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**Increased protein intake derived from leucine-enriched protein enhances the integrated myofibrillar protein synthetic response to short-term resistance training in untrained men and women: a 4-day randomized controlled trial**

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**Running Head:** Leucine-enriched protein and MyoPS

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## 1 **Abstract**

2 *Leucine* is a critical amino acid stimulating myofibrillar protein synthesis (MyoPS). The  
3 consumption of higher leucine-containing drinks stimulates MyoPS, but we know less about  
4 higher leucine solid foods. Here we examined the effect of short-term resistance exercise  
5 training (STRT) combined with supplementation of a protein and leucine-enriched bar,  
6 compared with STRT alone, on integrated (%/d) rates of MyoPS and anabolic protein  
7 signaling. In a non-blinded, randomized crossover trial, eight young adults performed four  
8 sessions of STRT without or while consuming the study bar (STRT+Leu, 16g of protein  
9 containing ~3g of leucine) for two 4d phases, separated by 2d non-exercise (Rest) washout.  
10 In combination with serial muscle biopsies, deuterated water permitted the measurement of  
11 myofibrillar protein synthesis and protein signaling phosphorylation. MyoPS during STRT  
12 ( $1.43 \pm 0.06$  %/d) and STRT+Leu ( $1.53 \pm 0.06$  %/d) were greater than Rest ( $1.31 \pm$   
13  $0.05$  %/d), and MyoPS during STRT+Leu ( $1.53 \pm 0.06$  %/d) was greater than STRT alone  
14 ( $1.43 \pm 0.06$  %/d). STRT+Leu increased the ratio of phosphorylated to total mTOR and  
15 4EBP1 compared to Rest. Engaging in STRT increased integrated MyoPS and protein  
16 signaling in young adults and was enhanced with increased protein intake derived from a  
17 leucine-enriched protein bar. This study was registered at [clinicaltrials.gov](https://clinicaltrials.gov) as NCT03796897.

## 19 **Keywords**

20 Amino acids, milk protein, skeletal muscle, protein bar, leucine, protein synthesis

## 21 **Introduction**

22 Resistance training (RT) stimulates pathways that ‘sensitize’ skeletal muscle to the anabolic  
23 responses of protein ingestion (Burd et al. 2011). Ingestion of an adequate dose of protein  
24 results in subsequent hyperaminoacidemia and stimulation of myofibrillar protein synthesis  
25 (MyoPS) in humans (Rennie et al. 2004). In addition, supplementation of fast and slow  
26 digestible proteins (e.g., whey and casein) enriched with crystalline leucine results in a rapid  
27 and sustained increase in circulating essential amino acids and effectively stimulates MyoPS  
28 in young and old adults (Churchward-Venne et al. 2012; Churchward-Venne et al. 2014;  
29 Kramer et al. 2015; Kramer et al. 2017). Milk-based proteins have the highest protein quality  
30 scores, whether measured as protein digestibility-corrected amino acid score or digestible  
31 indispensable amino acid score (FAO 2013). Thus, milk-derived proteins represent the most  
32 effective (leucine and essential amino acids per g of total protein) protein source to achieve  
33 an anabolic stimulation of MyoPS.

34 Leucine is an essential amino acid necessary for activating MyoPS via the  
35 mechanistic target of rapamycin (mTOR) signaling pathway in human skeletal muscle  
36 (Wolfson and Sabatini 2017; Wolfson et al. 2016). Recently, we reported that consuming a  
37 protein and leucine-enriched bar – formulated with 16 g of a blend of micellar casein, whey  
38 protein, and whey protein hydrolysate – induced aminoacidemia comparable to consuming a  
39 single higher-protein-containing meal (0.5g of protein/kg) (Traylor et al. 2021). Previous  
40 studies also reported that higher leucine-containing proteins enhance MyoPS and whole-body  
41 protein synthesis in young and older adults (Glynn et al. 2010; Zaremkyte et al. 2021). The  
42 effect of leucine on daily MyoPS can be independent of daily protein intake (Devries et al.  
43 2018a, 2018b; Murphy et al. 2016). Thus, further studies that evaluate the effect of protein-  
44 rich whole food — notably higher quality protein (i.e., higher leucine) — intake on protein  
45 synthesis in untrained young adults are needed.



46 The potential for short-term resistance training (STRT, 2 to 6 sessions) has  
47 applications in sports settings for athletic trainers and physical therapists who develop  
48 programs to help athletes return to practice and competition. Short resistance training may  
49 also provide clinicians with scientific justifications for recommendations regarding the design  
50 of resistance training programs, including the number of rehabilitative sessions necessary to  
51 obtain a specific strength outcome. Most STRT studies have examined strength, power, and  
52 neuromuscular adaptations in humans (Beck et al. 2007; Brown and Whitehurst 2003;  
53 Coburn et al. 2006). There is little information about the effect of STRT on integrated daily  
54 MyoPS. Some previous studies reported that early exercise-induced increases in MyoPS do  
55 not align with hypertrophic outcomes (Damas et al. 2016). Muscle hypertrophy, however, is  
56 known to result from accumulated increases in MyoPS after resistance exercise.

57 The present study aimed to examine the effect of STRT combined with high-leucine-  
58 containing protein bar supplementation (STRT+Leu), compared with STRT alone, on  
59 integrated (%/day) rates of MyoPS and purported anabolic signaling proteins. We  
60 hypothesized that STRT and STRT+Leu would increase MyoPS over resting values and that  
61 STRT+Leu would increase the MyoPS response more than STRT alone. Moreover, the acute  
62 phosphorylation of mTOR complex 1 (mTORC1)-related signaling proteins would be  
63 commensurate with the MyoPS responses.

## 64 65 **Materials and methods**

66 **Participants.** Eight young adults (five men and three women; Table 1) were recruited and  
67 provided written consent after being informed of the purpose, protocol, and risks of the study  
68 (Figure 1). Participants were recreationally active but had not been involved in a resistance  
69 training program for at least the previous three months and did not meet Canada's Physical  
70 Activity Guidelines (150 minutes of moderate-intensity exercise/week). Inclusion criteria

71 were as follows: men and women 18-29 years of age and generally healthy according to a  
72 standard health screening questionnaire. Exclusion criteria were the regular use of analgesic  
73 or anti-inflammatory drugs, history of neuromuscular problems, musculoskeletal disease, any  
74 acute or chronic illness, tobacco use or smoking, metabolic disorders, the use of  
75 corticosteroids, and not being able to perform lower-body resistance training. The Hamilton  
76 Integrated Research Ethics Board approved all experimental procedures (HIREB Project  
77 5706). This study was registered at clinicaltrials.gov as NCT03796897. Due to time  
78 constraints, this study did not achieve full recruitment.

79 **Experimental design.** The present study was a nonblinded, controlled randomized crossover  
80 design, consisting of STRT and STRT combined with protein and leucine-enriched bar  
81 supplementation (STRT+Leu). The participants visited the laboratory on eleven separate  
82 occasions, and specific details of the study design appear in Figure 2. At least one week  
83 before the oral deuterium oxide intake sessions for determining MyoPS and the underpinning  
84 mechanisms, study participants underwent familiarization with the study protocol and a  
85 whole-body dual-energy X-ray absorptiometry (DXA) scan (GE-Lunar iDXA; Aymes  
86 Medical, Newmarket, ON) to measure body composition. Additionally, participants  
87 performed unilateral/contralateral strength testing, which involved a five repetition maximum  
88 (RM) test of standard seated leg press and seated leg extension exercise in the same manner  
89 as previously described (Churchward-Venne et al. 2012; Churchward-Venne et al. 2014;  
90 Oikawa et al. 2020). Following baseline testing, participants began an 11 day controlled diet  
91 designed to meet energy requirements and to provide moderate daily protein intake (1.3  
92 g/kg/day) with all food provided by the study investigators. To monitor body-water  
93 deuterium enrichment, fasted state saliva samples were collected each morning of the study  
94 duration. During the visits for exercise training, the participants underwent  
95 unilateral/contralateral training sessions of the leg extensors with/without consumption of the

96 protein and leucine-enriched bars, respectively. During the Pre-STRT visits (day 1 and 7),  
97 muscle biopsies were taken from the *vastus lateralis* before a training session. During the  
98 post-test visits (day 4 and 10), muscle biopsies were taken 1 hour after the training session  
99 (STRT phase) or 1 hour after the training session and bar consumption (STRT+Leu phase) to  
100 determine the integrated MyoPS during each test phase and select molecular responses to  
101 acute resistance exercise. The leg for muscle biopsies were randomized for each treatment  
102 phase. There was a washout period for the crossover on days 5 and 6. Although it was  
103 impossible to blind the participants or investigators, all samples were analyzed, and data  
104 collated in a blinded fashion until statistical analysis was complete.

105 **Diets.** Each participant's energy requirement to maintain energy balance was determined  
106 using the Harris-Benedict equation and adjusted using a moderate activity factor of 1.7 for  
107 accounting for participants' reported physical activity patterns (Roza and Shizgal 1984). The  
108 study participants refrained from physical exercise 72 hours before testing and consumed  
109 their evening meal no later than 22.00 hours. The diet provided a protein intake in line with  
110 what Canadians generally consume (Auclair and Burgos 2021), which reflected a high  
111 acceptable protein distribution range and per the recent recommendations from several expert  
112 committees and researchers for optimal protein intakes (1.3 g/kg/day) (Auclair and Burgos  
113 2021; Moore et al. 2015; Traylor et al. 2018). To enhance compliance, investigators designed  
114 the study diets by meeting with each participant individually to customize meal plans  
115 according to each participant's personal food preferences. Participants were supplied with all  
116 of the required food, which consisted of pre-packaged, frozen meals (Heart-to-Home Meals,  
117 Hamilton, ON, Canada) containing animal/plant-based proteins and other items that required  
118 minimal preparation (i.e., granola bars, fruit cups, and juices). The participants consumed  
119 breakfast within 30 minutes of waking up and were instructed to space meals ~4-6 hours  
120 apart.

121 **Short-term resistance training.** The participants performed four separate unilateral  
122 resistance exercise sessions counterbalanced for their dominant leg and non-dominant leg.  
123 Twenty-four hours of rest were allowed between all training sessions. Each training session  
124 began with a leg press exercise and a warm-up of ten submaximal muscle contractions at  
125 ~50% of 1RM. Following a two-minute rest period, the participants performed four sets of  
126 eight to ten leg press and leg extension corresponding to 75% of 1RM values. The  
127 participants were verbally encouraged to produce maximal effort during the muscle action for  
128 each repetition. After completing each concentric muscle action, the participants were  
129 instructed to return their leg to full flexion in a controlled fashion. Two minutes of rest were  
130 allowed between each set. After the leg press exercise and two minutes of rest, the  
131 participants performed the leg extension exercise in the same fashion.

132 **Protein and leucine-enriched bar.** During the STRT+Leu phase, participants were  
133 administered the high-leucine-containing protein bars immediately before and after training  
134 (two bars in total per training session). The protein and leucine-enriched bar supplement  
135 contained ~16 g of a blend of micellar casein (AMCO, Burlington, NJ, USA), whey protein  
136 (Hilmar, Hilmar, CA, USA), whey protein hydrolysate (Hilmar, Hilmar, CA, USA), 1.5 g of  
137 free-leucine (Ajinomoto, Raleigh, NC, USA), 22 g low-glycemic carbohydrates (Ciranda,  
138 Hudson, WI, USA), and 11 g monounsaturated fat (Golden Barrel, Honey Brook, PA, USA).  
139 The total leucine content of the bar was ~3 g, which results in a robust elevation in  
140 aminoacidemia (particularly leucinemia) and previous studies indicate that leucinemia  
141 induces stimulation of MyoPS at the *vastus lateralis* (Churchward-Venne et al. 2014; Devries  
142 et al. 2018a; Traylor et al. 2021). Covance (Eurofins) produced the bars; their nutrient content  
143 is given in Table 2.

144 **Oral deuterated water protocol.** As previously described, deuterium can be labelled to non-  
145 essential amino acids (e.g., alanine) by intermediary reactions, so we utilized oral

146 consumption of deuterium oxide ( $[D_2O]$ ; 70% enriched; Cambridge Isotope Laboratories) to  
147 track the incorporation of newly labeled synthesized myofibrillar proteins (MacDonald et al.  
148 2013; Wilkinson et al. 2014). Per previous protocols, the present study determined body-  
149 water deuterium enrichment from saliva samples (MacDonald et al. 2013; Wilkinson et al.  
150 2014).

151 Participants reported to the laboratory in the fasted state on visit one (day 0 =  $D_2O$   
152 loading phase) and gave a pre-enrichment saliva sample, followed by ingestion of eight doses  
153 (0.8 ml/kg of lean mass) of 70%  $D_2O$  evenly spaced every hour through the day. Participants  
154 consumed one dose of  $D_2O$  every morning to maintain deuterium enrichment in the  
155 circulation of body water at a steady state throughout the study. Participants' saliva samples  
156 were collected in the fasted state to measure deuterium enrichment in body water. Samples  
157 were stored at  $-20\text{ }^\circ\text{C}$  before analysis (MacDonald et al. 2013; Wilkinson et al. 2014).

158 **Saliva sample analysis.** Each saliva sample was centrifuged at 1500 g for 10 minutes at  $4\text{ }^\circ\text{C}$ ,  
159 and the supernatant (water phase) was collected for further analysis. The sample was then  
160 diluted using double-distilled water to a ratio of 1:35 and analyzed for deuterium-enrichment  
161 utilizing a cavity ring-down spectroscopy and liquid isotope analyzer equipped with an  
162 automated injection system (Picarro L2130-I, Santa Clara, CA). The sample was injected six  
163 times per run, and data generated utilized the average deuterium isotopic concentrations of  
164 the last three injections resulting in a coefficient of variation of  $\leq 0.8\%$ . The deuterium  
165 enrichments were initially expressed as change in deuterium % relative to Vienna Standard  
166 Mean Ocean Water and were converted to APE using standard equations (Gasier et al. 2010).

167 **Muscle biopsy protocol.** Each participant reported to the laboratory after an overnight fast ( $>$   
168 10 h) on each biopsy testing day. Investigators created a sterile field over the biopsy site and  
169 injected  $\sim 5$  ml of a local anesthetic (2% xylocaine) under the skin and fascia. Muscle samples

170 were cleaned of visible blood, fat, and connective tissue before snap-freezing in liquid  
171 nitrogen and stored at -80°C for further analysis.

172 **Analytical methods.** Each muscle sample (~30-50 mg) was homogenized on ice in a buffer  
173 [10 µl of buffer per mg of muscle, 25 mM Tris-HCl (pH 7.4), 0.5% v/v Triton X-100 (Sigma  
174 Aldrich, Oakville, ON), and a complete protease/phosphatase inhibitor cocktail tablet  
175 (Complete Protease Inhibitor Mini-Tabs, Roche)]. The sample was then centrifuged at 700 g  
176 for 10 minutes at 4 °C, and the supernatant (sarcoplasmic fraction) and pellet were separated  
177 for western blotting and MyoPS analysis, respectively. The pellet containing muscle-bound  
178 alanine was solubilized and centrifuged as previously described (Dufner et al. 2005). Samples  
179 (myofibrillar proteins) were precipitated in 1 ml of 1 M perchloric acid and centrifuged at  
180 700 g for 10 minutes at 4 °C. The sample was washed twice with 70% ethanol and then  
181 hydrolyzed in 0.5 M HCL and Dowex (50WX8-200 resin; Sigma-Aldrich) at 110 °C for 72  
182 hours to liberate the muscle-bound amino acids. The free amino acids were then purified  
183 using Dowex ion-exchange chromatography, and myofibrillar <sup>2</sup>H-alanine enrichments were  
184 analyzed as previously described (MacDonald et al. 2013).

185 **Determination of myofibrillar protein synthesis.** The integrated rate of MyoPS was  
186 calculated via the precursor-product approach; the incorporation of deuterium-labeled alanine  
187 into the contractile proteins using body water enrichment (corrected for the average number  
188 of deuterium moieties incorporated per alanine) as the surrogate precursor labeling between  
189 subsequent biopsies as described previously (McGlory et al. 2018; Murphy et al. 2016).

190 **Western blotting.** The ratio of phosphorylation to total protein content was assessed by  
191 Western blotting. To standardize the protein concentration, 2 µg/µl of the sample  
192 (sarcoplasmic fraction) was mixed with double-distilled water in 4× Laemmli buffer  
193 containing 0.25 M Tris at 4% SDS, 20% glycerol, 0.015% bromophenol blue, and 10% 2-  
194 mercaptoethanol. Then each sample and a protein-ladder (Precision Plus Protein Standard,

195 Bio-Rad, Hercules, CA) were loaded into wells on a 26-well 4%-15% TGX Stain-Free  
196 Precast Gel (Bio-Rad, Hercules, CA). Four internal standards loaded in the last four wells on  
197 every gel and stain-free image on membranes were used as calibration curves to normalize  
198 the protein content (Mollica et al. 2009). To ensure a complete transfer, investigators  
199 examined membranes using ultraviolet activation of the gel pre-and post-transfer (ChemiDoc  
200 MP Imaging System, Bio-Rad). Membranes were then blocked with 5% bovine serum  
201 albumin for 90 minutes at room temperature before exposure to primary antibodies [1:1000;  
202 total form: mTOR (2972S), p70S6K (9202L), S6K (2217L), and 4EBP1 (9644S);  
203 phosphorylation form: mTOR<sup>Ser2448</sup> (5536S), p70S6K<sup>Thr389</sup> (9205L), S6K<sup>Ser235/236</sup> (2211S),  
204 and 4EBP1<sup>Thr37/46</sup> (2855C); Cell Signaling Technology] for 12 hours at 4°C. Additionally,  
205 membranes were then washed for 3 x 5 minutes in Tris-buffered saline and Tween 20 (TBS-  
206 T, Millipore Sigma) and incubated in secondary antibody (1:3000, anti-rabbit IgG conjugated  
207 with horseradish peroxidase, 7074S; Cell Signaling Technology) for 90 minutes at room  
208 temperature. After the secondary incubation, the membranes were washed for 3 x 5 minutes  
209 using TBS-T. Membrane bands (protein expression) were detected using a  
210 chemiluminescence solution (Clarity Western ECL substrate, Bio-Rad), and the bands were  
211 quantified using Image Lab 6.0.1 (Image Lab Software for Mac Version 6.0.1).

212 **Statistical analyses.** Normality for the distribution of data was assessed using the Shapiro-  
213 Wilk test. Non-normally distributed data (the ratio of phosphorylated to total p70S6K, S6K,  
214 and 4EBP1) were converted by log transformation (Curran-Everett 2018), and all the values  
215 were normally distributed. For comparing estimated 1RM and performed training volume  
216 between conditions, *t*-tests were used. One-way analysis of variance (ANOVA; intervention:  
217 REST, STRT, and STRT+Leu) was used to assess integrated MyoPS. Two-way repeated-  
218 measures ANOVA (condition by time) were applied to assess protein signaling. Follow-up  
219 analyses included *t*-tests with Bonferroni correction for multiple comparisons. An alpha level



220 of  $P \leq 0.05$  was considered statistically significant for all comparisons. Statistical analyses  
221 were completed using the SPSS Statistics Software package (Version 26.0 for Windows;  
222 IBM Corp., Chicago, IL).

223

## 224 **Results**

225 **Participant Characteristics.** Participants' anthropometric measures and body composition  
226 are presented in Table 1.

227 **Short-term resistance exercise training.** Participants' estimated 1RM and performed  
228 training volume are presented in Table 3. There were no differences in 1RM for leg press ( $P$   
229 = 0.92; 95% CI, 39.62-43.59) and leg extension ( $P = 0.72$ ; 95% CI, 11.11-15.65) between  
230 STRT and STRT+Leu phases. Participants adhered to 100% of the STRT sessions.

231 There were no differences in leg press ( $P = 0.92$ ; 95% CI, 282.07-257.34) and leg extension  
232 ( $P = 0.87$ ; 95% CI, 68.51-80.18) training volumes between STRT and STRT+Leu phases.

233 **Dietary intake.** Nutritional profiles for dietary intake and bar are presented in Table 2. There  
234 were no significant differences in the consumed energy ( $P = 0.33$ ; 95% CI, 15.30-6.10),  
235 carbohydrate ( $P = 0.75$ ; 95% CI, 2.74-2.07), and fat ( $P = 0.16$ ; 95% CI, 0.41-0.05) between  
236 STRT and STRT+Leu phases. Protein intake during STRT+Leu was greater than STRT  
237 phase ( $P < 0.001$ ; 95% CI, 0.39-0.24).

238 **Integrated myofibrillar protein synthesis.** Integrated daily MyoPS during STRT ( $1.43 \pm$   
239  $0.06$  %/d) and STRT+Leu ( $1.53 \pm 0.06$  %/d) phase were greater than Rest ( $1.31 \pm 0.05$  %/d)  
240 by  $8\% \pm 5\%$  ( $P = 0.001$ ; 95% CI, 0.05-0.19) and  $14\% \pm 5\%$  ( $P < 0.001$ ; 95% CI, 0.15-0.29),  
241 respectively (Figure 3). Protein and leucine-enriched bar consumption with resistance  
242 exercise (STRT+Leu) increased integrated MyoPS above STRT by  $7\% \pm 5\%$  ( $P = 0.004$ ;  
243 95% CI, 0.03-0.17; Figure 3).



244 **Phosphorylation status of proteins in the mTORC1 signaling pathway.** There was an  
245 interaction between condition and time for P/T mTOR ( $P=0.039$ ;  $\eta^2 = 0.48$ ) and 4EBP1  
246 ( $P=0.046$ ,  $\eta^2 = 0.26$ ). An acute session of resistance exercise and the ingestion of study bars  
247 (STRT+Leu) elevated the ratio of phosphorylated to total (P/T) mTOR ( $P = 0.030$ ; 95% CI,  
248 0.39-0.03) and 4EBP1 ( $P = 0.008$ ; 95% CI, 0.24-0.04) compared to Pre-STRT by  $13\% \pm 18\%$   
249 and  $13\% \pm 12\%$ , respectively, while STRT alone did not (Figure 4A and D). There were main  
250 effects for P/T p70S6K ( $P=0.002$ ;  $\eta^2 = 0.50$ ) and S6K ( $P=0.016$ ;  $\eta^2 = 0.35$ ). The ratio of  
251 P/T p70S6K was  $14\% \pm 16\%$  greater ( $P = 0.002$ ; 95% CI, 0.31-0.08) at Post-STRT compared  
252 to Pre-STRT with no difference between conditions ( $P = 0.25$ ; 95% CI, 0.57-0.16; Figure  
253 3B). The ratio of P/T S6K was  $20\% \pm 38\%$  greater ( $P = 0.016$ ; 95% CI, 0.47-0.06) at Post-  
254 STRT compared to Pre-STRT with no difference conditions ( $P = 0.054$ ; 95% CI, 0.72-0.01  
255 Figure 4C).

## 257 Discussion

258 The present study results indicate that STRT increased MyoPS and protein intake derived  
259 from twice-daily ingestion of a protein and leucine-enriched bar potentiated integrated rates  
260 of MyoPS. Corroborating the MyoPS data, the acute phosphorylation of mTOR and 4EBP1  
261 one hour after the STRT+Leu condition was more significant than Pre-STRT only in the  
262 STRT+Leu condition. These findings highlight that the consumption of the protein and  
263 leucine-enriched bars during four RE sessions led to an additional enhancement in the  
264 myofibrillar remodelling of RE.

265 In a previous study, we reported that ingestion of the study bar containing the isolated  
266 milk protein whey protein, and micellar casein resulted in a peak plasma leucine  
267 concentration of  $593 \pm 176 \mu\text{M}$  (range: 210-790  $\mu\text{M}$ ) when consumed as a snack two hours  
268 after consuming a low-protein breakfast (0.2 g protein/kg) in older adults (Traylor et al.

269 2021). The peak leucine concentration exceeded levels (~450  $\mu\text{M}$ ) reported to increase  
270 MyoPS in older adults (Murphy et al. 2016) and after consuming 40 g of whey protein-  
271 containing beverage (~320  $\mu\text{M}$ ) in younger adults (Yang et al. 2012). The protein and  
272 leucine-enriched study bar elevated EAA and BCAA concentrations comparable to  
273 consuming a higher-protein meal (0.5 g protein/kg) (Moore et al. 2015; Traylor et al. 2021).  
274 In concordance with the aminoacidemia and leucinemia in our previous work (Traylor et al.  
275 2021), the present study discovered that increasing protein intake through supplemental  
276 protein and leucine-enriched bar ingestion induced an additional increase in integrated rates  
277 of MyoPS during a unilateral lower-body resistance training protocol. We note that within a  
278 normal eating pattern a food matrix containing protein is consumed that blends whole food  
279 sources, and attenuates a rapid aminoacidemia/leucinemia response (Burd et al. 2019).  
280 Optimal stimulation of MyoPS, therefore, may not require hyperaminoacidemia (Mitchell et  
281 al. 2015). Thus, the results presented here align with previous studies indicating that  
282 increasing protein intake and periodically inducing hyperaminoacidemia, particularly  
283 leucinemia, induces further stimulation of MyoPS during STRT (Devries et al. 2018a, 2018b;  
284 Pennings et al. 2012; Yang et al. 2012).

285 In the present study, we controlled daily protein intake at 1.3 g of protein/kg/d, which  
286 is 62% higher than RDA for daily protein intake in healthy adults (Rand et al. 2003) and is  
287 within estimates of an optimal protein intake to potentiate RT-induced gains in lean mass  
288 (Morton et al. 2018). Our results indicated that while STRT induced a rise in MyoPS above  
289 resting, twice daily consumption of the study bar increased protein intake to 1.6 g/kg/d  
290 potentiating the anabolic effect of resistance exercise on the muscle (Figure 3). Although all  
291 essential amino acids are necessary to support MyoPS, leucine is the anabolic amino acid that  
292 activates MyoPS via the mTOR signalling pathway (Devries et al. 2018b; Phillips 2014). Our  
293 lab previously reported, despite low-protein consumption (6.25 g), that co-consumption of a

294 high amount of leucine (~5 g) increased acute MyoPS to the same extent as 25 g of protein in  
295 young men (Churchward-Venne et al. 2014). Also, higher leucine supplements (~4.5 g) or  
296 diets (~5 g) effectively enhanced acute (Devries et al. 2018a) and integrated MyoPS (Devries  
297 et al. 2018b; Murphy et al. 2016) in free-living older adults, regardless of their total daily  
298 protein intake. On the other hand, protein dose-response studies have shown an increase in  
299 MyoPS plateaus at approximately 0.3-0.35 g of protein/kg in young adults (Cuthbertson et al.  
300 2005; Moore et al. 2009; Yang et al. 2012). The present results indicated that twice-daily  
301 consumption of the study bar, which contained 6 g of leucine, potentiated day-to-day  
302 integrated MyoPS in young adults even when consuming a diet containing 1.3 g of  
303 protein/kg/d. We note that 6 g of leucine stimulates MyoPS (Churchward-Venne et al. 2012;  
304 Churchward-Venne et al. 2014; Devries et al. 2018a, 2018b). Given the previous leucine and  
305 protein dose-response studies (Devries et al. 2018a, 2018b), we speculated that the  
306 potentiated MyoPS response in the STRT+Leu condition was supported in part by an  
307 increased intake of leucine and essential amino acid as components of overall increased  
308 protein intake.

309 We investigated the mTORC1 signaling pathway in response to a resistance exercise  
310 session during STRT and STRT+Leu. Acute STRT+Leu resulted in a significant increase in  
311 phosphorylation of mTORC1 and 4EBP1 one hour following exercise and was not observed  
312 in the STRT condition (Figure 4). Furthermore, STRT with consumption of the study bars led  
313 to higher phosphorylation of 4EBP1 compared to STRT only (Figure 4D). Peri-workout  
314 protein ingestion results in MyoPS stimulation (Cermak et al. 2012; Schoenfeld et al. 2017).  
315 Pennings et al. showed that protein intake after exercise resulted in greater use of dietary-  
316 derived amino acids for MyoPS in young and older men (Pennings et al. 2011). Fujita et al.  
317 (Fujita et al. 2009) reported that protein consumption before resistance exercise increased  
318 MyoPS and phosphorylation of the mTORC1 signalling pathway post-exercise. Immediately

319 following resistance exercise, protein ingestion results in an additional increase in signalling  
320 and MyoPS (Dreyer et al. 2008). Thus, it is perhaps not surprising that consuming the study  
321 bar before and after resistance exercise in our study potentiated additional anabolic effects by  
322 activating the mTORC1 signalling pathway proteins.

323 Our findings highlight that the anabolic responses to STRT and a moderate daily  
324 protein intake over four days improved by ingesting a protein and leucine-enriched bar twice  
325 daily. Also, we analysed the acute phosphorylation of mTORC1 signalling proteins one hour  
326 following STRT or STRT+Leu. However, because this was a short-term measure of MyoPS  
327 and an acute measurement of protein signalling, we are limited in our ability to translate our  
328 findings to longer-term resistance training interventions. Due to the untrained nature of the  
329 study participants, subsequent 'damage-repair' was likely prevalent but did not necessarily  
330 align with functional benefits (Davies et al. 2020; Waskiw-Ford et al. 2020). We did not  
331 match total protein intake between conditions and did not directly measure plasma  
332 aminoacidemia after consuming the protein and leucine-enriched bar. Our previous study did  
333 show pronounced hyperaminoacidemia and leucinemia using the present study bar. (Traylor  
334 et al. 2021).

335 In conclusion, we showed that four lower-body resistance training sessions increased  
336 the daily rate of MyoPS. Increased protein intake derived from ingestion of the protein and  
337 leucine-enriched bar during STRT potentiated the integrated MyoPS response and stimulated  
338 mTOR signalling pathway protein phosphorylation. These findings highlight the effect of the  
339 protein and leucine-enriched bar to promote an anabolic response to resistance training and  
340 rationale for future research in developing enriched protein formulations for muscle recovery  
341 and growth with RT programs.

342

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349

### 350 **Competing interests statement**

351 Stuart M. Phillips reports having received competitive research funding from the US National  
352 Dairy Council (NDC) and Dairy Farmers of Canada during the time of this work. Stuart M.  
353 Phillips is listed as an inventor on Canadian patent 3052324 issued to Exerkine and a patent  
354 (US) 16/182891 pending to Exerkine (but reports no financial gains). Stuart M. Phillips  
355 reports receiving product from Enhanced Recovery but no payment outside the submitted  
356 work. CL, DAT, CM, SJ, JM, TG, JCM, TP, EAN and ML declare no conflicts of interest.

357

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361

### 362 **Contributors' statement**

363 CL and DAT contributed equally to this study; Conceptualization, CL, DAT, and SMP;  
364 Conducted the research, CL, DAT, CM, SJ, and JM; formal analysis, CL, DAT, JCM, and  
365 TP; Writing – original draft preparation, CL, DAT, EAN, and SMP; Writing – review and  
366 editing, all authors; Funding acquisition, DAT and SMP.

367

### 368 **Data availability**

369 The data underpinning the research are available from the corresponding author on  
370 reasonable request.

371

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533  
534

535 Tables

536 **Table 1. Characteristics of participants.**

Participants (n=8)	
Sex (M/W)	5/3
Age (yrs.)	22 ± 1
Height (cm)	181 ± 8
Body mass (kg)	87 ± 17
BMI (kg/m <sup>2</sup> )	27.1 ± 4.6
LM (kg)	61 ± 8
Body fat (%)	29 ± 11

537 Value are means ± SD, BMI, body mass index; LM, (fat- and bone-free) lean mass; M, men;

538 W, women.

539

540 **Table 2. Dietary intake during intervention.**

	STRT	STRT+Leu	Bar (2/d)	<i>P</i>
Energy (kcal/kg/d)	30±4	33±5	5±1	0.301
Protein (g/kg/d)	1.3±0.1	1.6±0.1*	0.4±0.1	< 0.001
Carbohydrate (g/kg/d)	5±1	5±1	1±0	0.438
Fat (g/kg/d)	1±0	1±0	0±0	0.163

541 Values are means ± SD. \* *P* < 0.05, significant difference from STRT. STRT, short-term

542 resistance training; STRT+Leu, STRT with protein and leucine-enriched bar

543 supplementation.

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548

549 **Table 3. Estimated 1RM and performed training volume for each test.**

	<b>STRT</b>	<b>STRT+Leu</b>	<b><i>P</i></b>
<b>Estimated 1RM</b>			
Leg press (kg)	93±39	91±39	0.920
Leg extension (kg)	41±39	39±13	0.722
<b>Training volume</b>			
Leg press (kg/set)	663±250	675±251	0.923
Leg extension (kg/set)	244±71	238±68	0.869

550 Values are means ± SD (n=8). STRT, short-term resistance training; STRT+Leu, short-term  
 551 resistance training combined with protein and leucine-enriched bar supplementation; 1RM,  
 552 one-repetition maximum.

553

554

555 **Figure captions**

556

557 **Figure 1.** Consort 2010 standards of reporting randomized human trials flow diagram of  
558 participant enrollment and random assignment to and analysis of study interventions.

559

560 **Figure 2. Schematic overview of study design.** STRT, short-term resistance training;  
561 STRT+Leu, short-term resistance training combined with high-leucine-containing protein bar  
562 supplementation; RE, resistance exercise; MB, muscle biopsy; PB, protein bar consumption;  
563 DXA, dual-energy X-ray absorptiometry; D<sub>2</sub>O, deuterium oxide.

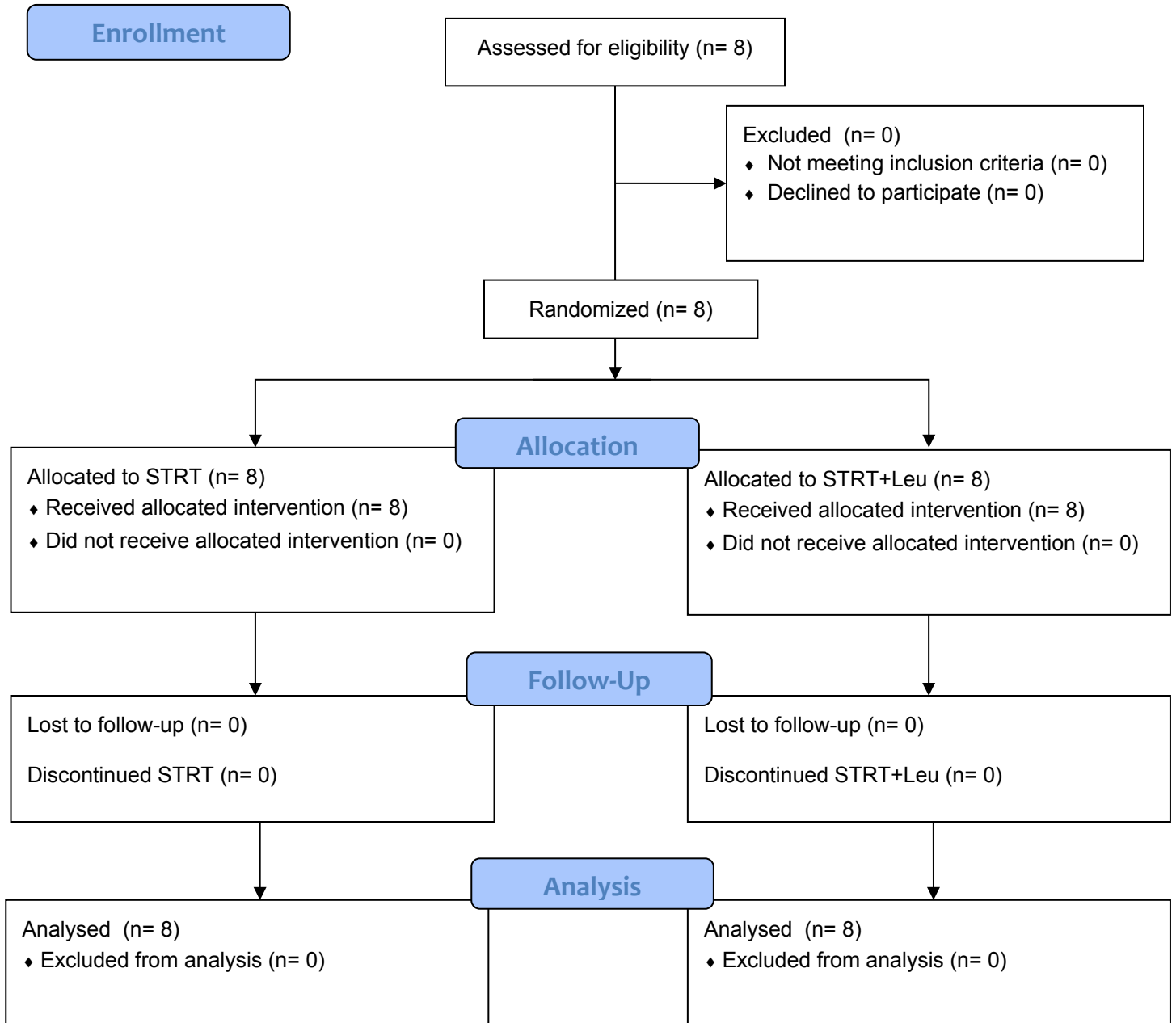
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565 **Figure 3. Integrated fractional synthesis rate (%/d) of vastus lateralis during Rest,**  
566 **STRT, and STRT+Leu.** Data are plotted as individual points (closed circles, men; open  
567 circles, women) with the mean  $\pm$  SD (n=8). \*  $P < 0.05$ , significantly different from Rest, #  $P$   
568  $< 0.05$ , significantly different from STRT. STRT, short-term resistance training; STRT+Leu,  
569 STRT with high-leucine-containing protein bar supplementation.

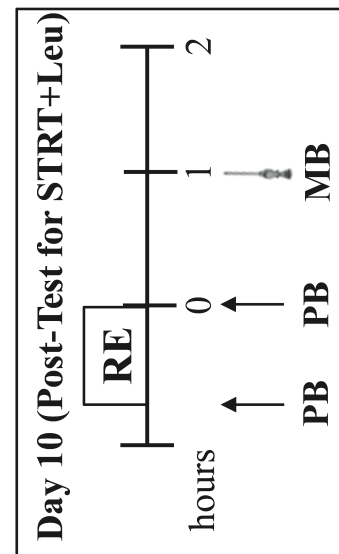
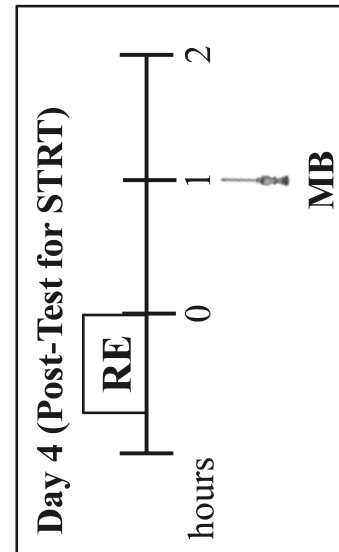
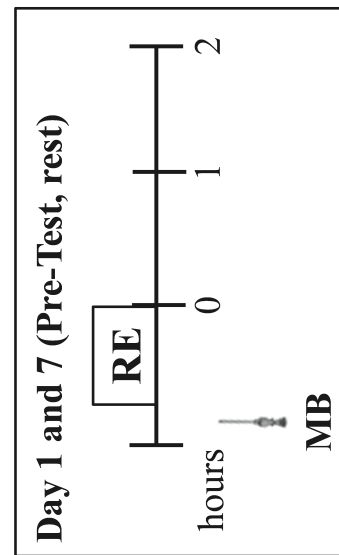
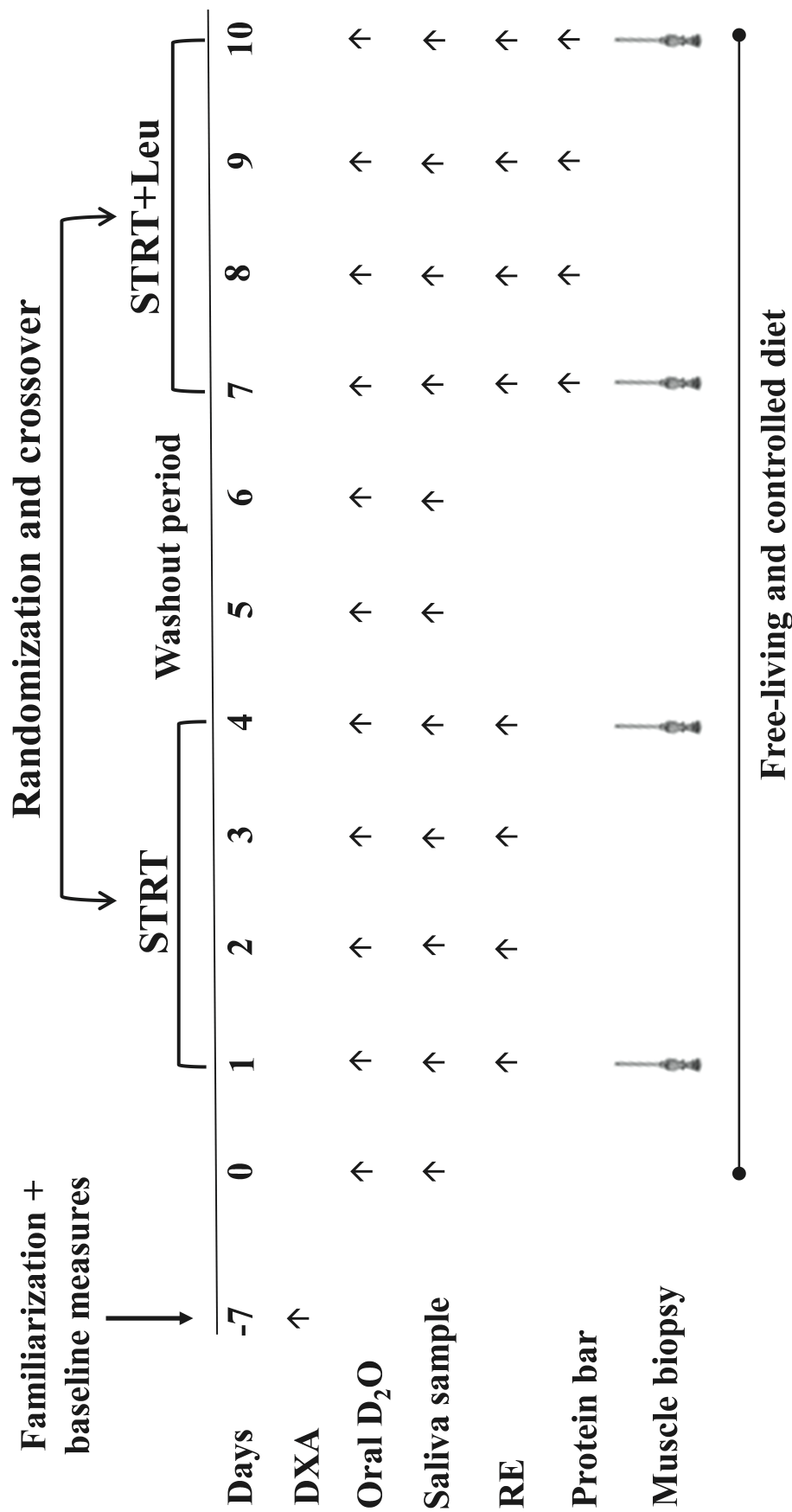
570

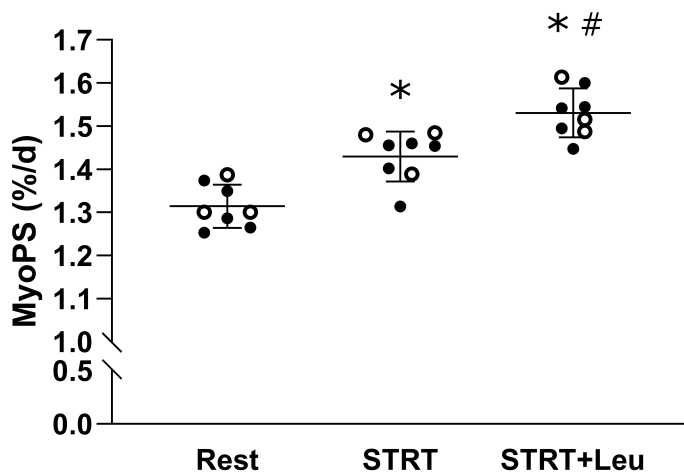
571 **Figure 4. Changes in phosphorylation status of mTOR signaling pathway proteins**  
572 **during STRT and STRT+Leu study phases.** The ratio of phosphorylated to total (A)  
573 mTOR, (B) p70S6K, (C) S6K, (D) 4EBP1, and (E) representative western blot bands. Data  
574 are expressed as mean  $\pm$  SD with individual data plots (circles, men; squares, women; n=8). \*  
575  $P < 0.05$ , significantly different from pre-test within conditions, #  $P < 0.05$ , main effect for  
576 time. STRT, short-term resistance training; STRT+Leu, STRT with high-leucine-containing  
577 protein bar supplementation; Ex. exercise; P/T, the ratio of phosphorylated to total protein  
578 expression.

## Enrollment of Eligible Participants









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● Pre-Ex (Men) ■ Pre-Ex (Women) □ Post-Ex (Women) ○ Post-Ex (Men)

