

Leucine co-ingestion augments the muscle protein synthetic response to the ingestion of 15 g protein following resistance exercise in older men

Andrew M. Holwerda^{1,4}, Kevin J.M. Paulussen¹, Maarten Overkamp¹, Joy P.B. Goessens¹, Irene-Fleur Kramer¹, Will K.W.H. Wodzig², Lex B. Verdijk^{1,4}, Lisette C.P.G.M. de Groot^{3,4} and Luc J.C. van Loon^{1,4*}

¹NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre+, the Netherlands, ²Central Diagnostic Laboratory, Maastricht University Medical Centre+, the Netherlands; ³Department of Human Nutrition, Wageningen University, Wageningen, The Netherlands; ⁴Top Institute Food and Nutrition (TIFN), Wageningen, the Netherlands;

Correspondence: Prof. L.J.C. van Loon, Ph.D.

Department of Human Biology

Maastricht University Medical Centre+

PO Box 616, 6200 MD

Maastricht, the Netherlands.

Tel: +31 43 388 1397

Fax: +31 43 367 0976

E-mail: L.vanLoon@maastrichtuniversity.nl;

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Disclosure Summary

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List of abbreviations and their definitions

1RM	1 repetition maximum
AUC	Area under the curve
DEXA	Dual-energy x-ray absorptiometry
ECG	Electrocardiogram
En%	Energy %
EndoRa	Endogenous phenylalanine rate of appearance
ExoRa	Exogenous phenylalanine rate of appearance
FSR	Fractional synthetic rate
MPE	Mole percent excess
OGTT	Oral glucose tolerance test
Phe Plasma	Dietary protein-derived amino acid availability in plasma
15G	15 g protein ingested following resistance exercise
15G+LEU	15 g protein with 1.5 g free leucine ingested following resistance exercise
SEM	Standard error of the mean
W	Watt

ABSTRACT (250 words)

1 **Background:** Older adults have shown an attenuated post-exercise increase in muscle protein
2 synthesis rates following ingestion of smaller amounts of protein when compared to younger
3 adults. Consequently, it has been suggested that older adults require the ingestion of more
4 protein to increase post-exercise muscle protein synthesis rates when compared to younger
5 adults.

6 **Objective:** We investigated whether co-ingestion of 1.5 g free leucine with a single, 15 g
7 bolus of protein further augments the post-prandial muscle protein synthetic response during
8 recovery from resistance-type exercise in older men.

9 **Design:** Twenty-four healthy older men (67 ± 1 y) were randomly assigned to ingest 15 g milk
10 protein concentrate (MPC80) with (15G+LEU; $n=12$) or without (15G; $n=12$) 1.5 g free
11 leucine after performing a single bout of resistance-type exercise. Post-prandial protein
12 digestion and amino acid absorption kinetics, whole-body protein metabolism, and post-
13 prandial myofibrillar protein synthesis rates were assessed using primed, continuous infusions
14 with L-[ring- 2 H $_5$]-phenylalanine, L-[ring- 2 H $_2$]-tyrosine and L-[1- 13 C]-leucine combined with
15 the ingestion of intrinsically L-[1- 13 C]-phenylalanine labeled milk protein.

16 **Results:** A total of $70\pm 1\%$ (10.5 ± 0.2 g) and $75\pm 2\%$ (11.2 ± 0.3 g) of the protein-derived amino
17 acids were released in the circulation during the 6 h post-exercise recovery phase in 15G+LEU
18 and 15G, respectively ($P<0.05$). Post-exercise myofibrillar protein synthesis rates were 16%
19 (0.058 ± 0.003 vs $0.049\pm 0.002\% \cdot h^{-1}$; $P<0.05$; based upon L-[ring- 2 H $_5$]-phenylalanine) and 19%
20 (0.071 ± 0.003 vs $0.060\pm 0.003\% \cdot h^{-1}$, $P<0.05$; based upon L-[1- 13 C]-leucine) greater in
21 15G+LEU when compared with 15G.

22 **Conclusion:** Leucine co-ingestion further augments the post-exercise muscle protein synthetic
23 response to the ingestion of a single 15 g bolus of protein in older men.

24
25 **Key words:** muscle protein synthesis, sarcopenia, aging, dietary protein, exercise, leucine.

26 **INTRODUCTION**

27 The age-related decline in skeletal muscle mass and strength, termed *sarcopenia*, is
28 accompanied by impairments in functional capacity and an increased risk of developing
29 chronic metabolic diseases (4). Whereas basal muscle protein synthesis and breakdown rates
30 appear to be unaffected by age (33), the muscle protein synthetic response to the main
31 anabolic stimuli, namely food intake and physical activity, seem to be blunted in older
32 individuals (47). This *anabolic resistance* is now considered as a central factor contributing
33 to the progression of sarcopenia.

34 A single session of resistance-type exercise strongly increases muscle protein synthesis rates
35 (36) and, therefore, represents an effective strategy to compensate for anabolic resistance. For
36 older individuals, ingestion of more than 20 g protein is required to augment post-exercise
37 muscle protein synthesis rates (10, 36, 53). Older individuals possess the capacity to further
38 increase the post-exercise muscle protein synthetic response by ingesting larger protein
39 doses, with the ingestion of 40 g protein further enhancing the muscle protein synthetic
40 response (53, 54). However, older individuals seldom consume 40 g protein in a single meal
41 (40, 41). Therefore, research is warranted to determine nutritional strategies that can augment
42 the muscle protein synthetic response to ingestion of small(er) amounts of protein during
43 recovery from resistance-type exercise in older adults.

44 Leucine has been established as one of the amino acids with greater anabolic properties due
45 to its ability to stimulate mTORC1 activity to phosphorylate key anabolic signaling proteins
46 (i.e., S6K1) in skeletal muscle tissue (3, 17). Previous work has demonstrated that co-
47 ingestion of free leucine augments the muscle protein synthetic response to protein or amino
48 acid ingestion in older individuals at rest (7, 9, 12, 13, 48) and after a single bout of
49 resistance-type exercise (2, 7, 9, 12, 13). It has also been demonstrated that leucine co-
50 ingested with the main meals augments the integrated muscle protein synthetic response to

51 resistance-type exercise assessed over multiple days (30). However, as leucine also stimulates
52 splanchnic tissue protein synthesis rates (29, 39), it could be speculated that free leucine co-
53 ingestion stimulates the uptake and incorporation of dietary protein derived amino acids in
54 the splanchnic tissues, thereby attenuating the post-prandial release of dietary protein derived
55 amino acids in the circulation. It remains to be established whether this would preclude the
56 impact of free leucine co-ingestion to further increase post-exercise muscle protein synthesis
57 rates. In short, it remains unclear whether or not free leucine co-ingestion impacts post-
58 prandial protein handling following the ingestion of a small amount of protein during post-
59 exercise recovery in older individuals. Therefore, in the present study we assessed post-
60 prandial protein handling and the muscle protein synthetic response following the ingestion
61 of a single 15 g bolus of protein with or without additional free leucine (1.5 g) during
62 recovery from a single bout of resistance-type exercise in older individuals.

63 We hypothesized that co-ingestion of 1.5 g free leucine with a single bolus of 15 g protein
64 attenuates the post-prandial release of protein derived amino acids in the circulation when
65 compared to the ingestion of 15 g protein. Furthermore, we hypothesized that despite a
66 potential attenuated rise in post-prandial plasma amino acid availability, free leucine co-
67 ingestion will further increase post-exercise muscle protein synthesis rates and allow for a
68 greater incorporation of the available dietary protein-derived amino acids into myofibrillar
69 protein. To test our hypothesis, we selected 24 healthy older (67 ± 1 y) men who ingested 15 g
70 protein with or without 1.5 g free leucine during recovery from a single bout of resistance-
71 type exercise. By combining the ingestion of specifically produced intrinsically L-[1- ^{13}C]-
72 phenylalanine and L-[1- ^{13}C]-leucine labeled milk protein concentrate with the administration
73 of primed continuous infusions of L-[*ring*- $^2\text{H}_5$]-phenylalanine, L-[1- ^{13}C]-leucine and L-[*ring*-
74 $^2\text{H}_2$]-tyrosine, we were able to assess protein digestion and amino acid absorption kinetics,

75 the increase in muscle protein synthesis rate, and the post-prandial incorporation of dietary
76 protein-derived amino acids during recovery from exercise in older individuals.

77 **MATERIALS AND METHODS**

78

79 *Subjects*

80 A total of 24 healthy, normoglycemic, older men (67 ± 1 y) were selected to participate in the
81 present study. Subjects' characteristics of the study participants are presented in **Table 1**.
82 Subjects were randomly assigned to ingest either 15 g protein (15G: $n=12$) or 15 g protein with
83 1.5 g crystalline free leucine (15G+LEU: $n=12$) in a double-blind fashion after completing a
84 single bout of whole-body resistance-type exercise. Randomization was performed by an
85 independent researcher who created a table in Excel (Microsoft, USA) using the random
86 number generator function, which was coupled to the different beverages before sorting the
87 number column in order of low to high. The independent researcher also prepared and masked
88 the test beverages on the test day. All subjects were informed of the nature and possible risks
89 of the experimental procedures before their written informed consent was obtained. The study
90 was approved by the Medical Ethical Committee of the Maastricht University Medical Centre,
91 The Netherlands, and conformed to standards for the use of human subjects in research as
92 outlined in the most recent version of the Helsinki Declaration. This study is part of a greater
93 project, which was registered at the Netherlands Trial Registry as NTR4492. Data from the
94 15G group have been published previously as part of a protein dose-response study conducted
95 in parallel within the same project (22).

96

97 *Pretesting*

98 Participants arrived at the laboratory at 0830 h by car or public transport in an overnight
99 fasted state. Upon arrival, body weight, body composition, and bone mineral content were
100 measured with DEXA (Dual-energy X-ray absorptiometry, DEXA; Discovery A; Hologic,
101 Bedford, MA). Thereafter, all participants performed an oral glucose tolerance test (OGTT).

102 Plasma glucose and insulin concentrations were measured to determine oral glucose
103 intolerance and/or the presence of type 2 diabetes according to 2006 American Diabetes
104 Association guidelines (1). All subjects were screened on medical issues and excluded if any
105 gastrointestinal, neurological or renal diseases were present.

106 Subjects were cleared to perform resistance-type exercise by a cardiologist who examined
107 electrocardiograms (ECG) measured at rest and during submaximal cycling (performed at 70
108 % of age-predicted heartrate max). The subjects were then familiarized with the exercise
109 equipment and physical activity protocol. Subjects first performed a 10-min cycling warm-up
110 at 70% of their age-predicted heart rate max before completing an estimation of their 1RM
111 (one repetition maximum) on the leg press and leg extension exercises using the multiple
112 repetitions testing procedure (28). For each exercise, subjects performed 10 submaximal
113 repetitions to warm-up and become familiarized with the equipment and to have lifting
114 technique critiqued and corrected. Subjects then performed sets at progressively increasing
115 loads until failing to complete a valid repetition, judged by their inability to complete the full
116 range of motion for an exercise. Ideally, subjects failed within 3–6 repetitions during the last
117 and heaviest set. A 2-min resting period between subsequent attempts was allowed. The
118 pretesting and experimental trials were separated by a period of at least 7 days.

119

120 *Diet and physical activity*

121 All volunteers were instructed to refrain from any exhaustive physical activity and to keep
122 their diet as consistent as possible 72 h prior to the trial. Subjects filled in dietary records for
123 48 h immediately before the experimental trial. Subjects consumed 8.6 ± 0.5 MJ·day⁻¹ on
124 average, with 47 ± 1 energy% (En%) as carbohydrate, 33 ± 1 En% as fat, and 18 ± 1 En% as
125 protein. Dietary protein intake averaged 1.1 ± 0.1 g·kg⁻¹ bodyweight. On the evening before

126 the experiment, all subjects consumed a standardized meal ($22.0 \pm 0.6 \text{ kJ} \cdot \text{kg}^{-1}$ bodyweight,
127 consisting of 55 En% as carbohydrate, 20 En% as protein, and 25 En% as fat).

128

129 *Experimental Protocol*

130 At 0800 h, participants reported to the lab in a fasted and rested state and had Teflon
131 catheters inserted into the antecubital veins of one arm and the top of the opposite hand. At
132 0830 h ($t = -150 \text{ min}$), a background blood sample was taken prior to the initiation of the
133 tracer infusion protocol. The plasma and intracellular phenylalanine and leucine pools were
134 primed with a single intravenous dose (priming dose) of L-[*ring*- $^2\text{H}_5$]-phenylalanine (3.6
135 $\mu\text{mol} \cdot \text{kg}^{-1}$), L-[*ring*- $^2\text{H}_2$]-tyrosine ($1.10 \mu\text{mol} \cdot \text{kg}^{-1}$), L-[$1\text{-}^{13}\text{C}$]-leucine ($7.19 \mu\text{mol} \cdot \text{kg}^{-1}$). Once
136 primed, the continuous stable isotope infusion was initiated (infusion rate: $0.06 \mu\text{mol} \cdot \text{kg}^{-1}$
137 $\cdot \text{min}^{-1}$ L-[*ring*- $^2\text{H}_5$]-phenylalanine, $0.018 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ L-[*ring*- $^2\text{H}_2$]-tyrosine, 0.12
138 $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}$ L-[$1\text{-}^{13}\text{C}$]-leucine; Cambridge Isotopes Laboratories, Andover, MA).
139 Participants rested for 1.5 h until 1000 h ($t = -60 \text{ min}$), when the participants completed the
140 resistance-type exercise session. At 1100 h ($t = 0 \text{ min}$), immediately after the resistance-type
141 exercise session, subjects had a blood sample and muscle biopsy collected from a randomized
142 leg. Subsequently, subjects ingested a 500 mL beverage containing 15 g intrinsically L-[1-
143 ^{13}C]-phenylalanine and L-[$1\text{-}^{13}\text{C}$]-leucine labeled milk protein (MPC80) alone (15G) or with
144 (15G+LEU) an added 1.5 g of crystalline free leucine (**Table 2**). The beverages contained 1.5
145 mL vanilla extract to improve palatability (Dr. Oetker, Amersfoort, the Netherlands). Blood
146 samples (10 mL) were subsequently taken at $t = 30, 60, 90, 120, 180, 240, 300, 360 \text{ min}$ after
147 protein ingestion. A second muscle biopsy was obtained from the contralateral leg at 1700 h
148 ($t = 360 \text{ min}$), signifying the end of the experimental trial.

149 Blood samples were collected in EDTA containing tubes and centrifuged at $1000g$ for 10 min
150 at $4 \text{ }^\circ\text{C}$. Aliquots of plasma were frozen in liquid nitrogen and stored at $-80 \text{ }^\circ\text{C}$. Muscle

151 biopsies were obtained from the middle region of the *M. vastus lateralis*, 15 cm above the
152 patella and approximately 4 cm below entry through the fascia, using the percutaneous needle
153 biopsy technique (5). Muscle samples were dissected carefully and freed from any visible
154 non-muscle material. The muscle samples were immediately frozen in liquid nitrogen and
155 stored at $-80\text{ }^{\circ}\text{C}$ until further analysis.

156

157 *Resistance-type exercise protocol*

158 The exercise protocol consisted of 60 min of moderate-to-high intensity whole-body
159 resistance-type exercise. After 10 min of self-paced cycling at 100 W with a cadence of 60–
160 80 RPM, subjects performed 5 sets of 10 repetitions on the horizontal leg press machine
161 (Technogym BV, Rotterdam, Netherlands), 2 sets of 10 repetitions on the lat pull down
162 machine (Technogym BV), 2 sets of 10 repetitions on the chest press machine and 5 sets of
163 10 repetitions on the leg extension machine (Technogym BV). The first set of the lower body
164 exercises were performed at 50 % 1RM and sets 2–5 were performed at 75–80 % 1RM. All
165 sets on the upper body exercises were performed at 75–80 % 1RM. Subjects were allowed to
166 rest for 2 min between all sets.

167

168 *Preparation of tracer and production of intrinsically labeled protein*

169 The stable isotope tracers L-*[ring- $^2\text{H}_5$]*-phenylalanine, L- $[1-^{13}\text{C}]$ -leucine and L-*[ring- $^2\text{H}_2$]*-
170 tyrosine were purchased from Cambridge Isotopes (Andover, MA) and dissolved in 0.9%
171 saline before infusion (Basic Pharma, Geleen, the Netherlands). Continuous intravenous
172 infusions were performed using a calibrated IVAC 598 pump (San Diego, CA, USA).
173 Intrinsically L- $[1-^{13}\text{C}]$ -phenylalanine and L- $[1-^{13}\text{C}]$ -leucine labeled milk protein (MPC80)
174 was extracted from whole milk obtained during the constant infusion of L- $[1-^{13}\text{C}]$ -
175 phenylalanine ($455\text{ }\mu\text{mol}\cdot\text{min}^{-1}$) and L- $[1-^{13}\text{C}]$ -leucine ($200\text{ }\mu\text{mol}\cdot\text{min}^{-1}$) for 96 h in a

176 lactating dairy cow (8, 44). The milk was collected, processed, and fractionated into the
177 MPC80 similarly to what has been previously described (19, 37, 44). The L-[1-¹³C]-
178 phenylalanine and L-[1-¹³C]-leucine enrichments in MPC80 were measured by gas
179 chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS; MAT 252,
180 Finnigan, Bremen, Germany) and averaged 38.3 mole percent excess (MPE) and 10.8 MPE,
181 respectively. The proteins met all chemical and bacteriological specifications for human
182 consumption.

183

184 *Plasma and muscle analysis*

185 Plasma glucose and insulin concentrations were analyzed using commercially available kits
186 (GLUC3, Roche, Ref: 05168791 190, and Immunologic, Roche, Ref: 12017547 122,
187 respectively). Plasma amino acid concentrations and enrichments were determined by gas
188 chromatography-mass spectrometry analysis (GC-MS; Agilent 7890A GC/5975C; MSD,
189 Wilmington, Delaware, USA). Myofibrillar protein-bound L-[*ring*-²H₅]-phenylalanine
190 enrichments were determined by GC-MS analysis, whereas the L-[1-¹³C]-phenylalanine and
191 L-[1-¹³C]-leucine enrichments were determined by GC-C-isotope ratio mass spectrometer
192 analysis (GC-C-IRMS; Trace GC Ultra, IRMS model MAT 253; Thermo Scientific) (20).

193

194 *Western blotting*

195 Muscle was homogenized as previously described (46), 10 μL of protein was loaded and
196 standard SDS-PAGE procedures were followed. Antibodies included total and
197 phosphorylated mTOR (Cat. no.: Total: #2972, Ser²⁴⁴⁸: #2971), S6K1 (Cat no.: Total: #9202,
198 Thr³⁸⁹: #9205), RS6 (Cat no.: Total: #2217, Ser^{235/236}: #4856), 4E-BP1 (Cat no.: Total:
199 #9452, Thr^{37/46}: #9459). α-tubulin (Cat no: #2125) was used as a loading control. All
200 antibodies were purchased from Cell Signaling Technology (Danvers, MA). All samples for a

201 given protein were detected on the same membrane using chemiluminescence and the
202 FluorChem HD imaging system (Alpha Innotech, Santa Clara, CA, USA).

203

204 *Calculations*

205 Ingestion of L-[1-¹³C]-phenylalanine labeled protein, intravenous infusion of L-[ring-²H₅]-
206 phenylalanine, and blood sample enrichment values were used to assess whole-body amino
207 acid kinetics in non-steady state conditions. Total, exogenous, and endogenous phenylalanine
208 rates of appearance and plasma availability of dietary protein-derived phenylalanine that
209 appeared in the systemic circulation as a fraction of total amount of phenylalanine that was
210 ingested, (Phe_{plasma}) were calculated using modified Steele's equations (6, 11, 52).
211 Myofibrillar protein fractional synthetic rate (FSR) was calculated using the standard
212 precursor-product method (Equation 1).

213

$$214 \quad FSR \left(\% \cdot h^{-1} \right) = \left(\frac{E_{m2} - E_{m1}}{E_{precursor} \times t} \right) \times 100 \quad (1)$$

215

216 $E_{m2} - E_{m1}$ represents the change in muscle protein bound L-[1-¹³C]-leucine or L-[ring-²H₅]-
217 phenylalanine enrichment. $E_{precursor}$ represents the average plasma L-[1-¹³C]-leucine or L-
218 [ring-²H₅]-phenylalanine enrichment during the tracer incorporation period. t indicates the
219 time interval (h) between biopsies.

220

221 *Statistics*

222 Data are expressed as means+SEMs or as box-whisker plots. Baseline characteristics between
223 groups were compared using a *student's* unpaired t-test. A two-factor repeated measures
224 ANOVA (time x treatment) with time as within-subjects factor and treatment group as
225 between-subjects factor was performed for the analysis of plasma amino acid concentrations,

226 plasma tracer enrichments, whole-body kinetics and glucose and insulin concentrations. The
227 analysis was carried out for the period starting at the time of protein administration, between t
228 = 0 and 360 min. Upon identification of a significant time X treatment interaction, Tukey
229 *post hoc* testing was used to identify time points in which the treatments differed. Non time-
230 dependent variables (*i.e.*, Whole-body metabolism, FSR values, L-[1-¹³C]-phenylalanine
231 myofibrillar enrichments) were compared between treatment groups using *student's* unpaired
232 t-tests. Statistical significance was set at $P < 0.05$. All calculations were performed using SPSS
233 21.0 (IBM, Chicago, Illinois, USA).

234 **RESULTS**

235

236 *Plasma concentrations*

237 Plasma glucose (**Figure 1A**) and insulin (**Figure 1B**) concentrations after protein ingestion
238 did not differ between the 15G and 15G+LEU groups ($P>0.05$). Plasma insulin
239 concentrations increased after protein ingestion in both treatments, reaching peak levels 30
240 min after protein ingestion.

241 Plasma leucine concentrations (**Figure 2A**) increased rapidly following protein ingestion
242 ($P<0.01$), but were greater in 15G+LEU (peak values: $407\pm 23 \mu\text{mol}\cdot\text{L}^{-1}$) when compared to
243 15G (peak values: $234\pm 16 \mu\text{mol}\cdot\text{L}^{-1}$, $P<0.01$). Area under the curve (AUC, **Figure 2B**)
244 analysis revealed that plasma leucine availability over the 6 h post-prandial was
245 approximately 1.8-fold greater in the 15G+LEU group when compared to the 15G group
246 ($P<0.001$). Plasma phenylalanine concentrations (**Figure 2C**) increased rapidly following
247 protein ingestion (Time effect: $P<0.01$) along with a main effect for treatment (Treatment
248 effect: $P<0.01$), but no time x treatment interaction ($P>0.05$). Plasma tyrosine concentrations
249 (**Figure 2D**) increased following protein ingestion (Time effect: $P<0.01$) along with a main
250 effect for treatment (Treatment effect: $P<0.01$), but no time x treatment interaction ($P>0.05$).

251

252 *Plasma amino acid enrichments*

253 Plasma enrichments from ingested (L-[1- ^{13}C]-phenylalanine), infused (L-[ring- $^2\text{H}_5$]-
254 phenylalanine) and ingested and infused (L-[1- ^{13}C]-leucine) amino acid tracers did not differ
255 between treatments before protein ingestion ($t = 0$ min; $P>0.05$). After protein ingestion,
256 plasma L-[1- ^{13}C]-phenylalanine enrichments, originating from the ingested protein, increased
257 in both groups reaching peak values at $t = 60$ min in 15G (9.6 ± 0.5 MPE) and $t = 120$ min in
258 15G+LEU (8.7 ± 0.5 MPE) in 15G+LEU. Plasma L-[ring- $^2\text{H}_5$]-phenylalanine enrichments

259 decreased after protein ingestion in both groups ($P<0.001$), but no significant group effect
260 was detected ($P>0.05$). Plasma L-[1- ^{13}C]-leucine enrichments increased after protein
261 ingestion ($P<0.001$), but no significant group effects were detected ($P>0.05$).

262

263 *Whole-body amino acid kinetics*

264 Exogenous phenylalanine appearance rates (**Figure 3A**) increased following protein ingestion
265 with peak levels being reached at $t = 60$ min in both treatment groups (15G: 0.19 ± 0.01 ,
266 15G+LEU: 0.16 ± 0.02 $\mu\text{mol Phe}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; $P>0.05$). Dietary protein-derived amino acid
267 availability, calculated as a fraction of the total amount of ingested protein (**Figure 3B**), was
268 higher in 15G ($75\pm 2\%$) when compared to 15G+LEU ($70\pm 1\%$; $P<0.05$).

269 Whole-body protein synthesis rates did not differ between the treatment groups (15G:
270 0.60 ± 0.01 , 15G+LEU: 0.59 ± 0.01 $\mu\text{mol Phe}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; $P>0.05$). Whole-body protein
271 breakdown rates did not differ between the treatment groups (15G: 0.49 ± 0.01 , 15G+LEU:
272 0.49 ± 0.01 $\mu\text{mol Phe}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; $P>0.05$). Protein ingestion resulted in a positive whole-body
273 protein net balance, with no differences observed between the treatment groups (15G:
274 0.108 ± 0.004 , 15G+LEU: 0.105 ± 0.003 $\mu\text{mol Phe}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; $P>0.05$). Furthermore, leucine
275 co-ingestion did not appear to influence whole-body phenylalanine oxidation rates (15G:
276 0.049 ± 0.003 , 15G+LEU: 0.046 ± 0.002 $\mu\text{mol Phe}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$; $P>0.05$).

277

278 *Myofibrillar fractional synthesis rates and protein-bound enrichments*

279 Myofibrillar L-[1- ^{13}C]-leucine and L-[ring- $^2\text{H}_5$]-phenylalanine enrichments were measured in
280 muscle samples collected immediately before protein ingestion and after the 6 h post-prandial
281 period. The post-prandial increase in myofibrillar protein bound L-[1- ^{13}C]-leucine
282 enrichments tended to be greater in 15G+LEU when compared to 15G (0.0360 ± 0.0016 vs
283 0.0314 ± 0.0016 MPE, respectively; $P=0.055$). The post-prandial increase in myofibrillar

284 protein bound L-[*ring*-²H₅]-phenylalanine enrichment was greater in 15G+LEU when
285 compared to 15G (0.0330±0.0015 vs 0.0278±0.0011 MPE, respectively; *P*<0.05).

286 Myofibrillar protein FSRs (in %·h⁻¹) were calculated using L-[*ring*-²H₅]-phenylalanine
287 plasma (**Figure 4A**) and muscle protein-bound enrichments and using L-[1-¹³C]-leucine
288 (**Figure 4B**) plasma and muscle protein-bound enrichments. When based upon L-[*ring*-²H₅]-
289 phenylalanine, myofibrillar protein FSR was approximately 16% greater in 15G+LEU
290 (0.0575±0.0032 %·h⁻¹) when compared with 15G (0.0495±0.0021 %·h⁻¹; *P*<0.05). When
291 based upon L-[1-¹³C]-leucine, myofibrillar protein FSR was approximately 19% greater in
292 15G+LEU (0.0710±0.0048 %·h⁻¹) when compared with 15G (0.0598±0.0030 %·h⁻¹; *P*<0.05).
293 L-[1-¹³C]-phenylalanine myofibrillar protein-bound enrichments (**Figure 5**) were not
294 different in 15G+LEU (0.0205±0.0022 MPE) when compared with 15G (0.0171±0.0017
295 MPE; *P*=0.24).

296

297 *Cellular signalling analyses*

298 The phosphorylation status (ratio of phosphorylated to total protein) of key proteins involved
299 in the initiation of muscle protein synthesis are presented in **Figure 6**. Phosphorylation of
300 S6K1 (**Figure 6B**) decreased in both groups over time (time effect, *P*<0.01). Phosphorylation
301 of 4E-BP1 (**Figure 6D**) increased over time and to a greater extent in 15G compared with
302 15G+LEU (*P*<0.01).

303 **DISCUSSION**

304 In the present study, we examined the impact of free leucine co-ingestion on post-prandial
305 protein handling and the subsequent muscle protein synthetic response following the
306 ingestion of 15 g protein during recovery from resistance-type exercise in older men. We
307 observed that 70-75 % of the dietary-derived amino acids were absorbed into the circulation
308 6 hours after the ingestion of 15 g protein. Co-ingesting 1.5 g free leucine with 15 g protein
309 further increased post-exercise myofibrillar protein synthesis rates, but did not significantly
310 increase the incorporation of dietary protein-derived amino acids in myofibrillar protein.

311 We administered a primed, continuous intravenous infusion of L-[ring-²H₅]-phenylalanine,
312 L-[ring-²H₂]-tyrosine and L-[1-¹³C]-leucine throughout a 6 h post-exercise recovery period in
313 older individuals. Following exercise, participants ingested either 15 g of intrinsically L-[1-
314 ¹³C]-phenylalanine labeled milk protein with or without 1.5 g free leucine. With this
315 experimental protocol, we were able to assess *in vivo* protein digestion and amino acid
316 absorption kinetics, whole-body protein metabolism, myofibrillar protein synthesis, and the
317 incorporation of dietary protein-derived amino acids in muscle protein (8). After protein
318 ingestion, we observed a rapid rise in circulating plasma amino acid concentrations (**Figure**
319 **2**) and an increase in the rate of exogenous phenylalanine appearance (**Figure 3A**),
320 demonstrating rapid protein digestion and subsequent absorption of dietary protein-derived
321 amino acids during recovery from exercise. As expected, fortification with 1.5 g free leucine
322 resulted in greater peak plasma leucine concentrations (407 ± 23 vs 234 ± 16 $\mu\text{mol} \cdot \text{L}^{-1}$, $P <$
323 0.01) at $t = 30$ min, and 1.8-fold greater plasma leucine availability over the entire 6 h post-
324 prandial period, when compared to the ingestion of 15 g protein ($P < 0.01$). We observed 70-
325 75 % of dietary protein-derived amino acid absorption into the circulation over the 6 h post-
326 prandial period in both groups. This seems to be much more when compared to recent work
327 from our lab using the same methodology (21, 34, 35). The apparent discrepancy is attributed

328 to the relatively small amount of dietary protein that was provided in the present study along
329 with the extended 6 h post-prandial assessment period, implying that more protein derived
330 amino acids will be absorbed during such an extended post-prandial period with a relatively
331 smaller bolus of protein being ingested (22). Free leucine fortification seemed to compromise
332 protein digestion and/or amino acid absorption as dietary protein-derived phenylalanine
333 availability was lower following leucine co-ingestion when assessed over the entire 6 h post-
334 prandial period (10.5 ± 0.2 vs 11.2 ± 0.3 g; $P < 0.05$). This was attributed to a mild attenuation
335 of exogenous amino acid appearance rates observed between $t = 30$ - 120 min (**Figure 3A**). It
336 could be speculated that the added free leucine may have stimulated splanchnic amino acid
337 retention of dietary-protein derived amino acids during first pass. In agreement, prior work in
338 neonatal pigs has demonstrated that free leucine co-ingested with a low protein dose
339 stimulates an increase in jejunum, but not liver protein synthesis (29, 39). Altogether, our
340 data demonstrate that free leucine co-ingestion further increases the post-prandial rise in
341 leucine concentrations but attenuates the rate of appearance of dietary protein-derived amino
342 acids into the circulation.

343 By administering a primed, continuous intravenous infusion of L-[ring- 2 H $_5$]-phenylalanine
344 and L-[ring- 2 H $_2$]-tyrosine and providing intrinsically L-[1- 13 C]-phenylalanine labeled protein,
345 we were able to assess post-prandial whole-body protein synthesis, breakdown, net balance
346 and oxidation. In both groups, protein ingestion resulted in a positive whole-body net protein
347 balance during post-exercise recovery. However, fortification with free leucine did not
348 further impact whole-body post-prandial protein synthesis, breakdown, or net balance. These
349 findings are in agreement with prior work in older men at rest (38) and in younger men
350 during post-exercise recovery (25). Despite previous reports that leucine administration
351 lowers whole-body amino acid oxidation rates (24, 32), we did not observe this effect. These
352 studies achieved far greater plasma leucine availability in comparison to the present study,

353 which may lead to a reduction in protein breakdown rates (32, 42) and thereby lowering the
354 availability of amino acids for oxidation (25, 42). Our present data align with recent work
355 administering similar, meal-like amounts of leucine (~4.5 g total) (38), and demonstrate that
356 leucine co-ingestion does not impact whole-body phenylalanine oxidation rates.

357 Changes in whole-body protein metabolism do not necessarily reflect changes on a muscle
358 tissue level. Therefore, we also collected skeletal muscle biopsies to directly assess the
359 impact of leucine fortification of a low protein dose on intramuscular signaling and the
360 muscle protein synthetic response to feeding. Resistance-type exercise and protein ingestion
361 activate intramuscular signaling proteins that regulate protein translation with mTOR and its
362 downstream targets, S6K1, RS6 and 4E-BP1 being of particular relevance. We observed no
363 differences in mTOR or RS6 phosphorylation, but detected a decrease in S6K1
364 phosphorylation over time. These findings align with previous work showing a rapid increase
365 in S6K1 activity following exercise, which subsides over 3-6 h (26, 51). Considering that
366 biopsy timing was intended to assess the muscle protein synthetic response during the entire
367 post-prandial period, it is most likely that transient increases in signaling activity had
368 subsided by 6 h. However, 4E-BP1 phosphorylation increased over time in both groups, and
369 to a greater extent after the ingestion of 15 g protein when compared with the ingestion of 15
370 g with leucine. We speculate that the higher leucine availability in 15G+LEU may have
371 transiently activated 4E-BP1 at an earlier time in comparison to 15G (14, 18, 23), which
372 steadily activated 4E-BP1 over the 6 h post-prandial period (26, 49).

373 Combining stable isotope labeled amino acid infusions with ingestion of intrinsically-labeled
374 protein, we were able to assess muscle protein synthesis rates under both steady-state (L-[1-
375 ¹³C]-leucine) as well as non-steady state (L-[ring-²H₅]-phenylalanine) precursor conditions
376 (8). Previous work has demonstrated that the ingestion of a low protein dose (< 20 g)
377 following resistance-type exercise does not further stimulate an increase in muscle protein

378 synthesis rates in older individuals (53, 54). In the present study, free leucine co-ingested
379 with a low protein dose (15 g) increased myofibrillar protein synthesis rates by 16% (L-[ring-
380 $^2\text{H}_5$]-phenylalanine, **Figure 4A**) and 19% (L-[1- ^{13}C]-leucine, **Figure 4B**) when compared
381 with the ingestion of 15 g protein. These findings are in line with multiple studies
382 demonstrating that free leucine co-ingestion can further increase the muscle protein synthetic
383 response to protein ingestion in older individuals at rest (2, 9, 12, 13, 30, 48) and during
384 recovery from resistance-type exercise (2, 7, 9, 12, 13, 30). In the present study, participants
385 ingested intrinsically L-[1- ^{13}C]-phenylalanine labeled protein, allowing us to directly assess
386 the metabolic fate of the dietary protein-derived amino acids (21, 44, 48). Despite the greater
387 post-prandial muscle protein synthetic response following the co-ingestion of free leucine, we
388 did not observe a significantly greater L-[1- ^{13}C]-phenylalanine enrichment in myofibrillar
389 protein in 15G+LEU compared with 15G (**Figure 5**). The absence of a difference in the
390 incorporation of dietary protein-derived amino acids in myofibrillar protein may be related to
391 the mild attenuation in dietary-protein derived phenylalanine availability in the circulation
392 when free leucine was co-ingested (**Figure 3B**). It has been reported that the increase in
393 muscle protein synthesis rates following leucine administration may become limited with
394 inadequate provision of exogenous amino acids as precursors (15, 16). More work will be
395 required to determine the optimal amount of dietary protein that should be co-ingested with
396 free leucine to ensure adequate plasma precursor availability to maximize post-prandial
397 muscle protein synthesis rates.

398 The muscle protein synthetic response to protein ingestion has been shown to be impaired in
399 older (47) and/or more clinically compromised populations (31, 50). Resistance-type exercise
400 is an effective strategy to improve the sensitivity of skeletal muscle to the anabolic properties
401 of dietary protein. However, recent work from our group has demonstrated that ingestion of
402 less than 30 g protein does not further increase the muscle protein synthetic response during

403 post-exercise recovery in older men (22). We (34) and others (53, 54) have shown that
404 increasing protein intake can compensate for this anabolic resistance, with as much as 45 g of
405 protein being required to achieve a robust anabolic response during exercise recovery in older
406 individuals. However, ingesting such large protein amounts may not be feasible in older
407 and/or more clinically compromised populations. The current data extend upon previous
408 findings and show that free leucine co-ingestion can further augment the post-exercise
409 muscle protein synthetic response to protein ingestion (2, 7, 9, 12, 13, 30). In particular, the
410 total amount of leucine provided in the 15G+LEU beverage (2.94 g) is equivalent to the
411 amount of leucine contained in 30 g of MPC80. Therefore, increasing the leucine content
412 through leucine co-ingestion may increase the efficiency by which the ingestion of smaller
413 protein doses can augment muscle protein synthesis rates during recovery from exercise.
414 Simply adding leucine to a post-exercise snack, to achieve approximately 3 g total leucine,
415 may represent an effective strategy to support muscle mass maintenance in the older
416 population without the need to ingest large(r) doses of protein. So far only few long-term
417 intervention studies have assessed the anabolic effect of prolonged leucine supplementation.
418 Whereas prolonged leucine supplementation does not seem to increase muscle mass in older
419 individuals (27, 45), it has been suggested that leucine supplementation may augment muscle
420 mass when combined with prolonged resistance-type exercise training (43). Nonetheless,
421 more work is needed to assess the long-term benefits of leucine supplementation in
422 combination with prolonged resistance-type exercise training in the older population.
423 In conclusion, leucine co-ingestion further augments the post-exercise muscle protein
424 synthetic response to the ingestion of a small amount of protein in older men.

DECLARATIONS

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Ethics approval and consent to participate.

This study was approved by the Medical Ethical Committee of the Maastricht University Medical Centre, The Netherlands (METC 14-3-052). All participants provided written informed consent before participation.

Authors' contributions

A.M.H., L.C.P.G.M.G, and L.J.C.L. designed the research; A.M.H., K.P., M.O. and I.F.K. conducted the research; A.M.H., J.P.B.G., W.K.W.H.W. and L.J.C.L. analyzed the data; A.M.H. and L.B.V. performed the statistical analysis; and A.M.H. and L.J.C.L. wrote the paper and hold primary responsibility for the final content. All authors read and approved the final manuscript.

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FIGURE LEGENDS

Figure 1. Plasma glucose (A, $\text{mmol}\cdot\text{L}^{-1}$) and insulin concentrations (B, $\text{mU}\cdot\text{L}^{-1}$) following ingestion of 15 g milk protein (15G; $n=12$) or 15 g milk protein co-ingested with 1.5 g free leucine (15G+LEU; $n=12$) after resistance-type exercise in older men. The dotted line represents the ingestion of the beverage. Values represent means+SEMs. Data were analyzed with repeated measures (time x treatment group) ANOVA. A; time effect: $P<0.01$, treatment effect: $P>0.05$, time x treatment group: $P>0.05$. B; time effect: $P<0.01$, treatment effect: $P>0.05$, time x treatment group: $P>0.05$.

Figure 2. Plasma leucine (A), phenylalanine (C) and tyrosine (D) concentrations ($\mu\text{mol}\cdot\text{L}^{-1}$) following ingestion of 15 g milk protein (15G; $n=12$) or 15 g milk protein co-ingested with 1.5 g free leucine (15G+LEU; $n=12$) during recovery from resistance-type exercise in older men. The dotted line represents the ingestion of the beverage. Values for panels A, C, and D represent means+SEMs. Data were analyzed with repeated measures (time x treatment group) ANOVA. A; time effect: $P<0.01$, treatment effect: $P<0.01$, time x treatment group: $P<0.01$. C; time effect: $P<0.01$, treatment effect: $P<0.01$, time x treatment group: $P>0.05$. D; time effect: $P<0.01$, treatment effect: $P<0.01$, time x treatment group: $P>0.05$. Plasma leucine area under the curves over 360 min (B, $\mu\text{mol}\cdot 360\text{ min}\cdot\text{L}^{-1}$) are presented as box and whisker plots. Boxes represent 25th to 75th percentiles. Horizontal lines and crosses within boxes represent medians and means, respectively. Whiskers represent minimum and maximum values. Data were analyzed with a student's *unpaired* t-test. *Significant difference ($P<0.05$) from 15G.

Figure 3. Exogenous phenylalanine rate of appearance (A, $\mu\text{mol Phe}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) following ingestion of 15 g milk protein (15G; $n=12$) or 15 g milk protein co-ingested with 1.5 g free

leucine (15G+LEU; $n=12$) during recovery from resistance-type exercise in older men. The dotted line represents the ingestion of the beverage. Values for panel A represent means+SEMs. Data were analyzed with repeated measures (time x treatment group) ANOVA. A; time effect: $P<0.01$, treatment effect: $P<0.05$, Time x treatment group: $P>0.05$. Dietary protein-derived amino acid plasma availability (panel B), calculated as a fraction of the total amount of ingested protein (% ingested protein), are presented as box and whisker plots. Boxes represent 25th to 75th percentiles. Horizontal lines and crosses within boxes represent medians and means, respectively. Whiskers represent minimum and maximum values. Data were analyzed with a student's *unpaired* t-test. *Significantly different ($P<0.05$) from 15G.

Figure 4. Myofibrillar protein fractional synthetic rates (FSR in $\% \cdot h^{-1}$) assessed using L-[ring- 2H_5]-phenylalanine (A) and L-[1- ^{13}C]-leucine (B) and following ingestion of 15 g milk protein (15G; $n=12$) or 15 g milk protein co-ingested with 1.5 g free leucine (15G+LEU; $n=12$) after resistance-type exercise in older men. Boxes represent 25th to 75th percentiles. Horizontal lines and crosses within boxes represent medians and means, respectively. Whiskers represent minimum and maximum values. Data were analyzed with a student's *unpaired* t-test. *Significantly different ($P<0.05$) from 15G.

Figure 5. L-[1- ^{13}C]-phenylalanine incorporation into myofibrillar protein following ingestion of 15 g milk protein (15G; $n=12$) or 15 g milk protein co-ingested with 1.5 g free leucine (15G+LEU; $n=12$) during recovery from resistance-type exercise in older men. Boxes represent 25th to 75th percentiles. Horizontal lines and crosses within boxes represent medians and means, respectively. Whiskers represent minimum and maximum values. Data

were analyzed with student's *unpaired* t-test. No significant difference between groups ($P=0.24$).

Figure 6. Muscle phosphorylation status (ratio of phosphorylated to total protein, AU) of mammalian target of rapamycin (mTOR; A) S6 protein kinase 1 (S6K1; B), ribosomal protein S6 (RS6; C), and eukaryotic translation initiation factor 4E-binding protein-1 (4E-BP1; D) in older men during recovery from resistance-type exercise (0 min) and 360 min after the ingestion of 15 g milk protein (15G; $n=12$) or 15 g milk protein co-ingested with 1.5 g free leucine (15G+LEU; $n=12$). Boxes represent 25th to 75th percentiles. Horizontal lines and crosses within boxes represent medians and means, respectively. Whiskers represent minimum and maximum values. Data were analyzed with repeated measures (time x treatment group) ANOVA. A; time effect: $P>0.05$, treatment effect: $P>0.05$, time x treatment group: $P>0.05$. B; time effect: $P<0.01$, treatment effect: $P>0.05$, time x treatment group: $P>0.05$. C; time effect: $P>0.05$, treatment effect: $P>0.05$, time x treatment group: $P>0.05$. D; time effect: $P<0.01$, treatment effect: $P>0.01$, time x treatment group: $P<0.01$ *Significantly different ($P<0.05$) compared to $t = 0$ min. †Significant difference ($P<0.05$) from 15G+LEU at the same time point.

TABLES

Table 1. Subjects' characteristics¹

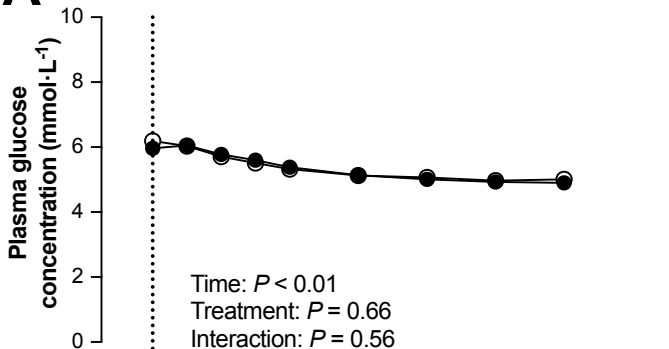
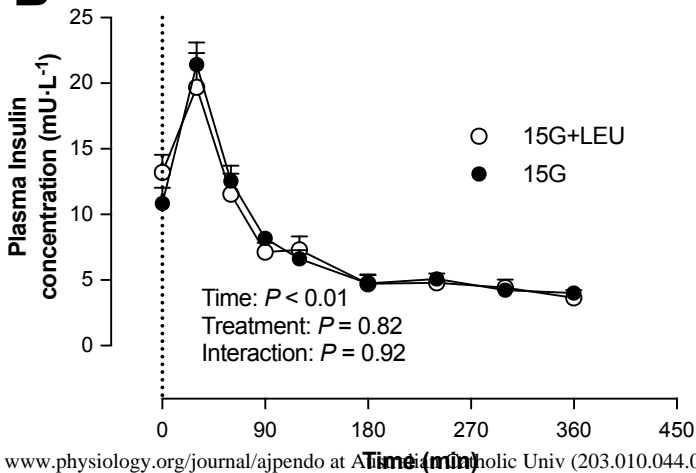
	15G (n = 12)	15G+LEU (n = 12)	P
Age, y	69 ± 2	66 ± 2	0.45
Total body mass, kg	78.8 ± 3.2	79.0 ± 2.4	0.96
Total lean mass, kg	57.6 ± 2.3	58.1 ± 1.5	0.86
Appendicular lean mass, kg	24.9 ± 1.1	25.6 ± 0.7	0.64
Percentage body fat, %	23.9 ± 0.9	23.2 ± 1.2	0.62
Height, m	1.75 ± 0.02	1.78 ± 0.01	0.23
BMI, kg·m ⁻²	25.8 ± 0.8	24.9 ± 0.8	0.43
HbA1c, %	5.3 ± 0.1	5.3 ± 0.1	0.80
Resting glucose, mmol·L ⁻¹	5.8 ± 0.2	6.2 ± 0.2	0.13
Resting insulin, mU·L ⁻¹	9.3 ± 0.9	8.4 ± 1.2	0.59
HOMA-IR	2.4 ± 0.2	2.4 ± 0.4	1.00
MVPA, min	145 ± 31	160 ± 33	0.95
1RM - Leg press, kg	179 ± 8	166 ± 6	0.23
1RM - Leg extension, kg	86 ± 6	88 ± 2	0.79
1RM - Lat pulldown, kg	60 ± 4	62 ± 4	0.78
1RM - Chest press, kg	60 ± 6	58 ± 5	0.77

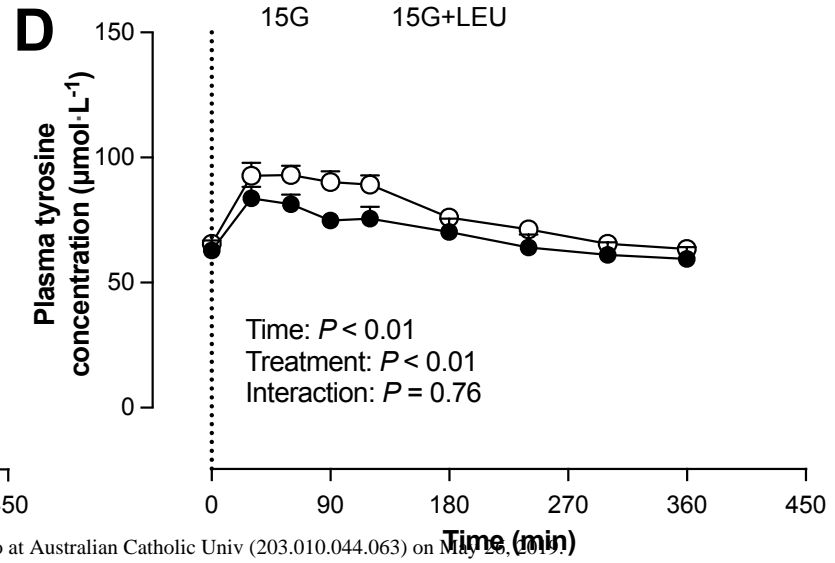
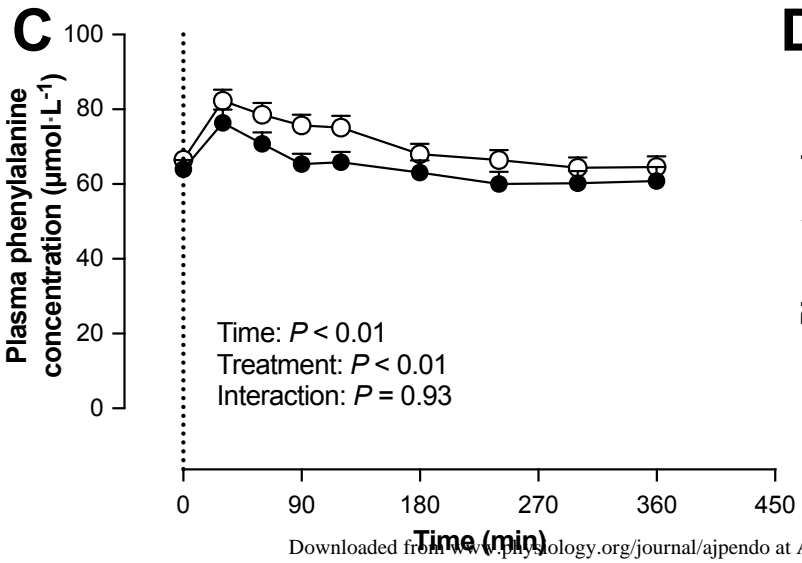
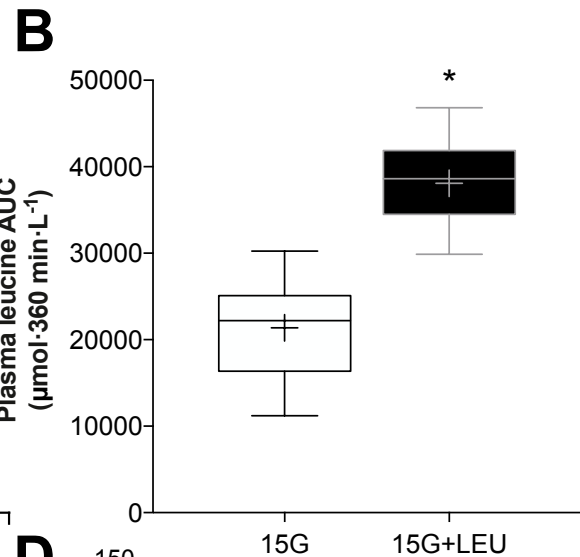
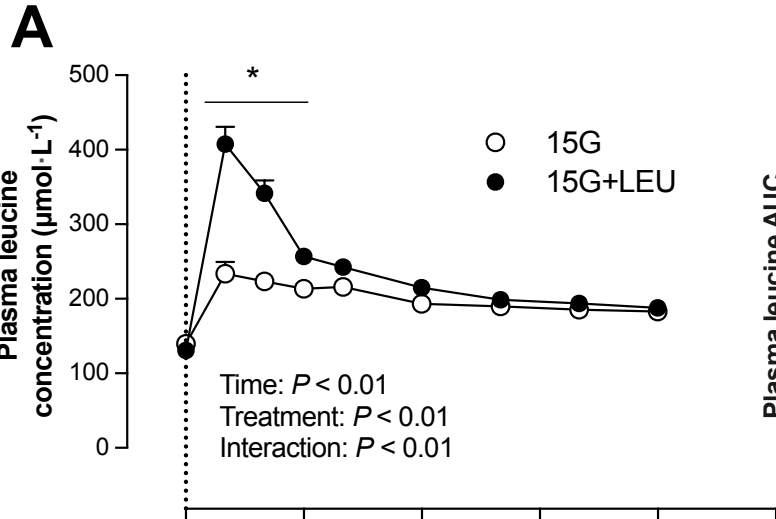
¹Values are mean ± SEM. *n* = 12 per treatment group. 15G: 15 g dietary protein, 15G+LEU: 15 g dietary protein + 1.5 g free crystalline leucine. 1RM: one repetition maximum, HbA1c: glycosylated hemoglobin, MVPA: moderate-to-vigorous physical activity, Resting: resting and fasted values. Data were analyzed with a student's unpaired t-test. No differences were detected between groups.

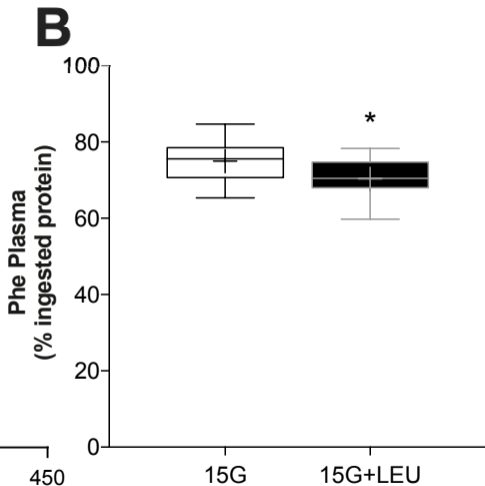
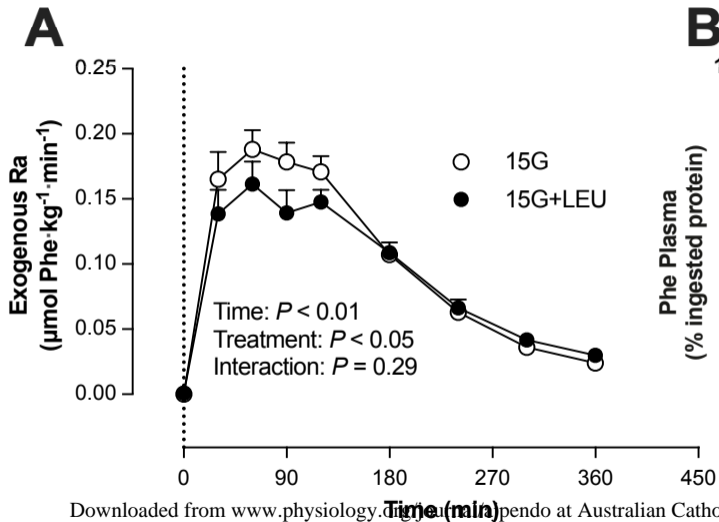
Table 2. Amino acid composition of the test beverages¹

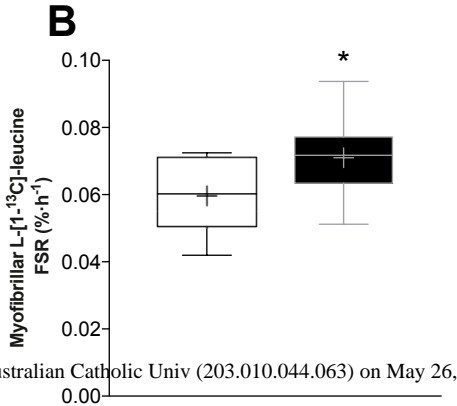
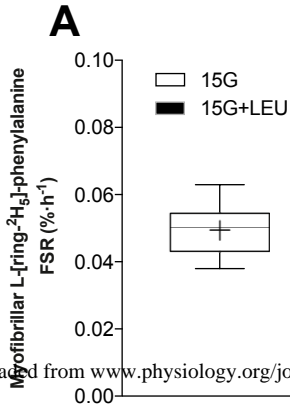
	15G	15G+LEU
	g	g
Alanine	0.45	0.45
Arginine	0.50	0.50
Aspartic acid	0.92	0.92
Glutamic acid	2.51	2.51
Glycine	0.23	0.23
Histidine	0.33	0.33
Isoleucine	0.66	0.66
Leucine	1.44	2.94
Lysine	1.19	1.19
Methionine	0.18	0.18
Phenylalanine	0.63	0.63
Proline	1.38	1.38
Serine	0.70	0.70
Threonine	0.57	0.57
Tyrosine	0.78	0.78
Valine	0.84	0.84

¹Values are expressed in g. 15G: 15 g dietary protein, 15G+LEU: 15 g dietary protein + 1.5 g free crystalline leucine.

A**B**







Myofibrillar L-[1-¹³C]-phenylalanine
enrichment (MPE)

