

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

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4 Ben Stocks¹, Jessica R. Dent¹, Henry B. Ogden¹, Martina Zemp² & Andrew Philp^{1,3}.

5 ¹ School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham,
 6 Birmingham, UK.

7 ² Institute of Human Movement Sciences and Sport, ETH Zurich, Zurich, CH.

8 ³ Mitochondrial Metabolism and Ageing Laboratory, Diabetes and Metabolism
 9 Division, Garvan Institute of Medical Research, Darlinghurst, AU.

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11 **Author Contributions:**

12 AP, BS and JRD conceived and designed the study. BS, JRD, HO and MZ
 13 contributed to acquisition of data. BS, JRD and AP analyzed and interpreted data. BS
 14 and AP wrote the manuscript. All authors revised the work for critical intellectual
 15 content and approved the final manuscript.

16

17 **Corresponding Author:**

18 Andrew Philp, Ph.D.

19 Mitochondrial Metabolism and Ageing Laboratory

20 Diabetes and Metabolism Division

21 Garvan Institute of Medical Research

22 384 Victoria Street, Darlinghurst, NSW, 2010, Australia

23 Phone: +61 (02) 9295 8249 | Fax: +61 (02) 9295 8201 | Email: a.philp@garvan.org.au

24

25 **Running Head:** Skeletal muscle signaling following fed and fasted exercise

26 **Abstract:**

27 Exercise performed in the fasted state acutely increases fatty acid availability and
 28 utilization. Furthermore, activation of energy-sensing pathways and fatty acid
 29 metabolic genes can be augmented by fasting and fasted exercise. However, whether
 30 a similar effect occurs at higher exercise intensities remains poorly understood. This
 31 study aimed to assess the effect of fed and fasted exercise upon post-exercise
 32 signaling and mRNA responses during moderate- to high-intensity steady-state
 33 exercise. Eight male participants (age: 25 ± 2 y, $\dot{V}O_{2\text{peak}}$: 47.9 ± 3.8 mL·kg⁻¹·min⁻¹)
 34 performed one hour of cycling at 70% W_{max} in the fasted (FAST) state or two hours
 35 following ingestion of a carbohydrate-rich mixed-macronutrient breakfast (FED).
 36 Muscle biopsies were collected pre-, immediately and three hours post-exercise from
 37 the *medial vastus lateralis*, whilst venous blood samples were collected throughout
 38 the trial. Plasma NEFA and glycerol concentrations were elevated during FAST
 39 compared to FED, although substrate utilization during exercise was similar.
 40 AMPK^{Thr172} phosphorylation was ~2.5-fold elevated post-exercise in both trials and
 41 was significantly augmented by ~30% during FAST. CREB^{Ser133} phosphorylation was
 42 elevated ~2-fold during FAST, although CREB^{Ser133} phosphorylation acutely
 43 decreased by ~50% immediately post-exercise. mRNA expression of *PK4* was ~3-
 44 4-fold augmented by exercise and ~2-fold elevated throughout FAST, whilst
 45 expression of *PPARGC1A* mRNA was similarly activated (~10-fold) by exercise in
 46 both FED and FAST. In summary, performing moderate- to high-intensity steady-
 47 state exercise in the fasted state increases systemic lipid availability, elevates
 48 phosphorylation of AMPK^{Thr172} and CREB^{Ser133}, and augments *PK4* mRNA
 49 expression without corresponding increases in whole body fat oxidation and the
 50 mRNA expression of *PPARGC1A*.

51 **Key words:**

52 AMPK, PGC1 α , substrate utilization, metabolism, breakfast.

53 **Introduction**

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

65

66 Performing exercise in the fasted state has the potential to produce favorable
67 metabolic adaptations over and above fed exercise training (53). For example,
68 moderate-intensity exercise, performed in the fasted state, increases fatty acid
69 availability and oxidation compared to exercise combined with carbohydrate ingestion
70 (1, 2, 10, 11, 16). At the cellular level, this response may be mediated in part via an
71 AMPK-dependent mechanism, as skeletal muscle AMPK α 2 activity is enhanced
72 during moderate-intensity fasted exercise compared to glucose ingestion (2). Despite
73 this, the optimal integration of nutrition and exercise to augment adaptive signaling
74 responses remains elusive. For example, AMPK and other exercise-sensitive signaling
75 molecules can be further activated at more vigorous exercise intensities (e.g. ~80%
76 $\dot{V}O_{2peak}$) (21). However, it remains unclear whether this represents a maximal
77 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 \pm SD: age, 25 ± 2 years; body mass, 74.6 ± 5.2 kg; peak oxygen uptake ($\dot{V}O_{2peak}$), 47.9 ± 3.8 mL \cdot kg⁻¹ \cdot 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

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

105

106 *Pre-testing*

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

122

123 *Experimental trials*

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

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

134

135 Participants arrived at the laboratory at ~ 8 am following an ~ 12 -hour overnight fast.
 136 Upon arrival, participants rested in the supine position for approximately ten minutes
 137 before a cannula was inserted into an antecubital forearm vein and a baseline venous
 138 blood sample was collected. Participants were then provided with a mixed-
 139 macronutrient breakfast (FED) ($0.9 \text{ g}\cdot\text{kg}^{-1}$ body mass (BM) of corn flakes cereal, 3.9
 140 $\text{mL}\cdot\text{kg}^{-1}$ BM of semi-skimmed milk, $1.1 \text{ g}\cdot\text{kg}^{-1}$ BM of toasted wholemeal bread, 0.3
 141 $\text{g}\cdot\text{kg}^{-1}$ BM of strawberry jam and $3.2 \text{ mL}\cdot\text{kg}^{-1}$ BM of orange juice (Sainsburys, UK);
 142 energy intake: $710 \pm 49 \text{ kcal}$; macronutrients: $1.75 \text{ g}\cdot\text{kg}^{-1}$ BM carbohydrate (of which
 143 $0.66 \text{ g}\cdot\text{kg}^{-1}$ BM is sugar), $0.1 \text{ g}\cdot\text{kg}^{-1}$ BM fat and $0.35 \text{ g}\cdot\text{kg}^{-1}$ BM protein; energy
 144 contribution: 75% carbohydrate, 10% fat and 15% protein), which they consumed
 145 within the first 15 minutes of the trial, or remained in the fasted state (FAST). The
 146 macronutrient composition of the breakfast reflected population trends for relatively
 147 greater consumptions of carbohydrates, and thus lowers fat and protein ingestion, at
 148 breakfast time (3, 59). Participants rested for two hours prior to providing a pre-
 149 exercise skeletal muscle biopsy from the *medial vastus lateralis*. Participants then
 150 cycled for one-hour at $70\% W_{\text{max}}$ before a second skeletal muscle biopsy was taken
 151 immediately post-exercise (completed within two minutes of exercise cessation).
 152 Participants then rested in a supine position prior to a third skeletal muscle biopsy

being obtained three-hours post-exercise. In order to minimize discomfort for the participant the first two skeletal muscle biopsies were taken from one leg with the third biopsy taken from the contralateral leg. Biopsies were taken from the opposite leg in the second trial. The leg receiving the initial biopsy was randomized and counterbalanced within the study. A new incision was made for each biopsy at least 2 cm from the previous site. Venous blood was collected throughout rest periods and during exercise. Respiratory variables were measured pre-exercise and at 15-minute intervals throughout exercise, heart rate was monitored continuously throughout exercise and RPE was determined at 15-minute intervals throughout exercise. Carbohydrate and fat oxidation were calculated from $\dot{V}O_2$ and $\dot{V}CO_2$ using the moderate-high exercise intensities equation of Jeukendrup and Wallis (31) during exercise and Frayn (23) at rest. Participants were allowed to drink water *ad libitum* during rest and exercise periods of trial one, with water intake matched during trial two to that consumed during each period of trial one (fluid intake during exercise: 618 ± 341 mL; post-exercise fluid intake: 635 ± 343 mL).

Muscle biopsies

Muscle biopsies were obtained from separate incision sites on the *medial vastus lateralis* under local anaesthesia (1% lidocaine; B. Braun, Melsungen, Germany) by a Bergström needle adapted with suction. Muscle was rapidly blotted to remove excess blood and flash frozen in liquid nitrogen. Muscle was powdered using a Cellcrusher tissue pulverizer on dry ice and stored at -80°C prior to analysis.

Immunoblotting

177 Tissue was homogenized in a 10-fold mass excess of ice-cold sucrose lysis buffer (50
 178 mM Tris, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM Na₄P₂O₇-10H₂O, 270 mM
 179 sucrose, 1 M Triton-X, 25 mM β -glycerophosphate, 1 μ M Trichostatin A, 10 mM
 180 Nicatinamide, 1mM 1,4-Dithiothreitol, 1% Phosphatase Inhibitor Cocktail 2; Sigma,
 181 1% Sigma Phosphatase Inhibitor Cocktail 2; Sigma, 4.8% cOmplete Mini Protease
 182 Inhibitor Cocktail; Roche) by shaking in a FastPrep 24 5G (MP Biomedicals) at 6.0
 183 m·s⁻¹ for 80 s and centrifuging at 4°C and 8000 g for 10 minutes to remove insoluble
 184 material. Protein concentrations were determined by the DC protein assay (Bio-Rad,
 185 Hercules, California, USA). Samples were boiled at 97°C for 5 min in laemmli
 186 sample buffer and an equal volume of protein (20-50 μ g) was separated by SDS-
 187 PAGE on 8 - 12.5% gels at a constant current of 23 mA per gel. Proteins were
 188 transferred on to BioTrace NT nitrocellulose membranes (Pall Life Sciences,
 189 Pensacola, Florida, USA) via wet transfer at 100 V for one hour. Membranes were
 190 then stained with Ponceau S (Sigma-Aldrich, Gillingham, UK) and imaged to check
 191 for even loading. Membranes were blocked in 3% dry-milk in tris-buffered saline
 192 with tween (TBST) for one hour before being incubated in primary antibody
 193 overnight at 4°C. Membranes were washed in TBST three times prior to incubation in
 194 appropriate horse radish peroxidase (HRP)-conjugated secondary antibody at room
 195 temperature for one hour. Membranes were then washed in TBST three times prior to
 196 antibody detection via enhanced chemiluminescence HRP substrate detection kit
 197 (Millipore, Watford, UK). Imaging and band quantification were undertaken using a
 198 G:Box Chemi-XR5 (Syngene, Cambridge, UK).

199

200 *Antibodies*

201 All primary antibodies were used at a concentration of 1:1000 in TBST unless
 202 otherwise stated. Antibodies: AMPK α (2603), p-AMPK^{Thr172} (2535), p-ACC^{Ser79}
 203 (3661), CAMKII (3362), p-CAMKII^{Thr268} (12716), CREB (1:500; 9197), p-
 204 CREB^{Ser133} (1:500; 9191), GAPDH (1:5000; 2118), p38 (9212), p-p38^{Thr180/Tyr182}
 205 (4511) were purchased from Cell Signaling Technology; ACC (05-1098) was
 206 purchased from Merck Millipore. Secondary antibodies were used at a concentration
 207 of 1:10000 in TBST. Anti-rabbit (7074) and anti-mouse (7076) antibodies were from
 208 Cell Signaling Technology

209

210 *Real-time RT-qPCR*

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

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

238

239 *Blood analyses*

240 Blood samples were collected into tubes containing ethylenediaminetetraacetic acid
 241 (EDTA; BD, Oxford, UK) for the collection of plasma and serum separator tubes for
 242 the collection of serum. EDTA plasma samples were placed immediately upon ice
 243 while serum samples were allowed to clot at room temperature for 25 minutes prior to
 244 both samples undergoing centrifugation at 1600 g at 4°C for 10 minutes before
 245 collection of plasma or serum from the supernatant. Samples were frozen at -80°C
 246 until further analysis. Plasma samples were subsequently analyzed on an autoanalyzer
 247 (iLAB650, Instrumentation Laboratory, Bedford, MA, USA) for glucose, lactate, non-
 248 esterified fatty acid (NEFA) and glycerol (Randox Laboratories, County Antrim, UK)
 249 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

275 treatment*time interaction effect ($p = 0.015$). Plasma glycerol was significantly
 276 elevated during exercise (135-180 minutes) in FAST compared to FED ($p < 0.05$).
 277 tAUC for plasma glycerol across the experimental period was significantly higher in
 278 FAST ($p = 0.006$). Plasma glucose displayed a treatment*time interaction effect ($p =$
 279 0.024), whereby plasma glucose was higher in FAST immediately prior to exercise
 280 (120 minutes; Figure 1C). However, a lack of treatment effect ($p = 0.866$) and no
 281 difference in tAUC ($p = 0.942$) shows plasma glucose was similar when the whole
 282 trial is considered. Plasma lactate displayed a significant effect for time ($p < 0.001$),
 283 increasing above baseline during exercise (135-180 minutes) and remained so during
 284 the first 40 minutes of recovery (180-220 minutes; $p < 0.05$; Figure 1D). No between-
 285 treatment differences were apparent for plasma lactate (main effect of treatment; $p =$
 286 0.774 , interaction; $p = 0.568$, tAUC; $p = 0.548$). Serum insulin displayed significant
 287 effects for treatment ($p < 0.001$), time ($p < 0.001$) and an interaction effect ($p <$
 288 0.001). Serum insulin was elevated above FAST from 20 minutes post-breakfast to
 289 immediately pre-exercise (120 minutes) in the FED trial ($p < 0.05$). Serum cortisol
 290 displayed a main effect of time ($p = 0.001$) but no treatment effect ($p = 0.277$). There
 291 was a trend towards an interaction effect ($p = 0.069$) for serum cortisol. Carbohydrate
 292 and fat oxidation during exercise were similar between trials (Table 1). $\dot{V}CO_2$ was
 293 significantly lower during FAST exercise ($p = 0.045$). $\dot{V}O_2$, RER, heart rate and RPE
 294 did not differ during exercise between trials (Table 1).

295

296 *Skeletal muscle signaling*

297 Exercise increased the phosphorylation of AMPK^{Thr172} (main effect of time; $p =$
 298 0.002); increasing ~2.5-fold immediately post-exercise ($p = 0.006$) and remaining
 299 ~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 ($p = 0.136$).

308

FAST also induced an ~2-fold elevation in the phosphorylation of CREB^{Ser133} (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 ($p = 0.113$).

315

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).

321

322 *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.

350

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

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

382

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

400 Mechanistically, augmented *PDK4* mRNA expression during FAST may be mediated
 401 by elevated $\text{PPAR}\alpha$ activation known to be responsive to exercise, NEFA
 402 concentration and energy-stress (40, 43).

403

404 Elevated activation of AMPK and PDK4 during fasting has the potential to acutely
 405 influence substrate metabolism. AMPK phosphorylates ACC thereby augmenting
 406 fatty acid oxidation (28), while PDK4 phosphorylates PDH-E1 α , inactivating the
 407 pyruvate dehydrogenase complex (PDC), and thereby inhibiting the decarboxylation
 408 of pyruvate to acetyl-CoA and, thus, carbohydrate oxidation (44). However, substrate
 409 utilization during exercise was similar between FED and FAST, despite elevated
 410 NEFA availability during FAST. This is likely due to the intensity of exercise
 411 dictating reliance upon carbohydrate oxidation and a sufficient endogenous supply of
 412 carbohydrate, i.e. muscle and liver glycogen, in the FAST trial. Indeed, plasma
 413 glucose concentration was unaffected by FAST, while phosphorylation of ACC^{Ser79}
 414 was also similar between treatments. Despite this, it cannot be ruled out that
 415 differences in substrate oxidation may arise if the exercise duration was extended,
 416 although this would likely require a reduction in exercise intensity. Overall this
 417 supports findings from a recent meta-analysis in which it was determined that fat
 418 oxidation was similar between fed and fasted aerobic exercise performed above 70%
 419 $\dot{V}\text{O}_{2\text{max}}$ (54). Nonetheless, elevated plasma NEFA and glycerol concentrations, despite
 420 similar rates of fat oxidation, indicate that increased lipolysis during fasted exercise is
 421 determined at the adipose tissue, independently of demand from the exercising
 422 musculature (14). Indeed, fasted exercise upregulates the expression of lipolytic genes
 423 adipose triglyceride lipase (*PNPLA2*) and hormone sensitive lipase (*LIPE*) in adipose
 424 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.

428

Metabolic adaptations within skeletal muscle are regulated by transcription factors and co-factors, including CREB and PGC1 α . 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 PGC1 α 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 PGC1 α 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 PGC1 α 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

474 expression suggests that similar results should be apparent when fasted exercise is
 475 compared to the ingestion of a mixed macronutrient breakfast. Regarding
 476 mitochondrial biogenic responses to fed vs fasted endurance exercise training the
 477 evidence is less consistent. Fasting has been demonstrated to augment citrate synthase
 478 activity (49, 53) but similar training-induced increases in succinate dehydrogenase
 479 activity has been shown in the fed and fasted state (15). PGC1 α is a key regulator of
 480 mitochondrial biogenesis within skeletal muscle (34, 38, 41). Thus our evidence of
 481 similar *PPARGC1A* mRNA expression may suggest that the mitochondrial biogenic
 482 responses to fed and fasted exercise training at a moderate-high exercise intensity
 483 may be similar. However, *PPARGC1A* mRNA expression does not necessarily
 484 correlate with training-induced increases in mitochondrial respiration within skeletal
 485 muscle (25). Utilization of high-resolution respirometry to directly examine the
 486 effects of fasting on exercise training induced mitochondrial biogenesis is warranted.

487

488 Overall, fasting and fasted exercise augments the phosphorylation of AMPK^{Thr172} and
 489 the mRNA expression of *PKD4* within human skeletal muscle. Furthermore, fasting
 490 increases the phosphorylation of CREB^{Ser133}, although CREB^{Ser133} phosphorylation is
 491 acutely decreased during exercise. However, *PPARGC1A* mRNA expression is
 492 similar between fed and fasted skeletal muscle in the resting and exercised states.
 493 Thus while fasting increases the systemic availability of NEFAs and the expression of
 494 genes associated with fatty acid metabolism, greater energy stress is required to
 495 augment the expression of the mitochondrial biogenic regulator *PPARGC1A*.

496

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501

502 **Disclosures**

503 The authors report no conflicts of interest.

504

505 **References**

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732 **Figure Captions**

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

740

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

748

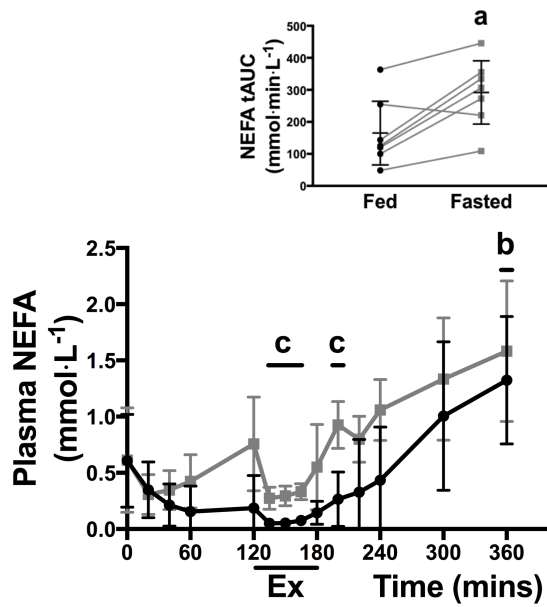
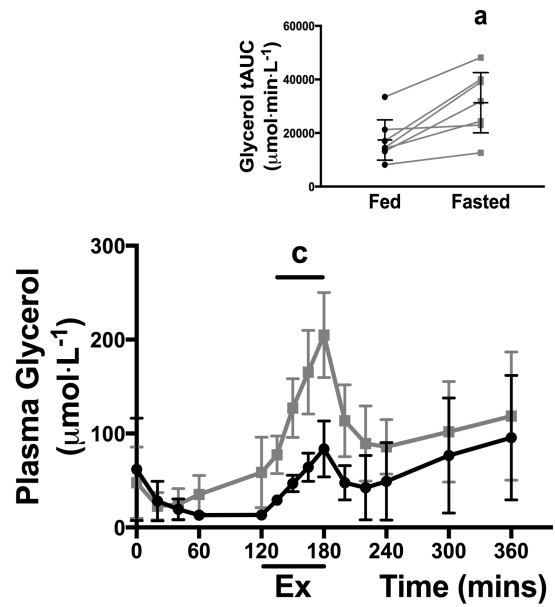
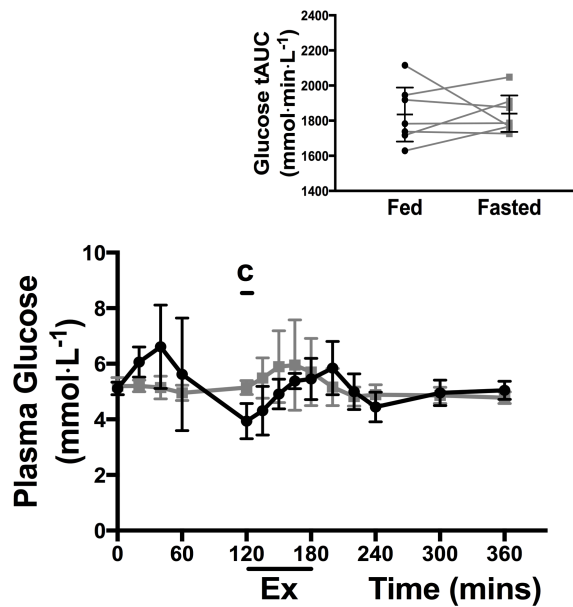
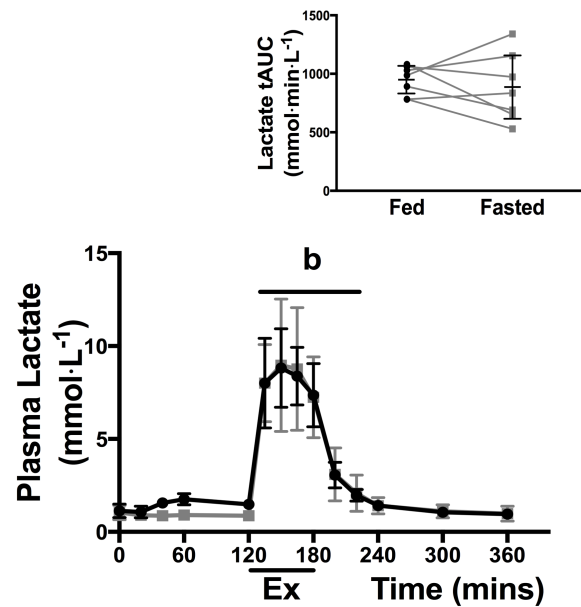
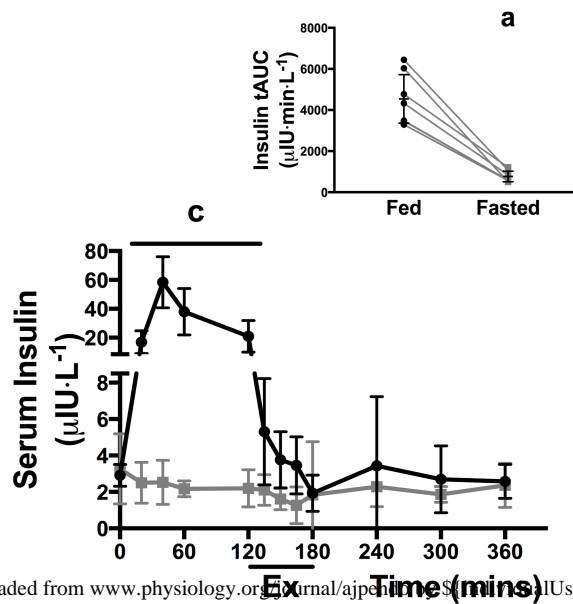
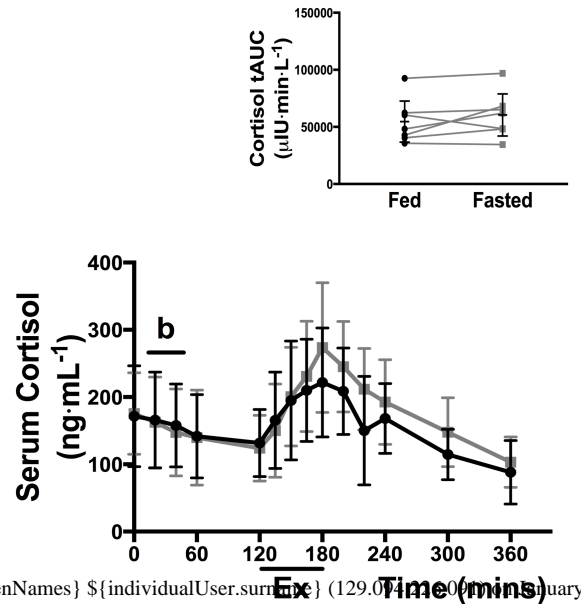
749 **Figure 3.** mRNA expression of *PPARGC1A* (A) and *PDK4* (B) immediately prior to
 750 (Pre), immediately post-exercise (+0h) and three hours post-exercise (+3h). All values
 751 are presented relative to individual pre-exercise values for the FED trial. **a:** main
 752 effect of treatment ($p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

754

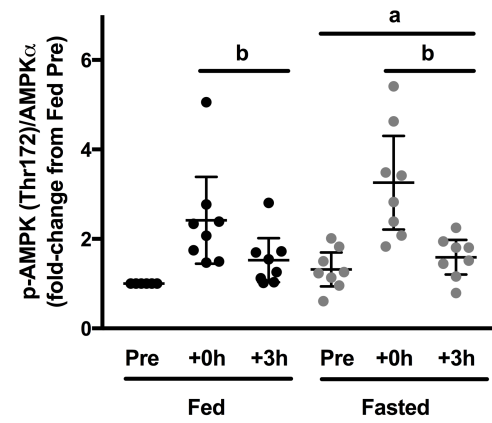
755 **Tables**756 Table 1. Physiological responses to 70%W_{max} cycling during FED and FAST.
757

		Time (minutes into exercise)						
		0	15	30	45	60	Mean	<i>p</i>
<i>Gas exchange</i>								
$\dot{V}O_2$ (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	0.537
	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	
$\dot{V}CO_2$ (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	0.045
	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	
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	0.269
	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	
<i>Oxidation rates</i>								
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	0.117
	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	
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	0.361
	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	
<i>Intensity</i>								
Heart rate (beats·min ⁻¹)	FED	67 ± 6	167 ± 4	172 ± 4	174 ± 3	177 ± 4	172 ± 4	0.687
	FAST	60 ± 7	166 ± 5	171 ± 5	174 ± 4	176 ± 3	172 ± 4	
RPE	FED		13 ± 1	14 ± 1	15 ± 1	16 ± 1	15 ± 1	0.111
	FAST		13 ± 2	15 ± 1	16 ± 1	17 ± 1	16 ± 1	

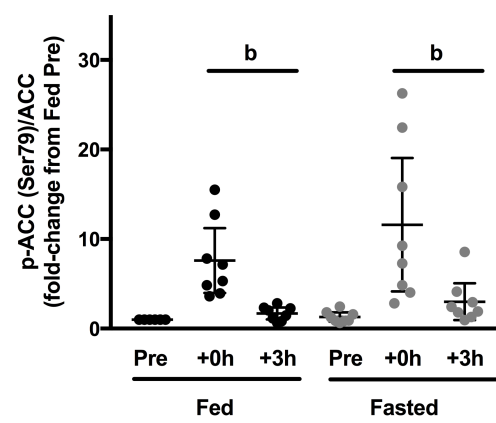
758 Data presented as means ± 95% confidence intervals (n = 8). Mean values represent the mean of the recorded values during exercise. *p* values
 759 represent repeated-measures t-test comparisons between exercising means for FED and FAST

A**B****C****D****E****F**

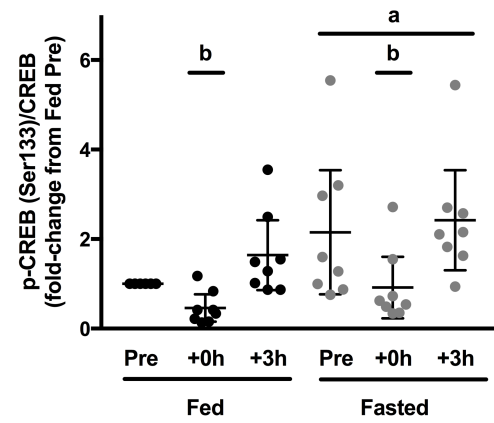
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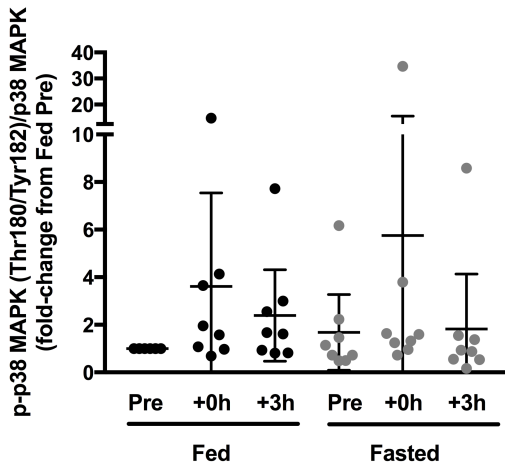
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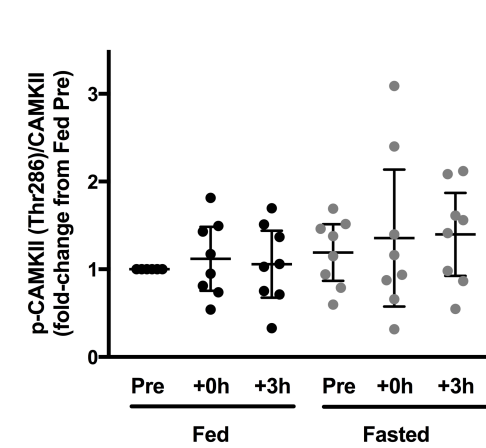
C



D



E



F

