AUSTRALIAN CATHOLIC UNIVERSITY

Research Bank
Journal article

Hot-water immersion does not increase postprandial muscle protein synthesis rates during recovery from resistance-type exercise in healthy, young males

Fuchs, Cas J., Smeets, Joey S. J., Senden, Joan M., Zorenc, Antoine H., Goessens, Joy P. B., van Marken Lichtenbelt, Wouter D., Verdijk, Lex B. and van Loon, Luc J. C.

This is the accepted manuscript version. For the publisher's version please see:

Fuchs, C. J., Smeets, J. S. J., Senden, J. M., Zorenc, A. H., Goessens, J. P. B., van Marken Lichtenbelt, W. D., Verdijk, L. B. and van Loon, L. J. C.. (2020). Hot-water immersion does not increase postprandial muscle protein synthesis rates during recovery from resistance-type exercise in healthy, young males. *Journal of Applied Physiology*, 128(4), pp. 1012-1022. https://doi.org/10.1152/japplphysiol.00836.2019

Hot-water immersion does not increase post-prandial muscle protein synthesis rates during recovery from resistance-type exercise in healthy, young males

Cas J. Fuchs[1], Joey S.J. Smeets[1], Joan M. Senden[1], Antoine H. Zorenc[1], Joy P. B. Goessens[1], Wouter D. van Marken Lichtenbelt[2], Lex B. Verdijk[1] and Luc J.C. van Loon[1*]

Department of [1]Human Biology, [2]Nutrition and Movement Sciences, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Centre+, Maastricht, the Netherlands

***Corresponding author:** L.J.C. van Loon, PhD., Department of Human Biology, Faculty of Health, Medicine and Life Sciences, Maastricht University, PO Box 616, 6200 MD Maastricht, the Netherlands. E-mail: l.vanloon@maastrichtuniversity.nl; Phone: +31-43-3881397; Fax: +31-43-3670976.

Author names for PubMed indexing: Fuchs, Smeets, Senden, Zorenc, Goessens, van Marken Lichtenbelt, Verdijk, van Loon

Word count (abstract through references): 7941
Number of figures (to print, not OSM): 8
Number of tables (to print, not OSM): 1
Running head: Post-exercise heating and muscle protein synthesis

Disclosure Summary: Cas J. Fuchs, Joey S.J. Smeets, Joan M. Senden, Antoine H. Zorenc, Joy P. B. Goessens, Wouter D. van Marken Lichtenbelt, Lex B. Verdijk and Luc J.C. van Loon declare no conflicts of interest related to this study.

List of abbreviations and their definitions

1RM	One repetition maximum
4E-BP1	Eukaryotic translation initiation factor 4E-binding protein 1
ANOVA	Analysis of variance

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

CON	Thermoneutral water immersion
DEXA	Dual-energy x-ray absorptiometry
FSR	Fractional synthetic rate
GC-C-IRMS	Gas chromatograph-combustion-isotope ratio mass spectrometer
GC-P-IRMS	Gas chromatograph-pyrolysis-isotope ratio mass spectrometer
HSP	Heat Shock Protein
HWI	Hot-water immersion
MPE	Mole percent excess
mTOR	Mammalian target of rapamycin
p70S6K	p70 ribosomal protein S6 kinase
rpS6	Ribosomal protein S6
SEM	Standard error of the mean
vs	Versus

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

1 **ABSTRACT**

2 **Purpose:** To assess the impact of post-exercise hot-water immersion on post-prandial
3 myofibrillar protein synthesis rates during recovery from a single bout of resistance-type
4 exercise in healthy, young males.

5 **Methods:** Twelve healthy, male adults (age: 23±1 y) performed a single bout of resistance-
6 type exercise followed by 20 min of water immersion of both legs. One leg was immersed in
7 hot water (46°C: HWI) while the other leg was immersed in thermoneutral water (30°C:
8 CON). After water immersion, a beverage was ingested containing 20 g intrinsically L-[1-
9 ^{13}C]-phenylalanine and L-[1-^{13}C]-leucine labelled milk protein with 45 g of carbohydrates. In
10 addition, primed continuous L-[*ring*-$^{2}H_{5}$]-phenylalanine and L-[1-^{13}C]-leucine infusions were
11 applied, with frequent collection of blood and muscle samples to assess myofibrillar protein
12 synthesis rates *in vivo* over a 5 h recovery period.

13 **Results:** Muscle temperature immediately after water immersion was higher in the HWI
14 compared to the CON leg (37.5±0.1 vs 35.2±0.2°C; P<0.001). Incorporation of dietary
15 protein-derived L-[1-^{13}C]-phenylalanine into myofibrillar protein did not differ between the
16 HWI and CON leg during the 5h recovery period (0.025±0.003 vs 0.024±0.002 MPE;
17 P=0.953). Post-exercise myofibrillar protein synthesis rates did not differ between the HWI
18 and CON leg based upon L-[1-^{13}C]-leucine (0.050±0.005 vs 0.049±0.002%·h^{-1}; P=0.815) and
19 L-[*ring*-$^{2}H_{5}$]-phenylalanine (0.048±0.002 vs 0.047±0.003%·h^{-1}; P=0.877), respectively.

20 **Conclusions:** Hot-water immersion during recovery from resistance-type exercise does not
21 increase the post-prandial rise in myofibrillar protein synthesis rates. In addition, post-
22 exercise hot-water immersion does not increase the capacity of the muscle to incorporate
23 dietary protein-derived amino acids in muscle tissue protein during subsequent recovery.

24

25 This study was registered at trialregister.nl as NL6221.

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

26

27 ***Keywords:*** heat stress; heating; hydrotherapy; recovery; adaptation; stable isotope tracers;

28 resistance exercise.

29 ***New and noteworthy:***

30 This is the first study to assess the effect of post-exercise hot-water immersion on post-

31 prandial myofibrillar protein synthesis rates and the incorporation of dietary protein-derived

32 amino acids into muscle protein. We observed that hot-water immersion during recovery from

33 a single bout of resistance-type exercise does not further increase myofibrillar protein

34 synthesis rates or augment the post-prandial incorporation of dietary protein-derived amino

35 acids in muscle throughout 5 hours of post-exercise recovery.

36

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

37 **INTRODUCTION**

38 Protein ingestion during recovery from exercise further augments the increase in muscle
39 protein synthesis rates and inhibits muscle protein breakdown, resulting in a positive net
40 muscle protein balance during the acute stages of post-exercise recovery (2, 4). Consequently,
41 post-exercise protein ingestion is widely applied by athletes as a strategy to increase post-
42 exercise muscle protein synthesis rates and, as such, to facilitate skeletal muscle
43 reconditioning. Based on the observation that ingestion of 20 g of a high-quality protein
44 source maximally stimulates post-exercise muscle protein synthesis rates in healthy, young
45 males (21, 37), athletes are advised to ingest 20 g protein during recovery from a single bout
46 of resistance-type exercise.

47 Currently, hot-water immersion (HWI) has been receiving a lot of attention as another
48 effective interventional strategy to facilitate post-exercise recovery (20, 34, 35). HWI has
49 been reported to increase (muscle) tissue temperature and stimulate limb blood flow during
50 recovery from exercise (26, 29). HWI during post-exercise recovery has also been reported to
51 improve performance recovery, such as attenuating the decrease in jump power (36) and
52 enhancing the recovery of isometric squat force (34). Recently, it was stated that there is
53 ample evidence to suggest that heating can promote muscle cell differentiation and alter the
54 expression of various genes, kinases and transcription factors involved in muscle remodeling
55 (20). Therefore, post-exercise HWI is proposed as an effective tool to facilitate skeletal
56 muscle reconditioning.

57 Increasing muscle temperature during recovery from exercise may increase enzyme activity
58 and increase skeletal muscle blood flow and, as such, augment nutrient delivery, uptake,
59 and/or subsequent metabolism. In agreement, a previous study in rats has shown that 30 min
60 of HWI increases phosphorylation of Akt and p70S6K, which are considered important
61 mediators of muscle protein synthesis and hypertrophy (38). Furthermore, Kakigi *et al.*

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

62 demonstrated that heat stress (applied 20 min before and during isokinetic knee extension
63 exercise) enhanced resistance exercise induced mTOR signaling in human skeletal muscle
64 (17). So far, there are no studies that have assessed the impact of hot-water immersion on
65 muscle protein synthesis rates. We hypothesized that hot-water immersion after a single bout
66 of resistance-type exercise increases post-prandial muscle protein synthesis rates during 5
67 hours of post-exercise recovery in young, healthy males.

68 In the present study, we combined contemporary stable isotope methodology with the
69 ingestion of specifically produced intrinsically L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine
70 labeled milk protein to assess the effects of post-exercise hot-water immersion on myofibrillar
71 protein synthesis rates as well as the incorporation of dietary protein-derived amino acids into
72 muscle tissue protein during 5 h of post-exercise recovery *in vivo* in healthy, young adults.

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

73 **METHODS**

74

75 *Subjects*

76 Twelve healthy, young males (age 23±1 y) participated in this study. All participants were
77 considered recreationally active (exercising ~3-4 times per week for a total duration of ~6 h)
78 and were familiar with resistance-type exercise. Subjects' characteristics are presented in
79 **Table 1**. Subjects were fully informed of the nature and possible risks of the experimental
80 procedures before their written informed consent was obtained. Participants had no prior
81 history of participating in stable isotope amino acid tracer experiments. This study was
82 approved by the Medical Ethics Committee of the Maastricht University Medical Centre+
83 (METC 15-3-038) and conforms to the principles outlined in the declaration of Helsinki for
84 use of human subjects and tissue. This study was registered at trialregister.nl as NL6221.

85

86 *General study design*

87 Each subject participated in one experiment, in which the effect of post-exercise hot-water
88 immersion on post-prandial muscle protein synthesis was studied after the ingestion of 20 g
89 intrinsically L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine labeled milk protein. The current
90 study was designed to determine the benefits of hot-water immersion to increase post-prandial
91 muscle protein synthesis rates during recovery from a single bout of resistance-type exercise
92 in a setting most relevant for athletes. At the start of the experiment, primed continuous L-
93 [*ring*-$^{2}H_5$]-phenylalanine and L-[1-^{13}C]-leucine infusions were applied together with repeated
94 blood sampling during the experimental day. After 1 h of rest, participants performed ~45 min
95 of resistance-type exercise training, after which they immersed both legs in water for 20 min

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

96 (1 leg at 46°C and 1 leg at 30°C). Thereafter, muscle biopsies were collected from both legs,
97 prior to ingesting 20 g intrinsically L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine labeled
98 milk protein with 45 g of glucose (polymers). Subsequent muscle biopsies were taken from
99 the *M. vastus lateralis* of both legs after 2 and 5 hours of post-exercise recovery.
100
101 *Pretesting*
102 All subjects participated in a screening session, which was performed at least 1 week prior to
103 the experiment. First, subjects' body weight and height were measured as well as body
104 composition by dual-energy X-ray absorptiometry (DEXA, Discovery A; Hologic, Bedford,
105 MA). The system's software package (Hologic-Apex software version 4.5.3 with viewer
106 software Hologic Physician's viewer, version 7.1) was used to determine whole body and
107 regional lean and fat mass. Subsequently, thigh skinfold thickness was measured using
108 Harpenden skinfold calipers (Baty International, Burgess Hill, England) and divided by 2 to
109 determine the thickness of the thigh subcutaneous fat layer over each participants' *M. vastus*
110 *lateralis*. In addition, participants were familiarized with the exercise equipment and
111 performed maximum strength tests as determined by their one repetition maximum (1RM) for
112 leg press and knee extension exercise (**Table 1**). Subjects first performed a 5 min cycling
113 exercise warm-up at 100 W. Thereafter, for both leg press and extension, subjects performed
114 2 sets with 10 submaximal or warm-up repetitions to become familiarized with the equipment
115 and to have lifting technique critiqued and corrected. Subjects then performed sets at
116 progressively increasing loads until failing to complete a valid repetition, judged by their
117 inability to complete the full range of motion for an exercise. A 2 min resting period between
118 subsequent attempts was allowed. Finally, participants were familiarized with the water
119 immersion procedure. One leg was immersed in hot water (46°C: HWI) while the other leg
120 was immersed in thermoneutral water (30°C: CON) for a total duration of 20 min. Both legs

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

121 were immersed to the level of the gluteal fold. The limb heated (HWI) was randomized
122 between subjects' dominant and nondominant leg. For the water immersion setup, two water
123 tanks were used that were completely open at the top and contained a tap at the bottom. This
124 allowed us to set and maintain water temperature (before the 20 min water immersion
125 procedure) by adding (hot) water from the top and remove water from below the tank. During
126 water immersion, temperature was monitored and kept constant and still at 46°C in HWI and
127 30°C in CON.

128

129 *Diet and activity prior to the experiment*

130 All subjects received the same standardized dinner (1710 kJ, consisting of 20.25 g protein,
131 51.75 g carbohydrate, 7.65 g dietary fiber and 11.25 g fat) the evening prior to the test day.
132 All volunteers refrained from alcohol and any sort of exhaustive physical labor and/or
133 exercise 2 days prior to the experimental day.

134

135 *Experimental protocol*

136 The experimental protocol is outlined in **Figure 1**. Each subject participated in one
137 experiment. At the start of the experimental day at 7:30 AM, following an overnight fast,
138 subjects reported to the laboratory. First, a telemetric pill (CorTemp HT150002; HQ Inc.) was
139 swallowed with tepid water for continuous measurements of body core temperature until the
140 end of the experiment. In addition, ibuttons (Maxim Integrated Products) were attached to the
141 skin on the left and right upper thigh (~10 cm above the patella) for continuous measurements
142 of skin temperature during the entire trial. Thereafter, a Teflon catheter was inserted into an
143 antecubital vein for intravenous isotope tracer infusion and a second catheter was inserted in a
144 dorsal hand vein of the contralateral arm, which was subsequently placed in a hot-box (60°C)
145 for arterialized blood sampling. After baseline blood sample collection (t = -125 min), the

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

146 plasma phenylalanine and leucine pools were primed with a single intravenous dose of L-
147 [*ring*-$^{2}H_5$]-phenylalanine (2 μmol·kg^{-1}) and L-[1-^{13}C]-leucine (4 μmol·kg^{-1}), respectively.
148 Subsequently, an intravenous infusion of L-[*ring*-$^{2}H_5$]-phenylalanine (infusion rate of 0.050
149 μmol·kg^{-1}·min^{-1}) and L-[1-^{13}C]-leucine (0.100 μmol·kg^{-1}·min^{-1}) was initiated and maintained
150 until the end of the trial using a calibrated IVAC 598 pump (San Diego, USA). After 1 h of
151 supine rest, another arterialized blood sample (t = -65 min) was obtained. Subsequently, the
152 participants performed a resistance-type exercise session. After a 5 min warm-up on a cycle
153 ergometer at self selected intensity (~114 W), the subjects performed 4 sets of 10 repetitions
154 (at 80% 1RM) on both the leg press and knee extension exercise. After completion of the
155 exercise bout (t = -20 min), another arterialized blood sample was obtained before the
156 participants immersed both legs in water for a total duration of 20 min. One leg was immersed
157 in hot water (46°C: HWI) while their contralateral leg was immersed in thermoneutral water
158 (30°C: CON). Immediately after water immersion, another arterialized blood sample was
159 obtained together with muscle temperature (MT23/5 probe; BAT-10, Physitemp, New Jersey,
160 USA) measurements and muscle biopsies from both legs. The muscle temperature probe was
161 inserted into the biopsy incision before each biopsy was collected from both legs.
162 Immediately afterwards, the subjects ingested 20 g intrinsically L-[1-^{13}C]-phenylalanine and
163 L-[1-^{13}C]-leucine labeled milk protein together with 45 g of carbohydrates (glucose polymers)
164 at t = 0 min. Thereafter, repeated blood samples (t = 30, 60, 90, 120, 180, 240, and 300 min)
165 were obtained together with muscle temperature measurements and biopsies from both legs at
166 t = 120 and 300 min. The muscle biopsies were collected from the middle region of the *M.*
167 *vastus lateralis* (~15 cm above the patella) with a Bergström needle under local anesthesia
168 (1). The first two biopsies in each leg (at t = 0 and 120 min) were taken from separate
169 incisions. The difference between the separate incisions was ~3 cm proximal from the
170 previous incision. The last biopsy (t = 300 min) was collected from the same incision as the

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

171 biopsy at t = 120 min. The biopsy at t = 300 min was collected with the needle inserted in a
172 proximal direction. This method ensured that all biopsy sites were separated by at least 3 cm
173 to minimize any artifact related to inflammation resulting from multiple biopsies. All biopsy
174 samples were freed from any visible adipose tissue and blood, immediately frozen in liquid
175 nitrogen, and stored at –80°C until subsequent analysis.

176

177 *Beverage*

178 Subjects received a total beverage volume of 400 mL. The beverage contained 20 g
179 intrinsically L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine labeled milk protein with 45 g of
180 a vanilla flavored proprietary carbohydrate blend consisting of dextrose and maltodextrin
181 (PepsiCo, Purchase, NY, USA). This was mixed in a bottle with water up to a total volume of
182 400 mL.

183

184 *Preparation of tracer*

185 The stable isotope tracers L-[*ring*-$^{2}H_{5}$]-phenylalanine and L-[1-^{13}C]-leucine were purchased
186 from Cambridge Isotopes (Andover, MA) and dissolved in 0.9 % saline before infusion
187 (Apotheek A15, Gorinchem, the Netherlands).

188

189 *Plasma and muscle analysis*

190 Blood samples (10 mL) were collected in EDTA containing tubes and centrifuged at 1000 *g*
191 and 4°C for 10 min. Aliquots of plasma were frozen in liquid nitrogen and stored at -80°C
192 until analysis. Plasma glucose and insulin concentrations were analyzed using commercially
193 available kits (ref. no. A11A01667, Glucose HK CP, ABX Diagnostics, Montpellier, France;
194 and ref. no. HI-14K, Millipore, Billerica, MA, respectively). Plasma amino acid
195 concentrations and enrichments were determined by gas chromatography-mass spectrometry

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

196 analysis (GC-MS; Agilent 7890A GC/5975C; MSD, Wilmington, Delaware, USA).
197 Specifically, internal standards of [U-$^{13}C_6$]-leucine, [U-$^{13}C_9{}^{15}N$]-phenylalanine, and [U-
198 $^{13}C_9{}^{15}N$]-tyrosine were added to the plasma samples. Plasma samples were deproteinized with
199 dry 5-sulfosalicylic acid. Free amino acids were purified using cation exchange
200 chromatography (AG 50W-X8 resin, mesh size: 100–200 µm, ionic form: hydrogen; Bio-Rad
201 Laboratories, Hercules, CA). The purified amino acids were converted into *tert*-
202 butyldimethylsilyl (*tert*-BDMS) derivatives with N-tert-butyldimethylsilyl-N-
203 methyltrifluoroacetamide (MTBSTFA) before analysis by GC-MS. The amino acid
204 concentrations and enrichments were determined using selective ion monitoring at
205 mass/charge (*m/z*) 302 and 308 for unlabeled and [U-$^{13}C_6$] labeled-leucine, 336 and 346 for
206 unlabeled and [U-$^{13}C_9{}^{15}N$] labeled phenylalanine respectively. The plasma leucine and
207 phenylalanine ^{13}C and ^{2}H enrichments were determined at m/z 302 and 303 for unlabeled and
208 labeled (1-^{13}C) leucine, respectively; m/z 336, 337, and 341 for unlabeled and labeled (1-^{13}C
209 and *ring*-$^{2}H_5$) phenylalanine, respectively. Standard regression curves were applied from a
210 series of known standard enrichment values against the measured values to assess the linearity
211 of the mass spectrometer and to account for any isotope fractionation.
212 Myofibrillar protein enriched fractions were extracted from ~60 mg of wet muscle tissue by
213 hand-homogenizing on ice using a pestle in a standard extraction buffer (7 µL·mg^{-1}). The
214 samples were spun at 700 *g* and 4ºC for 15 min. The pellet was washed with 500 µL ddH2O
215 and centrifuged at 700 *g* and 4ºC for 10 min. The myofibrillar protein was solubilized by
216 adding 1 mL of 0.3 M NaOH and heating at 50ºC for 30 min with vortex mixing every 10
217 min. Samples were centrifuged at 9500 *g* and 4ºC for 5 min, the supernatant containing the
218 myofibrillar proteins was collected and the collagen pellet was discarded. Myofibrillar
219 proteins were precipitated by the addition of 1 mL of 1 M perchloric acid (PCA) and spinning
220 at 700 *g* and 4ºC for 10 min. The myofibrillar protein was washed twice with 70 % ethanol

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

221 and hydrolyzed overnight in 2 mL of 6 M HCL at 110˚C. The free amino acids from the
222 hydrolyzed myofibrillar protein pellet were dried under a nitrogen stream while being heated
223 to 110ºC. The free amino acids were then dissolved in 25 % acetic acid solution, passed over
224 cation exchange AG 50W-X8 resin columns (mesh size: 100-200, ionic form: hydrogen; Bio-
225 Rad Laboratories, Hercules, CA), and eluted with 2 M NH_4OH. The purified amino acids (L-
226 [1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine enrichments) were analyzed by GC-C-IRMS
227 analysis. To determine myofibrillar protein L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine
228 enrichments by GC-C-IRMS analysis, the purified amino acids were converted into N-
229 ethoxycarbonyl ethyl ester derivatives with ethyl chloroformate (ECF). The derivatives were
230 then measured by GC-C-IRMS (Finnigan MAT 253, Bremen, Germany) using a DB5-MS-
231 column (no. 122-5532; Agilent J+W scientific GC Column, GC Isolink) and monitoring of
232 ion masses 44, 45, and 46. For measurement of L-[*ring*-$^2H_5$]-phenylalanine enrichment in the
233 myofibrillar protein pools, the eluate was dried, and the purified amino acids were also
234 derivatized into a N-ethoxycarbonyl ethyl ester. The derivatized samples were measured using
235 a gas chromatography-isotope ratio mass spectrometer (MAT 253; Thermo Fisher Scientific,
236 Bremen, Germany) equipped with a pyrolysis oven (GC-P-IRMS) using a 70-m DB-17MS
237 column, 5-m precolumn (No. 122–4762; Agilent), and GC-Isolink. Standard regression
238 curves and standards were applied to assess the linearity of the mass spectrometer and to
239 account for isotopic fractionation.
240 For western blot analysis, a portion of each muscle sample frozen for biochemical analyses
241 was homogenized in seven volumes Tris buffer (20 mM Tris-HCL, 5 mM EDTA, 10 mM Na-
242 pyrosphospate, 100 mM NaF, 2 mM Na3VO4, 1 % Nonident P-40; pH 7.4) supplemented
243 with the following protease and phosphatase inhibitors: Aprotinin 10 µg/mL, Leupeptin 10
244 µg/mL, Benzamidin 3 mM and PMSF 1 mM. After homogenization, each muscle extract was
245 centrifuged for 10 min at 10,000 *g* (4ºC) and sample buffer was added to the supernatant to

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

246 final concentrations of 60 mM Tris, 10 % glycerol, 20 mg/mL SDS, 0.1 mM DTT, 20 µg/mL
247 bromophenol blue. The supernatant was then heated for 5 min at 100°C and immediately
248 placed on ice. Immediately before analyses, the muscle extraction sample was warmed to
249 50ºC and centrifuged for 1 min at 1000 *g* (RT). The total amount of sample loaded on the gel
250 was based on protein content. After a Bradford assay, 30 µg protein were loaded in each lane.
251 With the exception of mTOR, protein samples were run on a Criterion Precast TGX 4-20 %
252 gel (Biorad Order No. 567-1094) ±90 min at 150 V (constant voltage) and transferred onto a
253 Trans-blot Turbo 0.2 µm nitrocellulose membrane (Biorad Order No. 170-4159) in 7 min at
254 2.5 A and 25 V. mTOR proteins were run and blotted for 10 min at 2.5 A and 25 V but on a
255 Criterion Precast XT 3-8 % Tris-acetate gel (Biorad order No. 345-0130). Specific proteins
256 were detected by overnight incubation at 4ºC on a shaker with specific antibodies in 50 % in
257 PBS Odyssey blocking buffer (Li-Cor Biosciences Part No. 927-40000) after blocking for 60
258 min at RT in 50 % in PBS Odyssey blocking buffer. Polyclonal primary phospho-specific
259 antibodies, anti-phospho-mTOR (Ser^{2448}), anti-phospho-S6K1 (Thr^{389}), anti-phospho-S6K1
260 (Thr^{421}/Ser^{424}), anti-phospho-rpS6 (Ser^{240}/Ser^{244}), anti-phospho-rpS6 (Ser^{235}/Ser^{236}), and anti-
261 phospho-4E-BP1 ($Thr^{37/46}$) were purchased from Cell Signaling Technology (Danvers, MA,
262 USA). In addition, anti-mTOR, anti-S6K1, anti-RS6, anti-4E-BP1, anti-HSP27, and anti-
263 HSP70 were also purchased from Cell Signaling Technology (Danvers, MA, USA).
264 Following incubation, membranes were washed three times 10 min in 0.1 % PBS-Tween 20
265 and once for 10 min in PBS. Next, samples were incubated on a shaker (1 h at RT) with
266 infrared secondary antibodies, donkey anti-rabbit IRDYE 800 (Rockland, Cat. No. 611-732-
267 127, dilution 1:10000) and donkey anti-mouse IRDYE 800CW (Li-Cor, Cat. No. 626-32212,
268 dilution 1:10000) dissolved in 50 % PBS Odyssey blocking buffer. After a final wash step (3
269 x 10 min) in 0.1 % Tween20-PBS and once 10 min in PBS, protein quantification was
270 performed by scanning on an Odyssey Infrared Imaging System (LI-COR Biotechnology,

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

271 Lincoln, NE). All measurements for phospho-specific and total proteins were performed on
272 the same membrane. Ponceau S staining was applied to assess and confirm equal total protein
273 loading. Phosphorylation status as a proxy of activation of the signaling proteins was
274 expressed relative to the total amount of each protein. For heat shock proteins (HSP27 and
275 HSP70) total protein content was expressed relative to ponceau S staining to correct for the
276 total amount of protein loaded.

277

278 *Calculations*

279 Ingestion of L-[1-^{13}C]-phenylalanine labeled protein, intravenous infusion of L-[*ring*-$^{2}H_5$]-
280 phenylalanine, and blood sample enrichment values were used to calculate total, and
281 exogenous phenylalanine rates of appearance (R_a), and plasma availability of dietary protein-
282 derived phenylalanine that appeared in the systemic circulation as a fraction of total amount
283 of phenylalanine that was ingested (Phe_{plasma}). For these calculations modified Steele's
284 equations (in non-steady state conditions) were used (3, 6). These parameters were calculated
285 as follows:

$$\text{Total}R_a = \frac{F_{iv} - \left[pV \cdot C(t) \cdot \frac{dE_{iv}}{dt}\right]}{E_{iv}(t)} \quad (1)$$

$$\text{Exo}R_a = \frac{\text{Total } R_a \cdot E_{po}(t) + \left[pV \cdot C(t) \cdot \frac{dE_{po}}{dt}\right]}{E_{prot}} \quad (2)$$

286

$$\text{Phe}_{\text{plasma}} = \left(\frac{\text{AUC}_{\text{ExoRa}}}{\text{Phe}_{\text{prot}}}\right) \cdot 100 \quad (3)$$

287 where F_{iv} is the intravenous tracer infusion rate (µmol·kg^{-1}·min^{-1}), *pV* (0.125 L·kg^{-1}) is the
288 distribution volume for phenylalanine (3). *C(t)* is the mean plasma phenylalanine
289 concentration between 2 consecutive time points. dE_{iv}/dt represents the time-dependent

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

290 variations of plasma phenylalanine enrichment derived from the intravenous tracer and E_{iv} *(t)*
291 is the mean plasma phenylalanine enrichment from the intravenous tracer between 2
292 consecutive time points. $ExoR_a$ represents the plasma entry rate of dietary phenylalanine, E_{po}
293 *(t)* is the mean plasma phenylalanine enrichment for the ingested tracer, dE_{po}/dt represents the
294 time-dependent variations of plasma phenylalanine enrichment derived from the oral tracer
295 and E_{prot} is the L-[1-^{13}C]-phenylalanine enrichment in the dietary protein. Phe_{plasma} is the
296 percentage of ingested dietary phenylalanine that becomes available in the plasma and is
297 calculated using Phe_{Prot} and AUC_{ExoRa}. Phe_{Prot} is the amount of dietary phenylalanine ingested
298 and AUC_{ExoRa} represents the area under the curve *(AUC)* of $ExoR_a$, which corresponds to the
299 amount of dietary phenylalanine that appeared in the blood over a 5 h period following
300 ingestion.
301 The fractional synthesis rate (*FSR*) of myofibrillar protein was calculated by dividing the
302 increment in enrichment in the product, i.e. protein-bound L-[1-^{13}C]-leucine or L-[*ring*-$^{2}H_5$]-
303 phenylalanine, by the enrichment of the respective precursor amino acid enrichments (i.e.,
304 plasma free amino acids). Weighted mean plasma L-[*ring*-$^{2}H_5$]-phenylalanine and L-[1-^{13}C]-
305 leucine enrichments were used as the preferred precursor pools to estimate myofibrillar
306 protein fractional synthesis rates from the continuously infused L-[*ring*-$^{2}H_5$]-phenylalanine,
307 and L-[1-^{13}C]-leucine tracers. Consequently, myofibrillar *FSR* was calculated as follows:
308

309 $$FSR\ (\%\cdot h^{-1}) = \left(\frac{E_{m2} - E_{m1}}{E_{precursor}\ x\ t}\right) x\ 100 \quad (4)$$

310

311 where $E_{m2} - E_{m1}$ represents muscle protein bound L-[*ring*-$^{2}H_5$]-phenylalanine or L-[1-^{13}C]-
312 leucine. $E_{precursor}$ represent the average plasma L-[*ring*-$^{2}H_5$]-phenylalanine or L-[1-^{13}C]-
313 leucine enrichment during the tracer incorporation period. *t* indicates the time interval (h)
314 between biopsies.

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

315

316 *Statistical Analysis*

317 Unless otherwise stated, all data are expressed as mean ± standard error of the mean (SEM).
318 Changes in blood glucose and insulin, plasma amino acid concentrations and enrichments,
319 exogenous phenylalanine $R_{a,}$ and core body temperature were analyzed using one-way
320 repeated-measures ANOVA with time as within-subjects factor. A two-factor (treatment X
321 time) repeated-measures ANOVA was performed for the analysis of L-[1-^{13}C]-phenylalanine
322 myofibrillar protein-bound enrichments, skeletal muscle and skin temperature, anabolic
323 signaling, and heat shock protein expression. A Student's paired *t* test was performed to
324 compare FSR values between the HWI and CON legs. In case of a significant main effect of
325 time or time X treatment interaction, Bonferroni corrected pairwise comparisons were
326 performed where appropriate. Statistical significance was set at $P<0.05$. All calculations were
327 performed using SPSS (version 24.0, IBM Corp., Armonk, NY, USA).

328

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

329 **RESULTS**

330

331 *Thermoregulatory responses*

332 Core temperature was slightly increased (from 37.0±0.1°C to 37.6±0.1°C) immediately after
333 water immersion (time effect, P<0.001). Within one hour after water immersion, core
334 temperature returned back to pre-immersion values (37.0±0.1°C), with no significant
335 differences during the remainder of the post-exercise recovery period (P>0.05). Thigh skin
336 temperatures over the entire experiment are shown in **Figure 2A**. A significant time X
337 treatment interaction was observed for thigh skin temperature (P<0.001). Thigh skin
338 temperature was significantly higher after exercise (t = -20 min) in both the HWI and CON
339 leg compared to rest (t = -60 min) (P<0.001). Only for the HWI leg, thigh skin temperature
340 was significantly increased immediately after water immersion by ~10°C (P<0.001). Thigh
341 skin temperature was significantly different between the HWI and CON legs immediately
342 after water immersion up to 2 h after ingestion of the recovery beverage (P<0.05).
343 Muscle temperature of both legs after water immersion are shown in **Figure 2B**. Considering
344 a ~0.5 cm skinfold thickness, the muscle temperature probe (5 cm) was inserted in the muscle
345 at a depth of ~4.5 cm. A significant time X treatment interaction was observed for muscle
346 temperature (P<0.001). Muscle temperature did not change significantly over time in the
347 CON leg, but was significantly higher immediately after water immersion (t = 0 min)
348 compared to t = 120 and 300 min in the HWI leg (P<0.001). After water immersion (t = 0
349 min), muscle temperature in the HWI leg was significantly higher (~2.3°C) when compared to
350 the CON leg (P<0.001). At time points 120 min and 300 min, muscle temperature was no
351 longer significantly different between legs.

352

353 *Plasma analyses*

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

354 Plasma glucose concentrations significantly increased from t = 0 to 30 min (from 5.0±0.2 to
355 7.9±0.3 mmol/L; time effect, P<0.001). At other time points, no significant differences were
356 observed when compared to baseline values (t = 0 min) (*data not shown*). Plasma insulin
357 concentrations were significantly increased from t = 0 (7.4±0.7 mU/L) to t = 30 (73.6±9.2
358 mU/L) and 60 (26.7±3.8 mU/L) min (time effect, P<0.001). At other time points following
359 drink ingestion, no significant differences were observed when compared to baseline values (t
360 = 0 min) (*data not shown*). Both plasma phenylalanine and leucine concentrations increased
361 following drink ingestion (time effect, P<0.001). Plasma phenylalanine concentrations
362 remained above basal levels (t = 0 min; 60.7±1.7 μmol/L) for 90 min (t = 90 min; 71.9±2.8
363 μmol/L; P<0.05) and were lower when compared to basal levels at t = 240 (52.2±1.4 μmol/L;
364 P<0.001) and 300 min (53.4±1.7 μmol/L; P=0.001). Plasma leucine concentrations were
365 significantly higher than basal levels (t = 0 min; 127±5 μmol/L) during the entire 5 h recovery
366 period (apart from t = 240 min; 143±5 μmol/L; P=0.069).

367 During the post-absorptive period, plasma L-[ring-$^{2}H_{5}$]-phenylalanine (**Figure 3A**) and L-[1-
368 ^{13}C]-leucine (**Figure 3B**) enrichments remained in a steady state. Following drink ingestion,
369 plasma L-[ring-$^{2}H_{5}$]-phenylalanine enrichments were significantly lower for the first 90 min,
370 not significantly different (compared to baseline) at t = 120 and 180 min, and significantly
371 higher at t = 240 and 300 min (time effect, P<0.001). Plasma L-[1-^{13}C]-leucine enrichments
372 increased in response to drink ingestion (time effect, P<0.001) and remained at an elevated
373 steady state of ~6.0-8.0 MPE for the duration of the 5 h post-prandial period. Following drink
374 ingestion, plasma L-[1-^{13}C]-phenylalanine enrichments increased rapidly (time effect,
375 P<0.001) from 0 to 14.3±0.4 MPE after 30 min and declined thereafter, albeit remaining
376 elevated above fasting levels for the remainder of the post-prandial period (**Figure 3C**).

377 Ingestion of the 20 g intrinsically labeled milk protein resulted in a rapid rise in exogenous
378 phenylalanine appearance rates (time effect, P<0.001; **Figure 4**) and this remained

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

379 significantly elevated over the entire post-prandial recovery period compared to baseline (t =
380 0 min). Over the entire 5 h period, 14.7±0.3 g (74±2%) of the ingested protein-derived amino
381 acids had been released into the circulation.
382
383 *Muscle tracer analysis*
384 Myofibrillar protein FSR based on intravenous L-[*ring*-$^{2}H_{5}$]-phenylalanine infusion with
385 plasma L-[*ring*-$^{2}H_{5}$]-phenylalanine enrichments as precursor pool (**Figure 5A**) or intravenous
386 L-[1-^{13}C]-leucine infusion combined with intrinsically L-[1-^{13}C]-leucine labeled protein
387 ingestion with plasma L-[1-^{13}C]-leucine enrichments as precursor pool (**Figure 5B**) are
388 displayed in **Figure 5**. Myofibrillar protein FSR as calculated over 0-2 h did not differ
389 between the HWI and CON leg based on the L-[*ring*-$^{2}H_{5}$]-phenylalanine tracer (0.065±0.002
390 vs 0.066±0.004%·h^{-1}, respectively; P=0.704) and the L-[1-^{13}C]-leucine tracer (0.065±0.008 vs
391 0.062±0.006%·h^{-1}, respectively; P=0.616). In addition, myofibrillar protein FSR as calculated
392 over the entire 5 h post-prandial period also did not differ between the HWI and CON leg
393 based on the L-[*ring*-$^{2}H_{5}$]-phenylalanine tracer (0.048±0.002 vs 0.047±0.003%·h^{-1},
394 respectively; P=0.877) as well as the L-[1-^{13}C]-leucine tracer (0.050±0.005 vs
395 0.049±0.002%·h^{-1}, respectively; P=0.815).
396 Myofibrillar L-[1-^{13}C]-phenylalanine enrichments are displayed in **Figure 6**. A significant
397 time effect was found (P<0.001). However, no significant treatment effect or time X
398 treatment interaction was observed (P>0.05), indicating higher myofibrillar L-[1-^{13}C]-
399 phenylalanine enrichments at 5 h vs 2 h for both the CON and HWI leg, with no differences
400 between legs.
401
402 *Muscle molecular signaling*

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

403 The phosphorylation status (ratio of phosphorylated protein to total protein) of key proteins
404 involved in the initiation of muscle protein synthesis are presented in **Figure 7 (A-F)**. No
405 significant differences were observed for muscle mTOR (Ser2448) phosphorylation status
406 (**Figure 7A**). A significant time effect, treatment effect, and time X treatment interaction was
407 observed for muscle p70S6K (Thr389) phosphorylation status (P<0.05; **Figure 7B**). Muscle
408 p70S6K (Thr389) phosphorylation status was significantly increased from 0 to 2 h (P=0.007)
409 in the HWI leg and significantly decreased from 2 to 5 h (P<0.01) in both the HWI and CON
410 leg. The phosphorylation status was significantly different between treatments at all time
411 points (P<0.05). At t = 0 h, the CON leg was significantly higher compared to the HWI leg
412 (P=0.031). At both t = 2 and 5 h, the HWI leg was significantly higher compared to the CON
413 leg (P<0.05). A significant time effect was found for muscle p70S6K (Thr421/Ser424)
414 phosphorylation status (P<0.001; **Figure 7C**). No significant treatment effect or time X
415 treatment interaction was observed for muscle p70S6K (Thr421/Ser424) phosphorylation
416 status. No significant differences were observed for muscle rpS6 (Ser240/244)
417 phosphorylation status (**Figure 7D**). A significant time effect was found for muscle rpS6
418 (Ser235/236) phosphorylation status (P=0.040; **Figure 7E**), with no significant treatment
419 effect or time X treatment interaction observed. No significant differences were observed for
420 muscle 4E-BP1 (Thr37/46) phosphorylation status (**Figure 7F**). Total protein content of
421 HSP27 and HSP70 (expressed relative to ponceau S staining) are presented in **Figure 8 (A-**
422 **B)**. No significant differences over time were observed for both HSP27 (**Figure 8A**) and
423 HSP70 (**Figure 8B**) contents.

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

424 **DISCUSSION**

425 In the present study, we assessed the impact of post-exercise hot-water immersion on post-
426 prandial myofibrillar protein synthesis rates during recovery from resistance-type exercise.
427 Hot-water immersion transiently increased skin and muscle temperature, but did not further
428 increase post-prandial myofibrillar protein synthesis rates or augment the incorporation of
429 dietary protein-derived amino acids in muscle tissue protein during 5 hours of post-exercise
430 recovery.

431 It has been well established that protein intake increases post-exercise muscle protein
432 synthesis rates (16, 21, 24, 31), thereby improving post-exercise skeletal muscle
433 reconditioning. It has previously been reported that ingestion of 20 g of a high-quality protein
434 maximizes post-exercise muscle protein synthesis rate during recovery from lower body
435 resistance exercise (21, 37). Therefore, in line with everyday practice, our (recreational)
436 athletes were provided with 20 g (of intrinsically labeled milk) protein and 45 g carbohydrates
437 following cessation of exercise. In line with previous work (7-9, 13, 32), we showed that the
438 dietary protein-derived amino acids were effectively being taken up and released in the
439 systemic circulation (Figure 4), thereby providing ample precursors to support the post-
440 exercise increase in myofibrillar protein synthesis. In total, more than 70% of the ingested
441 protein-derived amino acids were released in the circulation, thereby strongly increasing
442 plasma amino acid concentrations.

443 Hydrotherapy is a popular recovery strategy that is applied by many athletes to support their
444 post-exercise recovery (35). Two popular hydrotherapy strategies are cold-water immersion
445 (CWI) and hot-water immersion (HWI) (35). It has been reported that CWI is more effective

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

446 in improving markers of acute post-exercise recovery, such as reducing delayed onset muscle
447 soreness (DOMS), lowering limb swelling, and assisting in performance recovery compared
448 to HWI (18, 23, 33-35). However, CWI has been shown to lower tissue temperature and
449 reduce blood flow (12, 19), thereby reducing the incorporation of dietary protein-derived
450 amino acids into muscle protein and attenuating the post-exercise increase in muscle protein
451 synthesis rates by ~20% (7). Consequently, athletes aiming to improve post-exercise muscle
452 (re)conditioning are generally not recommended to apply CWI during recovery from exercise.
453 In contrast, it has been hypothesized that post-exercise HWI, by stimulating blood flow and/or
454 increasing muscle tissue temperature, can augment muscle protein synthesis during recovery
455 from exercise.

456 In the current study, exercise was followed by immersing one leg in hot water (46°C) while
457 the contralateral leg was immersed in thermoneutral water (30°C) for 20 min. This short hot-
458 water immersion regimen was selected based upon everyday practice by athletes as well as
459 previous studies (35) with the (hot) water temperature being selected based upon what was
460 perceived as tolerable for 20 min. Hot-water immersion increased skin (~10°C) as well as
461 muscle (~2.3°C) temperature, after which levels approximately returned to basal values
462 within the first 120 min of post-exercise recovery (Figure 2). Despite the observed increases
463 in skin and muscle temperature, post-exercise HWI did not modulate post-prandial
464 myofibrillar protein synthesis rates (Figure 5) or affect the incorporation of dietary protein
465 derived amino acids in muscle tissue during the early or later stages of post-exercise recovery
466 (Figure 6).

467 The current findings seem to be at odds with previous suggestions (based on anabolic
468 signaling responses) that heat stress may increase muscle protein synthesis rates (17, 38). It
469 has previously been shown in rodents that the application of 30 min of local heat stress
470 (HWI), activates the mTOR pathway by increasing the phosphorylation of Akt (at Ser473)

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

471 and p70S6K (at Thr389) (38). In line, Kakigi *et al.* (17) showed that local heat stress
472 performed 20 min before and during resistance exercise enhanced mTOR signaling in muscle
473 tissue in humans. However, those studies did not perform hot-water immersion during
474 recovery from exercise. In the present study, we also assessed the phosphorylation status of
475 several molecular markers that are important in the regulation of myofibrillar protein
476 synthesis during post-exercise recovery (Figure 7). We extend on previous findings by
477 showing that p70S6K phosphorylation at Thr389 was lower immediately after HWI, but
478 increased at 2 and 5 h into post-exercise recovery when compared with the CON leg (Figure
479 7B). The reason why p70S6K phosphorylation at Thr389 was lower in the HWI leg
480 immediately after post-exercise water immersion when compared to the CON leg remains
481 unclear. However, it is interesting to note that we have previously shown that p70S6K
482 phosphorylation at Thr389 was higher immediately after post-exercise CWI (8°C) when
483 compared with thermoneutral water immersion (30°C) for 20 min (7). Therefore, muscle
484 temperature seems to modulate p70S6K phosphorylation at Thr389. The fact that p70S6K
485 phosphorylation at Thr389 was increased at 2 and 5 h in the post-exercise recovery period in
486 the HWI compared with the CON leg may have compensated for the lower phosphorylation
487 status observed immediately after water immersion (t = 0 h). Other molecular markers did not
488 show differences between the HWI and CON leg (Figure 7). As evidence suggests that heat
489 stress can elicit protective effects to assist post-exercise recovery and adaptation by increasing
490 heat shock protein (HSP) expression (20), we also measured HSP27 and HSP70 expression.
491 We did not observe any differences in both HSPs between the HWI and CON leg during 5-h
492 of post-exercise recovery (Figure 8). This could potentially be explained by the fact that 5-h
493 of post-exercise recovery is not sufficient to pick up differences, as it is possible that longer
494 post-heating durations may be necessary to pick up changes in HSPs. Alternatively, our
495 heating strategy may not have been sufficient to induce changes in HSPs. Overall, these

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

496 observations seem to agree with the absence of differences in muscle protein synthesis rates
497 between the HWI and CON leg.

498 Our findings indicate that athletes seeking to accelerate post-exercise muscle reconditioning
499 do not benefit from the application of hot-water immersion during post-exercise recovery.
500 These findings seem to agree with Stadnyk *et al.* who did not observe greater gains in leg lean
501 mass or strength during more prolonged resistance-type exercise training when applying
502 heating (28). In contrast, two studies by Goto *et al.* showed that heating applied with (10) or
503 even without (11) exercise training over a period of 10 weeks increased gains in muscle cross-
504 sectional area and strength (10, 11). The apparent discrepancy between these studies may be
505 attributed to differences in (1) the applied exercise protocol, (2) heat modalities (i.e. heat pads
506 vs. heat- and steam-generating sheets) and duration, and (3) the applied methods to assess
507 muscle size. However, it is interesting to note that the study by Stadnyk *et al.* performed
508 exercise at a relatively high intensity (4 sets of 8 repetitions at 70% 1RM for 2-3 days a week)
509 (28), whereas the studies by Goto *et al.* performed low-intensity exercise training (3 sets of 30
510 repetitions against a resistance of less than 30 RM for 4 days a week) (10) or did not perform
511 exercise at all (11) over a 10-week intervention period. Therefore, it could be speculated that
512 heat stress may increase gains in muscle mass and strength when applied during low(er)-
513 intensity exercise or when applied without exercise throughout a more prolonged intervention
514 period, whereas heat stress performed around high-intensity exercise training does not
515 (further) augment gains in muscle mass and strength. In the present study, we clearly show
516 that short, hot-water immersion applied after a single bout of more intense resistance-type
517 exercise (80% 1RM) does not further augment muscle protein synthesis rates in (recreational)
518 athletes consuming ample protein during 5 hours of post-exercise recovery.

519 In the current study we observed a transient increase in muscle tissue temperature after HWI.
520 If we would extrapolate our data based on previous work (25), tissue temperature would

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

521 likely have returned to basal values within ~30 minutes after exercise. Therefore, it could be
522 speculated that our heating protocol (20 min of HWI at 46°C) did not elicit a sufficient,
523 continued increase in muscle temperature and that a stronger and/or more prolonged heating
524 protocol may have been more potent. In previous pilot work we established that HWI with
525 water at a temperature of ~46°C is the maximum tolerable temperature when applied for 20
526 min. With this protocol, we observed a muscle temperature of ~37.5°C in the HWI leg, which
527 was ~2.3°C higher compared with the CON leg. Previous studies have shown similar
528 increases in intramuscular temperature when applying other heating modalities, such as heat
529 pads and hot water-perfused limb cuffs (5, 27, 39). Muscle temperature may be further
530 increased (up to ~40°C) by applying diathermy (14, 15). However, it should be noted that the
531 application of diathermy for post-exercise recovery may not be practical for athletes, as this is
532 typically applied very locally with only a small amount of muscle being heated. Nonetheless,
533 further research may be warranted to investigate the impact of other heating strategies on
534 recovery during (low-intensity) exercise. Such future studies should focus on local, rather
535 than whole-body, heating strategies as prolonged whole-body heat stress can be detrimental
536 for other important aspects of post-exercise muscle recovery, such as glycogen synthesis (22).
537 In addition, future studies may want to address the impact of prolonged (post-exercise)
538 heating on the synthesis rates of more specific protein sub-fractions, such as mitochondrial
539 proteins (14, 15, 30).

540 In conclusion, short hot-water immersion during recovery from resistance-type exercise does
541 not increase post-prandial myofibrillar protein synthesis rates or augment the capacity of the
542 muscle to use dietary protein-derived amino acids for *de novo* myofibrillar protein accretion.
543 Post-exercise hot-water immersion, as often applied in practice, does not seem to enhance the
544 skeletal muscle adaptive response to exercise training and, thus, would unlikely improve
545 skeletal muscle conditioning.

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

ACKNOWLEDGEMENTS

We would like to acknowledge Lieke de Wit, Denise Tan and Annemie P. Gijsen for their (technical) assistance and the enthusiastic support of the subjects who volunteered to participate in this experiment.

GRANTS

This study was internally funded by Maastricht University.

DISCLOSURES

Cas J. Fuchs, Joey S.J. Smeets, Joan M. Senden, Antoine H. Zorenc, Joy P. B. Goessens, Wouter D. van Marken Lichtenbelt, Lex B. Verdijk, and Luc J.C. van Loon declare that they have no conflict of interest related to this study.

AUTHORS' CONTRIBUTIONS

C.J.F. and L.J.C.L. designed the research; C.J.F. and J.S.J.S. conducted the research; W.D.V.M.L. provided essential materials including assistance and instructions; C.J.F., J.M.S., A.H.Z and J.P.B.G. analyzed the data; C.J.F. and L.B.V. performed the statistical analysis; and C.J.F. and L.J.C.L. wrote the paper and hold primary responsibility for the final content. All authors read and approved the final manuscript.

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

REFERENCES

1. **Bergstrom J**. Percutaneous needle biopsy of skeletal muscle in physiological and clinical research. *Scand J Clin Lab Invest* 35: 609-616, 1975.
2. **Biolo G, Tipton KD, Klein S, and Wolfe RR**. An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein. *Am J Physiol* 273: E122-129, 1997.
3. **Boirie Y, Gachon P, Corny S, Fauquant J, Maubois JL, and Beaufrere B**. Acute postprandial changes in leucine metabolism as assessed with an intrinsically labeled milk protein. *Am J Physiol* 271: E1083-1091, 1996.
4. **Borsheim E, Tipton KD, Wolf SE, and Wolfe RR**. Essential amino acids and muscle protein recovery from resistance exercise. *Am J Physiol Endocrinol Metab* 283: E648-657, 2002.
5. **Cheng AJ, Willis SJ, Zinner C, Chaillou T, Ivarsson N, Ortenblad N, Lanner JT, Holmberg HC, and Westerblad H**. Post-exercise recovery of contractile function and endurance in humans and mice is accelerated by heating and slowed by cooling skeletal muscle. *J Physiol* 595: 7413-7426, 2017.
6. **Dangin M, Guillet C, Garcia-Rodenas C, Gachon P, Bouteloup-Demange C, Reiffers-Magnani K, Fauquant J, Ballevre O, and Beaufrere B**. The rate of protein digestion affects protein gain differently during aging in humans. *J Physiol* 549: 635-644, 2003.
7. **Fuchs CJ, Kouw IWK, Churchward-Venne TA, Smeets JSJ, Senden JM, Lichtenbelt W, Verdijk LB, and van Loon LJC**. Postexercise cooling impairs muscle protein synthesis rates in recreational athletes. *J Physiol* 598: 755-772, 2020.
8. **Gorissen SH, Burd NA, Hamer HM, Gijsen AP, Groen BB, and van Loon LJ**. Carbohydrate coingestion delays dietary protein digestion and absorption but does not modulate postprandial muscle protein accretion. *J Clin Endocrinol Metab* 99: 2250-2258, 2014.
9. **Gorissen SHM, Trommelen J, Kouw IWK, Holwerda AM, Pennings B, Groen BBL, Wall BT, Churchward-Venne TA, Horstman AMH, Koopman R, Burd NA, Fuchs CJ, Dirks ML, Res PT, Senden JMG, Steijns J, de Groot L, Verdijk LB, and van Loon LJC**. Protein Type, Protein Dose, and Age Modulate Dietary Protein Digestion and Phenylalanine Absorption Kinetics and Plasma Phenylalanine Availability in Humans. *J Nutr* 2020.
10. **Goto K, Oda H, Morioka S, Naito T, Akema T, Kato H, Fujiya H, Nakajima Y, Sugiura T, Ohira Y, and Yoshioka T**. Skeletal muscle hypertrophy induced by low-intensity exercise with heat-stress in healthy human subjects. *Jpn J Aerosp Environ Med* 44: 2007.
11. **Goto K, Oda H, Kondo H, Igaki M, Suzuki A, Tsuchiya S, Murase T, Hase T, Fujiya H, Matsumoto I, Naito H, Sugiura T, Ohira Y, and Yoshioka T**. Responses of muscle mass, strength and gene transcripts to long-term heat stress in healthy human subjects. *Eur J Appl Physiol* 111: 17-27, 2011.
12. **Gregson W, Black MA, Jones H, Milson J, Morton J, Dawson B, Atkinson G, and Green DJ**. Influence of cold water immersion on limb and cutaneous blood flow at rest. *Am J Sports Med* 39: 1316-1323, 2011.
13. **Groen BB, Horstman AM, Hamer HM, de Haan M, van Kranenburg J, Bierau J, Poeze M, Wodzig WK, Rasmussen BB, and van Loon LJ**. Post-Prandial Protein Handling: You Are What You Just Ate. *PLoS One* 10: e0141582, 2015.

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

14. **Hafen PS, Abbott K, Bowden J, Lopiano R, Hancock CR, and Hyldahl RD**. Daily heat treatment maintains mitochondrial function and attenuates atrophy in human skeletal muscle subjected to immobilization. *J Appl Physiol (1985)* 127: 47-57, 2019.
15. **Hafen PS, Preece CN, Sorensen JR, Hancock CR, and Hyldahl RD**. Repeated exposure to heat stress induces mitochondrial adaptation in human skeletal muscle. *J Appl Physiol (1985)* 125: 1447-1455, 2018.
16. **Holwerda AM, Paulussen KJM, Overkamp M, Goessens JPB, Kramer IF, Wodzig W, Verdijk LB, and van Loon LJC**. Dose-Dependent Increases in Whole-Body Net Protein Balance and Dietary Protein-Derived Amino Acid Incorporation into Myofibrillar Protein During Recovery from Resistance Exercise in Older Men. *J Nutr* 149: 221-230, 2019.
17. **Kakigi R, Naito H, Ogura Y, Kobayashi H, Saga N, Ichinoseki-Sekine N, Yoshihara T, and Katamoto S**. Heat stress enhances mTOR signaling after resistance exercise in human skeletal muscle. *J Physiol Sci* 61: 131-140, 2011.
18. **Kuligowski LA, Lephart SM, Giannantonio FP, and Blanc RO**. Effect of whirlpool therapy on the signs and symptoms of delayed-onset muscle soreness. *J Athl Train* 33: 222-228, 1998.
19. **Mawhinney C, Jones H, Low DA, Green DJ, Howatson G, and Gregson W**. Influence of cold-water immersion on limb blood flow after resistance exercise. *Eur J Sport Sci* 17: 519-529, 2017.
20. **McGorm H, Roberts LA, Coombes JS, and Peake JM**. Turning Up the Heat: An Evaluation of the Evidence for Heating to Promote Exercise Recovery, Muscle Rehabilitation and Adaptation. *Sports Med* 48: 1311-1328, 2018.
21. **Moore DR, Robinson MJ, Fry JL, Tang JE, Glover EI, Wilkinson SB, Prior T, Tarnopolsky MA, and Phillips SM**. Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. *Am J Clin Nutr* 89: 161-168, 2009.
22. **Naperalsky M, Ruby B, and Slivka D**. Environmental temperature and glycogen resynthesis. *Int J Sports Med* 31: 561-566, 2010.
23. **Pournot H, Bieuzen F, Duffield R, Lepretre PM, Cozzolino C, and Hausswirth C**. Short term effects of various water immersions on recovery from exhaustive intermittent exercise. *Eur J Appl Physiol* 111: 1287-1295, 2011.
24. **Rasmussen BB, Tipton KD, Miller SL, Wolf SE, and Wolfe RR**. An oral essential amino acid-carbohydrate supplement enhances muscle protein anabolism after resistance exercise. *J Appl Physiol (1985)* 88: 386-392, 2000.
25. **Rose S, Draper DO, Schulthies SS, and Durrant E**. The Stretching Window Part Two: Rate of Thermal Decay in Deep Muscle Following 1-MHz Ultrasound. *J Athl Train* 31: 139-143, 1996.
26. **Skurvydas A, Kamandulis S, Stanislovaitis A, Streckis V, Mamkus G, and Drazdauskas A**. Leg immersion in warm water, stretch-shortening exercise, and exercise-induced muscle damage. *J Athl Train* 43: 592-599, 2008.
27. **Slivka D, Tucker T, Cuddy J, Hailes W, and Ruby B**. Local heat application enhances glycogenesis. *Appl Physiol Nutr Metab* 37: 247-251, 2012.
28. **Stadnyk AMJ, Rehrer NJ, Handcock PJ, Meredith-Jones KA, and Cotter JD**. No clear benefit of muscle heating on hypertrophy and strength with resistance training. *Temperature (Austin)* 5: 175-183, 2018.
29. **Stephens JM, Halson SL, Miller J, Slater GJ, and Askew CD**. Influence of body composition on physiological responses to post-exercise hydrotherapy. *J Sports Sci* 36: 1044-1053, 2018.

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

30. **Tamura Y, Matsunaga Y, Masuda H, Takahashi Y, Takahashi Y, Terada S, Hoshino D, and Hatta H**. Postexercise whole body heat stress additively enhances endurance training-induced mitochondrial adaptations in mouse skeletal muscle. *Am J Physiol Regul Integr Comp Physiol* 307: R931-943, 2014.
31. **Tang JE, Manolakos JJ, Kujbida GW, Lysecki PJ, Moore DR, and Phillips SM**. Minimal whey protein with carbohydrate stimulates muscle protein synthesis following resistance exercise in trained young men. *Appl Physiol Nutr Metab* 32: 1132-1138, 2007.
32. **Trommelen J, Holwerda AM, Kouw IW, Langer H, Halson SL, Rollo I, Verdijk LB, and LJ VANL**. Resistance Exercise Augments Postprandial Overnight Muscle Protein Synthesis Rates. *Med Sci Sports Exerc* 48: 2517-2525, 2016.
33. **Vaile J, Halson S, Gill N, and Dawson B**. Effect of hydrotherapy on recovery from fatigue. *Int J Sports Med* 29: 539-544, 2008.
34. **Vaile J, Halson S, Gill N, and Dawson B**. Effect of hydrotherapy on the signs and symptoms of delayed onset muscle soreness. *Eur J Appl Physiol* 102: 447-455, 2008.
35. **Versey NG, Halson SL, and Dawson BT**. Water immersion recovery for athletes: effect on exercise performance and practical recommendations. *Sports Med* 43: 1101-1130, 2013.
36. **Viitasalo JT, Niemela K, Kaappola R, Korjus T, Levola M, Mononen HV, Rusko HK, and Takala TE**. Warm underwater water-jet massage improves recovery from intense physical exercise. *Eur J Appl Physiol Occup Physiol* 71: 431-438, 1995.
37. **Witard OC, Jackman SR, Breen L, Smith K, Selby A, and Tipton KD**. Myofibrillar muscle protein synthesis rates subsequent to a meal in response to increasing doses of whey protein at rest and after resistance exercise. *Am J Clin Nutr* 99: 86-95, 2014.
38. **Yoshihara T, Naito H, Kakigi R, Ichinoseki-Sekine N, Ogura Y, Sugiura T, and Katamoto S**. Heat stress activates the Akt/mTOR signalling pathway in rat skeletal muscle. *Acta Physiol (Oxf)* 207: 416-426, 2013.
39. **Zak RB, Hassenstab BM, Zuehlke LK, Heesch MWS, Shute RJ, Laursen TL, LaSalle DT, and Slivka DR**. Impact of local heating and cooling on skeletal muscle transcriptional response related to myogenesis and proteolysis. *Eur J Appl Physiol* 118: 101-109, 2018.

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

Figure legends

Figure 1. Schematic representation of the experimental design. Participants performed leg press and leg extension exercise followed by water immersion of both legs (1 leg was immersed in 46°C; the other leg was immersed in thermoneutral water at 30°C) for a total duration of 20 min. After muscle temperature measurements and collection of muscle biopsies from both legs, participants ingested 20 g intrinsically labeled milk protein with 45 g of carbohydrates. Thereafter at t = 120 and 300 min during post-exercise recovery, muscle temperature measurements and muscle biopsies were collected from both legs. Blood samples, skin and core temperature measurements were collected throughout the infusion day.

Figure 2. Skin temperature (A) during the entire experimental protocol and muscle temperature (B) immediately after water immersion (t = 0 min) and t = 120 and 300 min after drink ingestion in CON and HWI in healthy, young men (*n*=12). Values represent means±SEM. Data for skin and muscle temperature were analyzed with a two-way repeated measures ANOVA (time X treatment) with Bonferonni post hoc testing applied to locate differences. For skin and muscle temperature, time X treatment interaction, $P<0.001$. *, significantly different ($P<0.05$) from CON. HWI, Hot-water immersion (46°C) leg. CON, Thermoneutral water immersion (30°C) leg.

Figure 3. Plasma L-[ring-$^{2}H_{5}$]-phenylalanine (A), L-[1-^{13}C]-leucine (B), and L-[1-^{13}C]-phenylalanine (C) enrichments in MPE in healthy, young men (*n*=12). The dotted line represents the ingestion of 20 g intrinsically labeled milk protein with 45 g carbohydrate (at t = 0 min). Values represent means±SEM. Data were analyzed with a one-way repeated measures ANOVA with Bonferonni post hoc testing applied to locate differences. For all panels: time effect, $P<0.001$.

Figure 4. Exogenous phenylalanine rate of appearance (R_a) in healthy, young men (*n*=12). The dotted line represents the ingestion of 20 g intrinsically labeled milk protein with 45 g carbohydrate (at t = 0 min). Values represent means±SEM. Data were analyzed with a one-way repeated measures ANOVA with Bonferonni post hoc testing applied to locate differences. Time effect, $P<0.001$.

Figure 5. Myofibrillar protein FSRs as calculated with L-[ring-$^{2}H_{5}$]-phenylalanine (A) or L-[1-^{13}C]-leucine (B) as tracer during 5 h of post-exercise recovery with the ingestion of 20 g intrinsically labeled milk protein with 45 g carbohydrate in healthy, young men (*n*=12). Bars are means and dots represent individual values. Data were

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

analyzed with a paired Student's *t* test. FSR, fractional synthetic rate. HWI, Hot-water immersion (46°C) leg. CON, Thermoneutral water immersion (30°C) leg.

Figure 6. L-[1-^{13}C]-phenylalanine incorporation into myofibrillar protein after drink ingestion with intrinsically labeled L-[1-^{13}C]-phenylalanine in healthy, young men (*n*=12). Bars are means and dots represent individual values. Data were analyzed with a two-way repeated measures ANOVA (time X treatment). MPE, mole percent excess. HWI, Hot-water immersion (46°C) leg. CON, Thermoneutral water immersion (30°C) leg.

Figure 7. Skeletal muscle phosphorylation status (ratio of phosphorylated protein to total protein) of mTOR (Ser2448) (A), p70S6K (Thr389) (B), p70S6K (Thr421/Ser424) (C), rpS6 (Ser240/244) (D), rpS6 (Ser235/236) (E) and 4E-BP1 (Thr37/46) (F) immediately after post-exercise water immersion (t = 0 h) and after ingestion of 20 g intrinsically labeled milk protein with 45 g of carbohydrate (t = 2 and 5 h) in healthy, young men (*n*=12). Bars are means and dots represent individual values. Data were analyzed with a repeated measures ANOVA (time X treatment) with Bonferonni post hoc testing applied to locate differences. (A) No significant effects. (B) Significant time effect, treatment effect, and time X treatment interaction ($P<0.05$). (C) Significant time ($P<0.001$) effect. (D) No significant effects. (E) Significant time ($P=0.040$) effect. (F) No significant effects. *, significantly different ($P<0.05$) from CON. HWI, Hot-water immersion (46°C) leg. CON, Thermoneutral water immersion (30°C) leg.

Figure 8. Skeletal muscle heat shock protein content (expressed relative to ponceau S staining) of HSP27 (A) and HSP70 (B) immediately after post-exercise water immersion (t = 0 h) and after ingestion of 20 g intrinsically labeled milk protein with 45 g of carbohydrate (t = 2 and 5 h) in healthy, young men (*n*=12). Bars are means and dots represent individual values. Data were analyzed with a repeated measures ANOVA (time X treatment). No significant effects over time or between treatments were observed. HWI, Hot-water immersion (46°C) leg. CON, Thermoneutral water immersion (30°C) leg.

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

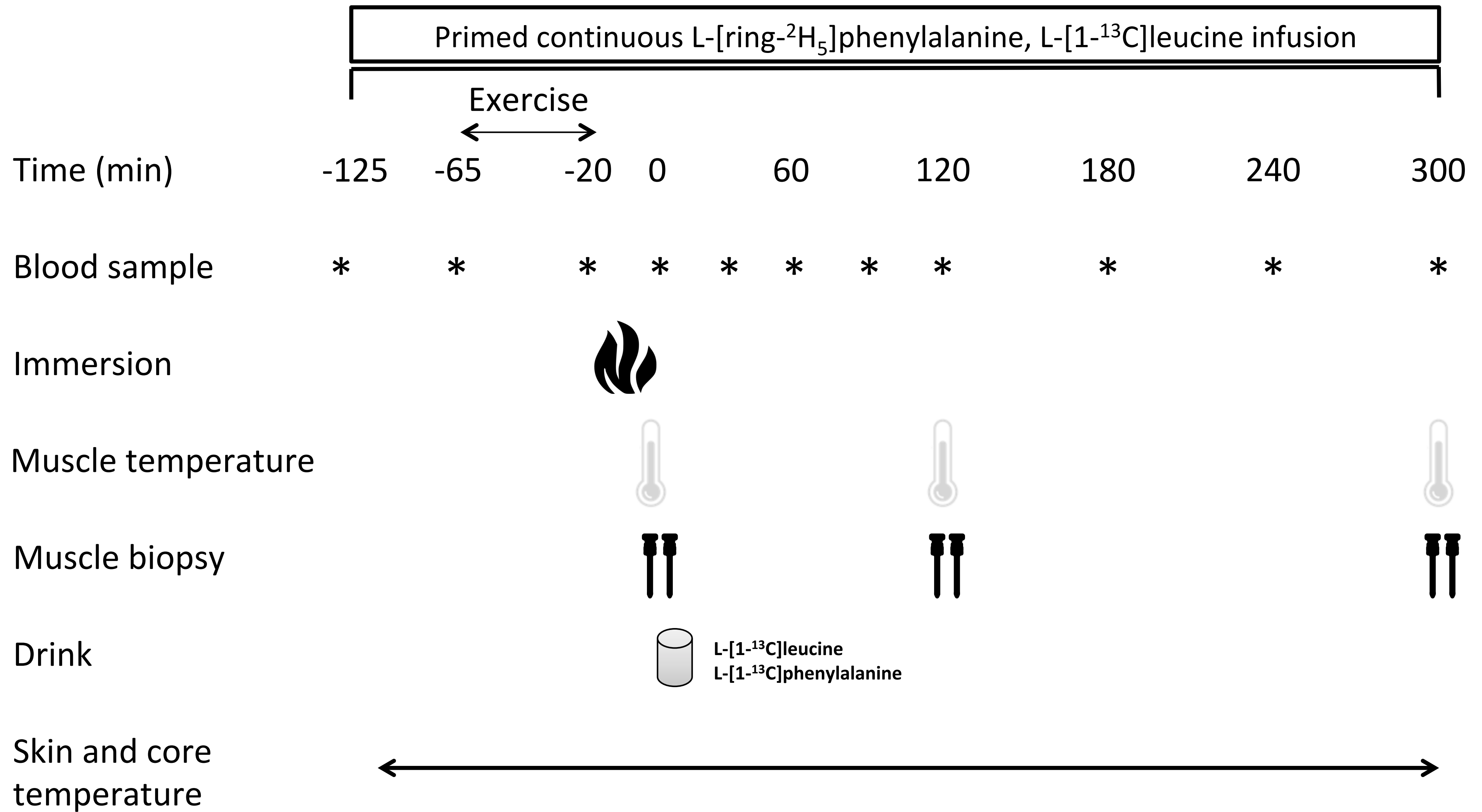
Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

Table 1. Subjects' characteristics

	Subjects (*n*= 12)
Age (y)	23 ± 1
Body mass (kg)	77.6 ± 1.8
Length (m)	1.83 ± 0.01
BMI (kg/m^2)	23.3 ± 0.6
LBM (kg)	62.1 ± 1.5
CON leg lean mass (kg)	10.7 ± 0.3
HWI leg lean mass (kg)	10.7 ± 0.3
Whole body fat mass (kg)	13.0 ± 1.1
CON leg fat mass (kg)	2.6 ± 0.3
HWI leg fat mass (kg)	2.6 ± 0.3
Whole body fat mass (%)	16.6 ± 1.2
Leg press 1RM (kg)	287 ± 22
Leg extension 1RM (kg)	127 ± 6

Values are expressed as means±SEM. BMI, body mass index; LBM, lean body mass; 1RM, one repetition maximum. HWI, Hot-water immersion (46°C) leg. CON, Thermoneutral water immersion (30°C) leg.

Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.



Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

A

Skin temperature (°C)

50
45
40
35
30
25
20
15
10
5
0

*
*
*

HWI
CON

-120 -60 0 60 120 180 240 300

B

Muscle temperature (°C)

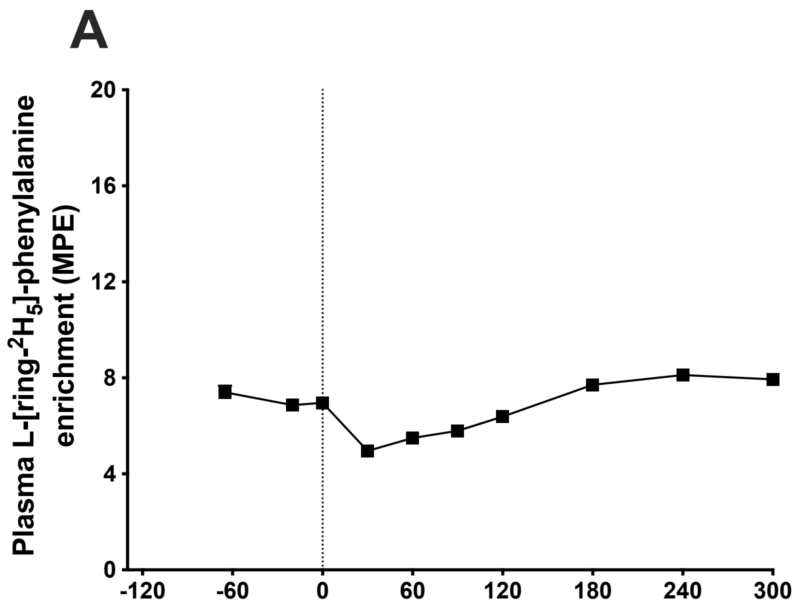
40
38
36
34
32
30

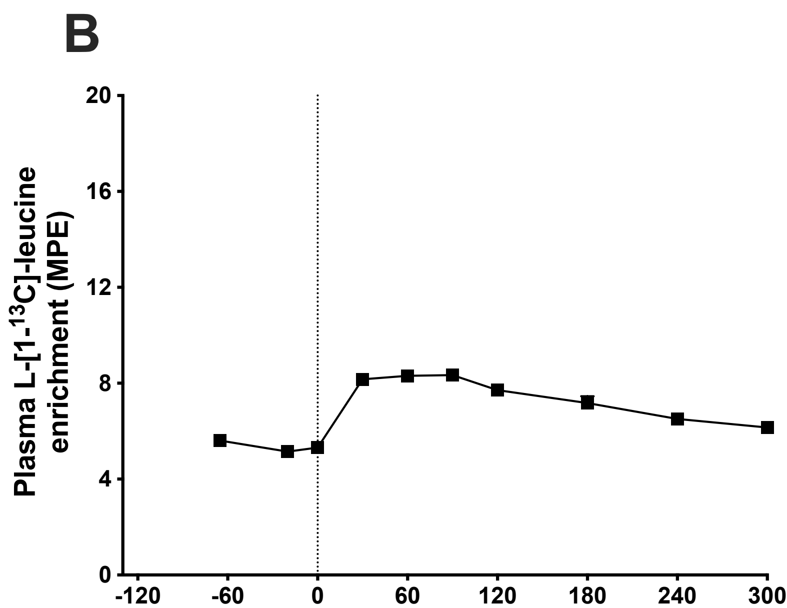
*

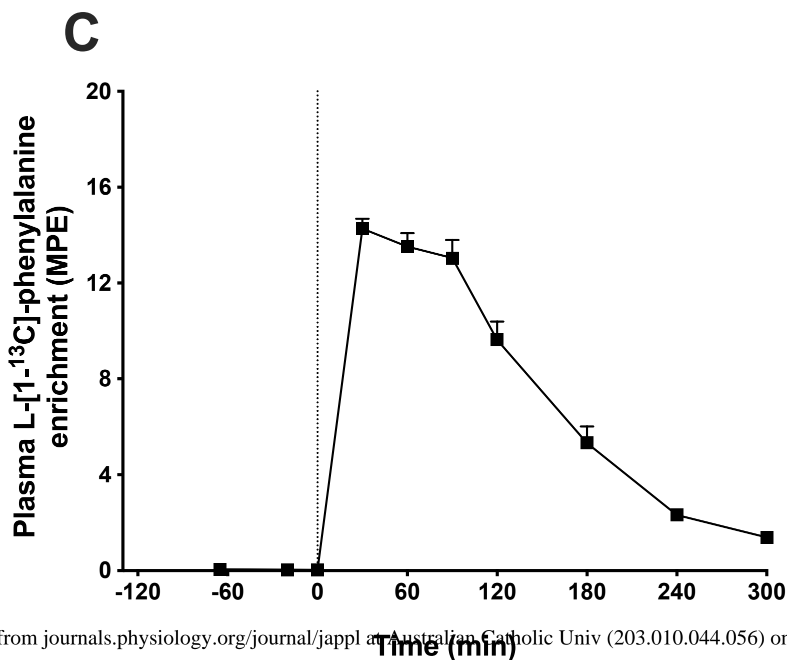
-120 -60 0 60 120 180 240 300

Time (min)

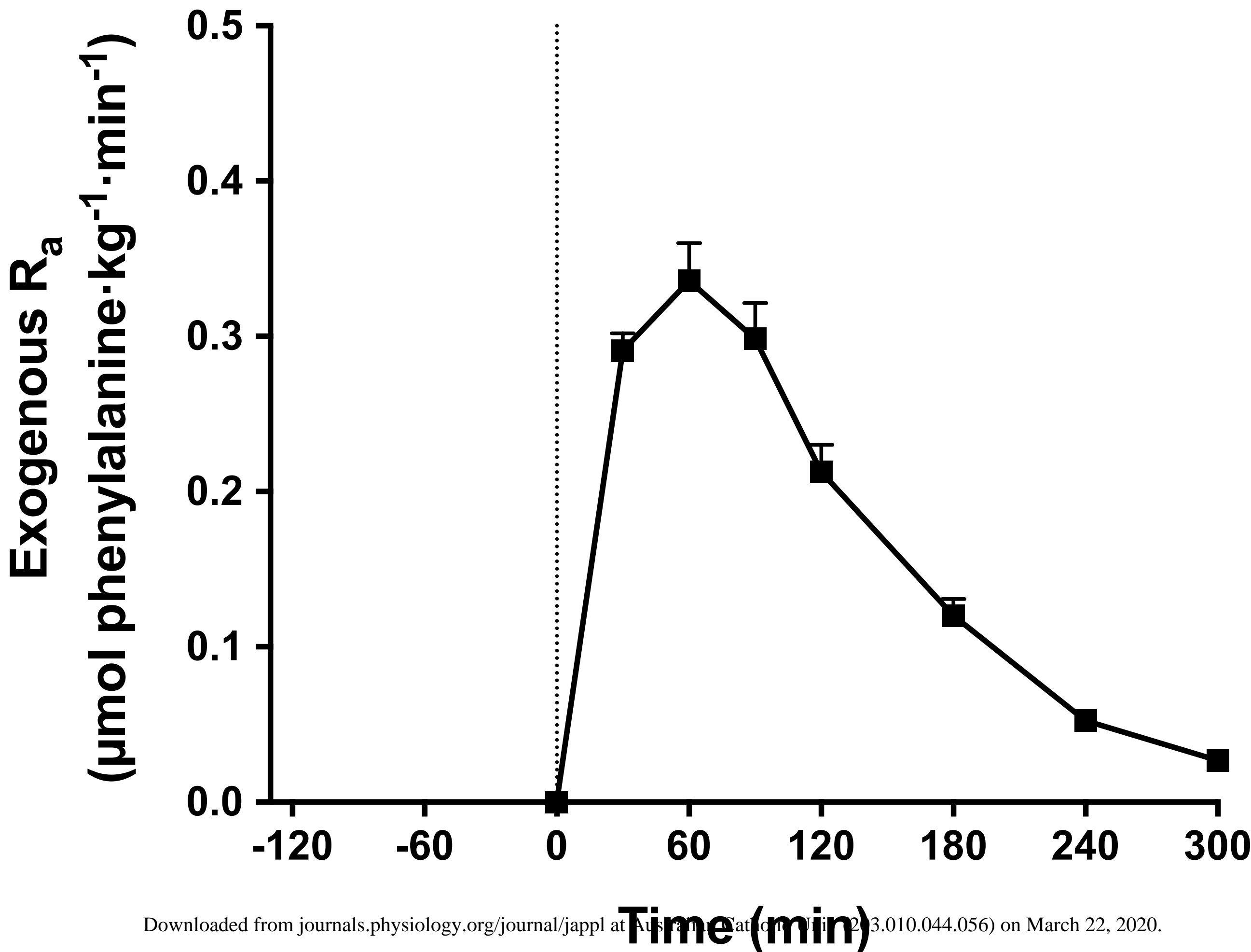
Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.







d from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on Marc



Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

A

L-[ring-$^2H_5$]-phenylalanine

CON
HWI

Myofibrillar protein FSR (%/h)

0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00

0-2 h
0-5 h

B

L-[1-^{13}C]-leucine

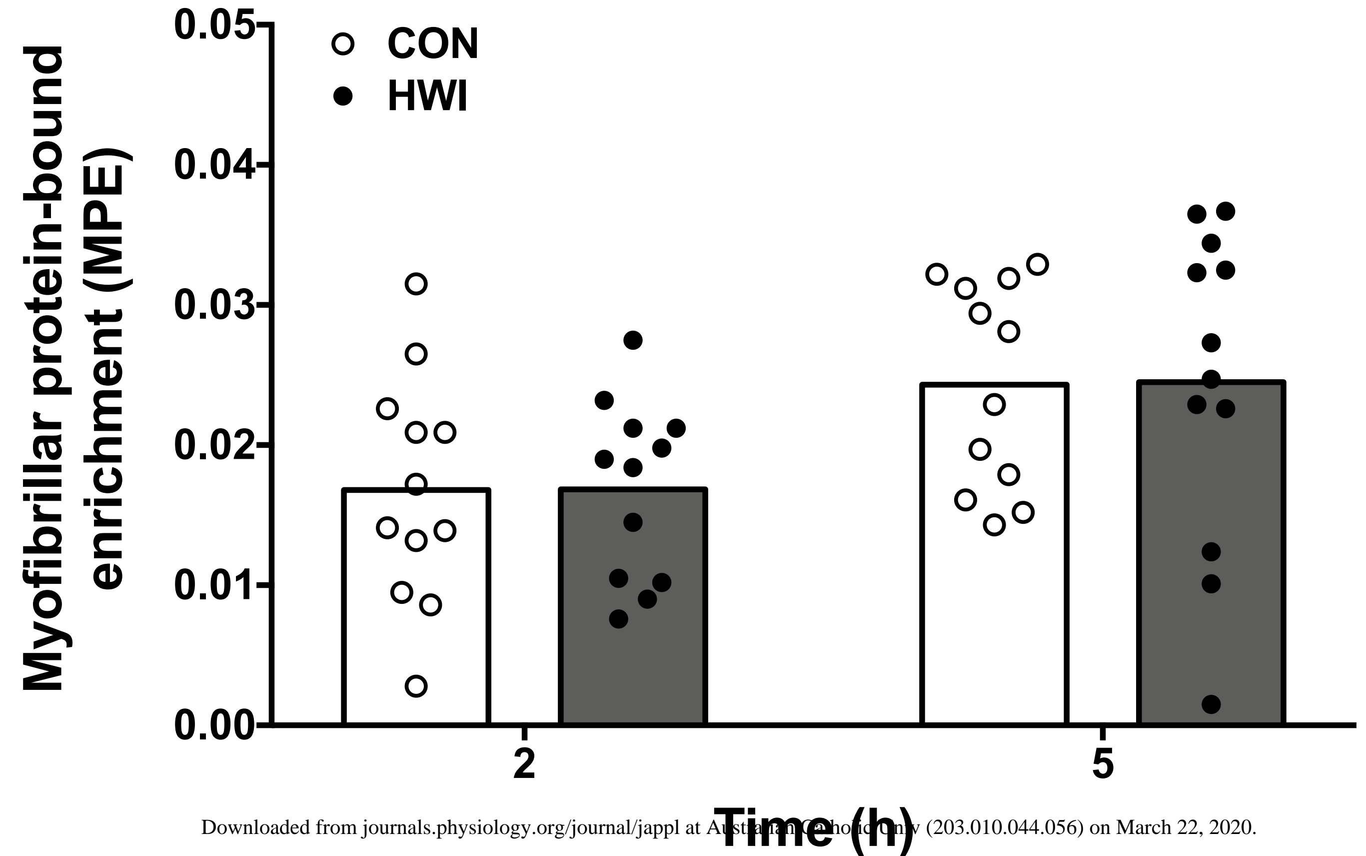
Myofibrillar protein FSR (%/h)

0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00

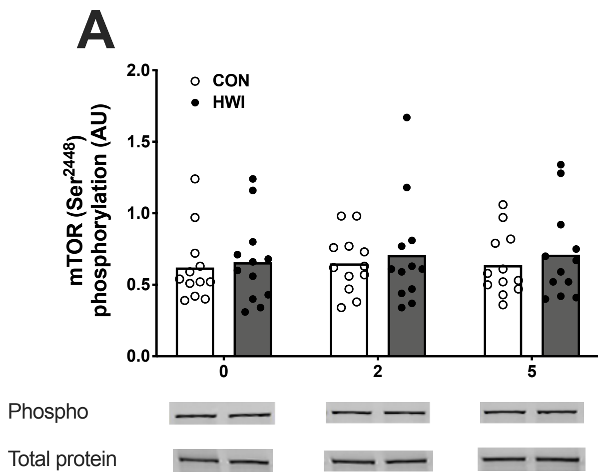
0-2 h
0-5 h

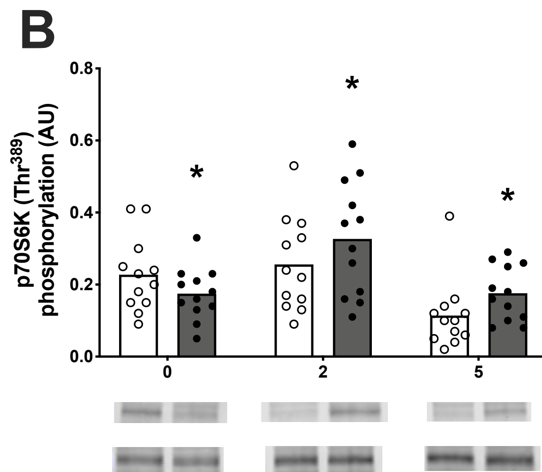
Time

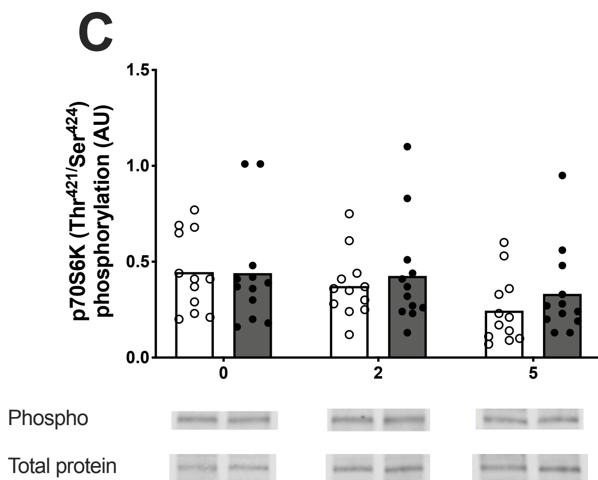
Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

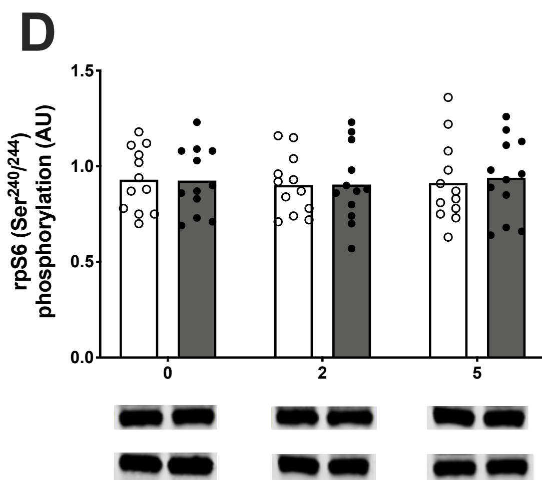


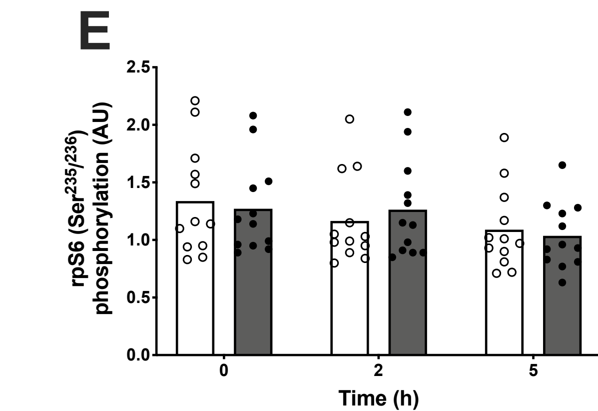
Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.

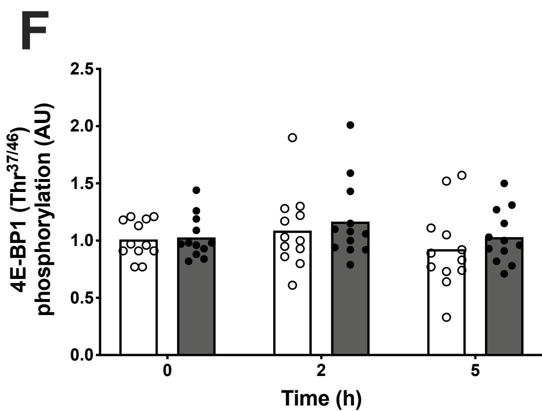




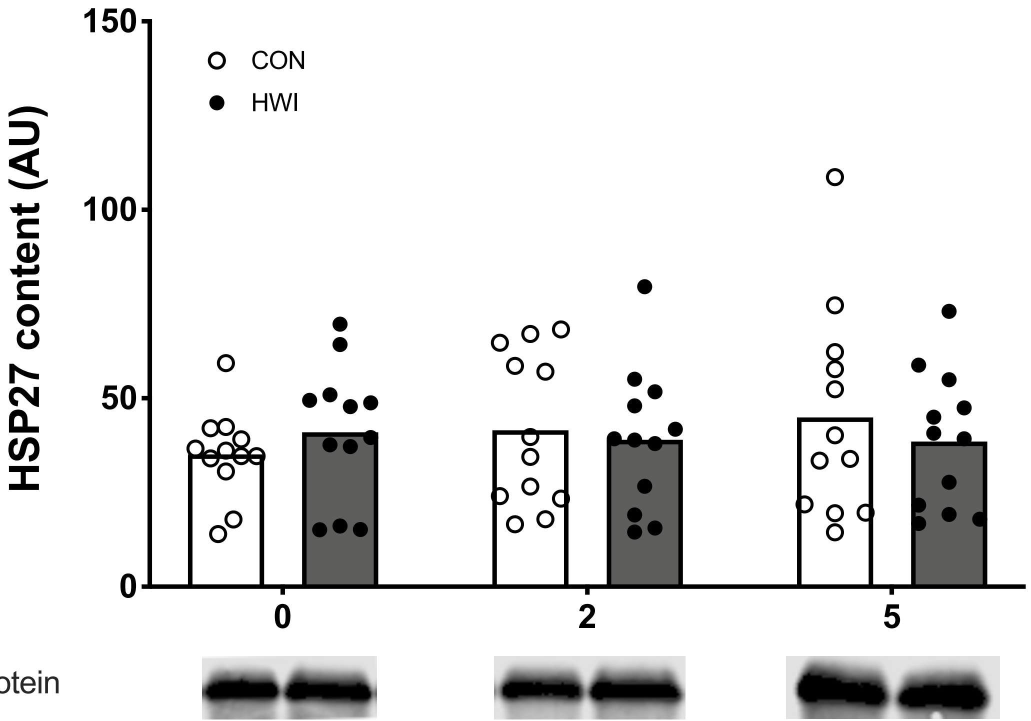


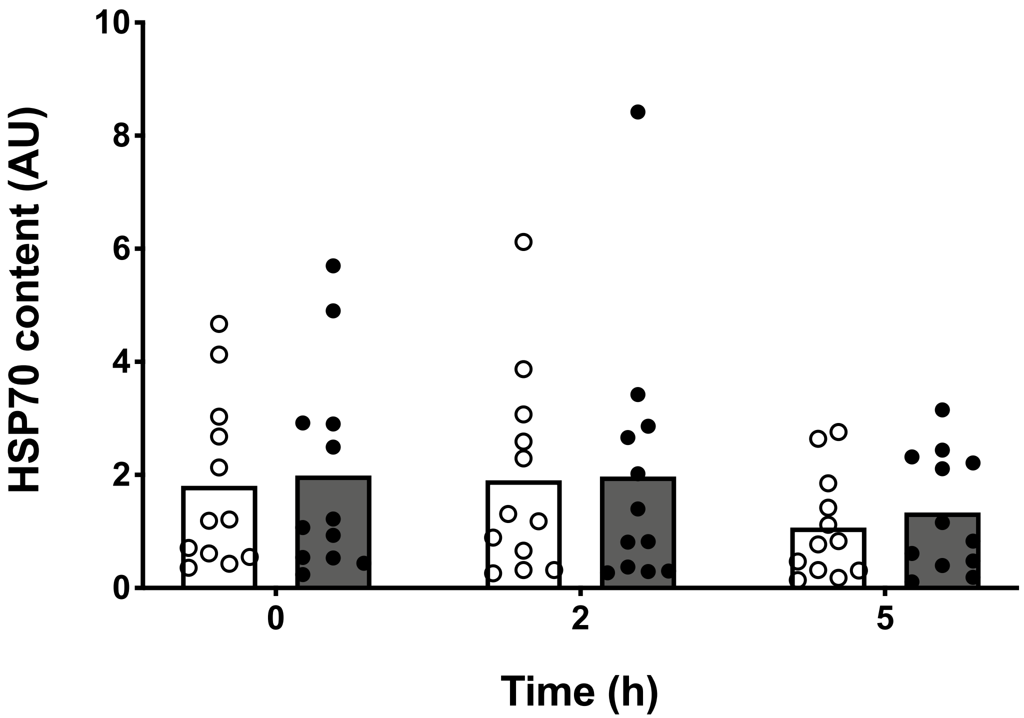






Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.





Downloaded from journals.physiology.org/journal/jappl at Australian Catholic Univ (203.010.044.056) on March 22, 2020.