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Whey protein augments leucinemia and post-exercise p70S6K1 activity compared to a hydrolysed collagen blend when in recovery from training with low carbohydrate availability

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45 **Abstract**

46 We examined the effects of whey versus collagen protein on skeletal muscle cell signalling
47 responses associated with mitochondrial biogenesis and protein synthesis in recovery from an
48 acute training session completed with low carbohydrate (CHO) availability. In a repeated
49 measures design (after adhering to a 36-h exercise-dietary intervention to standardise pre-
50 exercise muscle glycogen), eight males completed a 75-min non-exhaustive cycling protocol
51 and consumed 22 g of a hydrolysed collagen blend (COLLAGEN) or whey (WHEY) protein
52 45 min prior to exercise, 22 g during exercise and 22 g immediately post-exercise. Exercise
53 decreased ($P<0.05$) muscle glycogen content by comparable levels from pre-to post-exercise
54 in both trials (≈ 300 to 150 $mmol.kg^{-1}$ dw). WHEY protein induced greater increases in plasma
55 BCAAs ($P=0.03$) and leucine ($P=0.02$) than COLLAGEN. Exercise induced ($P<0.05$) similar
56 increases in PGC-1α (5-fold) mRNA at 1.5 h post-exercise between conditions though no affect
57 of exercise ($P>0.05$) was observed for p53, Parkin and Beclin1 mRNA. Exercise suppressed
58 ($P<0.05$) p70S6K1 activity in both conditions immediately post-exercise (≈ 25 $fmol.min^{-1}.mg^{-}$
59 ^{1}). Post-exercise feeding increased p70S6K1 activity at 1.5 h post-exercise ($P<0.05$), the
60 magnitude of which was greater ($P <0.05$) in WHEY (180 ± 105 $fmol.min^{-1}.mg^{-1}$) versus
61 COLLAGEN (73 ± 42 $fmol.min^{-1}.mg^{-1}$). We conclude that protein composition does not
62 modulate markers of mitochondrial biogenesis when in recovery from a training session
63 deliberately completed with low CHO availability. In contrast, whey protein augments post-
64 exercise p70S6K activity compared with hydrolysed collagen, as likely mediated via increased
65 leucine availability.

66 **Keywords:** autophagy, p70S6K1, CHO restriction, glycogen

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68

69 **Introduction**

70 The role of increased dietary protein intake in facilitating skeletal muscle adaptations
71 associated with endurance training is now gaining acceptance (Moore et al. 2014). Indeed,
72 consuming protein before (Coffey et al. 2011), during (Hulston et al. 2011) and/or after
73 (Rowlands et al. 2015) an acute training session stimulates muscle protein synthesis
74 (MPS). Post-exercise protein feeding has also been shown to modify skeletal muscle
75 transcriptome responses towards those supporting the endurance phenotype (Rowlands et al.
76 2011). In considering protein-feeding strategies for athletes, it is pertinent to consider the
77 absolute dose (Moore et al. 2009; Witard et al. 2014; Rowlands et al. 2015; MacNaughton et
78 al. 2016), feeding schedule (West et al., 2011; Areta et al. 2013), digestibility (Burke et al.
79 2012; Phillips, 2016) and source of protein (Tang et al. 2009; Wilkinson et al. 2007).
80 Contemporary guidelines recommend whey protein beverages due to its higher leucine content
81 and rapid aminoacidemia upon ingestion (Thomas et al. 2016), though hydrolysed collagen
82 beverages and gels are now commercially available and marketed to athletic populations.
83 Whilst the use of a gel delivery matrix appears particularly beneficial for endurance athletes
84 given the practical advantages of feeding while in locomotion (Impey et al. 2015), it is
85 noteworthy that collagen based formulations likely have lower leucine content and digestibility
86 compared with whey (Phillips, 2016).

87 With this in mind, the aim of the present study was to therefore examine the effects of two
88 practically relevant protein-feeding strategies (i.e. whey protein solution versus a hydrolysed
89 collagen blend in a gel format) in modulating skeletal muscle cell signalling responses
90 associated with mitochondrial biogenesis and MPS. Given the increased popularity of training
91 with low carbohydrate (CHO) availability (i.e. the train-low paradigm) in an attempt to enhance
92 mitochondrial related adaptations (Hawley and Morton, 2014; Bartlett et al. 2015; Impey et al.
93 2016; 2018), we adopted an experimental design whereby male cyclists completed a non-

94 exhaustive training session in which glycogen remained within an absolute concentration (i.e.
95 pre-and post-exercise concentrations of <350 and >100 mmol.kg dw^{-1}, respectively) considered
96 representative of train-low conditions (Impey et al. 2018).

97

98 Methodology

99 ***Subjects:*** After providing informed written consent, eight recreational male cyclists (age: 25 ±
100 3 years; height: 175 ± 0.1 cm; body mass: 74.4 ± 6.7 kg) who trained between 3 – 10 hours per
101 week took part in this study. Mean VO_{2peak} and peak power output (PPO) was 56.5 ± 3.8 $ml.kg^{-1}$
102 $.min^{-1}$ and 327 ± 26 W respectively. None of the participants had a history of neurological
103 disease or skeletal muscle abnormality and none were under pharmacological intervention
104 during the study. The study was approved by the Research Ethics Committee of Liverpool John
105 Moores University.

106 ***Design:*** In a repeated measures counterbalanced design separated by 7-9 days, subjects
107 completed two non-exhaustive acute exercise trials in conditions of reduced CHO availability
108 with whey (WHEY) or a hydrolysed collagen blend (COLLAGEN) provision before, during
109 and after exercise. At 36-40 h prior to the main experimental trials, all subjects performed a
110 glycogen depletion protocol followed by 36 h of low CHO (3 $g.kg.d^{-1}$) and energy intake (~7.58
111 ± 0.6 MJ.day^{-1}) (as replicated from Impey et al. 2016) in order to standardise pre-exercise
112 muscle glycogen content (see Figure 1). Subjects refrained from CHO intake on the morning
113 of the main experimental trial as well as during exercise, but consumed 1.2 g/kg body mass
114 (BM) of CHO split across two equal 0.6 g/kg doses at 30 min and 60 min post exercise in both
115 trials. Subjects consumed 22 g of whey or collagen protein at 45 minutes prior to exercise, 22
116 g during exercise and a further 22 g immediately post-exercise. Both trials represented
117 deliberate conditions of reduced CHO and absolute energy availability, but with high protein

118 availability in the form of whey or collagen throughout. Muscle biopsies were obtained from
119 the vastus lateralis immediately pre-, post- and at 1.5 h post-exercise.

120 ***Assessment of peak oxygen uptake:*** Participants were assessed for peak oxygen consumption
121 (VO_{2peak}) and peak aerobic power (PPO) as determined during an incremental cycle test
122 performed on an electromagnetically braked cycle ergometer as previously described (Impey
123 et al. 2015).

124 ***Experimental Protocol:***

125 ***Day 1 and 2:*** Participants arrived at the laboratory on the evening (17.00) of day 1. Subjects
126 then performed an intermittent glycogen-depleting cycling protocol lasting ~120 min (as
127 described by Impey et al. 2016). This protocol and all subsequent cycling protocols were
128 conducted on a fully adjustable electromagnetically braked cycle ergometer (Lode Excalibur,
129 Netherlands). The activity pattern and total time to exhaustion (115 ± 5 min; Energy expenditure:
130 1444 ± 107 kJ) were recorded and repeated exactly during the second experimental condition.
131 Participants then consumed a diet low in carbohydrate (3 $g.kg^{-1}$ BM) but high in protein (2
132 $g.kg^{-1}$ BM) over the next 36 h to minimise muscle glycogen replenishment to ~300-350
133 $mmol.kg^{-1}$ dw on the morning of the main experimental trial. During this 36 h period prior to
134 the main experimental trial, total energy intake equated to 7.58 ± 0.6 MJ. Estimated energy
135 expenditure (as calculated from resting metabolic rate using the Harris Benedict equation and
136 PAL level of 1.4 for the sedentary period on Day 2) was 15. 9 ± 1.1 MJ and hence energy
137 balance was – 8.4 ± 0.45 MJ.

138 ***Day 3:*** Subjects reported to the laboratory in a fasted state and an indwelling cannula (Safety
139 Lock 22G, BD Biosciences, West Sussex UK) was inserted into the anticubital vein in the
140 anterior crease of the forearm. Blood samples were collected immediately prior to and every
141 15 minutes during exercise as well as at 30 minute intervals in the recovery period from

142 exercise. Subjects consumed 22 g of protein from one of two commercially available products
143 consisting of a hydrolysed collagen blend in a gel format (COLLAGEN: Muscle Gel, Muscle
144 Pharm, USA; Ingredients: water, hydrolysed collagen, whey protein isolate, dietary fibre,
145 natural flavours, citric acid, ascorbic acid, malic acid, niacinamide, sodium benzoate,
146 potassium sorbate, sucralose, calcium D pantothenate, pyridoxine HCL, riboflavin) or a whey
147 protein solution (WHEY: Whey Protein, Science in Sport, Nelson, UK; Ingredients: whey
148 protein concentrate, whey protein isolate, fat reduced cocoa powder, natural flavourings,
149 xanthan gum, soy lecithin, sucralose) at 45 minutes prior to beginning exercise. Due to the
150 clear differences in delivery methods of protein sources (i.e. gel versus solutions), neither
151 single nor double blinding of treatments occurred. Fluid intake was matched in both conditions
152 to 500 ml at this time-point. Subjects then rested for 45 minutes prior to commencing exercise.
153 Protein was given 45 min prior to exercise in an attempt to maintain elevated circulatory amino
154 acid availability during the exercise protocol (Impey et al. 2015). Following a 5 min warm up
155 at 150 W, subjects then completed a prescribed cycling protocol consisting of 4 x 30 seconds
156 high intensity intervals at 200% PPO interspersed with 2.5 min active recovery at 40% PPO,
157 followed by 45 min steady state cycling at 60% PPO and finally, 3 x 3min intervals at 90%
158 PPO. During the HIT and steady state component, subjects ingested 7.3g of COLLAGEN or
159 WHEY protein every 20 min to provide 22 g of protein per hour. Physiological and perceptual
160 measures were recorded at regular intervals throughout exercise (e.g. heart rate, RPE) and
161 substrate utilisation was assessed during the steady state component of the exercise protocol
162 using online gas analysis (CPX Ultima, Medgraphics, Minnesota, US) according to Jeukendrup
163 and Wallis (2005). Following completion of the training session, subjects consumed an
164 additional 22 g of COLLAGEN or WHEY protein immediately post-exercise as well as
165 1.2g.kg^{-1} BM carbohydrate in the form of sports drinks (Science in Sport, Nelson, UK) and
166 snacks (Jaffacakes, UK) split as equal doses of 0.6 g.kg^{-1} BM at 30 and 60 minutes post-

167 exercise. Laboratory conditions remained constant across all experimental trials (19 – 21ºC, 40
168 – 50% humidity).

169 **Muscle biopsies:** Muscle biopsies were obtained from separate incision sites (2 – 3 cm apart)
170 from the lateral portion of the vastus lateralis muscle. Biopsies were obtained using a Bard
171 Monopty Disposable Core Biopsy Instrument (12 guage x 10 cm length, Bard Biopsy Systems,
172 Tempe, AZ, USA). Samples were obtained under local anaesthesia (0.5% marcaine) and
173 immediately frozen in liquid nitrogen and stored at – 80ºC for later analysis.

174 **Blood analysis:** Blood samples were collected in vacutainers containing K_2 EDTA, lithium
175 heparin or serum separation tubes, and stored on ice or at room temperature until centrifugation
176 at 1500 g for 15 min at 4 ℃. Serum and plasma were aliquoted and stored at -80 ℃ until analysis.
177 Plasma glucose, lactate, non-esterifed fatty acids (NEFA), glycerol, β-hydroxybutyrate (β-
178 OHB), insulin and amino acids were analysed as previously described (Impey et al. 2016).

179 **RNA extraction and analysis and Reverse transcriptase quantitative Real-Time**
180 **Polymerase Chain Reaction (rt-qRT-PCR):** Muscle samples (~ 20 mg) were immersed and
181 homogenized in 1ml TRIzol (Thermo Fisher Scientific, UK). RNA was extracted according to
182 the manufacturer's instructions. RNA concentration and purity were assessed by UV
183 spectroscopy at ODs of 260 and 280 nm using a Nanodrop 3000 (Fisher, Rosklide, Denmark).
184 70 ng RNA was used for each PCR reaction. Primer were purchased from Sigma (Suffolk, UK)
185 and forward (F) and reverse (R) sequences were as follows: PGC-1 (F:
186 TGCATGAGTGTGTGCTCTGT; R: CAGCACACTCGATGTCACTC), p53 (F:
187 ACCTATGGAAACTACTTCCTGAAA; R: CTGGCATTCTGGGAGCTTCA), Parkin (F:
188 TCCCAGTGGAGGTCGATTCT; R: GGAACCCCCTGTCGCTTAG), Beclin1 (F:
189 ATCTCGAGAAGGTCCAGGCT; R: TCTGGGCATAACGCATCTGG). rt-qRT-PCR
190 amplifications were performed using QuantiFast™ SYBR® Green RT-PCR one step kit on a

191 Rotogene 3000Q (Qiagen, Crawley, UK) supported by rotogene software (Hercules, CA,
192 USA). Detailed procedures are described by Impey et al. (2016).

193 **Muscle glycogen concentration:** Muscle glycogen concentration was determined from 10-20
194 mg muscle tissue according to the acid hydrolysis method described previously (Impey et al.
195 2016). Glucose concentrations were quantified using a commercially available kit (GLUC-HK,
196 Randox Laboratories, Antrim, UK).

197 **[γ-^{32}P] ATP Kinase Assay:** Twenty mg muscle tissue was used for the measurement of
198 p70S6K1 and PKB (Akt) activity as previously described (McGlory et al. 2014).

199 **Statistics:** Statistical analyses were performed using Statistical Package for the Social Scientist
200 (SPSS version 21). Changes in physiological and molecular responses between conditions (i.e.
201 muscle glycogen, circulatory metabolites, amino acids, mRNA and kinase activity) were
202 analysed using two way repeated measures General Linear Model, where the within factors
203 were time and condition. Where a significant main effect was observed, pairwise comparisons
204 were analysed according to Bonferoni post hoc tests in order to locate specific differences. A
205 P value < 0.05 was deemed significant and all data in text, figures and tables are presented as
206 mean ± SD.

207

208 **Results**

209 ***Physiological and metabolic responses to exercise***

210 Exercise intensity and substrate metabolism during the steady state component of the exercise
211 protocol is displayed in Table 1. No significant differences (P>0.05) were observed between
212 trials for any parameter. Exercise reduced (P<0.001) muscle glycogen stores to comparable
213 levels (150 mmol.kg^{-1} dw) with no difference (P=0.485) between conditions (Table 2). Plasma

214 NEFA, glycerol and β-OHB increased during exercise ($P<0.001$) though plasma glucose did
215 not display any change ($P = 0.112$) (Figure 2 A, B, C and D, respectively). Changes in plasma
216 NEFA availability across the whole sampling period were suppressed in WHEY compared with
217 the COLLAGEN trial ($P=0.046$) whereas no differences were observed between trials for
218 glycerol ($P=0.080$), β-OHB ($P = 0.070$) or glucose ($P=0.963$). Despite differences in NEFA
219 availability during exercise, no differences were observed in either CHO ($P=0.640$) or lipid
220 oxidation ($P=0.750$) during the steady state component of the exercise protocols (Table 1,
221 respectively).

222 ***Markers of mitochondrial adaptations***

223 The magnitude of the exercise-induced increase ($P = 0.001$) in PGC-1α mRNA expression at
224 90 min post-exercise was not different ($P = 0.731$) between trials (Figure 3A). Neither exercise
225 ($P = 0.354$) nor experimental condition ($P = 0.472$) affected p53 mRNA expression (Figure
226 3B). As markers of mitophagy, Parkin mRNA displayed no effect of exercise ($P = 0.417$) or
227 experimental condition ($P = 0.301$), whereas Beclin 1 displayed a trend towards an effect of
228 exercise ($P = 0.058$) but no effect of condition ($P = 0.968$).

229

230 ***Plasma amino acids, serum insulin and p70S6K1 related signalling***

231 Plasma leucine, BCAAs and EAAs all displayed a significant main effect of time ($P=0.043$,
232 0.028 and 0.021, respectively) during the sampling period (Figure 4 A, B, C respectively).
233 Pairwise comparisons demonstrated that leucine and BCAAs were significantly different from
234 pre-exercise after 30 and 45 minutes of exercise and that BCAAs were also different from pre-
235 exercise values after 30, 60 and 90 minutes of recovery. Such main effects of time appear to
236 be predominantly due to those changes occurring in the WHEY trial given that no differences

237 are apparent in the COLLAGEN trial. Additionally, leucine (P=0.02) and BCAA
238 concentrations (P=0.03) also demonstrated a main effect for condition such that WHEY was
239 greater than COLLAGEN whereas differences in EAA between trials only approached
240 statistical significance (P=0.060). When expressed as AUC data, only plasma leucine
241 (P=0.025) was different between trials whereas AUC for BCAA (P=0.135) and EAA (P=0.062)
242 were not different (data not shown).

243 In accordance with post-exercise CHO intake, insulin increased from pre- and post-exercise
244 values (P = 0.034) though the magnitude of change was not different between trials (P = 0.159)
245 (Figure 4D). As such, no difference (P=0.187) was apparent between trials for insulin AUC
246 data (data now shown)

247 PKB activity was elevated at 90 min post-exercise (P = 0.003) compared with pre-exercise
248 values, irrespective of nutritional condition (P=0.370) (Figure 4E). Exercise suppressed
249 (P=0.015) p70S6K activity to comparable levels immediately post-exercise (≈ 25 $fmol.min^{-}$
250 $^{1}.mg^{-1}$). However, post-exercise feeding increased p70S6K activity at 1.5 h post-exercise
251 (P=0.004), the magnitude of which was greater (P=0.046) in WHEY (180 ± 105 $fmol.min^{-1}.mg^{-}$
252 ^{1}) versus COLLAGEN (73 ± 42 $fmol.min^{-1}.mg^{-1}$) (Figure 4F).

253

254 **Discussion**

255 We examined the effects of whey versus collagen protein on skeletal muscle cell signalling
256 responses associated with mitochondrial biogenesis and protein synthesis in recovery from an
257 acute training session completed with low CHO availability. We deliberately studied two forms
258 of protein feeding that we consider have practical relevance for endurance athletes i.e. a whey
259 protein solution versus a hydrolysed collagen blend administered in a gel format. We also

260 adopted an acute training session intended to mimic situations in which endurance athletes
261 deliberately train with low endogenous and exogenous CHO availability in an attempt to
262 promote oxidative training adaptations (Impey et al. 2018). Whilst we observed no effects of
263 protein composition on acute adaptations associated with mitochondrial biogenesis, whey
264 protein induced greater leucinemia and post-exercise activity of p70S6K activity than collagen.

265 In accordance with the well-documented differences in amino acid composition between whey
266 and collagen (Castellanos et al. 2006), we observed marked differences in the extent of
267 leucinemia induced by the two protein feeding strategies. In this regard, leucine was elevated
268 to a greater extent with the whey protein solution when compared with the hydrolysed collagen
269 gel format. In agreement with previous reports from our laboratory (Taylor et al. 2013) and
270 others (Breen et al. 2011), we observed that amino acid availability does not apparently
271 modulate acute markers of mitochondrial adaptations. In contrast, we observed whey protein
272 induced greater increases in post-exercise p70S6K activity. The effects of post-exercise whey
273 protein consumption on activation of the mTOR-p70S6K pathway is well documented
274 (Phillips, 2016) and hence, the greater effect of whey compared with collagen on activation of
275 p70S6K is likely related to the increased leucine availability (Moberg et al. 2014; Apro et al.
276 2015a). Nonetheless, we acknowledge that direct assessment of muscle protein synthesis using
277 stable isotope or deuterium methods would have provided greater insight to the functional
278 relevance of the nutritional strategies used here. In addition, future studies could also assess if
279 such divergent signalling responses are still apparent if the collagen formulation was fortified
280 with additional leucine content to match that of the whey solution.

281 Although we readily acknowledge that the total leucine delivery in the WHEY trial may appear
282 excessive in terms of that required to facilitate protein synthesis as well as likely resulting in
283 elevated leucine oxidation (as suggested by the fall in leucine and BCAA after 30 and 45
284 minutes of exercise), we deliberately chose this dosing strategy for a number of practical

285 reasons. Firstly, given that exercising in CHO restricted states augments leucine oxidation
286 (Lemon and Mullin, 1980; Wagenmakers et al. 1991; Howarth et al. 2009), it was our deliberate
287 aim to administer higher exogenous leucine so as to deliver both substrate to promote muscle
288 protein synthesis (Breen et al. 2011; Pasiakos et al. 2011; Churchward-Venne et al. 2013) but
289 yet, also compensate for the higher levels of endogenous leucine oxidation (Lemon and Mullin,
290 1980; Howarth et al. 2009). Second, unpublished observations by the corresponding author on
291 elite professional cyclists indicated that this is the type of protein feeding strategy actually
292 adopted during morning training rides that are deliberately undertaken in the absence of CHO
293 intake before and during exercise. As such, our aim was to replicate these "real world"
294 strategies and determine the effects of such high protein availability on substrate metabolism
295 and post-exercise signalling responses. Finally, given that many elite cyclists are potentially
296 in daily energy deficits (Vogt et al. 2005) with low energy availability (Loucks et al. 2011),
297 and also that 3 g/kg body mass of daily protein has been recommended to maintain lean mass
298 during energy restriction (Stokes et al. 2018), we therefore considered this feeding strategy to
299 be in accordance with daily protein intakes for both quantity and frequency (Areta et al. 2013).
300 For example, over the 3.5 h data collection period (i.e. 9 am to 1230 pm), the present subjects
301 (ranging from 70-80 kg) consumed 66 g protein and hence for the daily target to be achieved
302 (i.e. approximately 210-240 g), our approach is therefore in accordance with a feeding strategy
303 where subsequent 30-40 g doses could be consumed at 3 h intervals (e.g. 1, 3, 6 and 9 pm if
304 required).

305 When considered in combination with our recent data (Impey et al. 2016; Hammond et al.
306 2016), the present study also adds to our understanding of the regulation of p70S6K activity
307 both during and after exercise. Indeed, whereas other researchers have reported that acute
308 endurance exercise does not suppress post-exercise p70S6K phosphorylation (Coffey et al.
309 2006) or activity (Apro et al. 2015b), we have consistently observed an exercise-induced

310 suppression in p70S6K activity. We suggest that such differences between studies may be due
311 to the magnitude of energy deficit associated with the CHO restriction and glycogen taxing
312 exercise protocols used both here and previously (Impey et al. 2016). Indeed, whilst it is
313 difficult to directly compare the total energy expenditure between this study and the data of
314 Apro et al. (2015b), the exercise intervention studied here elicited considerably lower muscle
315 glycogen concentrations (i.e. ~150 $mmol.kg^{-1}dw$ vs 350 $mmol.kg^{-1}dw$). The potential effects
316 of low muscle glycogen availability on post-exercise signalling (albeit in response to resistance
317 exercise) was also evidenced by Camera et al. (2012) who observed that low muscle glycogen
318 availability (i.e. 150-200 $mmol.kg^{-1}dw$) reduced mTOR phosphorylation compared with higher
319 glycogen concentration (i.e. 350-400 $mmol.kg^{-1}dw$). Nonetheless, these workers also observed
320 the apparent disconnect between snapshots of cell signalling and functional outcomes given
321 that glycogen concentrations did not affect myofibrillar protein synthesis.

322 In relation to the re-activation of p70S6K activity in the recovery period from exercise, it is
323 noteworthy that we previously observed that the sustained presence of reduced CHO (and
324 energy availability) and/or high post-exercise fat availability also suppresses the re-activation
325 of p70S6K1, even when leucine enriched whey protein was consumed in the post-exercise
326 period (Impey et al. 2016). Based on these studies, we therefore suggested that the apparent
327 suppression of p70S6K1 activity may be due to 1) reduced insulin and PKB signalling or, 2) a
328 direct effect of increased fat availability (Kimball et al. 2015) and/or reduced glycogen
329 mediating suppression of mTORC1 complex via energetic stress related mechanisms. The
330 present data lend support for the latter mechanism for several reasons. First, we observed that
331 the whey-induced increase in p70S6K1 activity when compared with collagen feeding was
332 independent of post-exercise insulin and PKB activity. Second, at the termination of exercise
333 (i.e. the 75 min time point that corresponds low muscle glycogen availability and energy
334 deficit) the absolute circulating NEFA concentrations observed in our collagen trial (i.e.

335 approximately 1.5 mmol.L^{-1}) was similar to that achieved with both CHO restriction (Impey et
336 al. 2016) and post-exercise high fat feeding protocols (Hammond et al. 2016). The apparent
337 suppression of NEFA in the WHEY trial may be due to the higher insulin responses associated
338 with feeding whey protein before and during exercise (Impey et al. 2015; Taylor et al. 2013),
339 thereby causing a reduction in lipolysis that manifests itself as reduced circulating NEFA
340 availability during the exercise period. Nonetheless, we acknowledge that the current
341 assessments of insulin concentration were limited to pre-and post-exercise time-points per se.
342 We also acknowledge the limitations associated with making inferences on muscle free fatty
343 acid (FFA) uptake on snapshot assessments of circulating NEFA per se. Nonetheless, given
344 recent data demonstrating that acute increases in fat availability (as achieved via lipid infusion
345 protocols) impairs MPS in human skeletal muscle despite similar circulating insulin and
346 leucine concentrations (Stephens et al. 2015), it remains possible that subtle alterations in FFA
347 availability (as caused by "acute" dietary manipulations) can have associated implications on
348 mTOR related signalling. When considered with previous studies (Impey et al. 2016;
349 Hammond et al. 2016), the present data suggest that in those exercise conditions in which
350 muscle glycogen is near depletion, the beneficial effects of whey protein (i.e. leucine mediated
351 activation of mTOR) are especially apparent when co-ingested with post-exercise CHO
352 feeding. Whilst there may be benefits of commencing training with reduced endogenous and
353 exogenous CHO availability, we suggest the post-exercise meal should contain a combination
354 of both protein and CHO, the latter to provide the necessary substrate, energy and metabolic
355 environment to stimulate cell signalling processes.

356 In summary, we demonstrate that when in recovery from an acute training session undertaken
357 with low CHO and energy availability, whey protein induces greater leucinemia and post-
358 exercise p70S6K activity compared with a hydrolysed collagen blend. Data suggest that
359 hydrolysed collagen blends are a sub-optimal protein source in relation to the goal of

360 stimulating those signalling pathways that regulate muscle protein synthesis. Future studies are
361 now required to directly assess the acute effects of whey versus collagen protein feeding on
362 muscle protein synthesis as well as to examine the long-term effects of such feeding strategies
363 on training-induced skeletal muscle adaptations and performance outcomes.

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510 were undertaken by SGI, APS, DLJ, GLC and JPM. All authors approved the final version of
511 the paper.

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513 **Figure 1**. Schematic representation of the experimental design. On the evening of day 1,
514 subjects completed a glycogen depleting protocol followed by consumption of 22 g of whey
515 protein. Throughout the entirety of day 2, subjects consumed a low CHO and low energy
516 dietary protocol that was matched for both protein and fat intake. During the main experimental
517 trial on day 3, subjects ingested 22 g of collagen (COLLAGEN) or whey (WHEY) protein
518 before, during and after completion of an acute train-low exercise protocol. In addition to
519 protein, subjects also consumed CHO (0.6 $g.kg^{-1}$ BM) at 30 min and 1 h post-exercise. Muscle
520 biopsies were obtained immediately pre-exercise, post-exercise and 1.5 h post-exercise. This
521 experimental protocol represents an amalgamation of train-low paradigms as subjects
522 effectively performed sleep low on the evening of day 1, consumed a low CHO diet on day 2
523 and finally, completed an acute training session on the morning of day 3 with CHO restricted
524 before and during exercise.

525

526 **Figure 2**. Plasma (A) NEFA, (B) glycerol, (C) βOHB and (D) Glucose during and in recovery
527 from exercise. Shaded area represents exercise duration. * $P<0.05$ significant difference from
528 pre-exercise (i.e. time-point 0), ^ $P<0.05$ significant main effect of condition.

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530 **Figure 3**. mRNA expression of (A) PGC-1α, (B) p53, (C) Parkin and (D) Beclin1. * $P<0.05$
531 significant difference from pre-exercise.

532

533 **Figure 4**. Plasma (A) leucine, (B) total BCAA, (C) total EAA and (D) insulin. Kinase activity
534 of (E) PKB and (F) p70S6K. Shaded area represents exercise duration. *$P<0.05$ significant
535 difference from pre-exercise, **$P<0.05$ significant difference from post-exercise, ^$P<0.05$
536 significant main effect of condition.

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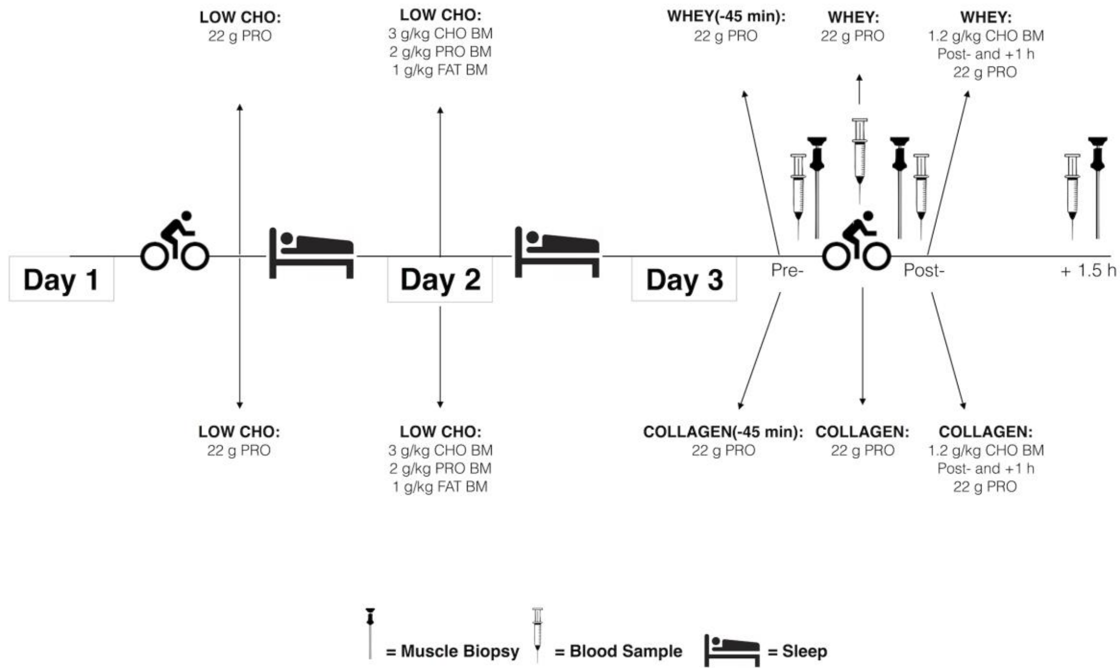
547 **Figure 1**



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558 **Figure 2**

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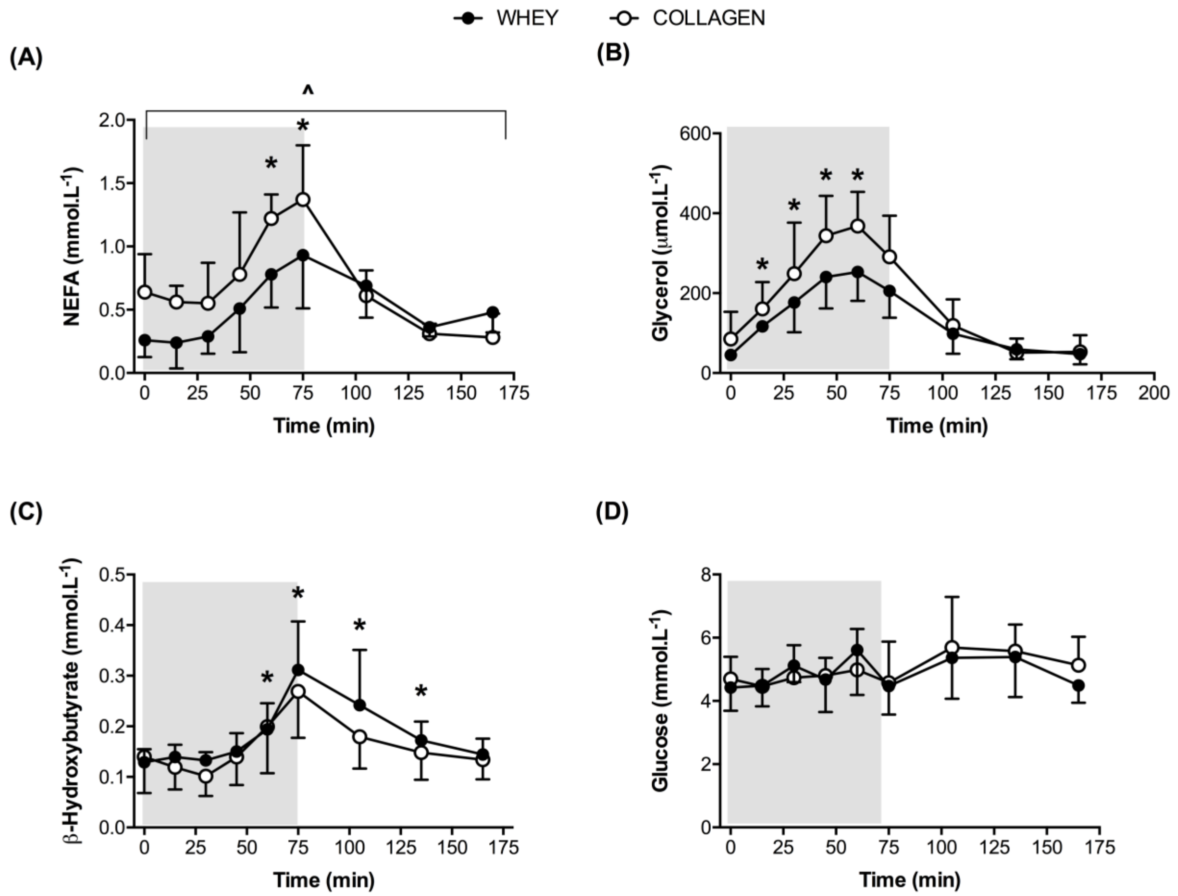

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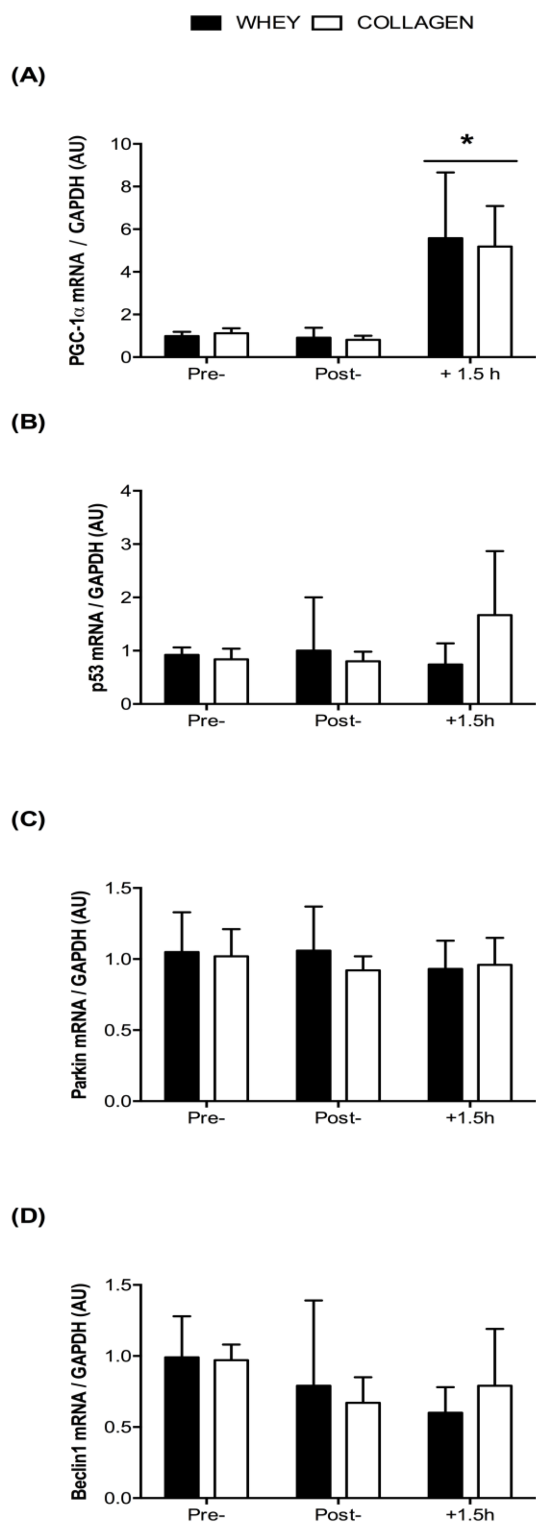
567 **Figure 3**



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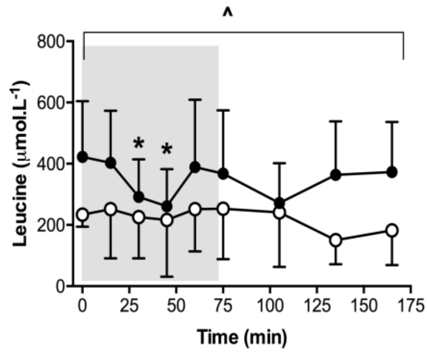
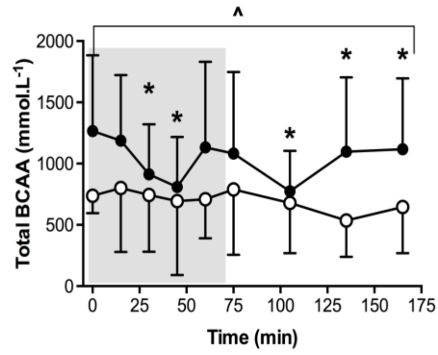
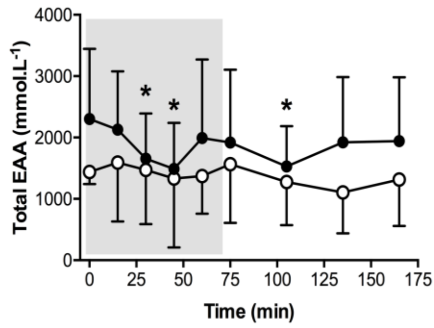
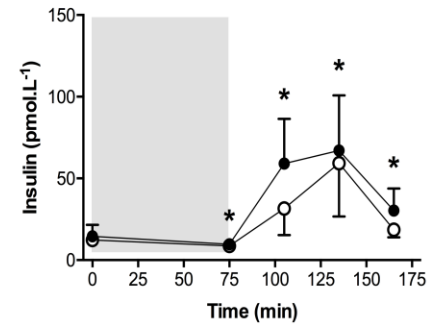
571 **Figure 4**

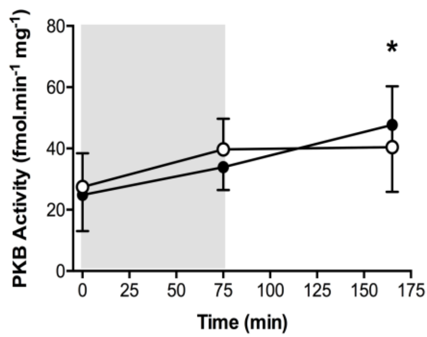
WHEY COLLAGEN

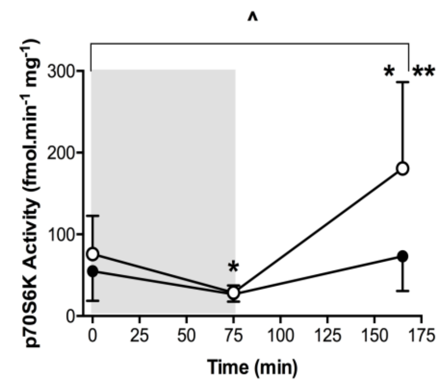












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576 **Table 1** – Exercise intensity and substrate metabolism during the steady state component of the
577 exercise protocol.

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	Time (min)		
	15	30	45
VO_2 (% VO_{2max})			
WHEY	66 ± 1	67 ± 2	68 ± 2
COLLAGEN	67 ± 2	67 ± 1	68 ± 1
Heart Rate ($b.min^{-1}$)			
WHEY	165 ± 12	167 ± 12	166 ± 12
COLLAGEN	167 ± 6	168 ± 8	168 ± 8
RER (AU)			
WHEY	0.86 ± 0.05	0.86 ± 0.05	0.86 ± 0.06
COLLAGEN	0.86 ± 0.05	0.87 ± 0.03	0.86 ± 0.03
CHO Oxidation ($g.min^{-1}$)			
WHEY	1.9 ± 0.8	1.9 ± 0.8	1.9 ± 0.9
COLLAGEN	2.1 ± 0.6	2.1 ± 0.6	2.1 ± 0.6
Lipid Oxidation ($g.min^{-1}$)			
WHEY	0.7 ± 0.4	0.7 ± 0.3	0.7 ± 0.4
COLLAGEN	0.6 ± 0.2	0.6 ± 0.2	0.7 ± 0.2

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598 **Table 2** – Muscle glycogen concentration before and after exercise. * denotes significant different
599 from pre-exercise, P<0.05.

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	Time (min)		
	Pre-	Post-	+ 90 min
Glycogen (mmol.kg^{-1} dw)			
WHEY	339 ± 66	158 ± 80 *	183 ± 35 *
COLLAGEN	356 ± 44	141 ± 25 *	173 ± 23 *

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