**RESEARCH PAPER**

# Achieving energy balance with a high-fat meal does not enhance skeletal muscle adaptation and impairs glycaemic response in a sleep-low training model

José L. Areta[1,2] | Juma Iraki[2] | Daniel J. Owens[1] | Sophie Joanisse[3] | Andrew Philp[4,5] | James P. Morton[1] | Jostein Hallén[2]

[1] Research Institute for Sport and Exercise Sciences, Liverpool John Moores University, Liverpool, UK

[2] Department of Physical Performance, Norwegian School of Sport Sciences, Oslo, Norway

[3] Department of Kinesiology, McMaster University, Hamilton, Ontario, Canada

[4] Mitochondrial Metabolism and Ageing Laboratory, Diabetes and Metabolism Division, Garvan Institute of Medical Research, Darlinghurst, Australia

[5] St Vincent's Medical School, UNSW Medicine, UNSW Sydney, Sydney, Australia

**Correspondence**
José L. Areta, Research Institute for Sport and Exercise Sciences, Liverpool, John Moores University, Tom Reilly Building, Byrom Street Campus, Liverpool L3 3AF, UK.
Email: j.l.areta@ljmu.ac.uk

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**Abstract**

Training with low carbohydrate availability (LCHO) has been shown to acutely enhance endurance training skeletal muscle response, but the concomitant energy deficit (ED) in LCHO interventions has represented a confounding factor in past research. This study aimed at determining if achieving energy balance with high fat (EB-HF) acutely enhances the adaptive response in LCHO compared to ED with low fat (ED-LF). In a crossover design, nine well-trained males completed a 'sleep-low' protocol: on day 1 they cycled to deplete muscle glycogen while reaching a set energy expenditure (30 kcal (kg of fat free mass (FFM))$^{-1}$). Post-exercise, low carbohydrate, protein-matched meals completely (EB-HF, 30 kcal (kg FFM)$^{-1}$) or partially (ED-LF, 9 kcal (kg FFM)$^{-1}$) replaced the energy expended, with the majority of energy derived from fat in EB-HF. In the morning of day 2, participants exercised fasted, and skeletal muscle and blood samples were collected and a carbohydrate–protein drink was ingested at 0.5 h recovery. Muscle glycogen showed no treatment effect ($P < 0.001$) and decreased from $350 \pm 98$ to $192 \pm 94$ mmol (kg dry mass)$^{-1}$ between rest and 0.5 h recovery. Phosphorylation status of the mechanistic target of rapamycin and AMP-activated protein kinase pathway proteins showed only time effects. mRNA expression of p53 increased after exercise ($P = 0.005$) and was higher in ED-LF at 3.5 h compared to EB-HF ($P = 0.027$). Plasma glucose and insulin area under the curve ($P < 0.04$) and peak values ($P \leq 0.05$) were higher in EB-HF after the recovery drink. Achieving energy balance with a high-fat meal in a 'train-low' ('sleep-low') model did not enhance markers of skeletal muscle adaptation and impaired glycaemia in response to a recovery drink following training in the morning.

**KEYWORDS**
endurance, energy availability, high-fat feeding, muscle glycogen, train low

# 1 | INTRODUCTION

Training with low endogenous and exogenous carbohydrate availability (LCHO) by means of strategically restricting carbohydrate intake can acutely enhance skeletal muscle response to endurance exercise (Bartlett, Hawley, & Morton, 2015). The physiological response to LCHO has been researched intensely over the past decade and the improved adaptation has been attributed to enhanced intracellular events increasing muscle oxidative capacity, triggered by low glycogen regulation of key kinases (e.g. AMP-activated protein kinase (AMPK)), transcription factors (e.g. p53) and transcriptional co-activators (e.g. peroxisome proliferator-activated receptor $\gamma$ coactivator $1\alpha$ (PGC-$1\alpha$)) (Bartlett et al., 2015). However, the majority of the studies reporting beneficial metabolic adaptations to LCHO have concomitantly induced an acute state of low energy intake in the LCHO experimental groups (Bartlett et al., 2013; Impey et al., 2016; Lane et al., 2015; Morton et al., 2009; Yeo et al., 2008, 2010). Provided that energy deficit can increase muscle oxidative capacity (Civitarese et al., 2007; Coen et al., 2015), it is unclear if a negative energy balance plays a role skeletal muscle adaptation during LCHO training.

Chronic energy deficit (ED)/low energy availability (LEA) has also been associated with systemic physiological effects that negatively affect health and may impair training adaptation (De Souza et al., 2014; De Souza, Koltun, & Williams, 2019; Mountjoy et al., 2018). ED can affect skeletal muscle physiological response and we have shown that 5 days of ED down-regulates skeletal muscle protein synthesis (Areta et al., 2014), but how ED affects adaptation to endurance-type training is unclear. If ED impairs training adaptation during LCHO-training, increasing fat intake could rescue the energy balance to ultimately restore the normal adaptive response. Moreover, increasing circulating fatty acids through diet could enhance skeletal muscle oxidative capacity through up-regulation of AMPK activity (Yeo et al., 2008), carnitine-acyl transferase activity (Goedecke et al., 1999), and fatty-acid translocase (FAT/CD36) (Cameron-Smith et al., 2003).

Short-term high-fat diets have, however, also been shown to negatively affect metabolism and training adaptation to endurance-type training. Research using various experimental protocols shows that short-term high-fat diets down-regulate pyruvate dehydrogenase (PDH) activity and skeletal muscle capacity for glycogenolysis (Stellingwerff et al., 2006) and mitochondrial respiration (Leckey et al., 2018); ultimately impairing high-intensity exercise capacity (Havemann et al., 2006). Research from our group has also shown that an acute (15 h) high-fat low-carbohydrate diet impairs the early adaptive response during recovery from endurance training, compared to a high-CHO energy-matched group (Hammond et al., 2016).

Based on these previous findings, therefore, it is not possible to rule out if achieving energy balance from ingested fat can enhance the early response to skeletal muscle in LCHO training. Recent findings from our group show no difference in markers of skeletal muscle adaptation between LCHO low energy and LCHO high fat (energy balance) during two consecutive aerobic training sessions

## New Findings

- **What is the central question of this study?**
  Does achieving energy balance mainly with ingested fat in a 'sleep-low' model of training with low muscle glycogen affect the early training adaptive response during recovery?

- **What is the main finding and its importance?**
  Replenishing the energy expended during exercise mainly from ingested fat to achieve energy balance in a 'sleep-low' model does not enhance the response of skeletal muscle markers of early adaptation to training and impairs glycaemic control the morning after compared to training with low energy availability. These findings are important for optimizing post-training dietary recommendations in relation to energy balance and macronutrient intake.

(Hammond et al., 2019). However, the lack of differences observed in this study can be due to the overriding effect of the short time frame (2.5 h) between the two consecutive training sessions of the 'twice-a-day' model used (Andrade-Souza et al., 2019). To eliminate this confounding factor, the 'sleep-low, train-low' model has emerged as a particularly potent strategy to prolong the period of CHO and energy restriction between two sessions (Bartlett et al., 2013; Lane et al., 2015).

The aim of this study was, therefore, to determine if achieving energy balance, with energy derived mostly from dietary fat, would enhance the metabolic and skeletal muscle response after a second LCHO training session compared to an energy deficit LCHO training session using a 'sleep-low, train-low' model with ~12 h between two sessions. Our hypothesis was that achieving energy balance with high fat would not enhance the adaptive response in response to LCHO training.

# 2 | METHODS

## 2.1 | Ethical approval

The study was approved by the Norwegian School of Sport Sciences Ethics Committee (Application ID 01-020517) and conformed to the standards of the *Declaration of Helsinki*. The study was registered in the Norwegian Center for Research Data (NSD) with reference number 54131/3/ASF. All subjects were informed about the nature of the study and possible risks involved and gave written consent.

## 2.2 | Subjects

Nine well-trained male endurance cyclists/triathletes completed the study. The participants characteristics were as follows: age: 30 ± 7 years, height: 185 ± 5 cm, body mass: 81 ± 8 kg, body fat 17 ± 5%, fat free mass: 68 ± 6 kg, peak aerobic power output (PPO): 407 ± 38 W and 5.1 ± 0.6 W kg$^{-1}$, $\dot{V}_{O_2 max}$: 5.3 ± 0.4 l min$^{-1}$ and 66 ± 6 ml kg$^{-1}$ min$^{-1}$, gross efficiency (average value from measurements at 50, 67, and 85% PPO): 20.3 ± 0.8%.

## 2.3 | Baseline testing

### 2.3.1 | Incremental cycling test

Approximately 1 week prior to the first experimental trial, subjects' PPO and $\dot{V}_{O_2 max}$ (60 s average) were determined on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands; also used in all subsequent exercise sessions) as previously described (Torrens, Areta, Parr, & Hawley, 2016). For respiratory gases assessment during exercise, subjects breathed through a Hans Rudolph (Shawnee, KS, USA) two-way non-rebreathing valve and mouth piece attached to a mixing chamber and linked to a calibrated Oxycon Pro metabolic cart (Jaeger, Hochberg, Germany). Subjects were fan-cooled during all exercise sessions.

### 2.3.2 | Cycling gross efficiency

Following 10–15 min recovery after PPO test, subjects cycled for three consecutive 5-min stages at 50, 67 and 85% of PPO, respectively, for determination of cycling gross efficiency as reported by Moseley & Jeukendrup (2001) using the respiratory data of the last minute of each stage.

### 2.3.3 | Resting metabolic rate

On a separate day, resting metabolic rate (RMR) was assessed in the morning (6.30–7.30 am). Participants were instructed to keep exercise to ~1 h of moderate intensity in the afternoon at the latest the day prior, remain fasted in the morning, not to consume caffeine or nicotine on the day, and to arrive at the laboratory by car or public transport. Following 10 min of lying supine, a face-mask was fitted to the subjects and breath-by-breath respiratory data were collected for 25 min using a calibrated Oxycon Pro metabolic system, while the subjects remained still. The last 20 min of data were used to estimate 24 h RMR, calculated using oxygen equivalents (Weir, 1949). Pilot testing of this protocol in 10 individuals tested twice on consecutive days showed a coefficient of variation of 4.8 ± 2.7% for RMR and 2.2 ± 2.8% for respiratory exchange ratio (RER).

### 2.3.4 | Body composition

Following RMR assessment, body composition was assessed with dual-energy X-ray absorptiometry (Lunar iDXA, GE Healthcare, Madison, WI, USA with GE Healthcare enCORE software version 14.10.022).

## 2.4 | Experimental protocol

### 2.4.1 | Study overview

In a randomized, counterbalanced, cross-over design, participants visited the laboratory twice for overnight 'sleep-low' interventions (Lane et al., 2015), with a period of 1–2 weeks between the two visits (Figure 1). Briefly, an evening cycling session (day 1) had the double aim of depleting glycogen and increasing energy expenditure to a set amount (30 kcal (kg FFM)$^{-1}$), while the second cycling session in the morning (day 2) had the aim of inducing a training stimulus while in low carbohydrate availability. After the glycogen-depleting session, participants were provided with low-carbohydrate meals ('dinner') for two different interventions aiming at completely (energy balance–high fat (EB-HF), 30 kcal (kg FFM)$^{-1}$) or partially (energy deficit–low fat (ED-LF), 9 kcal (kg FFM)$^{-1}$) restoring the energy expended during the session. Partially replacing energy expenditure in ED-LF was unavoidable when providing a CHO and protein-matched intervention. Subjects slept overnight in our premises (~20.30 h to 06.30–06.45 h). Upon waking, sleep quality rating was assessed, and RMR immediately assessed using the same protocol as in baseline testing in a dedicated room. Afterwards, a catheter (18 G, BD, Franklin Lakes, NJ, USA) was placed in the antecubital vein for serial blood sampling, and a baseline muscle biopsy taken from the vastus lateralis using a 6 mm Bergström needle modified for manual suction, following local anaesthesia (1% lidocaine, AstraZeneca, Cambridge, UK). Then, participants completed a 75 min exercise session, during which respiratory gases and venous blood were sampled. A recovery drink was provided at 30 min recovery, immediately after the second muscle biopsy was taken. Blood was collected 30, 60, 120 and 180 min post-drink, and at 3.5 h recovery a final muscle biopsy was collected. Muscle biopsies were therefore taken pre-exercise and at 0.5 and 3.5 h recovery. The blood sampling, biopsies and recovery drink timing are all reported relative to the end of the 75 min exercise session, which is the time 'zero' (0 min). All blood samples were collected in 6 ml EDTA-containing vacuum sealed tubes (BD), immediately spun at 3000 g for 10 min at 4°C, and plasma aliquoted and stored at −80°C for later analysis.

## 2.5 | Exercise sessions

### 2.5.1 | Glycogen-depleting session

Following 10 min at 50% PPO, intensity was manually alternated every 2 min, between 85 and 50% of PPO until reaching the target metabolic gross energy expenditure (30 kcal (kg FFM)$^{-1}$, approx. 2000 kcal).
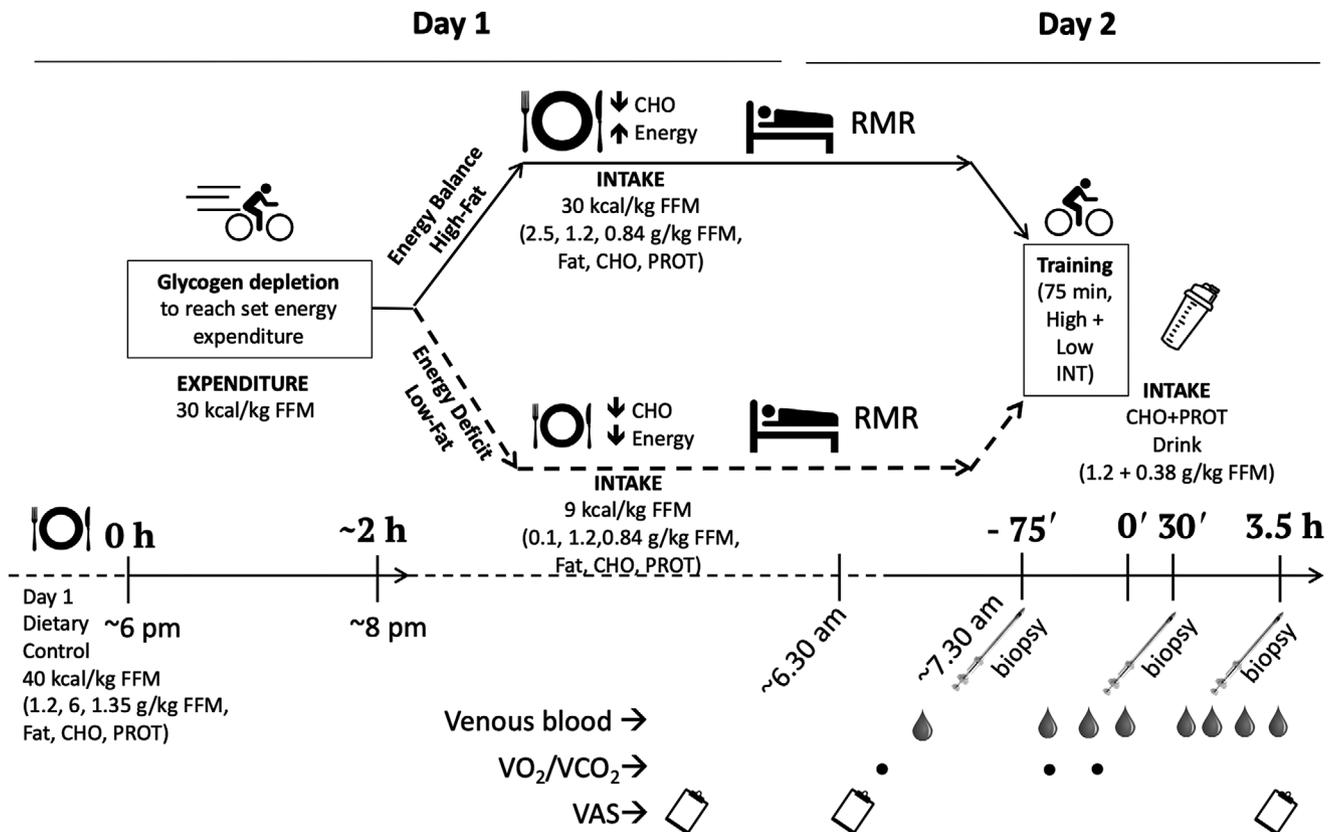
**Day 1**                          **Day 2**



**FIGURE 1** Schematic overview of experimental trials. On the evening of day 1, participants undertook a glycogen-depleting session until reaching 30 kcal kg$^{-1}$ fat free mass (FFM) of metabolic energy expenditure. Immediately after the session, participants were provided with low carbohydrate (CHO) meals to completely (energy balance, high-fat) or partially (energy deficit, low-fat) restore the energy expended during exercise. In the morning of day 2, resting metabolic rate (RMR) was assessed upon waking. Subsequently a 75 min training session incorporating changes of intensity was completed and a CHO + protein (PROT) drink was ingested to optimize recovery. Skeletal muscle biopsies and venous blood samples were obtained during the morning. Visual analogue scales (VAS), at indicated time points were used to assess subjective appreciation of hunger and sleeping quality (upon waking only)

Metabolic energy expenditure was estimated with a custom-made automated spreadsheet based on the duration, absolute power output of each interval and gross efficiency of each individual determined during baseline testing.

If an individual could not complete a 2 min interval at 85% of PPO, intensity was decreased 5% for each subsequent higher intensity interval. This pattern was repeated up until the participant failed to maintain 60% PPO, after which intensity was clamped at 50% PPO.

Note that the total metabolic workload of 30 kcal (kg FFM)$^{-1}$ is not related to the alleged threshold for energy availability (Ihle & Loucks, 2004). This workload was determined from piloting as a manageable workload capable of both significantly increasing total energy expenditure and depleting muscle glycogen given the duration and intensity of the session (Areta & Hopkins, 2018).

### 2.5.2 | Morning exercise session

The exercise session undertaken on day 2 lasted a total of 75 min. Provided the participants would have not been able to complete a full

high intensity session due to the lack of fuel substrate to match the energy demands (i.e. glycogen) (Yeo et al., 2008), short high intensity intervals (HIIs) were used to increase the stimulus of the session above what a steady-state low intensity session only would have achieved (MacInnis & Gibala, 2017). A 5 min warm-up at 50% PPO was followed by 4 × 30 s HIIs at 130% PPO and 2 min recovery at 100 W. After HIIs, participants cycled at 50% PPO for 45 min and respiratory gases were collected for 5 min periods at 25–30 and 55–60 min, coinciding with blood collection and rate of perceived exertion assessment. The session ended with 5 × 1 min at 80% PPO with 1 min recovery at 50% PPO, ending with 5 min at 50% PPO.

### 2.6 | Dietary control and treatments

### 2.6.1 | Dietary control

Subjects were instructed to maintain their regular diet on the days prior to testing and pre-packaged custom-made diets were provided for day 1. The diets contained 1.2, 6 and 1.35 g (kg FFM)$^{-1}$ day$^{-1}$

**TABLE 1** Energy balance and macronutrient content plan for treatments

| | EB-HF | ED-LF |
|---|---|---|
| Energy (total day 1) | | |
| RMR energy expenditure (kcal (kg FFM)$^{-1}$)* | $35 \pm 5$ | $35 \pm 5$ |
| Exercise energy expenditure (kcal (kg FFM)$^{-1}$)† | $30 \pm 0.1$ | $30 \pm 0.1$ |
| Total energy intake (kcal (kg FFM)$^{-1}$) | 70 | 49 |
| Energy balance (kcal (kg FFM)$^{-1}$) | $5 \pm 5$ | $-15 \pm 5$ |
| Energy availability (kcal (kg FFM)$^{-1}$) | 40 | 19 |
| Treatment meals macronutrient intake | | |
| Fat (g (kg FFM)$^{-1}$) (% total energy) | 2.5 (73%) | 0.1 (10%) |
| Carbohydrates (g (kg FFM)$^{-1}$) (% total energy) | 1.2 (16%) | 1.2 (53%) |
| Protein (g (kg FFM)$^{-1}$) (% total energy) | 0.84 (11%) | 0.84 (37%) |

Values of RMR, exercise energy expenditure and energy balance are means ± SD. Energy availability is defined as (total exercise energy intake – exercise energy expenditure)/FFM. *Based on baseline resting metabolic rate measurement. †Estimated for each individual during glycogen-depletion session based on individual efficiency, does not include estimation of incidental physical activity during the day. EB, energy balance; ED, energy deficit; FFM, fat free mass; RMR, resting metabolic rate.

of fat, carbohydrates and protein, respectively, providing 40 kcal (kg FFM)$^{-1}$ day$^{-1}$. The last meal was consumed in the laboratory ~45–60 min prior to the glycogen-depleting session and contained 0.1, 2 and 0.3 g (kg FFM)$^{-1}$ of fat, carbohydrates and protein, respectively.

### 2.6.2 | Dietary treatments

The EB-HF or ED-LF treatment meals were protein and carbohydrate-matched meals that were approximately isovolumetric. Details of diets including the average energy intake of day 1 in addition to macronutrient composition of treatment meals are reported in Table 1. All calculations were made to target divergent energy availability values in each group, provided energy availability is likely to be a key parameter in relation to the physiological effect of energy restriction (Loucks, Kiens, & Wright, 2011). References to 'energy balance' values are made due to its being a more widespread concept and for ease of interpretation to the reader and these were calculated retrospectively.

### 2.6.3 | Recovery drink

A recovery drink was provided after the second muscle biopsy in the morning exercise session, and contained 1.2 and 0.38 g (kg FFM)$^{-1}$ of carbohydrates and protein (maltodextrin and whey protein isolate), respectively. The purpose of this drink was to stimulate recovery processes, as supported by current evidence (Moore, Camera, Areta, & Hawley, 2014), which is also in accordance to current practice in endurance sports.

### 2.6.4 | Subjective hunger and sleep quality ratings

Visual analogue scales (VAS) were used for assessment of participants' feeling of hunger 45–60 min post-dinner (day 1), immediately post-RMR assessment and at 2.5 h recovery from exercise (day 2), and sleep quality rating immediately upon waking. The VAS were 10 cm lines displaying the extremes 'no hunger at all' and 'worst possible hunger' and 'worse sleep I ever had' and 'best sleep I ever had' for hunger and sleep ratings, respectively.

### 2.6.5 | Calculation of substrate utilization

Substrate utilization was determined using non-protein RER calculations and described in detail elsewhere (Torrens et al., 2016).

## 2.7 | Analytical techniques

### 2.7.1 | Plasma

Glucose and lactate were analysed using a Biosen C-Line (EKF Diagnostics, Magdeburg, Germany). Free fatty acid (FFA) concentrations were measured using a non-esterified fatty acid assay kit (NEFA-HR (2), Wako Pure Chemical Industries, Ltd, Osaka, Japan). Glycerol was analysed using a kit coupling an enzyme assay involving glycerol kinase and glycerol phosphate oxidase (MAK117, Sigma-Aldrich, St Louis, MO, USA). $\beta$-Hydroxybutyrate ($\beta$HB) was analysed using a colorimetric assay kit (No. 700190, Cayman Chemical Co., Ann Arbor, MI, USA).

### 2.7.2 | Muscle glycogen content

Freeze-dried muscle samples were hydrolysed with 1.8 M HCl (100°C, 2.5 h), neutralized with 6 M NaOH and determined fluorometrically as glucose units using an enzymatic assay (Jensen et al., 2012).

### 2.7.3 | Immunoblotting

Muscle was processed and analysed as previously described (Stocks, Dent, Ogden, Zemp, & Philp, 2019). All primary antibodies were prepared in Tris-buffered saline–Tween 20 at a dilution of 1:1000 except for p-p38, which was prepared in 5% BSA. Antibodies used were as follows: Acetyl-CoA Carboxylase (ACC; Cell Signaling Technology (CST), Danvers, MA, USA, no. 3676), p-ACC$^{Ser79}$ (CST, no. 3661), AMPK $\alpha$2 (CST, no. 2757), p-AMPK$^{Thr172}$ (CST, no. 2535), Akt (CST, no. 4691), p-Akt$^{Ser473}$ (CST, no. 4060), eukaryotic elongation factor 2 (eEF2; CST, no. 2332), p-eEF2$^{Thr56}$ (CST, no. 2331), p38 (CST, no. 9212), p-p38$^{Thr180/Tyr182}$ (CST, no. 4511), p70 ribosomal protein S6 kinase 1 (S6K1; CST, no. 2708), p-S6K1$^{Thr389}$ (CST, no. 9205), rukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1;CST, no. 9644) and p-4E-BP1$^{Thr37/46}$ (CST, no. 9459).

## 2.7.4 | RT-qPCR

RT-qPCR was employed for the determination of relative mRNA abundance of targeted genes (Table 2). Total RNA extraction was achieved by phenol–chloroform extraction and cDNA synthesis was performed with Precision nanoScript2 Reverse Transcription Kit as per the manufacturer's guidelines (Primer Design, Camberley, UK). The cDNA was stored at −80°C until required for qPCR. Quantitative PCR was performed for all reference genes and genes of interest using the Precision PLUS SYBR green kit (Primer Design). Melt curve analysis was performed to confirm all PCR products demonstrated a clear single peak melt temperature showing that only one gene target had been amplified and that primer–dimer issues were not present.

The determination of stable reference genes was conducted in line with the geNorm geometric averaging method. The geometric mean of the reference targets *18S*, *GAPDH* and *TOP1* was used (geNorm $V < 0.15$) after determining the three most stable targets comparing out of six reference genes, which showed a high reference target stability of $M < 0.5$. Relative mRNA abundance for genes of interest was calculated using the $\Delta\Delta C_t$ method (Livak & Schmittgen, 2001). Commercially available geNorm reference gene kit information is as follows (official gene symbol, accession no., anchor nucleotide): *18S*, M10098, 234; *ACTB*, NM_001101, 1194; *GAPDH*, NM_002046, 1087; *RPL13A*, NM_012423, 727; *TOP1*, NM_003286, 2361; *B2M*, NM_004048, 362. Full sequence information is not available due to containing commercially sensitive details.

## 2.7.5 | Statistical analyses

Data were analysed using one- or two-way repeated-measures ANOVA, as appropriate, with Student–Newman–Keuls *post hoc* analysis to correct for the family-wise error during multiple *post hoc* tests (SigmaPlot for Windows Version 13, Systat Software, Inc., San Jose, CA, USA). Grouped data were analysed using Student's paired *t* test and magnitudes of difference in area under the curve for blood parameters were analysed with effect sizes (ES) with an on-line-available tool (sportsci.org/resource/stats/xcrossover.xls) following guidelines explained therein. All data are presented as means ± standard deviation (SD) and the level of statistical significance was set at $P < 0.05$.

## 3 | RESULTS

### 3.1 | Glycogen depleting/energy expenditure sessions

The glycogen-depleting sessions were completed with average values of: $266 \pm 26$ W absolute power, $65.3 \pm 1.3\%$ of PPO, $15.2 \pm 1.2$ RPE at $82 \pm 4\%$ of maximal heart rate with a total duration of $109 \pm 8$ min, and estimated metabolic energy expended was $2031 \pm 177$ kcal

$(30 \pm 0.1$ kcal (kg FFM)$^{-1}$), displaying no differences in any of these variables between groups.

### 3.2 | Resting metabolic rate

We did not detect differences in RMR between conditions in absolute values (baseline $2350 \pm 272$ kcal day$^{-1}$; EB-HF $2409 \pm 150$ kcal day$^{-1}$; ED-LF $2284 \pm 248$ kcal day$^{-1}$) or relative to fat-free mass (baseline $34.9 \pm 4.7$ kcal (kg FFM)$^{-1}$ day$^{-1}$; EB-HF $35.3 \pm 4.0$ kcal (kg FFM)$^{-1}$ day$^{-1}$; ED-LF $34.0 \pm 5.3$ kcal (kg FFM)$^{-1}$ day$^{-1}$). RER was significantly lower compared to baseline ($0.85 \pm 0.03$) both in EB-HF ($0.83 \pm 0.02$; $P = 0.004$) and in ED-LF ($0.81 \pm 0.02$; $P < 0.001$).

### 3.3 | Physiological parameters during morning exercise

The average heart rate for the morning exercise session was $142 \pm 10$ bpm ($76 \pm 4\%$ max), and the maximal values were $166 \pm 10$ bpm ($88 \pm 3\%$ max). Physiological and metabolic parameters during the submaximal portion of exercise are reported in Table 3. None of these variables showed a treatment or time effect.

### 3.4 | Free fatty acids, glycerol and $\beta$HB

There was a main effect of time for plasma FFAs ($P < 0.001$) and a time × treatment interaction ($P = 0.003$) (Figure 2a). During the ED exercise, there was a faster increase in free fatty acids which resulted in higher peak values during ED ($646 \pm 241$ $\mu$mol l$^{-1}$) *versus* EB ($466 \pm 215$ $\mu$mol l$^{-1}$; $P = 0.005$). Glycerol, instead, showed only a main effect of time ($P < 0.001$) with no differences between treatments (Figure 2b). $\beta$HB showed no significant changes (Figure 2c).

### 3.5 | Glucose, insulin and lactate

There was a main effect of time for glucose ($P < 0.001$) and a time × treatment interaction ($P = 0.033$) with no significant main effect of treatment ($P = 0.069$). Thirty minutes post-recovery drink, plasma glucose reached higher values in EB *versus* ED ($8.4 \pm 1.6$ *vs.* $7.6 \pm 1.6$ mmol l$^{-1}$; $P = 0.027$, Figure 2d) and remained higher 60 min post-drink ($8.0 \pm 1.2$ *vs.* $6.8 \pm 1.5$ mmol l$^{-1}$, $P = 0.001$). The area under the curve for glucose between post-exercise and until 2.5 h post-exercise was higher in EB *versus* ED and there were large effect sizes ($113 \pm 15$ *vs.* $100 \pm 15$ arbitrary units (AU), respectively; ES, 0.82, 95% CL 0.16–1.48, $P = 0.02$). Plasma insulin showed a main effect of time ($P < 0.001$), and a time × treatment interaction ($P < 0.001$), with no significant main effect of treatment ($P = 0.078$). Insulin increased above baseline at 30–120 min post-recovery drink ($P < 0.001$). The peak value was reached at 30 min post-drink in ED ($373 \pm 147$ pmol l$^{-1}$) and at

**TABLE 2** Primer information for all primers used in PCR analysis

| Gene | Gene symbol | GenBank accession no. | Primer sequence | Product length (bp) |
|---|---|---|---|---|
| Citrate synthase | CS | NM_004077.2 | F: TTGGCTGCTGGTAACTGGAC  R: TCCAGCATGGTGACCACATG | 108 |
| Cluster of differentiation 36 | CD36 | NM_00100154 | F: AAGTTAAGCAAAGAGAGGTCCTTATACG  R: GCAGGAAAGAGACTGTGTTGTC | 96 |
| Cytochrome c oxidase 4 | COX4 | NM_001861.4 | F: CGAGCAATTTCCACCTCTGT  R: GGTCACGCCGATCCATATAA | 94 |
| Cytochrome c, somatic | CYCS | NM_018947 | F: CCAGTGCCACACCGTTGAA  R: GTGTATCCTCTCCCCAGATGATG | 146 |
| Dynamin like 1 | DRP1 | | F: CACCCGGAGACCTCTCATTC  R: CCCCATTCTTCTGCTTCCAC | 99 |
| Mitofusin 2 | MFN2 | | F: CCCCCTTGTCTTTATGCTGATGTT  R: TTTTGGGAGAGGTGTTGCTTATTTC | 162 |
| Nuclear factor, erythroid 2 like 2 | NRF2 | NM_002040.3 | F: AAATTGAGATTGATGGAACAGAGAA  R: TATGGCCTGGCTTACACATTCA | 95 |
| Parkin | PRKN | NM_004562.2 | F: CACTGCCCTGGGACTAGTG  R: CGATCAGGTGCAAAGCTACTG | 91 |
| Peroxisome proliferator-activated receptor γ coactivator 1α | PPARGC1A | NM_00133075 | F: TGCTAAACGACTCCGAGAA  R: TGCAAAGTTCCCTCTGCT | 67 |
| Pyruvate dehydrogenase kinase 4 | PDK4 | NM_002612.3 | F: CATCGTGTATGTTCCTTCTCACC  R: ACCTCTATTGGTGTAAGGGAAGG | 114 |
| SCO2, cytochrome c oxidase assembly protein | SCO2 | | F: CTTCACTCACTGCCCTGACA  R: CGGTCAGACCCAACAGCTT | 191 |
| Sirtuin 1 | SIRT1 | NM_012238 | F: GAGCCATGAAGTATGACAAAGATGA  R: GGCACTTCATGGGGTATGGAA | 109 |
| Transcription factor A, mitochondrial | TFAM | NM_003201.2 | F: CTACAGAACTAATTAGAAGAATTGCCC  R: ACTTGGAGTTAGCTGTTCTTTAAATC | 143 |
| Tumour protein 53 | TP53 | NM_000546 | F: ACCTATGGAAACTACTTCCTGAAA  R: CTGGCATTCTGGGAGCTTCA | 141 |

**TABLE 3** Physiological and metabolic variables during low intensity portion of morning exercise on day 2

| | 25–30 min | | 55–60 min | |
| --- | --- | --- | --- | --- |
| | **EB-HF** | **ED-LF** | **EB-HF** | **ED-LF** |
| $\dot{V}_{O_2}$ (l min$^{-1}$) | 3.1 ± 0.3 | 3.2 ± 0.4 | 3.1 ± 0.3 | 3.2 ± 0.3 |
| $\dot{V}_{O_2}$ (% max) | 59 ± 3.4 | 59.4 ± 3.9 | 59 ± 3.6 | 59.5 ± 3.3 |
| RER | 0.8 ± 0.02 | 0.78 ± 0.03 | 0.8 ± 0.02 | 0.78 ± 0.03 |
| Fat oxidation (g min$^{-1}$) | 1.1 ± 0.1 | 1.2 ± 0.2 | 1.1 ± 0.1 | 1.1 ± 0.2 |
| CHO oxidation (g min$^{-1}$) | 1.4 ± 0.4 | 1.1 ± 0.4 | 1.3 ± 0.3 | 1.2 ± 0.4 |
| Energy from fat (%) | 67 ± 7 | 72 ± 10 | 67 ± 6 | 71 ± 11 |
| Energy from CHO (%) | 33 ± 7 | 28 ± 10 | 33 ± 6 | 29 ± 11 |
| Gross efficiency (%) | 19.4 ± 1 | 19.3 ± 1.4 | 19.4 ± 1.2 | 19.3 ± 1.3 |
| RPE | 11.7 ± 1.6 | 11.4 ± 1.1 | 12.5 ± 1.5 | 11.6 ± 0.9 |

Values are means ± SD. CHO, carbohydrate; EB, energy balance; ED, energy deficit; RER, respiratory exchange ratio; RMR, resting metabolic rate; RPE, rate of perceived exertion (Borg scale).
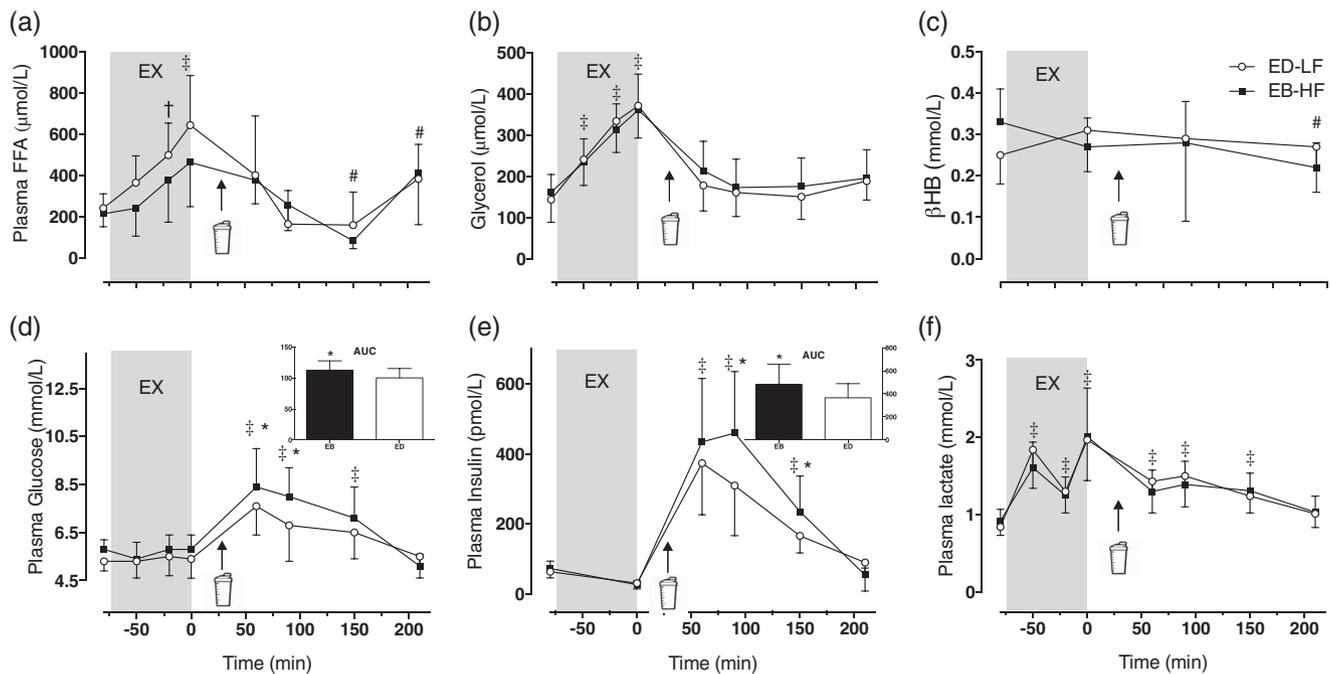


**FIGURE 2** Plasma free fatty acids (a), glycerol (b), $\beta$-hydroxybutyrate (c), glucose (d), insulin (e) and lactate (f) at base-line, during morning exercise and during 3.5 h recovery, including a recovery drink (1.2 and 0.38 g (kg FFM)$^{-1}$ of carbohydrates and protein, respectively) at 30 min recovery (bottle and arrow). Glucose and insulin panels include insets for post-exercise to 150 min area under the curve (arbitrary units). Values are means ± SD. Significantly different ($P < 0.05$): ‡from baseline in both groups; †from baseline in ED-LF; #from baseline in EB-HF; *between groups at indicated time points. AUC, area under the curve; EB-HF, energy balance–high fat; ED-LF, energy deficit–low fat; EX, exercise; FFA, free fatty acids; FFM, fat free mass

60 min post-drink in EB (459 ± 176 pmol l$^{-1}$). At this time point, EB was higher than ED (459 ± 176 vs. 310 ± 143 pmol l$^{-1}$, respectively; $P < 0.001$), and remained elevated in EB compared to ED at 120 min post-drink (235 ± 102 vs. 166 ± 49 pmol, respectively; $P = 0.038$; Figure 2e). The area under the curve for insulin between post-exercise and 2.5 h recovery was higher in EB than ED and there were large effect sizes (801 ± 293 vs. 611 ± 204 AU, respectively; ES, 0.93, 95% CL 0.08–1.79, $P = 0.035$).

## 3.6 | Skeletal muscle

### 3.6.1 | Muscle glycogen

There was a main effect of time on muscle glycogen ($P < 0.001$) but no differences between treatments (Figure 3a). Resting muscle glycogen (350 ± 98 mmol (kg dry mass (DM))$^{-1}$) was decreased by 45% between pre-exercise and 0.5 h post-exercise ($\Delta$ 159 ± 64 mmol (kg DM)$^{-1}$,
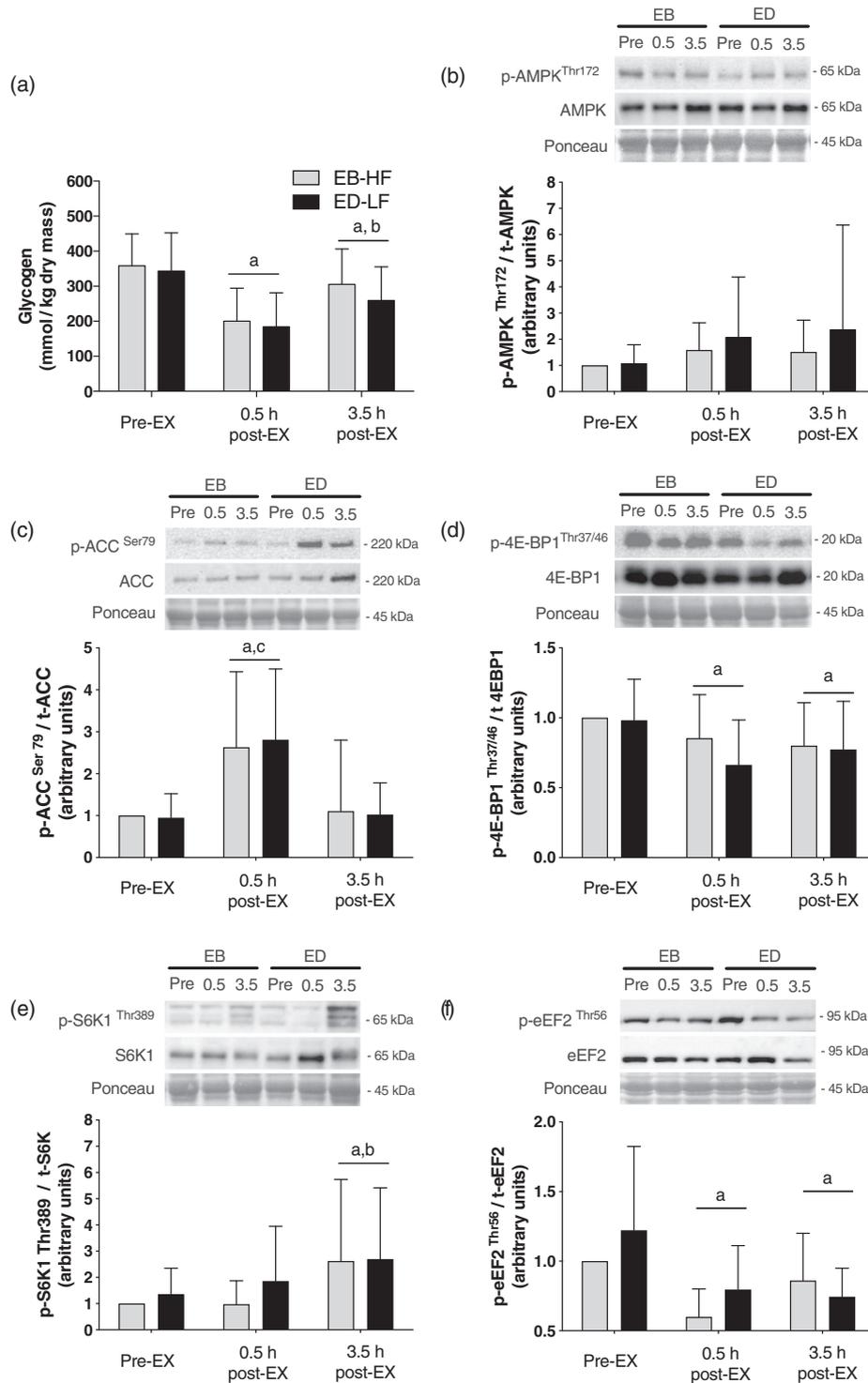
**FIGURE 3** Skeletal muscle glycogen (a), and phosphorylation status of AMPK$^{Thr172}$ (b), ACC$^{Ser79}$ (c), 4EBP-1$^{Thr47/46}$ (d), S6K$^{Thr389}$ (e) and eEF2$^{Thr56}$ (f) relative to pre-ex EB and representative blots. Samples were taken pre-exercise, and at 0.5 and 3.5 h recovery from exercise, including a recovery drink (1.2 and 0.38 g (kg FFM)$^{-1}$ of carbohydrates and protein, respectively) at 0.5 h recovery. Values are means ± SD. Signalling data are fold change relative to pre-EX EB-HF. Time point difference from: [a]Pre-EX; [b]0.5 h post-EX and [c]3.5 h post-EX ($P < 0.05$). EB-HF, energy balance–high fat; ED-LF, energy deficit–low fat

**TABLE 4** mRNA content of different genes relative to 0 h EB-HF time point

| Gene | 0 h ED-LF | 3.5 h EB-HF | ED-LF | Treatment | Time | Interaction |
|------|-----------|-------------|-------|-----------|------|-------------|
| PPARGC1A | 0.91 ± 0.47 | 3.09 ± 2.31 | 3.73 ± 3.50 | 0.550 | **0.020** | |
| P53 | 1.00 ± 0.33 | 1.14 ± 0.34 | 1.59 ± 0.56* | 0.073 | **0.005** | **0.027** |
| CS | 1.02 ± 0.09 | 0.94 ± 0.16 | 0.98 ± 0.11 | 0.280 | 0.223 | |
| CYCS | 0.94 ± 0.38 | 0.98 ± 0.56 | 0.92 ± 0.54 | 0.557 | 0.910 | |
| PRKN | 1.06 ± 0.34 | 0.83 ± 0.35 | 0.93 ± 0.33 | 0.372 | 0.112 | |
| NRF2 | 1.11 ± 0.51 | 0.96 ± 0.6 | 0.95 ± 0.51 | 0.519 | 0.388 | |
| TFAM | 1.00 ± 0.45 | 1.05 ± 0.58 | 1.06 ± 0.52 | 1.000 | 0.654 | |
| COX4 | 1.06 ± 0.47 | 1.04 ± 0.52 | 1.51 ± 0.90 | 0.112 | 0.160 | |
| SIRT1 | 0.91 ± 0.32 | 0.99 ± 0.28 | 1.06 ± 0.36 | 0.920 | 0.294 | |
| MFN2 | 1.01 ± 0.70 | 0.89 ± 0.46 | 1.25 ± 0.95 | 0.490 | 0.548 | |
| DRP1 | 1.27 ± 0.81 | 1.08 ± 0.58 | 1.40 ± 0.68 | 0.128 | 0.315 | |
| SCO2 | 1.04 ± 0.60 | 1.21 ± 0.79 | 1.30 ± 0.60 | 0.486 | 0.103 | |
| CPT1 | 0.90 ± 0.31 | 1.01 ± 0.69 | 0.90 ± 0.51 | 0.240 | 0.970 | |
| CD36 | 1.12 ± 0.59 | 1.09 ± 0.57 | 1.12 ± 0.80 | 0.712 | 0.716 | |
| PDK4 | 0.94 ± 0.56 | 2.18 ± 1.47 | 1.73 ± 1.87 | 0.254 | 0.068 | |

Values are means ± SD. Main effects that are significant ($P < 0.05$) are indicated in bold. *Significantly different from EB within time point, $P = 0.005$.

$P < 0.001$), and 47% of the difference was resynthesized at 3.5 h post-exercise ($\Delta$ 91 ± 74 mmol (kg DM)$^{-1}$, $P < 0.001$) returning to 80% of starting values (282 ± 116 mmol (kg DM)$^{-1}$).

### 3.6.2 | Muscle intracellular signalling

There were no differences between treatments in any of the measured protein phosphorylation statuses but there were main effects of time for p-ACC$^{Ser79}$ ($P < 0.001$), p-4EBP-1$^{Thr47/46}$ ($P = 0.019$), p-S6K$^{Thr389}$ ($P = 0.04$) and p-eEF2$^{Thr56}$ ($P = 0.003$; Figure 3). The phosphorylation of AMPK$^{Thr172}$ (Figure 3b), Akt$^{Ser473}$ and p38$^{Thr180/Tyr182}$ showed no changes. Representative blots are shown in Figure 3.

### 3.6.3 | Muscle mRNA expression

Genes related to mitochondrial biogenesis and mitochondrial proteins were mainly responsive to exercise (Table 4). PGC-1$\alpha$ showed a time effect ($P = 0.02$) and p53 showed a time effect ($P = 0.005$), and a time × treatment interaction ($P = 0.027$) with no significant treatment effect ($P = 0.073$). For p53, within ED, mRNA was higher at 3.5 h compared to 0 h ($P < 0.001$), and within the 3.5 h time point ED-LF was higher than EB-HF ($P = 0.005$).

### 3.7 | Subjective ratings of hunger and sleep quality

There was a main effect of time for subjective ratings of hunger ($P < 0.001$) with values increasing from 21 ± 18 mm post-dinner to

60 ± 17 mm post-RMR and 65 ± 19 mm at 3.5 h recovery. There was no significant difference in perception of increased sleep quality in EB-HF (64 ± 20 mm) *versus* ED-LF (52 ± 21 mm; $P = 0.078$).

## 4 | DISCUSSION

The main finding of this study is that restoring the energy expended after glycogen-depleting exercise with an EB-HF meal has no positive effect on training adaptations and impairs glycaemic regulation post-training the following day compared to an ED-LF meal. Specifically, there were no meaningful or large differences between acute ED-LF and EB-HF on early markers of muscle adaptation to endurance-type training such as intracellular signalling and mRNA expression of pathways associated to substrate utilization and mitochondrial biogenesis. In addition, glucose regulation and the insulin response were impaired in EB-HF compared to ED-LF after a post-training carbohydrate-containing meal. To our knowledge this is the first study addressing if replenishing the energy expended during exercise during a low-carbohydrate with fat benefits adaptation to endurance training using a 'sleep-low' model. This extends our recent findings using a 'twice-a-day' model where we showed no differences in the adaptive response between low-CHO energy deficit and low-CHO energy balance (high fat) treatment groups (Hammond et al., 2019). Moreover, these results suggest that replenishing energy expended during exercise with a high-fat meal is more likely to impair than enhance the adaptive response to training with low skeletal muscle glycogen.

Using a sleep-low train-low model in which a high-fat meal was consumed prior to sleep, we studied trained cyclists who 12 h

later commenced morning exercise with reduced but comparable pre-exercise muscle glycogen but divergent amounts of energy availability (Table 1), all undertaken in a strictly controlled setting. The morning skeletal muscle glycogen concentrations group means of ~350 mmol kg$^{-1}$ dry mass (Figure 3) were in line with expected values for this population after training and low carbohydrate diet (Areta & Hopkins, 2018), and are in accordance with the diminished morning RERs indicating low carbohydrate availability (Bergström, Hermansen, Hultman, & Saltin, 1967). Despite the divergent energy availability between groups (Table 1), there were no differences in RMR, sleep or hunger, and substrate utilization, and perceptual responses were comparable between trials. There was, additionally, a large use of fat of ~1.1 g min$^{-1}$ exercising at 60% of $\dot{V}_{O_2 max}$ (Table 3) in both groups, which is close to twofold that of the maximal rate of fat oxidation we observed in athletes under normal conditions (Areta, Austarheim, Wangensteen, & Capelli, 2018). Data therefore are in agreement with classic literature (Bergström et al., 1967) which shows that local glycogen and carbohydrate availability are key factors in regulating substrate metabolism, which was the key common factor in both of our groups. Despite the clear effect of LCHO modulating the metabolic response and substrate use, the large difference in energy availability and fat intake had only small effects on markers of metabolic adaptation.

Genes associated to carbohydrate metabolism (pyruvate dehydrogenase kinase 4 (PDK4)) and mitochondrial biogenesis (PGC-1$\alpha$ and p53) were up-regulated post-training in both groups and despite no effect of diet on genes associated to fat metabolism, with a small response of the p53 gene in the energy deficit group (Table 4). The magnitude in the change of PDK4 mRNA was lower than that observed in previous studies (Lane et al., 2015; Pilegaard et al., 2005) probably due to being dampened by the carbohydrate ingested in the recovery drink, which has been shown to completely blunt the exercise-induced increase in PDK4 mRNA 4 h post-exercise (Cluberton, McGee, Murphy, & Hargreaves, 2005). This may also have affected the fat metabolism genes post-exercise, as the lack of change in CD36 or carnitine palmitoyltransferase I (CPT1) mRNA in EB-HF was surprising given the previously documented increase of these in high-fat interventions (Hammond et al., 2016; Pilegaard et al., 2005). Instead, the most prominent gene responses to exercise was that of p53 and PGC-1$\alpha$, which is similar to previous observations in response to exercise (Bartlett et al., 2013; Hammond et al., 2016; Impey et al., 2016). Overexpression of p53 has been linked to increased mitochondrial content and aerobic capacity (Park et al., 2009) and is thought to play a key role in mitochondrial biogenesis (Bartlett et al., 2013). Additionally, as a further benefit in the ED-LF intervention we also observed improved glucose control in the morning.

We show for the first time that glucose regulation is impaired after a second bout of exercise when a high-fat meal is ingested following a glycogen-depleting exercise bout the day before. Prior research has shown that an acute exercise session followed by a 36 h high-fat diet (Sparti & Décombaz, 1992) or 7 h intralipid infusion (Pehmoller et al., 2012) impaired exogenous glucose and insulin regulation, but these studies incorporated no exercise prior the provision of carbohydrates.

Using a similar design to these, Newsom et al. (2010) showed that low muscle glycogen, rather than energy deficit, was related to improved intravenous glucose disposal. Our findings were unexpected given we show no difference in muscle glycogen between groups and other studies showed no effect of large amounts of fat provision intravenously and orally after exercise on glucose regulation (Fox, Kaufman, & Horowitz, 2004; Schenk, Cook, Kaufman, & Horowitz, 2005). Therefore, we did not expect to observe this effect from a single high-fat meal given the large energy expenditure on both training sessions and the training status of our participants, allegedly with a superior insulin sensitivity compared to the normal population (Steenberg et al., 2019). Our data may partially be explained by the recent findings that chronic training reduces the acute insulin-sensitizing effect of acute exercise, which has been linked to AMPK signalling in muscle (Steenberg et al., 2019). However, we could not link AMPK signalling to blood glucose regulation.

In relation to the activation of the energy-sensing AMPK pathway, we observed no time or treatment effect on the phosphorylation of AMPK$^{Thr172}$ after exercise, similar to what has been reported previously (Yeo et al., 2010). It is possible that the 'well-trained' status of our participants explains at least partially the lack of response in this parameter. Training has been shown to reduce the AMPK response at an absolute training load (McConell et al., 2005) and it has recently been shown that AMPK activity and p-AMPK$^{Thr172}$ are increased in untrained but not trained individuals in response to a 2 h endurance-type session (McConell, Wadley, Le plastrier, & Linden, 2020). However, ACC$^{Ser79}$, which is directly phosphorylated by AMPK, showed increased phosphorylation early post-exercise in both groups (Figure 3c) returning to baseline afterwards as we (Bartlett et al., 2013) and others (Wojtaszewski et al., 2003) have previously shown. Altogether, these results suggest that ED-LF, did not seem to further up-regulate the 'energy-sensing' AMPK signalling at these time points suggesting no interference, synergistic or additive effect between muscle glycogen and energy status in the phosphorylation status of key proteins in this pathway.

The energy status or macronutrient intake did not affect skeletal muscle protein synthesis intracellular markers as evidenced by the phosphorylation status of the mechanistic target of rapamycin (mTOR) pathway. We have previously shown that 5 days of reduced energy availability had no effect on mTOR or its downstream effector p70S6K (Areta et al., 2014), and the current results expand those findings by showing also no effect with endurance exercise. However, in that study (Areta et al., 2014) there was a disconnect between the early signalling response and a measured reduction in myofibrillar protein synthesis. Moreover, it is possible that p70S6K activity may have been down-regulated by high-fat feeding as we previously documented (Hammond et al., 2016). Nevertheless, p-eEF2$^{Thr56}$ decreased at all time points post-exercise likely responding to exercise, and p-S6K$^{Thr389}$ increased 3.5 h post-exercise likely due to increased amino-acidaemia from the recovery drink. However, 4EBP-1$^{Thr47/46}$ phosphorylation remained suppressed throughout recovery. In conclusion, these signalling pathways showed no differences between groups, but our data expand current knowledge on the effects

of LEA and are the first to directly investigate its effect on status of adaptation markers for oxidative phenotype shift in skeletal muscle.

Concordant with our hypothesis, achieving energy balance with a high fat meal did not enhance skeletal muscle response and whole-body metabolism in response to endurance training compared to energy deficit–low fat. While there is growing evidence that LEA can have negative effects on health and potentially adaptation to training through down-regulating muscle protein synthesis (Areta et al., 2014), bone metabolism (Ihle & Loucks, 2004), reproductive function and other physiological systems (Mountjoy et al., 2018), its effect on oxidative capacity and endurance capacity is less clear. Our findings suggest that, while LEA may down-regulate some physiological systems, this might happen at the expense of maintaining (or even improving) functional capacity of other physiological systems (e.g. maintaining tissues' oxidative capacity). If such is the case, it may be possible that LEA/energy deficit could be a stressor independent from, and additive to, training and macronutrient manipulation as a trigger for adaptation. While there can be negative psycho-physiological effects of chronic LEA such as those associated with relative energy deficiency in sport syndrome (Mountjoy et al., 2018) and the triad models (De Souza et al., 2014), evidence suggests that the severity of energy deficit and the duration (Ihle & Loucks, 2004) may be key factors in determining a positive or negative outcome of LEA as a stressor. We, therefore, do not advocate inducing LEA chronically, but highlight the potential for periodized energy/nutrition interventions as part of a balanced training/nutrition plan (Areta, 2020; Stellingwerff, 2018), taking care not to induce a chronic state of LEA which may be detrimental in the short and long-term for the individual (Areta, 2020; Mountjoy et al., 2018).

We believe this is the first study examining the effect of LEA and macronutrient composition on endurance-type training and muscle oxidative capacity early adaptive response and provides an initial observation on the responses to short-term (<24 h) LEA using a 'sleep-low' model. This study informs the best choice of macronutrient composition to maximize adaptations to endurance-type training. As such, our findings indicate that when training with low carbohydrate availability, transient energy deficit may be more beneficial than replacing the expended energy with ingested fat and supports and extends previous findings using isoenergetic low-CHO high-protein *versus* low-CHO high-fat diets (Leckey et al., 2018) and using a twice-a-day model (Hammond et al., 2019). Future studies should address whether more prolonged duration of LEA would result on a different response, provided the 'dose' and duration of reduced energy availability to affect physiological responses in males is still unknown. Additionally, the inclusion of a high-carbohydrate energy balance group in future studies would provide further insights for unravelling the effects of interaction between macronutrient and energy. Finally, it is important to highlight that the current findings represent preliminary results focusing on the early responses of markers associated to key metabolic pathways. Further research will be required to determine if these early-response results are representative of real-world outcomes, which are a product of complex interactions

between an intricate network of metabolic responses and other factors.

In conclusion, our findings are the first to indicate that replenishing energy after glycogen-depleting exercise with a low-carbohydrate high-fat meal in a 'sleep-low' model does not enhance skeletal muscle adaptation and metabolic response in comparison to a low-carbohydrate low-fat (low energy) meal. Interventions aiming at maximizing aerobic training adaptation through restricting carbohydrate availability should incorporate the concept that achieving energy balance through means of increasing fat intake will likely impair rather than enhance the response to training.

## COMPETING INTERESTS

All authors report no conflict of interest.

## AUTHOR CONTRIBUTIONS

Trials and sample collection were performed at the Norwegian School of Sport Sciences (NSSS). Sample analysis was performed at NSSS, Liverpool John Moores University and at the University of Birmingham. Author contributions are as follows: (1) conception or design of the work: J.L.A., J.H. and J.P.M.; (2) acquisition, analysis or interpretation of data for the work: J.L.A., D.O., J.H., S.J., A.P., J.P.M. and J.I. (3) drafting of the work or revising it critically for important intellectual content: J.L.A., J.I., S.J., J.H., A.P., J.P.M. and D.O. All authors approved the final version of the manuscript agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

## ORCID

*José L. Areta* https://orcid.org/0000-0001-6918-1223
*Andrew Philp* https://orcid.org/0000-0003-3860-4136

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