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### Article

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**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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52 **Abstract**

53 Given that the enhanced oxidative adaptations observed when training in carbohydrate (CHO) restricted states  
54 are potentially regulated through free fatty acid (FFA) mediated signalling and that leucine rich protein elevates  
55 muscle protein synthesis, the present study aimed to test the hypothesis that leucine enriched protein feeding  
56 enhances circulating leucine concentration but does not impair FFA availability nor whole body lipid oxidation  
57 during exercise. Nine males cycled for 2 h at 70%  $VO_{2peak}$  when fasted (PLACEBO) or having consumed a  
58 whey protein solution (WHEY) or a leucine enriched whey protein gel (GEL), administered as 22 g 1 hour pre-  
59 exercise, 11 g/h during and 22 g thirty minutes post-exercise. Total leucine administration was 14.4 g and 6.3 in  
60 GEL and WHEY, respectively. Mean plasma leucine concentrations were elevated in GEL ( $P= 0.001$ ) compared  
61 with WHEY and PLACEBO ( $375 \pm 100$ ,  $272 \pm 51$ ,  $146 \pm 14 \mu\text{mol.L}^{-1}$  respectively). No differences ( $P= 0.153$ )  
62 in plasma FFA (WHEY  $0.53 \pm 0.30$ , GEL  $0.45 \pm 0.25$ , PLACEBO  $0.65 \pm 0.30$ ,  $\text{mmol.L}^{-1}$ ) or whole body lipid  
63 oxidation during exercise (WHEY  $0.37 \pm 0.26$ , GEL  $0.36 \pm 0.24$ , PLACEBO  $0.34 \pm 0.24$  g/min) were apparent  
64 between trials, despite elevated ( $P= 0.001$ ) insulin in WHEY and GEL compared with PLACEBO ( $38 \pm 16$ ,  $35 \pm$   
65  $16$ ,  $22 \pm 11 \text{ pmol.L}^{-1}$  respectively). We conclude that leucine enriched protein feeding does not impair FFA  
66 availability nor whole body lipid oxidation during exercise, thus having practical applications for athletes who  
67 deliberately train in CHO restricted states to promote skeletal muscle adaptations.

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

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## 77 **Introduction**

78 Traditional nutritional approaches for endurance training have typically promoted high CHO availability before,  
79 during and after training sessions in order to ensure high daily training intensities and volumes as well as  
80 promoting recovery (Cermak and Van Loon, 2013; Hawley et al. 1997). However, during the last decade, we  
81 and others have consistently observed a potent effect of *reduced* CHO availability (i.e. fasted and/or glycogen  
82 depleted training) in modulating training-induced adaptations in skeletal muscle (Hawley and Morton, 2014).  
83 For example, reducing endogenous and/or exogenous CHO availability during short-term (e.g. 3-10 week)  
84 endurance training increases mitochondrial enzyme activity and protein content (Morton et al., 2009; Yeo et al.,  
85 2008; Van Proeyen et al., 2011), increases both whole body (Yeo et al., 2008) and intramuscular lipid oxidation  
86 (Hulston et al., 2010), and in some instances, improves exercise capacity (Hansen et al., 2005). These data have  
87 therefore led to the innovative “*train-low, compete-high*” model surmising that athletes deliberately complete a  
88 portion of their training programme with reduced CHO availability so as to augment training adaptation but yet  
89 always ensure high CHO availability prior to and during competition in an attempt to promote maximal  
90 performance (Burke, 2010). The augmented training response observed with training-low strategies are  
91 currently thought to be regulated via the enhanced activation of upstream cell signalling kinases including both  
92 AMPK (Yeo et al. 2010) and p38MAPK (Cochran et al. 2010) that ultimately converge on the downstream  
93 regulation of key transcription factors and co-activators such as PGC-1 $\alpha$  (Psilander et al. 2013), p53 (Bartlett et  
94 al. 2013) and PPAR $\delta$  (Philp et al. 2013). In this way, training with low CHO availability thereby leads to a co-  
95 ordinated upregulation of both the nuclear and mitochondrial genomes.

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

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

112 It is noteworthy, however, that the provision of high protein availability could hinder additional pathways that  
113 may be involved in regulation of the training response associated with training-low. Indeed, we observed that  
114 the elevated insulin induced by amino acid ingestion (albeit administered as a casein hydrolysate solution)  
115 attenuated circulating free fatty acid (FFA) availability and whole body rates of lipid oxidation (Taylor et al.  
116 2013). This effect may be problematic given that FFA may also act as signalling intermediates involved in  
117 regulating training adaptation in addition to providing substrate for oxidation (Zbinden et al. 2014; Philp et al.  
118 2013; Fyffe, et al. 2006). Any feeding strategy intended to achieve high protein availability (in an attempt to  
119 reduce protein breakdown and promote protein synthesis) during acute train-low sessions should therefore be  
120 simultaneously administered with the goal of minimising reductions in lipolysis such that FFA mediated cell  
121 signalling can still occur.

122 Accordingly, the aim of the current study was to therefore test the hypothesis that leucine enriched protein  
123 feeding enhances circulating leucine availability but does not impair FFA availability and lipid oxidation during  
124 exercise. To this end, we utilised two protein feeding strategies consisting of a traditional whey protein solution  
125 but also a novel leucine enriched protein gel. The use of the latter is considered particularly advantageous for  
126 endurance athletes given that gels are a highly practical approach to feeding when in locomotion (Pfeiffer et al.,  
127 2010, 2012; Lee et al. 2014). When taken together, it is hoped that our data will be of immediate practical  
128 application for those athletes who deliberately train in CHO restricted states in order to promote skeletal muscle  
129 adaptations that are reflective of both mitochondrial and myofibrillar protein synthesis.

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## 133 **Methodology**

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

142 **Overview of Experimental Design:** In a repeated measures counter-balanced design (using a Latin Squares  
143 approach) and after having previously completed an assessment of  $\text{VO}_{2\text{peak}}$  and PPO, subjects reported to the  
144 laboratory on the morning of the experimental trial (following an overnight fast) on 3 occasions separated 5-7  
145 days. To determine the sequence of testing (i.e. the order of which subjects were tested from 1-9), subjects were  
146 drawn from a random number generator. Subjects consumed a standardised diet (5 g.kg<sup>-1</sup> CHO, 2 g.kg<sup>-1</sup> Protein,  
147 1 g.kg<sup>-1</sup> Fat) and refrained from exercise for the 48 h prior to all of the testing procedures. With the exception of  
148 the breakfast and pre-bedtime snack (both of which were made by the subject themselves and consumed in their  
149 own home after appropriate dietary instruction), all food was provided in pre-packaged containers and  
150 consumed on the university premises in view of author one so as to verify compliance. On arrival at the  
151 laboratory on the morning of the experimental trial, subjects consumed a protein (in the form of whey or gel) or  
152 placebo supplement 1 h prior to commencing 2 h of cycling at 60% PPO (~ 70%  $\text{VO}_{2\text{peak}}$ ), at 30 min intervals  
153 during exercise and a final dose at 30 minutes post-exercise. Venous blood samples were taken at regular  
154 intervals prior to, during and after exercise. Measurements of heart rate, substrate oxidation and subjective  
155 ratings of perceived exertion, enjoyment and gastrointestinal discomfort were obtained during exercise.

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

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

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



192 recorded every 15 min during exercise. Upon completion of exercise, subjects dismounted from the ergometer,  
193 towel dried and nude mass was recorded. Thirty minutes after exercise, subjects consumed a further 22 g of  
194 protein or placebo with 500 ml water in the form of WHEY or GEL and and two more blood samples were  
195 taken at 1 h and 2 h after completion of exercise. Laboratory conditions remained constant across all trials (19 -  
196 21 °C, 40 – 50% humidity). In this way, subjects consumed a total of 66 g protein during the protein trials (i.e. 22  
197 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  
198 leucine enriched protein gel (total leucine intake of 14.4 g). Upon exercise completion, subjects also rated their  
199 perceived ratings of enjoyment as quantified by the Physical Activity Enjoyment Scale (Kenzierski and DiCarlo,  
200 1991).

201  
202 **Blood analysis:** Blood samples were collected in vacutainers containing K<sub>2</sub> EDTA, lithium heparin or serum  
203 separation tubes, and stored on ice or at room temperature until centrifugation at 1500 g for 15 min at 4 °C.  
204 Serum and plasma were aliquoted and stored at -80 °C until analysis. Plasma glucose, lactate, FFA and glycerol  
205 were analysed using the Randox Daytona spectrophotometer with commercially available kits (Randox, Ireland),  
206 as per the manufacturer's instructions. Serum insulin concentrations were analysed using commercially  
207 available ELISA (Cobas, Roche Diagnostics, USA) as per the manufacturer's instructions. Plasma amino acids  
208 were quantified as their *tert*-butyldimethylsilyl (*t*-BDMS) derivatives after the addition of suitable internal  
209 standards by Gas Chromatography-Mass Spectrometry: briefly, 200 µl of plasma was deproteinized with 1 ml of  
210 100% ethanol, the supernatant dried under nitrogen at 90 °C, re-dissolved in 500 µl 0.5 M HCl, extracted with  
211 2ml ethyl acetate (to remove lipid fraction), and the aqueous layer was then dried and derivatized (with 100 µl  
212 Acetonitrile and 100 µl MTBSTFA) at 90 °C for 90 min. The *t*-BDMS BCAA derivatives were separated on  
213 RTX5-ms (15m x 0.25 id, 0.25 µ film thickness) capillary column, initial column temp 70 °C, then ramped at  
214 12 °C/min to 280 °C, injector temperature was 240 °C, Helium carrier gas was 1.2 ml/min. Selected ion  
215 monitoring of the (M-57) fragment was performed for each amino analysed, and the area under the peak  
216 determined relative to the mass isotopomer of the stable isotopically labelled internal standard used for each  
217 amino acid e.g. we monitored m/z 302 for Val and Leu and the isotopomer 303 (for <sup>13</sup>C Valine) and 304 (for  
218 1,2 <sup>13</sup>C<sub>2</sub> Leucine).

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

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

229

## 230 **Results**

### 231 *Physiological and perceptual responses during exercise*

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

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

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

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

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

254

### 255 *Plasma amino acid responses*

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

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

268

### 269 **Discussion**

270 The aim of the present study was to test the hypothesis that leucine enriched protein feeding enhances  
271 circulating leucine availability but does not impair FFA availability and lipid oxidation during exercise. To this  
272 end, we utilised two protein feeding strategies consisting of a traditional whey protein solution but also a novel  
273 leucine enriched protein gel. We provide novel data by demonstrating that provision of protein (in either of the  
274 aforementioned forms) before, during and after two hours of endurance exercise undertaken in the absence of  
275 CHO feeding pre- and during exercise does not impair FFA availability or lipid oxidation. Given that the  
276 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-1 $\alpha$ ) 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,

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

310 Although we readily acknowledge that the total leucine delivery in both trials may appear as excessive in terms  
311 of that required to facilitate maximal protein synthesis as well as likely resulting in elevated leucine oxidation  
312 (Bowtell et al. 1998), we deliberately chose this dosing strategy for a number of practical reasons. Firstly, given  
313 that exercising in CHO restricted states augments leucine oxidation (Lemon and Mullin, 1980; Wagenmakers et  
314 al. 1991; Howarth et al. 2009), it was our deliberate aim to administer higher exogenous leucine so as to deliver  
315 both substrate to promote muscle protein synthesis (Breen et al. 2011; Pasiakos et al. 2011; Churchward-Venne  
316 et al. 2013) but yet, also compensate for the higher levels of endogenous leucine oxidation (Lemon and Mullin,  
317 1980; Wagenmakers et al. 1991; Howarth et al. 2009). Second, unpublished observations by the corresponding  
318 author on elite professional cyclists indicated that this is the type of protein feeding strategy actually adopted  
319 during morning training rides that are deliberately undertaken in the absence of CHO intake before and during  
320 exercise. As such, our initial aim was to replicate these “real world” strategies and determine if such doses of  
321 protein actually impairs FFA availability and lipid oxidation. Finally, given that many elite cyclists are  
322 potentially in daily energy deficits (Vogt et al. 2005) with low energy availability (Loucks et al. 2011), and also  
323 that 2-3 x RDA for daily protein is required to maintain lean mass during energy restriction (Pasiakos et al.  
324 2013), we therefore considered this feeding strategy to be in accordance with daily protein intakes for both  
325 quantity and frequency (Areta et al. 2013). For example, over the 5 h data collection period (i.e. 8 am to 1 pm),  
326 the present subjects (ranging from 67-86 kg) consumed 66 g protein and hence for the daily target to be  
327 achieved (i.e. approximately 160-200 g), our approach is therefore in accordance with a feeding strategy where  
328 subsequent 30 g doses could be consumed at 3 h intervals (e.g. 2, 5, 8 and 11 pm if required).

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

335 blunted by pharmacological reduction of circulating FFA availability (Zbinden et al. 2014). Furthermore,  
336 exercise undertaken in a glycogen-depleted state increases PPAR $\delta$  binding to the CPT1 promoter (Philp et al.  
337 2013), the latter considered as a rate limiting enzyme for long chain fatty acid oxidation. The observation of no  
338 apparent reduction in lipolysis with protein feeding in the present study disagrees with previous observations  
339 from our laboratory where we demonstrated that a casein hydrolysate solution (administered as 20 g 45 min pre-  
340 exercise, 10 g during exercise and 20 g post-exercise) attenuated plasma FFA availability and whole body rates  
341 of lipid oxidation during exercise. Such discrepancies may be due in part to differences in timing of pre-  
342 exercise feeding (i.e. 60 versus 45 minutes before exercise) as well as type of protein consumed (i.e. casein  
343 hydrolysate versus predominantly whey isolate proteins). The latter point is particularly relevant given that  
344 hydrolysate proteins induce greater insulin responses compared with isolated protein sources (Tang et al. 2009;  
345 Pennings et al. 2011). As such, the effects of protein ingestion on metabolic responses during exercise are likely  
346 dependent upon the interplay of a number of important factors including type and timing of protein ingestion.  
347 Such factors should therefore be taken into consideration when designing real world feeding strategies.

348 To achieve our delivery for the leucine enriched protein trial, we chose to develop a novel protein based gel.  
349 Indeed, considering that leucine is often not readily soluble in water, we reasoned that a gel format provided a  
350 more convenient approach for which to deliver a leucine enriched protein source. Moreover, gels are a highly  
351 practical approach to carrying fuel and feeding whilst in locomotion as opposed to conventional approaches of  
352 fluid delivery (Pfeiffer et al., 2010; Lee et al. 2014). Accordingly, gels are a highly utilised form of supplement  
353 by endurance athletes (Pfeiffer et al., 2012). On the basis that caffeine can partially restore training intensity  
354 when in glycogen depleted states (Lane et al. 2013) and also that beta-hydroxy beta-methylbutyrate (HMB) can  
355 reduce muscle protein breakdown (Wilkinson et al. 2013), we also chose to fortify our GEL with additional  
356 caffeine (100 mg per 22 g of protein) and HMB (1 g per 22 g of protein). Although we did not measure  
357 parameters related to these products given that our exercise protocol was clamped (i.e. 2 h fixed intensity of 70%  
358  $VO_{2peak}$ ) and no indices of protein breakdown were obtained, our observations of no differences in  
359 gastrointestinal discomfort between trials supports the notion that multiple ingredients in the gel format were  
360 well tolerated. Interestingly, subjects' ratings of perceived enjoyment (as quantified by the Physical Activity  
361 Enjoyment Scale) also tended to be higher in GEL ( $90 \pm 5$ ) versus both WHEY ( $84 \pm 5$ ) and PLACEBO ( $79 \pm$   
362  $5$ ). Given that this scale takes into account subjective measurements of mood status related to vigor and arousal,  
363 this observation maybe related to the higher plasma concentrations of leucine observed in the GEL trial and is

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

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

378

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### 381 **Conflicts of Interest**

382 The authors declare no conflicts of interest.

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

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

519

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

524

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

530

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

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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

	<u>Time (min)</u>			
	<b>30</b>	<b>60</b>	<b>90</b>	<b>120</b>
<b>CHO Oxidation (g/min)</b>				
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*
<b>Lipid Oxidation (g/min)</b>				
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*
<b>HR (b/min)</b>				
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*
<b>VO<sub>2</sub> (%max)</b>				
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
<b>RPE (AU)</b>				
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*
<b>GI (AU)</b>				
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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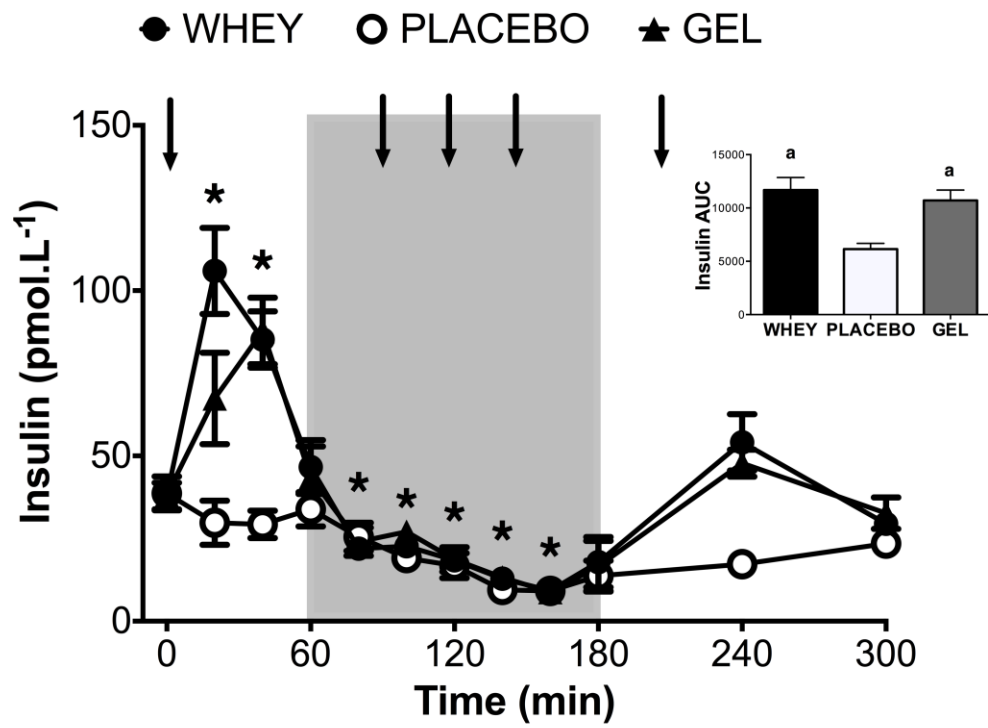
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580 FIGURE 1

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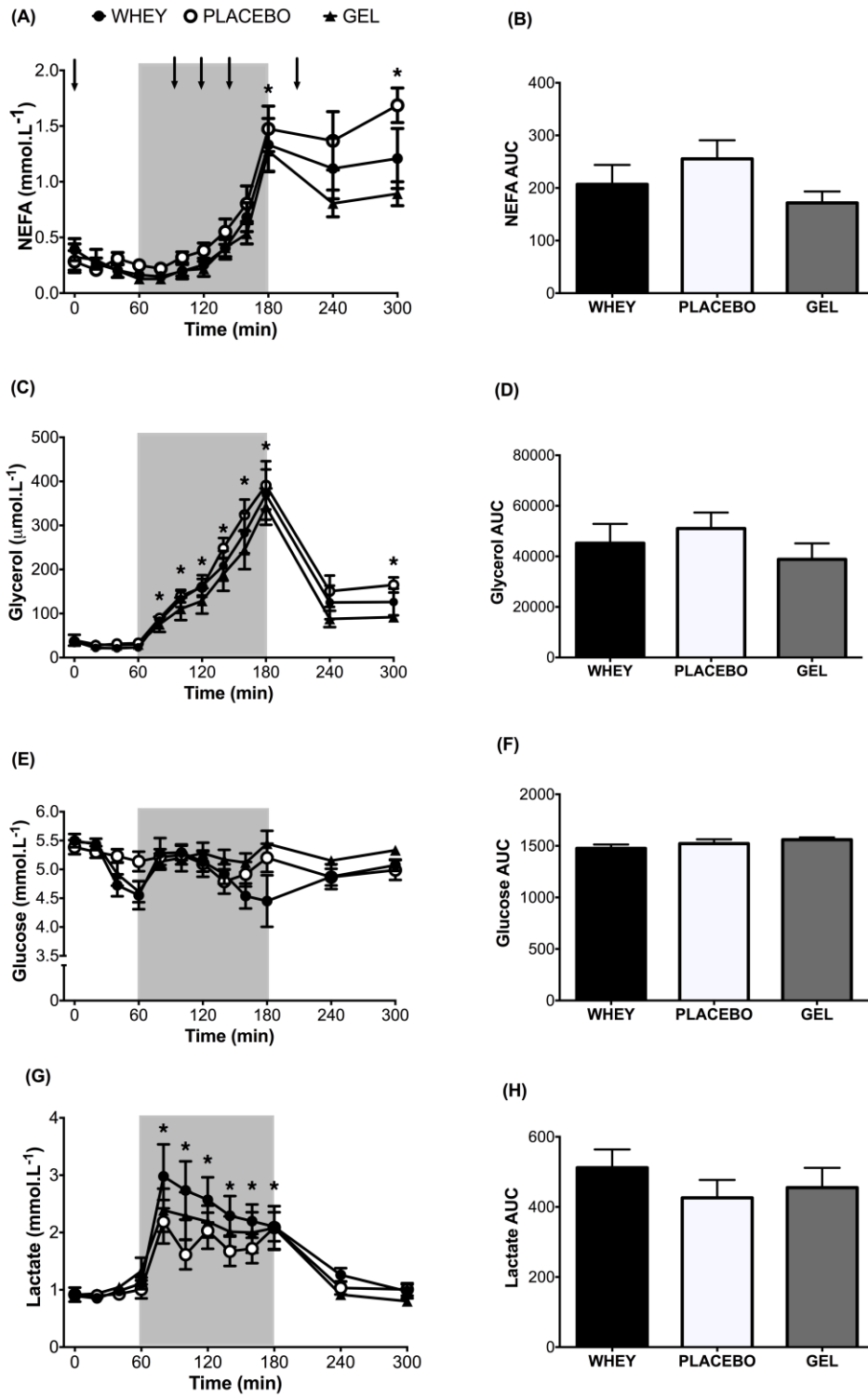
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597 FIGURE 2

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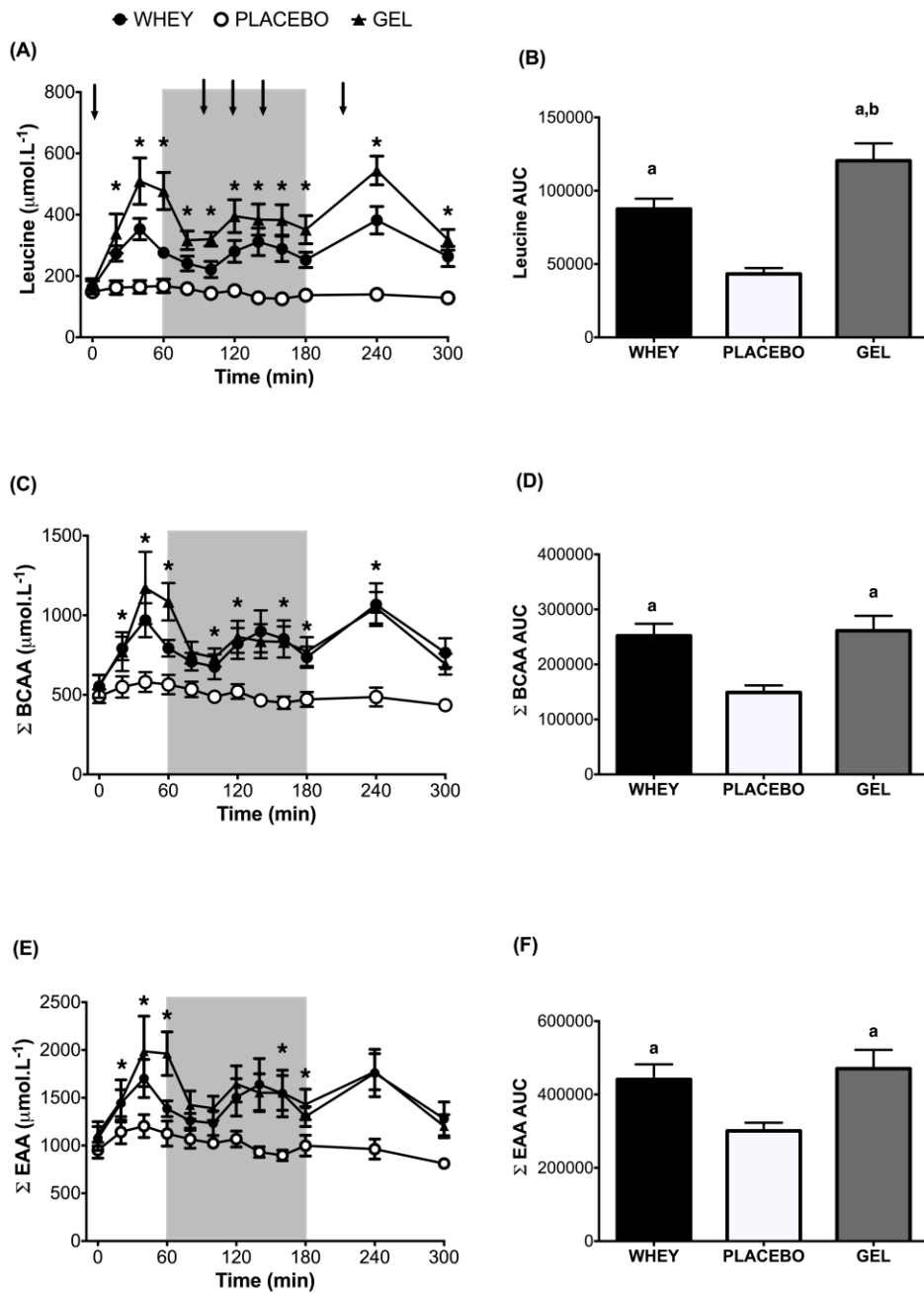


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603 FIGURE 3



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