Real-Time PCR System (Applied Biosystems, Thermo Fisher, UK). The qPCR reaction was run as per the manufacturers instructions (Primerdesign) and followed by a melt curve (Applied Biosystems) to ascertain specificity. 2-4 ng of cDNA was added to each well in a 20 uL reaction volume. qPCR results were analysed using Experiment Manager (Thermo Fisher). mRNA expression was expressed relative to the expression in the pre-exercise sample during FED for each individual using the $2^{-\Delta\Delta CQ}$ method (35) with the C_q value for *GAPDH* used as an internal control. Statistical analysis was performed on the $2^{-\Delta\Delta CQ}$ transformed data.

Blood analyses

Blood samples were collected into tubes containing ethylenediaminetetraacetic acid (EDTA; BD, Oxford, UK) for the collection of plasma and serum separator tubes for the collection of serum. EDTA plasma samples were placed immediately upon ice while serum samples were allowed to clot at room temperature for 25 minutes prior to both samples undergoing centrifugation at 1600 g at 4°C for 10 minutes before collection of plasma or serum from the supernatant. Samples were frozen at -80°C until further analysis. Plasma samples were subsequently analyzed on an autoanalyzer (iLAB650, Instrumentation Laboratory, Bedford, MA, USA) for glucose, lactate, non-esterified fatty acid (NEFA) and glycerol (Randox Laboratories, County Antrim, UK) using commercially available kits. Serum samples were analyzed by commercially

250 available ELISA kits for insulin (Ultrasensitive insulin ELISA, Mercodia, Uppsala,
251 SWE) and cortisol (Cortisol ELISA, IBL International, Hamburg, GER).
252
253 *Statistics*
254 Two-way repeated measures ANOVAs assessed effects of time, treatment and
255 time*treatment interaction effects for all time-course data. Ryan-Holm-Bonferroni
256 multiple comparison corrections were applied *post-hoc* where applicable. Total area
257 under the curve (tAUC) was determined for blood metabolites using the trapezoid
258 method. Differences in tAUC for blood metabolites and means for exercising $\dot{V}O_2$,
259 substrate utilization, heart rate and RPE were assessed using repeated-measures t-
260 tests. Due to issues with blood sampling from one participant, blood analyses are
261 performed with a sample size of 7. All statistics were performed using the Statistical
262 Package for the Social Sciences (SPSS) version 22.0. Data are presented as means
263 with 95% confidence intervals. Statistical significance was accepted as $p \leq 0.05$.
264
265 **Results**
266 *Substrate availability and utilization*
267 Plasma NEFA and glycerol concentrations were elevated during FAST (Figures 1A &
268 1B), indicative of elevated lipolysis and fatty acid availability. Plasma NEFA
269 concentration displayed main effects for treatment ($p = 0.004$), time ($p = 0.001$) and a
270 treatment*time interaction effect ($p < 0.001$). Plasma NEFA was significantly
271 elevated in FAST compared to FED during exercise (135-165 minutes) and at twenty
272 minutes into recovery (200 minutes; $p < 0.05$). tAUC for plasma NEFA across the
273 experimental period was significantly elevated in FAST ($p = 0.011$). Plasma glycerol
274 concentration showed main effects for treatment ($p = 0.007$), time ($p < 0.001$) and a

treatment*time interaction effect (p = 0.015). Plasma glycerol was significantly elevated during exercise (135-180 minutes) in FAST compared to FED ($p < 0.05$). tAUC for plasma glycerol across the experimental period was significantly higher in FAST (p = 0.006). Plasma glucose displayed a treatment*time interaction effect (p = 0.024), whereby plasma glucose was higher in FAST immediately prior to exercise (120 minutes; Figure 1C). However, a lack of treatment effect (p = 0.866) and no difference in tAUC (p = 0.942) shows plasma glucose was similar when the whole trial is considered. Plasma lactate displayed a significant effect for time ($p < 0.001$), increasing above baseline during exercise (135-180 minutes) and remained so during the first 40 minutes of recovery (180-220 minutes; $p < 0.05$; Figure 1D). No between-treatment differences were apparent for plasma lactate (main effect of treatment; p = 0.774, interaction; p = 0.568, tAUC; p = 0.548). Serum insulin displayed significant effects for treatment ($p < 0.001$), time ($p < 0.001$) and an interaction effect ($p < 0.001$). Serum insulin was elevated above FAST from 20 minutes post-breakfast to immediately pre-exercise (120 minutes) in the FED trial ($p < 0.05$). Serum cortisol displayed a main effect of time (p = 0.001) but no treatment effect (p = 0.277). There was a trend towards an interaction effect (p = 0.069) for serum cortisol. Carbohydrate and fat oxidation during exercise were similar between trials (Table 1). $\dot{V}CO_2$ was significantly lower during FAST exercise (p = 0.045). $\dot{V}O_2$, RER, heart rate and RPE did not differ during exercise between trials (Table 1).

Skeletal muscle signaling

Exercise increased the phosphorylation of $AMPK^{Thr172}$ (main effect of time; p = 0.002); increasing ~2.5-fold immediately post-exercise (p = 0.006) and remaining ~1.5-fold above baseline three hours post-exercise (p = 0.006 vs pre-exercise; p =

0.005 vs immediately post-exercise; Figure 2A). Furthermore, AMPK^{Thr172} phosphorylation was elevated during the FAST trial (by ~30% at pre- and immediately post-exercise time points), displaying a main effect for treatment (p = 0.048) and a trend towards a treatment*time interaction effect (p = 0.067). Phosphorylation of ACC^{Ser79} (Figure 2B) increased ~9-fold immediately post-exercise and remained ~2-fold elevated 3-h post-exercise (main effect of time; p = 0.005), however unlike AMPK there was no effect of treatment (p = 0.165) or a treatment*time interaction (p = 0.136).

FAST also induced an ~2-fold elevation in the phosphorylation of CREB$^{Ser\ 133}$ (main effect of treatment; p = 0.034; Figure 2C). CREB^{Ser133} phosphorylation also displayed a main effect for time (p = 0.002); decreasing in phosphorylation by ~50% immediately post-exercise (p = 0.001) before tending to increase above baseline at 3h post-exercise (p = 0.086 vs pre-exercise; p = 0.001 vs immediately post-exercise). No treatment*time interaction effect was apparent (p = 0.113).

Neither p38 MAPK$^{Thr180/Tyr182}$ (main effect of treatment; p = 0.534, time; p = 0.240, interaction; p = 0.325) or CAMKII^{Thr286} (main effect of treatment; p = 0.176, time; p = 0.573, interaction; p = 0.722) phosphorylation were significantly altered by exercise or feeding, which both displayed large inter-individual variance in post-exercise responses (Figures 2D & 2E).

Acute mRNA response

PPARGC1A mRNA expression increased ~10-fold three hours after exercise (main effect of time; p = 0.001; p = 0.001 vs pre-exercise; p = 0.001 vs immediately post-

exercise, Figure 3A). *PPARGC1A* mRNA expression was similar in FED and FAST trials (main effect of treatment; $p = 0.248$). There was no treatment*time interaction effect ($p = 0.237$).

Expression of *PDK4* increased with time (main effect of time; $p < 0.001$) and was elevated by ~2 - 3.5-fold throughout the FAST trial (main effect of treatment; $p = 0.003$, Figure 3B). There was a trend for a treatment*time interaction effect ($p = 0.083$), whereby at the immediately post-exercise time point *PDK4* expression increased ~2-fold from pre-exercise values only in the FAST trial (FAST immediately post-exercise vs FAST pre-exercise; $p = 0.035$, FED immediately post-exercise vs FED pre-exercise, $p = 0.153$). Furthermore, *PDK4* expression was higher in the FAST trial compared to the FED trial three hours post-exercise (~8-fold vs. ~3-fold above FED pre-exercise values, respectively; $p = 0.066$).

Discussion

Our results build on previous studies (2, 10, 11, 27) to demonstrate that moderate- to high-intensity steady-state endurance exercise (70% W_{max}) performed in the fasted state increases fatty acid availability, augments $AMPK^{Thr172}$ phosphorylation and increases *PDK4* mRNA expression compared to exercise performed following the ingestion of a standard breakfast. Furthermore, while endurance exercise acutely decreased phosphorylation of $CREB^{Ser133}$, fasting increased the phosphorylation of this activation site. However, despite elevated phosphorylation of AMPK and the transcription factor CREB during fasted exercise, the exercise-induced increases in ACC^{Ser79} phosphorylation, *PPARGC1A* mRNA and whole-body fat oxidation were similar between treatments.

FAST elevated AMPK^{Thr172} phosphorylation throughout the experimental period. Furthermore, a trend (p = 0.067) towards an interaction effect indicates that the exercise-activated AMPK^{Thr172} phosphorylation was likely augmented during fasted exercise. This supports previous research finding elevated AMPKα activity or AMPK^{Thr172} phosphorylation following moderate-intensity or sprint exercise performed in the fasted state compared to when glucose is consumed (2, 27). However, this is not a universal finding. Similar post-exercise AMPK^{Thr172} phosphorylation following fed and fasted exercise has been reported (2, 16, 33, 47, 52), while elevated AMPK^{Thr172} phosphorylation following fed exercise can also be apparent (20). It is unclear why these differences occur, although some of the discrepancies may be due to the amount of carbohydrate ingested in each study. For example, the breakfast provided in the study of Edinburgh *et al* (20), where AMPK^{Thr172} phosphorylation is augmented in the fed state, included only 65g of carbohydrate. However, the total carbohydrate ingestion in the current study (~130 g) was similar to studies that have shown no effect of fasting on exercise-induced AMPK^{Thr172} phosphorylation (2, 33), although the pattern of ingestion did differ (i.e. one large dose versus several smaller doses throughout exercise, respectively). Ingestion of a carbohydrate-rich breakfast, albeit one providing more carbohydrate than in the current study, can increase skeletal muscle glycogen content within several hours (9). Thus in the current study, unlike when carbohydrate drinks are provided throughout exercise, exercise may have been commenced with differing levels of skeletal muscle glycogen, which has the potential to influence AMPK phosphorylation and activity within skeletal muscle (5, 32, 43, 50, 61, 63). Furthermore, elevated AMPK^{Thr172} phosphorylation during FAST could be a result of

increased allosteric activation by AMP (17, 26, 39, 60). Indeed, skeletal muscle AMP and the AMP/ATP ratio is elevated during exercise performed in the fasted compared to the fed state (33). Nonetheless, it remains inconclusive as to why AMPK phosphorylation is likely enhanced during FAST in the present study. Additionally, despite elevated $AMPK^{Thr172}$ phosphorylation, no difference was apparent in ACC^{Ser79} phosphorylation, a downstream substrate of AMPK. Thus whether fasting influenced AMPK activity remains unclear.

Consistent with previous literature, fasting and fasted exercise augmented the expression of *PDK4* mRNA (10, 11, 19, 45, 48, 57, 58). However, conversely to continuous glucose ingestion throughout exercise and recovery (10, 11), ingestion of a standard carbohydrate-rich breakfast two-hours prior to exercise did not completely ablate the exercise-induced expression of *PDK4* mRNA. Given that the total carbohydrate and energy intake were similar between this study and those of Civitarese et al (10) and Cluberton et al (11), this suggests that the timing and frequency of ingestion and/or the type of carbohydrate ingested (i.e. complex starches versus simple sugars) may be important in the regulation of *PDK4* mRNA expression. Substrate availability is known to play an important role in skeletal muscle *PDK4* mRNA expression (44). In the current study, elevated NEFA availability during FAST may explain activation of *PDK4* mRNA immediately post-exercise (i.e. during exercise) in the FAST trial only, especially as glycogen concentrations are likely to be similar between treatments (2, 16, 19, 37, 52). Furthermore, elevating circulating NEFA post-exercise in both trials could explain why *PDK4* mRNA expression is elevated post-exercise following fed exercise in the current study and not when NEFA remains suppressed by continued carbohydrate supplementation (10, 11).

Mechanistically, augmented *PDK4* mRNA expression during FAST may be mediated by elevated PPARα activation known to be responsive to exercise, NEFA concentration and energy-stress (40, 43).

Elevated activation of AMPK and PDK4 during fasting has the potential to acutely influence substrate metabolism. AMPK phosphorylates ACC thereby augmenting fatty acid oxidation (28), while PDK4 phosphorylates PDH-E1α, inactivating the pyruvate dehydrogenase complex (PDC), and thereby inhibiting the decarboxylation of pyruvate to acetyl-CoA and, thus, carbohydrate oxidation (44). However, substrate utilization during exercise was similar between FED and FAST, despite elevated NEFA availability during FAST. This is likely due to the intensity of exercise dictating reliance upon carbohydrate oxidation and a sufficient endogenous supply of carbohydrate, i.e. muscle and liver glycogen, in the FAST trial. Indeed, plasma glucose concentration was unaffected by FAST, while phosphorylation of ACC^{Ser79} was also similar between treatments. Despite this, it cannot be ruled out that differences in substrate oxidation may arise if the exercise duration was extended, although this would likely require a reduction in exercise intensity. Overall this supports findings from a recent meta-analysis in which it was determined that fat oxidation was similar between fed and fasted aerobic exercise performed above 70% $\dot{V}O_{2max}$ (54). Nonetheless, elevated plasma NEFA and glycerol concentrations, despite similar rates of fat oxidation, indicate that increased lipolysis during fasted exercise is determined at the adipose tissue, independently of demand from the exercising musculature (14). Indeed, fasted exercise upregulates the expression of lipolytic genes adipose triglyceride lipase (*PNPLA2*) and hormone sensitive lipase (*LIPE*) in adipose tissue of overweight males (8). Furthermore, elevated circulating insulin prior to

exercise, and trends for elevated insulin during the first 45 minutes of exercise, in the FED trial would contribute to the suppression of lipolysis (14, 18, 51) and the reduced circulating NEFA and glycerol apparent following breakfast consumption.

Metabolic adaptations within skeletal muscle are regulated by transcription factors and co-factors, including CREB and PGC1α. Elevated phosphorylation of $CREB^{Ser133}$, purported to increase the transcriptional activity of CREB (24), has been reported within skeletal muscle in the context of fasting (57) and exercise (21, 46). Thus it was examined here whether an additive or synergistic effect of fasted exercise may be apparent on $CREB^{Ser133}$ phosphorylation. Compared to FED, $CREB^{Ser133}$ phosphorylation was elevated throughout the trial during FAST. Whilst, similarly as reported by Egan et al (21), exercise reduced phosphorylation of $CREB^{Ser133}$ immediately post-exercise and tended to increase above baseline levels by three-hours post-exercise. However, this is certainly not a universal finding with increased $CREB^{Ser133}$ phosphorylation (46) or no change in phosphorylation (55, 56) also apparent immediately post-exercise. This variation in results could be due to methodological differences in exercise modality and intensity. Indeed, $CREB^{Ser133}$ phosphorylation decreases to a larger extent following cycling at 80% $\dot{V}O_{2peak}$ compared to at 40% $\dot{V}O_{2peak}$ (21). Furthermore, the metabolic stress induced from single-legged exercise (55, 56) and two-legged cycling (21, 46) is likely to be different. Interestingly, during unilateral exercise $CREB^{Ser133}$ phosphorylation can be elevated in the non-exercising contralateral leg whilst remaining unchanged in the exercising leg (55). This suggests that while exercise-induced systemic factors may activate skeletal muscle CREB, local contraction-mediated factors may oppose this

449 and even cause dephosphorylation if the intensity is sufficient (21). Overall it remains
450 unclear why $CREB^{Ser133}$ phosphorylation may decrease during exercise.
451
452 Despite elevated phosphorylation of the upstream kinase AMPK and transcription
453 factor CREB, *PPARGC1A* mRNA expression was similar between FED and FAST.
454 This corroborates previous data finding similar *PPARGC1A* mRNA expression
455 following exercise performed in the fasted versus glucose-supplemented state (10,
456 11). Interestingly, this differs from the glycogen-depleted state, where basal and
457 exercise-induced *PPARGC1A* mRNA expression is augmented (5). This indicates that
458 greater metabolic stress is apparent during glycogen-depleted exercise rather than
459 fasting and fasted-exercise. As *PPARGC1A* mRNA expression is, to a degree, self-
460 regulated (29) this data suggests that PGC1α co-transcriptional activity may be
461 similar when exercise is performed in the fed or fasted states. It is intriguing that this
462 is the case despite elevated AMPK and CREB phosphorylation in the fasted state. It
463 could be speculated that the degree of additional AMPK and CREB phosphorylation
464 in FAST is not large enough to augment *PPARGC1A* mRNA expression. Another
465 explanation may be that PGC1α integrates a large number of signals from many
466 converging pathways and as such differential activation of the AMPK pathway
467 without activation of other pathways, e.g. p38 MAPK and CAMKII, is insufficient to
468 alter PGC1α co-transcriptional activity.
469
470 Performing exercise training in the fasted compared to carbohydrate-supplemented
471 state augments training-induced increases in fatty acid metabolism during exercise
472 and the content and activity of fatty acid metabolic proteins (15, 53). Indeed, our
473 acute data showing elevated fatty acid availability and augmented *PDK4* mRNA

expression suggests that similar results should be apparent when fasted exercise is compared to the ingestion of a mixed macronutrient breakfast. Regarding mitochondrial biogenic responses to fed vs fasted endurance exercise training the evidence is less consistent. Fasting has been demonstrated to augment citrate synthase activity (49, 53) but similar training-induced increases in succinate dehydrogenase activity has been shown in the fed and fasted state (15). PGC1α is a key regulator of mitochondrial biogenesis within skeletal muscle (34, 38, 41). Thus our evidence of similar *PPARGC1A* mRNA expression may suggest that the mitochondrial biogenic responses to fed and fasted exercise training at a moderate-high exercise intensity may be similar. However, *PPARGC1A* mRNA expression does not necessarily correlate with training-induced increases in mitochondrial respiration within skeletal muscle (25). Utilization of high-resolution respirometry to directly examine the effects of fasting on exercise training induced mitochondrial biogenesis is warranted.

Overall, fasting and fasted exercise augments the phosphorylation of $AMPK^{Thr172}$ and the mRNA expression of *PDK4* within human skeletal muscle. Furthermore, fasting increases the phosphorylation of $CREB^{Ser133}$, although $CREB^{Ser133}$ phosphorylation is acutely decreased during exercise. However, *PPARGC1A* mRNA expression is similar between fed and fasted skeletal muscle in the resting and exercised states. Thus while fasting increases the systemic availability of NEFAs and the expression of genes associated with fatty acid metabolism, greater energy stress is required to augment the expression of the mitochondrial biogenic regulator *PPARGC1A*.

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Disclosures

The authors report no conflicts of interest.

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731

Figure Captions

Figure 1. Time-course and tAUC (inset) for NEFA (A), glycerol (B), glucose (C) and lactate (D) in plasma and insulin (E) and cortisol (F) in serum in FED (black circles) and FAST (grey squares). Exercise (Ex) was performed between minutes 120 and 180. **a**: main effect of treatment ($p \leq 0.05$); **b**: main effect of time (significantly different to 0 minutes (pre-breakfast); $p \leq 0.05$); **c**: interaction effect (significantly different between FED and FAST; $p \leq 0.05$). Data presented as means ± 95% confidence intervals (n = 7).

Figure 2. Phosphorylation of $AMPK^{Thr172}$ (A), ACC^{ser79} (B), $CREB^{Ser133}$ (C), p38 $MAPK^{Thr180/Tyr182}$ (D) and $CAMKII^{Thr286}$ (E) immediately prior to (Pre), immediately post-exercise (+0h) and three hours post-exercise (+3h). (F) Representative immunoblot images. All values are presented relative to individual pre-exercise values for the FED trial. **a**: main effect of treatment ($p \leq 0.05$); **b**: main effect of time (significantly different to pre-exercise; $p \leq 0.05$). Data presented as means ± 95% confidence intervals (n = 8).

Figure 3. mRNA expression of *PPARGC1A* (A) and *PDK4* (B) immediately prior to (Pre), immediately post-exercise (+0h) and three hours post-exercise (+3h). All values are presented relative to individual pre-exercise values for the FED trial. **a**: main effect of treatment ($p \leq 0.05$); **b**: main effect of time (significantly different to pre-exercise; $p \leq 0.05$). Data presented as means ± 95% confidence intervals (n = 8).

Tables

Table 1. Physiological responses to 70%W_{max} cycling during FED and FAST.

		Time (minutes into exercise)						
		0	15	30	45	60	Mean	*p*
Gas exchange								
$\dot{V}O_2$ (L·min^{-1})	FED	0.41 ± 0.03	2.99 ± 0.23	3.12 ± 0.28	3.13 ± 0.32	3.12 ± 0.30	3.09 ± 0.28	
	FAST	0.46 ± 0.04	2.98 ± 0.21	3.08 ± 0.22	3.08 ± 0.21	3.00 ± 0.23	3.04 ± 0.21	0.537
$\dot{V}CO_2$ (L·min^{-1})	FED	0.35 ± 0.03	2.83 ± 0.21	2.84 ± 0.24	2.82 ± 0.27	2.85 ± 0.26	2.84 ± 0.24	
	FAST	0.38 ± 0.04	2.78 ± 0.19	2.74 ± 0.21	2.70 ± 0.20	2.68 ± 0.20	2.73 ± 0.19	0.045
RER	FED	0.87 ± 0.04	0.95 ± 0.02	0.91 ± 0.03	0.90 ± 0.02	0.91 ± 0.02	0.92 ± 0.02	
	FAST	0.82 ± 0.06	0.93 ± 0.03	0.89 ± 0.05	0.88 ± 0.03	0.89 ± 0.03	0.90 ± 0.03	0.269
Oxidation rates								
Carbohydrate (g·min^{-1})	FED	0.30 ± 0.07	3.07 ± 0.32	2.70 ± 0.26	2.62 ± 0.30	2.76 ± 0.34	2.79 ± 0.27	
	FAST	0.24 ± 0.13	2.87 ± 0.43	2.41 ± 0.43	2.26 ± 0.41	2.40 ± 0.42	2.48 ± 0.39	0.117
Fat (g·min^{-1})	FED	0.09 ± 0.02	0.26 ± 0.11	0.46 ± 0.11	0.50 ± 0.13	0.44 ± 0.14	0.42 ± 0.11	
	FAST	0.14 ± 0.05	0.34 ± 0.17	0.56 ± 0.18	0.62 ± 0.17	0.53 ± 0.20	0.51 ± 0.17	0.361
Intensity								
Heart rate (beats·min^{-1})	FED	67 ± 6	167 ± 4	172 ± 4	174 ± 3	177 ± 4	172 ± 4	
	FAST	60 ± 7	166 ± 5	171 ± 5	174 ± 4	176 ± 3	172 ± 4	0.687
RPE	FED		13 ± 1	14 ± 1	15 ± 1	16 ± 1	15 ± 1	
	FAST		13 ± 2	15 ± 1	16 ± 1	17 ± 1	16 ± 1	0.111

Data presented as means ± 95% confidence intervals (n = 8). Mean values represent the mean of the recorded values during exercise. *p* values represent repeated-measures t-test comparisons between exercising means for FED and FAST

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