# Leucine enriched protein feeding does not impair exercise-induced free fatty acid availability and lipid oxidation: beneficial implications for training in carbohydrate restricted states

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## Abstract

Given that the enhanced oxidative adaptations observed when training in carbohydrate (CHO) restricted states are potentially regulated through free fatty acid (FFA) mediated signalling and that leucine rich protein elevates muscle protein synthesis, the present study aimed to test the hypothesis that leucine enriched protein feeding enhances circulating leucine concentration but does not impair FFA availability nor whole body lipid oxidation during exercise. Nine males cycled for 2 h at 70% $VO_{2peak}$ when fasted (PLACEBO) or having consumed a whey protein solution (WHEY) or a leucine enriched whey protein gel (GEL), administered as 22 g 1 hour pre-exercise, 11 g/h during and 22 g thirty minutes post-exercise. Total leucine administration was 14.4 g and 6.3 in GEL and WHEY, respectively. Mean plasma leucine concentrations were elevated in GEL (P= 0.001) compared with WHEY and PLACEBO (375 ± 100, 272 ± 51, 146 ± 14 $\mu mol.L^{-1}$ respectively). No differences (P= 0.153) in plasma FFA (WHEY 0.53 ± 0.30, GEL 0.45 ± 0.25, PLACEBO 0.65 ± 0.30, $mmol.L^{-1}$) or whole body lipid oxidation during exercise (WHEY 0.37 ± 0.26, GEL 0.36 ± 0.24, PLACEBO 0.34 ± 0.24 g/min) were apparent between trials, despite elevated (P= 0.001) insulin in WHEY and GEL compared with PLACEBO (38 ± 16, 35 ± 16, 22 ± 11 $pmol.L^{-1}$ respectively). We conclude that leucine enriched protein feeding does not impair FFA availability nor whole body lipid oxidation during exercise, thus having practical applications for athletes who deliberately train in CHO restricted states to promote skeletal muscle adaptations.



**Keywords:** mitochondria, endurance training, lipolysis, whey

## Introduction

Traditional nutritional approaches for endurance training have typically promoted high CHO availability before, during and after training sessions in order to ensure high daily training intensities and volumes as well as promoting recovery (Cermak and Van Loon, 2013; Hawley et al. 1997). However, during the last decade, we and others have consistently observed a potent effect of *reduced* CHO availability (i.e. fasted and/or glycogen depleted training) in modulating training-induced adaptations in skeletal muscle (Hawley and Morton, 2014). For example, reducing endogenous and/or exogenous CHO availability during short-term (e.g. 3-10 week) endurance training increases mitochondrial enzyme activity and protein content (Morton et al., 2009; Yeo et al., 2008; Van Proeyen et al., 2011), increases both whole body (Yeo et al., 2008) and intramuscular lipid oxidation (Hulston et al., 2010), and in some instances, improves exercise capacity (Hansen et al., 2005). These data have therefore led to the innovative *"train-low, compete-high"* model surmising that athletes deliberately complete a portion of their training programme with reduced CHO availability so as to augment training adaptation but yet always ensure high CHO availability prior to and during competition in an attempt to promote maximal performance (Burke, 2010). The augmented training response observed with training-low strategies are currently thought to be regulated via the enhanced activation of upstream cell signalling kinases including both AMPK (Yeo et al. 2010) and p38MAPK (Cochran et al. 2010) that ultimately converge on the downstream regulation of key transcription factors and co-activators such as PGC-1α (Psilander et al. 2013), p53 (Bartlett et al. 2013) and PPARδ (Philp et al. 2013). In this way, training with low CHO availability thereby leads to a co-ordinated upregulation of both the nuclear and mitochondrial genomes.

Despite the emergence of the train-low paradigm, its practical application in athletic populations is not without limitations, most notably a potential reduction in absolute training intensities (Yeo et al. 2008) as well as increased skeletal muscle protein oxidation and breakdown (Lemon and Mullin, 1980; Howarth et al. 2010). An obvious solution to compensate for the latter is to consume high quality protein in close proximity to the exercise stimulus given that protein provision before (Coffey et al. 2011), during (Hulston et al. 2011) and after exercise (Breen et al. 2011; Howarth et al. 2010) is facilitative of a positive net protein balance. In this regard, Pasiakos et al. (2011) also observed that enhanced concentration of leucine (3.5 g in 10 g EAA) in an essential amino acid mixture consumed during two hours of endurance exercise significantly enhanced post-exercise muscle protein synthesis rates when compared with the same total intake of essential amino acids containing reduced leucine concentration (1.87 g in 10 g EAA). We also observed that consuming protein before, during

and after acute exercise undertaken in a glycogen depleted state did not impair the activation of the AMPK-PGC-1α pathway and positively affected molecular regulators of protein synthesis (e.g. eEF2 phosphorylation status), thus demonstrating that high amino acid availability still permits activation of key cell signalling cascades thought to regulate the training-low response (Taylor et al. 2013). When taken together, such data suggest that reduced CHO but high protein availability (especially leucine rich protein) may therefore be a strategic approach to stimulate training-induced adaptations of skeletal muscle.

It is noteworthy, however, that the provision of high protein availability could hinder additional pathways that may be involved in regulation of the training response associated with training-low. Indeed, we observed that the elevated insulin induced by amino acid ingestion (albeit administered as a casein hydrolysate solution) attenuated circulating free fatty acid (FFA) availability and whole body rates of lipid oxidation (Taylor et al. 2013). This effect may be problematic given that FFA may also act as signalling intermediates involved in regulating training adaptation in addition to providing substrate for oxidation (Zbinden et al. 2014; Philp et al. 2013; Fyffe, et al. 2006). Any feeding strategy intended to achieve high protein availability (in an attempt to reduce protein breakdown and promote protein synthesis) during acute train-low sessions should therefore be simultaneously administered with the goal of minimising reductions in lipolysis such that FFA mediated cell signalling can still occur.

Accordingly, the aim of the current study was to therefore test the hypothesis that leucine enriched protein feeding enhances circulating leucine availability but does not impair FFA availability and lipid oxidation during exercise. To this end, we utilised two protein feeding strategies consisting of a traditional whey protein solution but also a novel leucine enriched protein gel. The use of the latter is considered particularly advantageous for endurance athletes given that gels are a highly practical approach to feeding when in locomotion (Pfeiffer et al., 2010, 2012; Lee et al. 2014). When taken together, it is hoped that our data will be of immediate practical application for those athletes who deliberately train in CHO restricted states in order to promote skeletal muscle adaptations that are reflective of both mitochondrial and myofibrillar protein synthesis.

## Methodology

**Subjects:** Nine males (age 29 ± 4 years, height 179.7 ± 2.9 cm and body mass 79.4 ± 3.3 kg) volunteered to participate in the study after providing informed written consent. Subjects were recreational and competitive cyclists and tri-athletes who trained between 3 – 7 hours per week and had been cycling regularly for > 1 year. Mean $VO_{2peak}$ and peak power output (PPO) for the cohort was 53.0 ± 2.1 $ml.kg^{-1}.min^{-1}$ and 334 ± 13 W respectively. None of the subjects had a history of neurological disease or skeletal muscle abnormality and none were under pharmacological intervention during the course of the study. Subjects were asked to maintain habitual activity levels during the course of the study. The study was approved by the Research Ethics Committee of Liverpool John Moores University.

**Overview of Experimental Design*:*** In a repeated measures counter-balanced design (using a Latin Squares approach) and after having previously completed an assessment of $VO_{2peak}$ and PPO, subjects reported to the laboratory on the morning of the experimental trial (following an overnight fast) on 3 occasions separated 5-7 days. To determine the sequence of testing (i.e. the order of which subjects were tested from 1-9), subjects were drawn from a random number generator. Subjects consumed a standardised diet (5 $g.kg^{-1}$ CHO, 2 $g.kg^{-1}$ Protein, 1 $g.kg^{-1}$ Fat) and refrained from exercise for the 48 h prior to all of the testing procedures. With the exception of the breakfast and pre-bedtime snack (both of which were made by the subject themselves and consumed in their own home after appropriate dietary instruction), all food was provided in pre-packaged containers and consumed on the university premises in view of author one so as to verify compliance. On arrival at the laboratory on the morning of the experimental trial, subjects consumed a protein (in the form of whey or gel) or placebo supplement 1 h prior to commencing 2 h of cycling at 60% PPO (~ 70% $VO_{2peak}$), at 30 min intervals during exercise and a final dose at 30 minutes post-exercise. Venous blood samples were taken at regular intervals prior to, during and after exercise. Measurements of heart rate, substrate oxidation and subjective ratings of perceived exertion, enjoyment and gastrointestinal discomfort were obtained during exercise.

**Assessment of maximal oxygen uptake:** Peak oxygen consumption ($VO_{2peak}$) and peak aerobic power (PPO) were determined during an incremental cycle test performed on an electromagnetically braked cycle ergometer (SRM, Julich, Germany). The subjects completed a 10 min warm up at 100 W and self-selected cadence, before commencing the test which consisted of 2 min stages with 30 W increments in power until volitional exhaustion. Breath-by-breath measurements were obtained throughout exercise using a CPX Ultima series online gas analysis system (Medgraphics, Minnesota, US). $VO_{2peak}$ was stated as being achieved by the following end-point

criteria: 1) heart rate within 10 beats.min$^{-1}$ of age-predicted maximum, 2) respiratory exchange ratio > 1.1, and 3) plateau of oxygen consumption despite increased workload. Peak aerobic power was taken as the final stage completed during the incremental test.

**Experimental protocol*:*** Participants arrived at the laboratory in the morning of the trial (08:00) having followed a prescribed diet for two days prior and following an overnight fast. After obtaining measures of nude body mass, an indwelling cannula (Safety Lock 22G, BD Biosciences, West Sussex UK) was inserted into the anticubital vein in the anterior crease of the forearm and a resting blood sample drawn. After the resting blood sample was taken, the cannula was flushed with ~ 5 ml of sterile saline (Kays Medical supplies. Liverpool UK) to keep the cannula patent and sterile, this procedure was repeated after each subsequent blood draw. Having obtained a resting sample, subjects' ingested ether a non-caloric placebo gel and 500 ml of water (PLACEBO), a leucine enriched protein gel that is not currently commercially available (GEL) providing 22 g protein + 2.4 g leucine (total leucine of 4.8 g) with 500 ml of water, or 22 g of commercially available whey protein (total leucine of 2.1 g; REGO Protein, Science in Sport, UK) mixed in 500 ml water (WHEY) and then rested in the laboratory for 60 min prior to the start of exercise. The nutritional compositions of the protein supplements are shown in Table 1. Both the protein gel and placebo gel (containing Water, Gellan Gum, Sodium Citrate, Sodium Benzoate, Potassium Sorbate, Sodium Chloride, Xanthan Gum, Acesulfame K, Ascorbic Acid and Citric Acid) are not currently commercially available and were manufactured flavour matched in-house in an Informed Sport accredited laboratory (Science in Sport, SiS, Nelson, UK). On the basis that caffeine can partially restore training intensity when in glycogen depleted states (Lane et al. 2013) and also that beta-hydroxy beta-methylbutyrate (HMB) can reduce muscle protein breakdown (Wilkinson et al. 2013), we also chose to fortify our GEL with additional caffeine (100 mg per 22 g of protein) and HMB (1 g per 22 g of protein). Blood samples were obtained repeatedly at 20 min intervals during the pre-exercise period and immediately before exercise commenced. Subjects then cycled for 120 min at 60% PPO (~70% $VO_{2peak}$) on a fully adjustable electromagnetically braked cycle ergometer (SRM, Julich, Germany). Each subject used their own pedals, cleats and shoes during each trial. During exercise, feeding of 11 g whey protein with 250 ml water occurred at 30 min, 5.5 g at 60 and 90 min in 125 ml water, or a volume matched placebo or protein gel and water. Blood samples were taken every 20 minutes during exercise and at termination. Substrate oxidation (g.min$^{-1}$) was determined during exercise using CPX Ultima series online gas analysis system (Medgraphics, Minnesota, US) using equations of Jeukendrup and Wallis (2005). Ratings of perceived exertion (RPE) (Borg 1970), gastrointestinal discomfort (GI) (Pfieffer, et al. 2012) and heart rate (Polar Kempele 610i, Finland) were

recorded every 15 min during exercise. Upon completion of exercise, subjects dismounted from the ergometer, towel dried and nude mass was recorded. Thirty minutes after exercise, subjects consumed a further 22 g of protein or placebo with 500 ml water in the form of WHEY or GEL and and two more blood samples were taken at 1 h and 2 h after completion of exercise. Laboratory conditions remained constant across all trials (19 - 21℃, 40 – 50% humidity). In this way, subjects consumed a total of 66 g protein during the protein trials (i.e. 22 g before, 22 g during and 22 g after exercise) in the form of whey protein (total leucine intake of 6.3 g) or a leucine enriched protein gel (total leucine intake of 14.4 g). Upon exercise completion, subjects also rated their perceived ratings of enjoyment as quantified by the Physical Activity Enjoyment Scale (Kenzierski and DiCarlo, 1991).

**Blood analysis:** Blood samples were collected in vacutainers containing $K_2$ EDTA, lithium heparin or serum separation tubes, and stored on ice or at room temperature until centrifugation at 1500 g for 15 min at 4℃. Serum and plasma were aliquoted and stored at -80℃ until analysis. Plasma glucose, lactate, FFA and glycerol were analysed using the Randox Daytona spectrophotometer with commercially available kits (Randox, Ireland), as per the manufacturer's instructions. Serum insulin concentrations were analysed using commercially available ELISA (Cobas, Roche Diagnostics, USA) as per the manufacturer's instructions. Plasma amino acids were quantified as their *tert*-butyldimethylsilyl (*t*-BDMS) derivatives after the addition of suitable internal standards by Gas Chromatography-Mass Spectrometry: briefly, 200 µl of plasma was deproteinized with 1 ml of 100% ethanol, the supernatant dried under nitrogen at 90ºC, re-dissolved in 500 µl 0.5 M HCl, extracted with 2ml ethyl acetate (to remove lipid fraction), and the aqueous layer was then dried and derivatized (with 100 µl Acetonitrile and 100 µl MTBSTFA) at 90ºC for 90 min. The *t*-BDMS BCAA derivatives were separated on RTX5-ms (15m x 0.25 id, 0.25 µ film thickness) capillary column, initial column temp 70ºC, then ramped at 12ºC/min to 280ºC, injector temperature was 240ºC, Helium carrier gas was 1.2 ml/min. Selected ion monitoring of the (M-57) fragment was performed for each amino analysed, and the area under the peak determined relative to the mass isotopomer of the stable isotopically labelled internal standard used for each amino acid e.g. we monitored m/z 302 for Val and Leu and the isotopomer 303 (for $13^C$ Valine) and 304 (for 1,2 $13^C_2$ Leucine).

**Statistical analysis.** Statistical analysis was conducted using the Statistical Package for the Social Sciences software program (SPSS, version 18). Changes in physiological and metabolic responses (i.e. physiological variables, amino acids, metabolites and substrate oxidation rates) were analysed using a two-way repeated

measures general linear model (GLM) where the within factors were time and condition. Additionally, area under the curve (AUC) for metabolites, amino acids and insulin were also calculated (using Graph Pad Prism, version 6) and differences between conditions were assessed using a one-way repeated measures GLM. A comparison of subjects' perceived rating of enjoyment during exercise was also analysed according to a one-way repeated measures GLM where the. Where a significant main effect was observed, pairwise comparisons were analysed according to Bonferoni post-hoc tests in order to locate specific differences. Statistical significance was set at $P < 0.05$ and all data in text, figures, and tables are presented as means ± SEM.

## Results

### *Physiological and perceptual responses during exercise*

A comparison of subjects' physiological and perceptual responses during the exercise protocol is shown in Table 2. Heart rate ($P<0.01$), lipid oxidation ($P<0.01$) and RPE ($P<0.01$) all displayed progressive increases during exercise, whereas CHO oxidation exhibited a significant decline ($P<0.01$). In contrast, GI discomfort displayed no change during exercise ($P=0.14$). However, there was no difference in heart rate ($P=0.84$), oxygen uptake ($P=0.67$), CHO oxidation ($P=0.97$), lipid oxidation ($P=0.90$), RPE ($P=0.11$) and GI discomfort ($P=0.19$) between the PLACEBO, GEL and WHEY trials. Subjects also reported a tendency for a higher rating of perceived enjoyment ($P=0.073$) in the GEL (89 ± 5 AU) versus the WHEY (84 ± 5 AU) and PLACEBO (79 ± 5 AU) trials.

### *Metabolic responses and substrate oxidation during exercise*

Serum insulin significantly increased in the pre-exercise period and also showed significant declines during exercise ($P<0.01$) in both the WHEY and GEL trials (see Figure 1). Accordingly, insulin displayed significant differences between conditions ($P<0.01$) where both WHEY ($P<0.01$) and GEL ($P=0.01$) were significantly higher than PLACEBO (see Figure 1) though no differences were apparent between the WHEY and GEL trials ($P=1.0$). Total AUC for insulin also displayed differences ($P<0.01$) between conditions such that both WHEY and GEL were different from PLACEBO ($P<0.01$ and $=0.01$, respectively) but no differences occurred between WHEY and GEL ($P=1.0$)

Exercise induced significant increases in plasma NEFA ($P<0.01$), glycerol ($P<0.01$) and lactate ($P<0.01$) whereas glucose displayed no significant ($P=0.09$) change (see Figure 2 A, C, E and F, respectively).

Despite significant changes in insulin in the protein fed trials, plasma NEFA (P=0.12), glycerol (P=0.42), glucose (P=0.19) and lactate (P=0.06) were not different between the PLACEBO, WHEY and GEL trials at rest or during exercise. Accordingly, total AUC for NEFA (P=0.09), glycerol (P=0.38), glucose (P=0.1) and lactate (P=0.07) also displayed no differences between treatments.

***Plasma amino acid responses***

Plasma leucine, BCAAs and EAAs are displayed in Figure 3 A, C and E, respectively. Feeding-induced increases in plasma leucine (P<0.01) were significantly different between conditions (P<0.01) such that a significant interaction effect was observed (P=0.02). Specifically, plasma leucine was significantly greater in GEL versus both WHEY (P<0.01) and PLACEBO (P<0.01). As such, total AUC for leucine was also different between treatments with differences evident across all pair-wise comparisons (all P<0.01) (see Figure 3 B).

Similar to leucine, feeding also induced a significant increase (both P<0.01) in plasma BCAAs and EAAs for both WHEY and GEL where both a significant effect of condition (both P<0.01) and interaction (P<0.01 and P=0.04, respectively) was observed. However, pairwise comparisons revealed there to be no differences in total BCAAs and EAAs between GEL and WHEY (P=1.0 and 0.6, respectively) though both were different from PLACEBO (P<0.01). In accordance, although total AUC for BCAAs and EAAs displayed significant main effects between treatments (both P<0.01), significant pairwise effects (all P<0.01) were only apparent when comparing GEL and WHEY from PLACEBO (see Figure 3 D and F).

## Discussion

The aim of the present study was to test the hypothesis that leucine enriched protein feeding enhances circulating leucine availability but does not impair FFA availability and lipid oxidation during exercise. To this end, we utilised two protein feeding strategies consisting of a traditional whey protein solution but also a novel leucine enriched protein gel. We provide novel data by demonstrating that provision of protein (in either of the aforementioned forms) before, during and after two hours of endurance exercise undertaken in the absence of CHO feeding pre- and during exercise does not impair FFA availability or lipid oxidation. Given that the enhanced training response observed when training in CHO restricted states is potentially regulated through

277 FFA mediated signalling and also that leucine enriched protein elevates muscle protein synthesis, we therefore
278 consider our data to have practical implications (i.e. feeding strategies) for those athletes who deliberately train
279 in CHO restricted states in order to enhance skeletal muscle adaptations to endurance training.

280 Despite the apparent advantage to carefully scheduling periods of fasted (Van Proeyen et al. 2011) and/or
281 glycogen depleted endurance training (Yeo et al. 2008; Morton et al. 2009), such approaches may be limited in
282 that skeletal muscle protein oxidation and breakdown is increased (Lemon and Mullin, 1980; Howarth et al.
283 2009) and hence, net protein balance becomes negative if amino acids are also not ingested (Hulston et al. 2011).
284 If performed chronically (especially in the presence of reduced daily caloric intake), this approach could
285 therefore lead to a loss of skeletal muscle mass (Cabone et al. 2013; Mettler et al. 2010; Pasiakos et al. 2013)
286 and potentially, a de-training effect (Breen et al. 2011). To this end, we recently demonstrated that consuming a
287 casein hydrolysate solution before, during and after glycogen depleted exercise does not impair activation of
288 mitochondrial related signalling pathways (e.g. AMPK- PGC-1α) as well as positively affecting molecular
289 regulators of protein synthesis (e.g. eEF2 phosphorylation status) (Taylor et al. 2013). As such, many elite
290 endurance cyclists now perform prolonged morning rides in CHO restricted states but with protein rich
291 breakfasts and additional protein during exercise (typically in the form of whey / casein drinks) in a deliberate
292 attempt to promote oxidative adaptations of skeletal muscle (Walsh, 2014).

293 In terms of modulating protein synthesis, however, current evidence also suggests that both rapidly digestible
294 but leucine rich proteins are the optimal protein source. For example, Pasiakos et al. (2011) observed that
295 enhanced concentration of leucine (3.5 g in 10 g EAA) in an essential amino acid mixture consumed during two
296 hours of endurance exercise significantly enhanced post-exercise muscle protein synthesis rates when compared
297 with the same total intake of essential amino acids but containing reduced leucine concentration (1.87 g in 10 g
298 EAA). Furthermore, Churchward-Venne et al. (2014) demonstrated comparable post-exercise myofibrillar
299 synthesis rates (albeit from a resistance exercise stimulus) from mixed macronutrient drinks containing 25 g
300 whey versus a bolus of 6.25 g whey supplemented with 5 g leucine. Such observations therefore formed the
301 underlying rationale for the present study in terms of studying whey protein (naturally high in leucine) but also,
302 a novel leucine enriched protein gel. Total leucine administration during the data collection period (i.e. 5 hours)
303 was 14.4 and 6.3 g in WHEY and GEL, respectively. Accordingly, mean plasma leucine levels were higher in
304 GEL versus WHEY and in both feeding strategies, plasma leucinemia increased to levels that would be expected
305 to promote post-exercise muscle protein synthesis (Breen et al. 2011; Pasiakos et al. 2011). Unfortunately,

direct estimates of muscle protein synthesis (and related molecular regulators) were not obtained in the present study nor did we quantify rates of leucine oxidation. Future studies would therefore benefit from direct measures of both mixed muscle protein synthesis (and in sub-cellular fractions) as well as leucine oxidation, both achieved via the inclusion of muscle biopsies and stable isotope methodology.

Although we readily acknowledge that the total leucine delivery in both trials may appear as excessive in terms of that required to facilitate maximal protein synthesis as well as likely resulting in elevated leucine oxidation (Bowtell et al. 1998), we deliberately chose this dosing strategy for a number of practical reasons. Firstly, given that exercising in CHO restricted states augments leucine oxidation (Lemon and Mullin, 1980; Wagenmakers et al. 1991; Howarth et al. 2009), it was our deliberate aim to administer higher exogenous leucine so as to deliver both substrate to promote muscle protein synthesis (Breen et al. 2011; Pasiakos et al. 2011; Churchward-Venne et al. 2013) but yet, also compensate for the higher levels of endogenous leucine oxidation (Lemon and Mullin, 1980; Wagenmakers et al. 1991; Howarth et al. 2009). Second, unpublished observations by the corresponding author on elite professional cyclists indicated that this is the type of protein feeding strategy actually adopted during morning training rides that are deliberately undertaken in the absence of CHO intake before and during exercise. As such, our initial aim was to replicate these "real world" strategies and determine if such doses of protein actually impairs FFA availability and lipid oxidation. Finally, given that many elite cyclists are potentially in daily energy deficits (Vogt et al. 2005) with low energy availability (Loucks et al. 2011), and also that 2-3 x RDA for daily protein is required to maintain lean mass during energy restriction (Pasiakos et al. 2013), we therefore considered this feeding strategy to be in accordance with daily protein intakes for both quantity and frequency (Areta et al. 2013). For example, over the 5 h data collection period (i.e. 8 am to 1 pm), the present subjects (ranging from 67-86 kg) consumed 66 g protein and hence for the daily target to be achieved (i.e. approximately 160-200 g), our approach is therefore in accordance with a feeding strategy where subsequent 30 g doses could be consumed at 3 h intervals (e.g. 2, 5, 8 and 11 pm if required).

In terms of modulating the train-low response, the important aspect of the present paper is that neither the whey protein solution nor the leucine enriched protein gel attenuated circulating FFA availability or whole body rates of lipid oxidation during exercise, despite elevated serum insulin levels in the pre-exercise period. This finding is especially relevant considering that the enhanced training response observed when training with low CHO availability (particularly the elevated capacity for lipid oxidation during customary exercise) is likely regulated, in part, through FFA mediated signalling. Indeed, the exercise-induced increase in p38MAPK activity is

blunted by pharmacological reduction of circulating FFA availability (Zbinden et al. 2014). Furthermore, exercise undertaken in a glycogen-depleted state increases PPARδ binding to the CPT1 promoter (Philp et al. 2013), the latter considered as a rate limiting enzyme for long chain fatty acid oxidation. The observation of no apparent reduction in lipolysis with protein feeding in the present study disagrees with previous observations from our laboratory where we demonstrated that a casein hydrolysate solution (administered as 20 g 45 min pre-exercise, 10 g during exercise and 20 g post-exercise) attenuated plasma FFA availability and whole body rates of lipid oxidation during exercise. Such discrepancies may be due in part to differences in timing of pre-exercise feeding (i.e. 60 versus 45 minutes before exercise) as well as type of protein consumed (i.e. casein hydrolysate versus predominantly whey isolate proteins). The latter point is particularly relevant given that hydrolysate proteins induce greater insulin responses compared with isolated protein sources (Tang et al. 2009; Pennings et al. 2011). As such, the effects of protein ingestion on metabolic responses during exercise are likely dependent upon the interplay of a number of important factors including type and timing of protein ingestion. Such factors should therefore be taken into consideration when designing real world feeding strategies.

To achieve our delivery for the leucine enriched protein trial, we chose to develop a novel protein based gel. Indeed, considering that leucine is often not readily soluble in water, we reasoned that a gel format provided a more convenient approach for which to deliver a leucine enriched protein source. Moreover, gels are a highly practical approach to carrying fuel and feeding whilst in locomotion as opposed to conventional approaches of fluid delivery (Pfeiffer et al., 2010; Lee et al. 2014). Accordingly, gels are a highly utilised form of supplement by endurance athletes (Pfeiffer et al., 2012). On the basis that caffeine can partially restore training intensity when in glycogen depleted states (Lane et al. 2013) and also that beta-hydroxy beta-methylbutyrate (HMB) can reduce muscle protein breakdown (Wilkinson et al. 2013), we also chose to fortify our GEL with additional caffeine (100 mg per 22 g of protein) and HMB (1 g per 22 g of protein). Although we did not measure parameters related to these products given that our exercise protocol was clamped (i.e. 2 h fixed intensity of 70% $VO_{2peak}$) and no indices of protein breakdown were obtained, our observations of no differences in gastrointestinal discomfort between trials supports the notion that multiple ingredients in the gel format were well tolerated. Interestingly, subjects' ratings of perceived enjoyment (as quantified by the Physical Activity Enjoyment Scale) also tended to be higher in GEL (90 ± 5) versus both WHEY (84 ± 5) and PLACEBO (79 ± 5). Given that this scale takes into account subjective measurements of mood status related to vigor and arousal, this observation maybe related to the higher plasma concentrations of leucine observed in the GEL trial and is

accordance with the hypothesis that elevated BCAAs (especially in conditions of low CHO availability) may alleviate symptoms of central fatigue (Blomstrand et al., 2005; Blomstrand, 2006).

In summary, we provide novel data by demonstrating that neither a whey protein solution nor a leucine enriched protein gel impairs circulating FFA availability or rates of whole body lipid oxidation during two hours of endurance exercise undertaken in a CHO restricted state. Given that the augmented training response observed when training with reduced CHO availability (i.e. absence of CHO provision before/during exercise and/or glycogen depleted conditions) is potentially regulated through FFA mediated signalling and also that leucine rich protein elevates muscle protein synthesis, we consider our data to have practical implications for those athletes who deliberately train in CHO restricted states in order to enhance skeletal muscle adaptations to endurance training (as reflective of both mitochondrial and myofibrillar protein synthesis). Further studies are now required to examine both the acute (e.g. cell signalling, gene expression responses and direct measures of protein turnover) and chronic effects (e.g. mitochondrial adaptations) of reduced CHO but high protein availability on skeletal muscle adaptation to endurance training as well as potential impact on measures of exercise performance.

**Acknowledgements**

This work was supported by a research grant from Science in Sport (SiS) UK, plc.

**Conflicts of Interest**

The authors declare no conflicts of interest.

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TABLE 1 – Overview of the nutritional compositions of the GEL and WHEY products (protein in both products was from whey isolate sources).

TABLE 2 – Physiological and perceptual responses during exercise. * denotes significant difference from 30 min, $P<0.05$.

FIGURE 1 – Serum insulin concentrations before, during and after exercise. Total area under the curve for insulin is also shown inset. Shaded area represents the exercise bout. Downward arrows denote timing of treatment ingestion. * denotes significant difference from baseline values i.e. minute 0, $P<0.05$. [a] denotes significant difference from PLACEBO, $P<0.05$.

FIGURE 2 – Plasma (A) NEFA, (C) glycerol, (E) glucose and (G) lactate before, during and after exercise. Shaded area represents the exercise bout. Downward arrows denote timing of treatment ingestion. Total area under the curve (AUC) for NEFA (B), glycerol (D), glucose (F) and lactate (H) are also shown in the right hand panels. * denotes significant difference from baseline values i.e. minute 0, $P<0.05$.

FIGURE 3 – Plasma (A) leucine, (C) BCAAs, and (E) EAAs before, during and after exercise. Shaded area represents the exercise bout. Downward arrows denote timing of treatment ingestion. * denotes significant difference from baseline values i.e. minute 0, $P<0.05$. Total area under the curve (AUC) for leucine (B), BCAAs (D) and EAAs (F) are also shown in the right hand panels. [a] denotes significant difference from PLACEBO, $P<0.05$. [b] denotes significant difference from WHEY, $P<0.05$.

TABLE 1

| | GEL | WHEY |
|---|---|---|
| Total Protein (g) | 22.0 | 22.0 |
| Additional Leucine (g) | 2.4 | 0.0 |
| Total Leucine (g) | 4.8 | 2.1 |
| Total BCAA (g) | 7.5 | 4.9 |
| Total EAA (g) | 13.1 | 9.3 |
| Caffeine (mg) | 100.0 | 0.0 |
| HMB (g) | 1.0 | 0.0 |

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574 TABLE 2

| | Time (min) | | | |
|---|---|---|---|---|
| | **30** | **60** | **90** | **120** |
| **CHO Oxidation (g/min)** | | | | |
| Gel | 3.14 ± 0.18 | 2.70 ± 0.23* | 2.25 ± 0.15* | 1.98 ± 0.15* |
| Placebo | 3.13 ± 0.19 | 2.61 ± 0.18* | 2.38 ± 0.15* | 2.20 ± 0.13* |
| Whey | 3.13 ± 0.24 | 2.64 ± 0.22* | 2.24 ± 0.17* | 1.97 ± 0.18* |
| **Lipid Oxidation (g/min)** | | | | |
| Gel | 0.12 ± 0.08 | 0.31 ± 0.11* | 0.51 ± 0.06* | 0.63 ± 0.05* |
| Placebo | 0.05 ± 0.07 | 0.34 ± 0.08* | 0.47 ± 0.07* | 0.61 ± 0.07* |
| Whey | 0.11 ± 0.09 | 0.40 ± 0.08* | 0.47 ± 0.07* | 0.60 ± 0.07* |
| **HR (b/min)** | | | | |
| Gel | 149 ± 4 | 154 ± 3 | 158 ± 4* | 164 ± 4* |
| Placebo | 148 ± 3 | 153 ± 3 | 157 ± 4* | 164 ± 4* |
| Whey | 152 ± 5 | 156 ± 3 | 157 ± 4* | 163 ± 3* |
| **$VO_2$ (%max)** | | | | |
| Gel | 67± 2 | 68 ± 2 | 68 ± 2 | 67 ± 3 |
| Placebo | 67 ± 1 | 67± 1 | 68± 2 | 70 ± 2 |
| Whey | 66 ± 2 | 69 ± 1 | 67 ± 2 | 68 ± 3 |
| **RPE (AU)** | | | | |
| Gel | 11 ± 1 | 13 ± 1* | 13 ± 1* | 14 ± 1* |
| Placebo | 12 ± 1 | 14 ± 1* | 15 ± 1* | 16 ± 1* |
| Whey | 12 ± 1 | 14 ± 1* | 14 ± 1* | 15 ± 1* |
| **GI (AU)** | | | | |
| Gel | 10 ± 1 | 10 ± 1 | 10 ± 1 | 10 ± 1 |
| Placebo | 9 ± 1 | 10 ± 1 | 10 ± 1 | 9 ± 1 |
| Whey | 10 ± 1 | 11 ± 1 | 11 ± 1 | 10 ± 1 |

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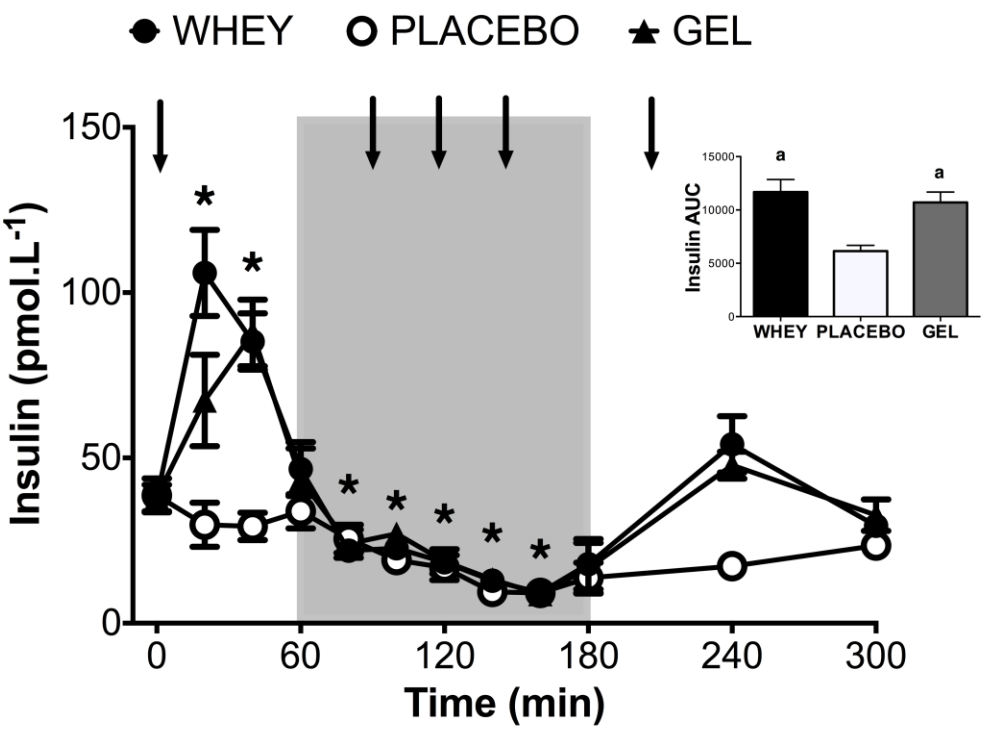
FIGURE 1


WHEY
PLACEBO
GEL
Insulin (pmol.L-1)
150
100
50
0
0
60
120
180
240
300
Time (min)
*
Insulin AUC
15000
10000
5000
0
a
a
WHEY
PLACEBO
GEL

597 FIGURE 2

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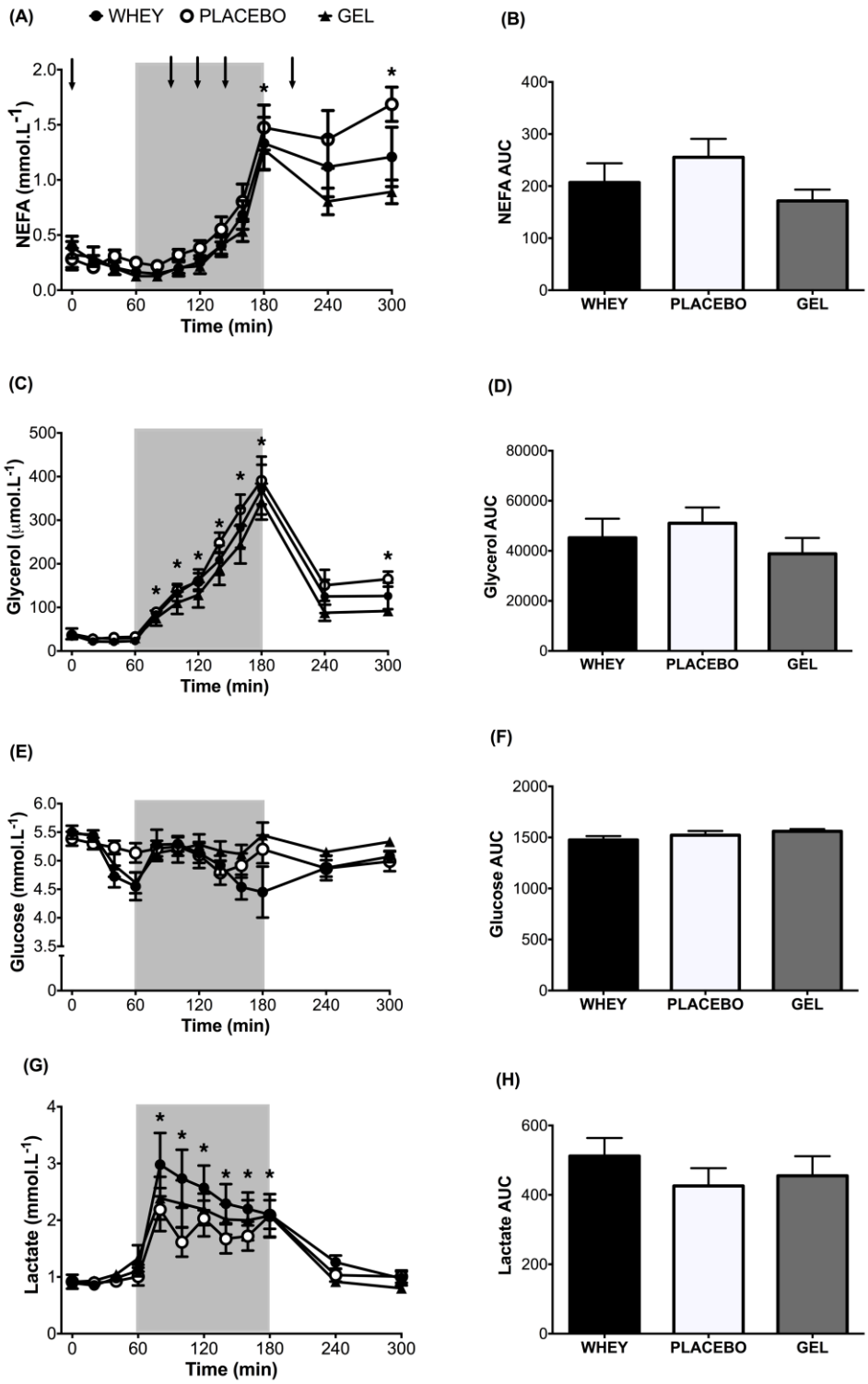


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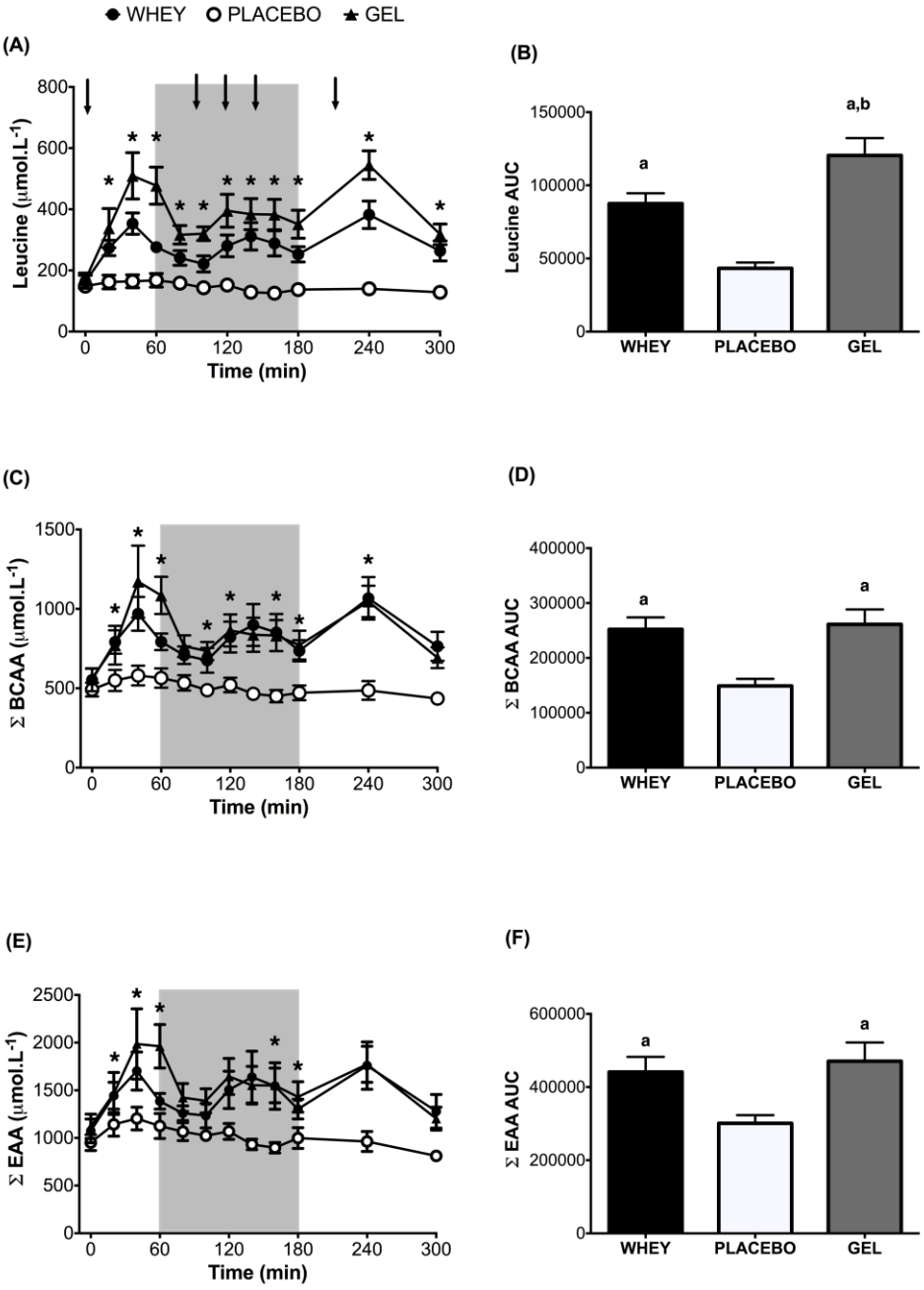
603 FIGURE 3

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