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:

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

Cas J. Fuchs¹, Joey S.J. Smeets¹, Joan M. Senden¹, Antoine H. Zorenc¹, Joy P. B. Goessens¹, Wouter D. van Marken Lichtenbelt², Lex B. Verdijk¹ and Luc J.C. van Loon¹*

Department of ¹Human Biology, ²Nutrition 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
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>CON</td>
<td>Thermoneutral water immersion</td>
</tr>
<tr>
<td>DEXA</td>
<td>Dual-energy x-ray absorptiometry</td>
</tr>
<tr>
<td>FSR</td>
<td>Fractional synthetic rate</td>
</tr>
<tr>
<td>GC-C-IRMS</td>
<td>Gas chromatograph-combustion-isotope ratio mass spectrometer</td>
</tr>
<tr>
<td>GC-P-IRMS</td>
<td>Gas chromatograph-pyrolysis-isotope ratio mass spectrometer</td>
</tr>
<tr>
<td>HSP</td>
<td>Heat Shock Protein</td>
</tr>
<tr>
<td>HWI</td>
<td>Hot-water immersion</td>
</tr>
<tr>
<td>MPE</td>
<td>Mole percent excess</td>
</tr>
<tr>
<td>mTOR</td>
<td>Mammalian target of rapamycin</td>
</tr>
<tr>
<td>p70S6K</td>
<td>p70 ribosomal protein S6 kinase</td>
</tr>
<tr>
<td>rpS6</td>
<td>Ribosomal protein S6</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard error of the mean</td>
</tr>
<tr>
<td>vs</td>
<td>Versus</td>
</tr>
</tbody>
</table>
ABSTRACT

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

Methods: Twelve healthy, male adults (age: 23±1 y) performed a single bout of resistance-type exercise followed by 20 min of water immersion of both legs. One leg was immersed in hot water (46°C: HWI) while the other leg was immersed in thermoneutral water (30°C: CON). After water immersion, a beverage was ingested containing 20 g intrinsically L-[1-\(^{13}\)C]-phenylalanine and L-[1-\(^{13}\)C]-leucine labelled milk protein with 45 g of carbohydrates. In addition, primed continuous L-[ring-\(^{2}\)H\(_5\)]-phenylalanine and L-[1-\(^{13}\)C]-leucine infusions were applied, with frequent collection of blood and muscle samples to assess myofibrillar protein synthesis rates in vivo over a 5 h recovery period.

Results: Muscle temperature immediately after water immersion was higher in the HWI compared to the CON leg (37.5±0.1 vs 35.2±0.2°C; \(P<0.001\)). Incorporation of dietary protein-derived L-[1-\(^{13}\)C]-phenylalanine into myofibrillar protein did not differ between the HWI and CON leg during the 5h recovery period (0.025±0.003 vs 0.024±0.002 MPE; \(P=0.953\)). Post-exercise myofibrillar protein synthesis rates did not differ between the HWI and CON leg based upon L-[\(^{1}\)C]-leucine (0.050±0.005 vs 0.049±0.002%·h\(^{-1}\); \(P=0.815\)) and L-[ring-\(^{2}\)H\(_5\)]-phenylalanine (0.048±0.002 vs 0.047±0.003%·h\(^{-1}\); \(P=0.877\)), respectively.

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

This study was registered at trialregister.nl as NL6221.
Keywords: heat stress; heating; hydrotherapy; recovery; adaptation; stable isotope tracers; resistance exercise.

New and noteworthy:

This is the first study to assess the effect of post-exercise hot-water immersion on post-prandial myofibrillar protein synthesis rates and the incorporation of dietary protein-derived amino acids into muscle protein. We observed that hot-water immersion during recovery from a single bout of resistance-type exercise does not further increase myofibrillar protein synthesis rates or augment the post-prandial incorporation of dietary protein-derived amino acids in muscle throughout 5 hours of post-exercise recovery.
INTRODUCTION

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

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

Increasing muscle temperature during recovery from exercise may increase enzyme activity and increase skeletal muscle blood flow and, as such, augment nutrient delivery, uptake, and/or subsequent metabolism. In agreement, a previous study in rats has shown that 30 min of HWI increases phosphorylation of Akt and p70S6K, which are considered important mediators of muscle protein synthesis and hypertrophy (38). Furthermore, Kakigi et al.
demonstrated that heat stress (applied 20 min before and during isokinetic knee extension
exercise) enhanced resistance exercise induced mTOR signaling in human skeletal muscle
(17). So far, there are no studies that have assessed the impact of hot-water immersion on
muscle protein synthesis rates. We hypothesized that hot-water immersion after a single bout
of resistance-type exercise increases post-prandial muscle protein synthesis rates during 5
hours of post-exercise recovery in young, healthy males.

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

Subjects

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

General study design

Each subject participated in one experiment, in which the effect of post-exercise hot-water immersion on post-prandial muscle protein synthesis was studied after the ingestion of 20 g intrinsically L-[1-13C]-phenylalanine and L-[1-13C]-leucine labeled milk protein. The current study was designed to determine the benefits of hot-water immersion to increase post-prandial muscle protein synthesis rates during recovery from a single bout of resistance-type exercise in a setting most relevant for athletes. At the start of the experiment, primed continuous L-[ring-2H₅]-phenylalanine and L-[1-13C]-leucine infusions were applied together with repeated blood sampling during the experimental day. After 1 h of rest, participants performed ~45 min of resistance-type exercise training, after which they immersed both legs in water for 20 min
(1 leg at 46°C and 1 leg at 30°C). Thereafter, muscle biopsies were collected from both legs, prior to ingesting 20 g intrinsically L-[1-\textsuperscript{13}C]-phenylalanine and L-[1-\textsuperscript{13}C]-leucine labeled milk protein with 45 g of glucose (polymers). Subsequent muscle biopsies were taken from the \textit{M. vastus lateralis} of both legs after 2 and 5 hours of post-exercise recovery.

\textit{Pretesting}

All subjects participated in a screening session, which was performed at least 1 week prior to the experiment. First, subjects’ body weight and height were measured as well as body composition by dual-energy X-ray absorptiometry (DEXA, Discovery A; Hologic, Bedford, MA). The system’s software package (Hologic-Apex software version 4.5.3 with viewer software Hologic Physician’s viewer, version 7.1) was used to determine whole body and regional lean and fat mass. Subsequently, thigh skinfold thickness was measured using Harpenden skinfold calipers (Baty International, Burgess Hill, England) and divided by 2 to determine the thickness of the thigh subcutaneous fat layer over each participants’ \textit{M. vastus lateralis}. In addition, participants were familiarized with the exercise equipment and performed maximum strength tests as determined by their one repetition maximum (1RM) for leg press and knee extension exercise (\textbf{Table 1}). Subjects first performed a 5 min cycling exercise warm-up at 100 W. Thereafter, for both leg press and extension, subjects performed 2 sets with 10 submaximal or warm-up repetitions to become familiarized with the equipment and to have lifting technique critiqued and corrected. Subjects then performed sets at progressively increasing loads until failing to complete a valid repetition, judged by their inability to complete the full range of motion for an exercise. A 2 min resting period between subsequent attempts was allowed. Finally, participants were familiarized with the water immersion procedure. One leg was immersed in hot water (46°C: HWI) while the other leg was immersed in thermoneutral water (30°C: CON) for a total duration of 20 min. Both legs
were immersed to the level of the gluteal fold. The limb heated (HWI) was randomized
tween subjects’ dominant and nondominant leg. For the water immersion setup, two water
tanks were used that were completely open at the top and contained a tap at the bottom. This
allowed us to set and maintain water temperature (before the 20 min water immersion
procedure) by adding (hot) water from the top and remove water from below the tank. During
water immersion, temperature was monitored and kept constant and still at 46°C in HWI and
30°C in CON.

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

Experimental protocol
The experimental protocol is outlined in Figure 1. Each subject participated in one
experiment. At the start of the experimental day at 7:30 AM, following an overnight fast,
subjects reported to the laboratory. First, a telemetric pill (CorTemp HT150002; HQ Inc.) was
swallowed with tepid water for continuous measurements of body core temperature until the
end of the experiment. In addition, ibuttons (Maxim Integrated Products) were attached to the
skin on the left and right upper thigh (~10 cm above the patella) for continuous measurements
of skin temperature during the entire trial. Thereafter, a Teflon catheter was inserted into an
antecubital vein for intravenous isotope tracer infusion and a second catheter was inserted in a
dorsal hand vein of the contralateral arm, which was subsequently placed in a hot-box (60°C)
for arterialized blood sampling. After baseline blood sample collection (t = -125 min), the
plasma phenylalanine and leucine pools were primed with a single intravenous dose of L-
[ring-2H5]-phenylalanine (2 μmol·kg\(^{-1}\)) and L-[1-\(^{13}\)C]-leucine (4 μmol·kg\(^{-1}\)), respectively. Subsequently, an intravenous infusion of L-[ring-2H5]-phenylalanine (infusion rate of 0.050 μmol·kg\(^{-1}\)·min\(^{-1}\)) and L-[1-\(^{13}\)C]-leucine (0.100 μmol·kg\(^{-1}\)·min\(^{-1}\)) was initiated and maintained until the end of the trial using a calibrated IVAC 598 pump (San Diego, USA). After 1 h of supine rest, another arterialized blood sample (t = -65 min) was obtained. Subsequently, the participants performed a resistance-type exercise session. After a 5 min warm-up on a cycle ergometer at self selected intensity (~114 W), the subjects performed 4 sets of 10 repetitions (at 80% 1RM) on both the leg press and knee extension exercise. After completion of the exercise bout (t = -20 min), another arterialized blood sample was obtained before the participants immersed both legs in water for a total duration of 20 min. One leg was immersed in hot water (46°C: HWI) while their contralateral leg was immersed in thermoneutral water (30°C: CON). Immediately after water immersion, another arterialized blood sample was obtained together with muscle temperature (MT23/5 probe; BAT-10, Physitemp, New Jersey, USA) measurements and muscle biopsies from both legs. The muscle temperature probe was inserted into the biopsy incision before each biopsy was collected from both legs. Immediately afterwards, the subjects ingested 20 g intrinsically L-[1-\(^{13}\)C]-phenylalanine and L-[1-\(^{13}\)C]-leucine labeled milk protein together with 45 g of carbohydrates (glucose polymers) at t = 0 min. Thereafter, repeated blood samples (t = 30, 60, 90, 120, 180, 240, and 300 min) were obtained together with muscle temperature measurements and biopsies from both legs at t = 120 and 300 min. The muscle biopsies were collected from the middle region of the \textit{M. vastus lateralis} (~15 cm above the patella) with a Bergström needle under local anesthesia (1). The first two biopsies in each leg (at t = 0 and 120 min) were taken from separate incisions. The difference between the separate incisions was ~3 cm proximal from the previous incision. The last biopsy (t = 300 min) was collected from the same incision as the
biopsy at t = 120 min. The biopsy at t = 300 min was collected with the needle inserted in a proximal direction. This method ensured that all biopsy sites were separated by at least 3 cm to minimize any artifact related to inflammation resulting from multiple biopsies. All biopsy samples were freed from any visible adipose tissue and blood, immediately frozen in liquid nitrogen, and stored at –80°C until subsequent analysis.

**Beverage**

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

**Preparation of tracer**

The stable isotope tracers L-[ring-2H5]-phenylalanine and L-[1-13C]-leucine were purchased from Cambridge Isotopes (Andover, MA) and dissolved in 0.9 % saline before infusion (Apotheek A15, Gorinchem, the Netherlands).

**Plasma and muscle analysis**

Blood samples (10 mL) were collected in EDTA containing tubes and centrifuged at 1000 g and 4°C for 10 min. Aliquots of plasma were frozen in liquid nitrogen and stored at -80°C until analysis. Plasma glucose and insulin concentrations were analyzed using commercially available kits (ref. no. A11A01667, Glucose HK CP, ABX Diagnostics, Montpellier, France; and ref. no. HI-14K, Millipore, Billerica, MA, respectively). Plasma amino acid concentrations and enrichments were determined by gas chromatography-mass spectrometry.
analysis (GC-MS; Agilent 7890A GC/5975C; MSD, Wilmington, Delaware, USA). Specifically, internal standards of [U-\(^{13}\)C\(_6\)]-leucine, [U-\(^{13}\)C\(_9\)\(^{15}\)N]-phenylalanine, and [U-\(^{13}\)C\(_9\)\(^{15}\)N]-tyrosine were added to the plasma samples. Plasma samples were deproteinized with dry 5-sulfosalicylic acid. Free amino acids were purified using cation exchange chromatography (AG 50W-X8 resin, mesh size: 100–200 µm, ionic form: hydrogen; Bio-Rad Laboratories, Hercules, CA). The purified amino acids were converted into tert-butylidimethylsilyl (tert-BDMS) derivatives with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) before analysis by GC-MS. The amino acid concentrations and enrichments were determined using selective ion monitoring at mass/charge (m/z) 302 and 308 for unlabeled and [U-\(^{13}\)C\(_6\)] labeled-leucine, 336 and 346 for unlabeled and [U-\(^{13}\)C\(_9\)\(^{15}\)N] labeled phenylalanine respectively. The plasma leucine and phenylalanine \(^{13}\)C and \(^2\)H enrichments were determined at m/z 302 and 303 for unlabeled and labeled (1-\(^{13}\)C and ring-\(^2\)H\(_5\)) phenylalanine, respectively. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation.

Myofibrillar protein enriched fractions were extracted from ~60 mg of wet muscle tissue by hand-homogenizing on ice using a pestle in a standard extraction buffer (7 µL·mg\(^{-1}\)). The samples were spun at 700 g and 4°C for 15 min. The pellet was washed with 500 µL ddH\(_2\)O and centrifuged at 700 g and 4°C for 10 min. The myofibrillar protein was solubilized by adding 1 mL of 0.3 M NaOH and heating at 50°C for 30 min with vortex mixing every 10 min. Samples were centrifuged at 9500 g and 4°C for 5 min, the supernatant containing the myofibrillar proteins was collected and the collagen pellet was discarded. Myofibrillar proteins were precipitated by the addition of 1 mL of 1 M perchloric acid (PCA) and spinning at 700 g and 4°C for 10 min. The myofibrillar protein was washed twice with 70% ethanol...
and hydrolyzed overnight in 2 mL of 6 M HCL at 110°C. The free amino acids from the
hydrolyzed myofibrillar protein pellet were dried under a nitrogen stream while being heated
to 110°C. The free amino acids were then dissolved in 25% acetic acid solution, passed over
cation exchange AG 50W-X8 resin columns (mesh size: 100-200, ionic form: hydrogen; Bio-
Rad Laboratories, Hercules, CA), and eluted with 2 M NH₄OH. The purified amino acids (L-
[1-¹³C]-phenylalanine and L-[1-¹³C]-leucine enrichments) were analyzed by GC-C-IRMS
analysis. To determine myofibrillar protein L-[1-¹³C]-phenylalanine and L-[1-¹³C]-leucine
enrichments by GC-C-IRMS analysis, the purified amino acids were converted into N-
ethoxycarbonyl ethyl ester derivatives with ethyl chloroformate (ECF). The derivatives were
then measured by GC-C-IRMS (Finnigan MAT 253, Bremen, Germany) using a DB5-MS-
column (no. 122-5532; Agilent J+W scientific GC Column, GC Isolink) and monitoring of
ion masses 44, 45, and 46. For measurement of L-[ring-²H₅]-phenylalanine enrichment in the
myofibrillar protein pools, the eluate was dried, and the purified amino acids were also
derivatized into a N-ethoxycarbonyl ethyl ester. The derivatized samples were measured using
a gas chromatography-isotope ratio mass spectrometer (MAT 253; Thermo Fisher Scientific,
Bremen, Germany) equipped with a pyrolysis oven (GC-P-IRMS) using a 70-m DB-17MS
column, 5-m precolumn (No. 122–4762; Agilent), and GC-Isolink. Standard regression
curves and standards were applied to assess the linearity of the mass spectrometer and to
account for isotopic fractionation.

For western blot analysis, a portion of each muscle sample frozen for biochemical analyses
was homogenized in seven volumes Tris buffer (20 mM Tris-HCL, 5 mM EDTA, 10 mM Na-
pyrophosphate, 100 mM NaF, 2 mM NaVO₃, 1 % Nonident P-40; pH 7.4) supplemented
with the following protease and phosphatase inhibitors: Aprotinin 10 μg/mL, Leupeptin 10
μg/mL, Benzamidin 3 mM and PMSF 1 mM. After homogenization, each muscle extract was
centrifuged for 10 min at 10,000 g (4°C) and sample buffer was added to the supernatant to
final concentrations of 60 mM Tris, 10 % glycerol, 20 mg/mL SDS, 0.1 mM DTT, 20 µg/mL bromophenol blue. The supernatant was then heated for 5 min at 100ºC and immediately placed on ice. Immediately before analyses, the muscle extraction sample was warmed to 50ºC and centrifuged for 1 min at 1000 g (RT). The total amount of sample loaded on the gel was based on protein content. After a Bradford assay, 30 µg protein were loaded in each lane. With the exception of mTOR, protein samples were run on a Criterion Precast TGX 4-20 % gel (Biorad Order No. 567-1094) ±90 min at 150 V (constant voltage) and transferred onto a Trans-blot Turbo 0.2 µm nitrocellulose membrane (Biorad Order No. 170-4159) in 7 min at 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 Criterion Precast XT 3-8 % Tris-acetate gel (Biorad order No. 345-0130). Specific proteins were detected by overnight incubation at 4ºC on a shaker with specific antibodies in 50 % in PBS Odyssey blocking buffer (Li-Cor Biosciences Part No. 927-40000) after blocking for 60 min at RT in 50 % in PBS Odyssey blocking buffer. Polyclonal primary phospho-specific antibodies, anti-phospho-mTOR (Ser 2448), anti-phospho-S6K1 (Thr 389), anti-phospho-S6K1 (Thr 421/Ser 424), anti-phospho-rpS6 (Ser 240/Ser 244), anti-phospho-rpS6 (Ser 235/Ser 236), and anti-phospho-4E-BP1 (Thr 37/46) were purchased from Cell Signaling Technology (Danvers, MA, USA). In addition, anti-mTOR, anti-S6K1, anti-RS6, anti-4E-BP1, anti-HSP27, and anti-HSP70 were also purchased from Cell Signaling Technology (Danvers, MA, USA). Following incubation, membranes were washed three times 10 min in 0.1 % PBS-Tween 20 and once for 10 min in PBS. Next, samples were incubated on a shaker (1 h at RT) with infrared secondary antibodies, donkey anti-rabbit IRDYE 800 (Rockland, Cat. No. 611-732-127, dilution 1:10000) and donkey anti-mouse IRDYE 800CW (Li-Cor, Cat. No. 626-32212, dilution 1:10000) dissolved in 50 % PBS Odyssey blocking buffer. After a final wash step (3 x 10 min) in 0.1 % Tween20-PBS and once 10 min in PBS, protein quantification was performed by scanning on an Odyssey Infrared Imaging System (LI-COR Biotechnology,
Lincoln, NE). All measurements for phospho-specific and total proteins were performed on the same membrane. Ponceau S staining was applied to assess and confirm equal total protein loading. Phosphorylation status as a proxy of activation of the signaling proteins was expressed relative to the total amount of each protein. For heat shock proteins (HSP27 and HSP70) total protein content was expressed relative to ponceau S staining to correct for the total amount of protein loaded.

**Calculations**

Ingestion of L-[1-\(^{13}\)C]-phenylalanine labeled protein, intravenous infusion of L-[\(\text{ring-}^{2}\text{H}_3\)]-phenylalanine, and blood sample enrichment values were used to calculate total, and exogenous phenylalanine rates of appearance (\(R_a\)), and plasma availability of dietary protein-derived phenylalanine that appeared in the systemic circulation as a fraction of total amount of phenylalanine that was ingested (Phe\(_{\text{plasma}}\)). For these calculations modified Steele’s equations (in non-steady state conditions) were used (3, 6). These parameters were calculated as follows:

\[
\text{Total}\, R_a = \frac{F_{iv} \cdot [pV \cdot C(t) \cdot \frac{dE_{iv}}{dt}]}{E_{iv}(t)}
\]

(1)

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

(2)

\[
\text{Phe}_{\text{plasma}} = \left(\frac{\text{AUC}_{\text{Exo}Ra}}{\text{Phe}_{\text{prot}}}\right) \cdot 100
\]

(3)

where \(F_{iv}\) is the intravenous tracer infusion rate (\(\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}\)), \(pV\) (0.125 \(\text{L} \cdot \text{kg}^{-1}\)) is the distribution volume for phenylalanine (3). \(C(t)\) is the mean plasma phenylalanine concentration between 2 consecutive time points. \(\frac{dE_{iv}}{dt}\) represents the time-dependent
variations of plasma phenylalanine enrichment derived from the intravenous tracer and $E_{iv}(t)$ is the mean plasma phenylalanine enrichment from the intravenous tracer between 2 consecutive time points. $ExoR_a$ represents the plasma entry rate of dietary phenylalanine, $E_{po}(t)$ is the mean plasma phenylalanine enrichment for the ingested tracer, $dE_{po}/dt$ represents the time-dependent variations of plasma phenylalanine enrichment derived from the oral tracer and $E_{prot}$ is the L-[1-13C]-phenylalanine enrichment in the dietary protein. $Phe_{plasma}$ is the percentage of ingested dietary phenylalanine that becomes available in the plasma and is calculated using $Phe_{Prot}$ and $AUC_{ExoRa}$. $Phe_{Prot}$ is the amount of dietary phenylalanine ingested and $AUC_{ExoRa}$ represents the area under the curve (AUC) of $ExoR_a$, which corresponds to the amount of dietary phenylalanine that appeared in the blood over a 5 h period following ingestion.

The fractional synthesis rate (FSR) of myofibrillar protein was calculated by dividing the increment in enrichment in the product, i.e. protein-bound L-[1-13C]-leucine or L-[ring-$^2$H$_5$]-phenylalanine, by the enrichment of the respective precursor amino acid enrichments (i.e., plasma free amino acids). Weighted mean plasma L-[ring-$^2$H$_5$]-phenylalanine and L-[1-13C]-leucine enrichments were used as the preferred precursor pools to estimate myofibrillar protein fractional synthesis rates from the continuously infused L-[ring-$^2$H$_5$]-phenylalanine, and L-[1-13C]-leucine tracers. Consequently, myofibrillar FSR was calculated as follows:

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

(4)

where $E_{m2} - E_{m1}$ represents muscle protein bound L-[ring-$^2$H$_5$]-phenylalanine or L-[1-13C]-leucine. $E_{precursor}$ represent the average plasma L-[ring-$^2$H$_5$]-phenylalanine or L-[1-13C]-leucine enrichment during the tracer incorporation period. $t$ indicates the time interval (h) between biopsies.
Statistical Analysis

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

Thermoregulatory responses
Core temperature was slightly increased (from 37.0±0.1°C to 37.6±0.1°C) immediately after water immersion (time effect, \(P<0.001\)). Within one hour after water immersion, core temperature returned back to pre-immersion values (37.0±0.1°C), with no significant differences during the remainder of the post-exercise recovery period (\(P>0.05\)). Thigh skin temperatures over the entire experiment are shown in Figure 2A. A significant time X treatment interaction was observed for thigh skin temperature (\(P<0.001\)). Thigh skin temperature was significantly higher after exercise (t = -20 min) in both the HWI and CON leg compared to rest (t = -60 min) (\(P<0.001\)). Only for the HWI leg, thigh skin temperature was significantly increased immediately after water immersion by \(\sim\)10°C (\(P<0.001\)). Thigh skin temperature was significantly different between the HWI and CON legs immediately after water immersion up to 2 h after ingestion of the recovery beverage (\(P<0.05\)).

Muscle temperature of both legs after water immersion are shown in Figure 2B. Considering a \(\sim\)0.5 cm skinfold thickness, the muscle temperature probe (5 cm) was inserted in the muscle at a depth of \(\sim\)4.5 cm. A significant time X treatment interaction was observed for muscle temperature (\(P<0.001\)). Muscle temperature did not change significantly over time in the CON leg, but was significantly higher immediately after water immersion (t = 0 min) compared to t = 120 and 300 min in the HWI leg (\(P<0.001\)). After water immersion (t = 0 min), muscle temperature in the HWI leg was significantly higher (\(\sim\)2.3°C) when compared to the CON leg (\(P<0.001\)). At time points 120 min and 300 min, muscle temperature was no longer significantly different between legs.

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

During the post-absorptive period, plasma L-[ring-\( ^2 \)H\(_5 \)]-phenylalanine \((\text{Figure 3A})\) and L-[\( ^13 \)C]-leucine \((\text{Figure 3B})\) enrichments remained in a steady state. Following drink ingestion, plasma L-[ring-\( ^2 \)H\(_5 \)]-phenylalanine enrichments were significantly lower for the first 90 min, not significantly different (compared to baseline) at t = 120 and 180 min, and significantly higher at t = 240 and 300 min (time effect, \( P<0.001 \)). Plasma L-[\( ^13 \)C]-leucine enrichments increased in response to drink ingestion (time effect, \( P<0.001 \)) and remained at an elevated steady state of ~6.0-8.0 MPE for the duration of the 5 h post-prandial period. Following drink ingestion, plasma L-[\( ^13 \)C]-phenylalanine enrichments increased rapidly (time effect, \( P<0.001 \)) from 0 to 14.3±0.4 MPE after 30 min and declined thereafter, albeit remaining elevated above fasting levels for the remainder of the post-prandial period \((\text{Figure 3C})\).

Ingestion of the 20 g intrinsically labeled milk protein resulted in a rapid rise in exogenous phenylalanine appearance rates (time effect, \( P<0.001 \); \textbf{Figure 4}) and this remained
significantly elevated over the entire post-prandial recovery period compared to baseline (t = 0 min). Over the entire 5 h period, 14.7±0.3 g (74±2%) of the ingested protein-derived amino acids had been released into the circulation.

Muscle tracer analysis

Myofibrillar protein FSR based on intravenous L-[ring-2H5]-phenylalanine infusion with plasma L-[ring-2H5]-phenylalanine enrichments as precursor pool (Figure 5A) or intravenous L-[1-13C]-leucine infusion combined with intrinsically L-[1-13C]-leucine labeled protein ingestion with plasma L-[1-13C]-leucine enrichments as precursor pool (Figure 5B) are displayed in Figure 5. Myofibrillar protein FSR as calculated over 0-2 h did not differ between the HWI and CON leg based on the L-[ring-2H5]-phenylalanine tracer (0.065±0.002 vs 0.066±0.004%·h⁻¹, respectively; P=0.704) and the L-[1-13C]-leucine tracer (0.065±0.008 vs 0.062±0.006%·h⁻¹, respectively; P=0.616). In addition, myofibrillar protein FSR as calculated over the entire 5 h post-prandial period also did not differ between the HWI and CON leg based on the L-[ring-2H5]-phenylalanine tracer (0.048±0.002 vs 0.047±0.003%·h⁻¹, respectively; P=0.877) as well as the L-[1-13C]-leucine tracer (0.050±0.005 vs 0.049±0.002%·h⁻¹, respectively; P=0.815).

Myofibrillar L-[1-13C]-phenylalanine enrichments are displayed in Figure 6. A significant time effect was found (P<0.001). However, no significant treatment effect or time X treatment interaction was observed (P>0.05), indicating higher myofibrillar L-[1-13C]-phenylalanine enrichments at 5 h vs 2 h for both the CON and HWI leg, with no differences between legs.

Muscle molecular signaling
The phosphorylation status (ratio of phosphorylated protein to total protein) of key proteins involved in the initiation of muscle protein synthesis are presented in Figure 7 (A-F). No significant differences were observed for muscle mTOR (Ser2448) phosphorylation status (Figure 7A). A significant time effect, treatment effect, and time X treatment interaction was observed for muscle p70S6K (Thr389) phosphorylation status ($P<0.05$; Figure 7B). Muscle p70S6K (Thr389) phosphorylation status was significantly increased from 0 to 2 h ($P=0.007$) in the HWI leg and significantly decreased from 2 to 5 h ($P<0.01$) in both the HWI and CON leg. The phosphorylation status was significantly different between treatments at all time points ($P<0.05$). At $t = 0$ h, the CON leg was significantly higher compared to the HWI leg ($P=0.031$). At both $t = 2$ and 5 h, the HWI leg was significantly higher compared to the CON leg ($P<0.05$). A significant time effect was found for muscle p70S6K (Thr421/Ser424) phosphorylation status ($P<0.001$; Figure 7C). No significant treatment effect or time X treatment interaction was observed for muscle p70S6K (Thr421/Ser424) phosphorylation status. No significant differences were observed for muscle rpS6 (Ser240/244) phosphorylation status (Figure 7D). A significant time effect was found for muscle rpS6 (Ser235/236) phosphorylation status ($P=0.040$; Figure 7E), with no significant treatment effect or time X treatment interaction observed. No significant differences were observed for muscle 4E-BP1 (Thr37/46) phosphorylation status (Figure 7F). Total protein content of HSP27 and HSP70 (expressed relative to ponceau S staining) are presented in Figure 8 (A-B). No significant differences over time were observed for both HSP27 (Figure 8A) and HSP70 (Figure 8B) contents.
In the present study, we assessed the impact of post-exercise hot-water immersion on post-prandial myofibrillar protein synthesis rates during recovery from resistance-type exercise. Hot-water immersion transiently increased skin and muscle temperature, but did not further increase post-prandial myofibrillar protein synthesis rates or augment the incorporation of dietary protein-derived amino acids in muscle tissue protein during 5 hours of post-exercise recovery.

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

Hydrotherapy is a popular recovery strategy that is applied by many athletes to support their post-exercise recovery (35). Two popular hydrotherapy strategies are cold-water immersion (CWI) and hot-water immersion (HWI) (35). It has been reported that CWI is more effective
in improving markers of acute post-exercise recovery, such as reducing delayed onset muscle
soreness (DOMS), lowering limb swelling, and assisting in performance recovery compared
to HWI (18, 23, 33-35). However, CWI has been shown to lower tissue temperature and
reduce blood flow (12, 19), thereby reducing the incorporation of dietary protein-derived
amino acids into muscle protein and attenuating the post-exercise increase in muscle protein
synthesis rates by ~20% (7). Consequently, athletes aiming to improve post-exercise muscle
(re)conditioning are generally not recommended to apply CWI during recovery from exercise.
In contrast, it has been hypothesized that post-exercise HWI, by stimulating blood flow and/or
increasing muscle tissue temperature, can augment muscle protein synthesis during recovery
from exercise.
In the current study, exercise was followed by immersing one leg in hot water (46°C) while
the contralateral leg was immersed in thermoneutral water (30°C) for 20 min. This short hot-
water immersion regimen was selected based upon everyday practice by athletes as well as
previous studies (35) with the (hot) water temperature being selected based upon what was
perceived as tolerable for 20 min. Hot-water immersion increased skin (~10°C) as well as
muscle (~2.3°C) temperature, after which levels approximately returned to basal values
within the first 120 min of post-exercise recovery (Figure 2). Despite the observed increases
in skin and muscle temperature, post-exercise HWI did not modulate post-prandial
myofibrillar protein synthesis rates (Figure 5) or affect the incorporation of dietary protein
derived amino acids in muscle tissue during the early or later stages of post-exercise recovery
(Figure 6).
The current findings seem to be at odds with previous suggestions (based on anabolic
signaling responses) that heat stress may increase muscle protein synthesis rates (17, 38). It
has previously been shown in rodents that the application of 30 min of local heat stress
(HWI), activates the mTOR pathway by increasing the phosphorylation of Akt (at Ser473)
and p70S6K (at Thr389) (38). In line, Kakigi et al. (17) showed that local heat stress performed 20 min before and during resistance exercise enhanced mTOR signaling in muscle tissue in humans. However, those studies did not perform hot-water immersion during recovery from exercise. In the present study, we also assessed the phosphorylation status of several molecular markers that are important in the regulation of myofibrillar protein synthesis during post-exercise recovery (Figure 7). We extend on previous findings by showing that p70S6K phosphorylation at Thr389 was lower immediately after HWI, but increased at 2 and 5 h into post-exercise recovery when compared with the CON leg (Figure 7B). The reason why p70S6K phosphorylation at Thr389 was lower in the HWI leg immediately after post-exercise water immersion when compared to the CON leg remains unclear. However, it is interesting to note that we have previously shown that p70S6K phosphorylation at Thr389 was higher immediately after post-exercise CWI (8°C) when compared with thermoneutral water immersion (30°C) for 20 min (7). Therefore, muscle temperature seems to modulate p70S6K phosphorylation at Thr389. The fact that p70S6K phosphorylation at Thr389 was increased at 2 and 5 h in the post-exercise recovery period in the HWI compared with the CON leg may have compensated for the lower phosphorylation status observed immediately after water immersion (t = 0 h). Other molecular markers did not show differences between the HWI and CON leg (Figure 7). As evidence suggests that heat stress can elicit protective effects to assist post-exercise recovery and adaptation by increasing heat shock protein (HSP) expression (20), we also measured HSP27 and HSP70 expression. We did not observe any differences in both HSPs between the HWI and CON leg during 5-h of post-exercise recovery (Figure 8). This could potentially be explained by the fact that 5-h of post-exercise recovery is not sufficient to pick up differences, as it is possible that longer post-heating durations may be necessary to pick up changes in HSPs. Alternatively, our heating strategy may not have been sufficient to induce changes in HSPs. Overall, these
observations seem to agree with the absence of differences in muscle protein synthesis rates between the HWI and CON leg.

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

In the current study we observed a transient increase in muscle tissue temperature after HWI. If we would extrapolate our data based on previous work (25), tissue temperature would...
likely have returned to basal values within ~30 minutes after exercise. Therefore, it could be speculated that our heating protocol (20 min of HWI at 46°C) did not elicit a sufficient, continued increase in muscle temperature and that a stronger and/or more prolonged heating protocol may have been more potent. In previous pilot work we established that HWI with water at a temperature of ~46°C is the maximum tolerable temperature when applied for 20 min. With this protocol, we observed a muscle temperature of ~37.5°C in the HWI leg, which was ~2.3°C higher compared with the CON leg. Previous studies have shown similar increases in intramuscular temperature when applying other heating modalities, such as heat pads and hot water-perfused limb cuffs (5, 27, 39). Muscle temperature may be further increased (up to ~40°C) by applying diathermy (14, 15). However, it should be noted that the application of diathermy for post-exercise recovery may not be practical for athletes, as this is typically applied very locally with only a small amount of muscle being heated. Nonetheless, further research may be warranted to investigate the impact of other heating strategies on recovery during (low-intensity) exercise. Such future studies should focus on local, rather than whole-body, heating strategies as prolonged whole-body heat stress can be detrimental for other important aspects of post-exercise muscle recovery, such as glycogen synthesis (22). In addition, future studies may want to address the impact of prolonged (post-exercise) heating on the synthesis rates of more specific protein sub-fractions, such as mitochondrial proteins (14, 15, 30).

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


**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 Bonferroni 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-2H$_5$]-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 Bonferroni post hoc testing applied to locate differences. For all panels: time effect, $P<0.001$.

**Figure 4.** Exogenous phenylalanine rate of appearance (Ra) 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 Bonferroni post hoc testing applied to locate differences. Time effect, $P<0.001$.

**Figure 5.** Myofibrillar protein FSRs as calculated with L-[ring-2H$_5$]-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
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-[1-13C]-phenylalanine incorporation into myofibrillar protein after drink ingestion with intrinsically labeled L-[1-13C]-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 Bonferroni 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.
Table 1. Subjects’ characteristics

<table>
<thead>
<tr>
<th></th>
<th>Subjects (n= 12)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (y)</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>Body mass (kg)</td>
<td>77.6 ± 1.8</td>
</tr>
<tr>
<td>Length (m)</td>
<td>1.83 ± 0.01</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.3 ± 0.6</td>
</tr>
<tr>
<td>LBM (kg)</td>
<td>62.1 ± 1.5</td>
</tr>
<tr>
<td>CON leg lean mass (kg)</td>
<td>10.7 ± 0.3</td>
</tr>
<tr>
<td>HWI leg lean mass (kg)</td>
<td>10.7 ± 0.3</td>
</tr>
<tr>
<td>Whole body fat mass (kg)</td>
<td>13.0 ± 1.1</td>
</tr>
<tr>
<td>CON leg fat mass (kg)</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>HWI leg fat mass (kg)</td>
<td>2.6 ± 0.3</td>
</tr>
<tr>
<td>Whole body fat mass (%)</td>
<td>16.6 ± 1.2</td>
</tr>
<tr>
<td>Leg press 1RM (kg)</td>
<td>287 ± 22</td>
</tr>
<tr>
<td>Leg extension 1RM (kg)</td>
<td>127 ± 6</td>
</tr>
</tbody>
</table>

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.
<table>
<thead>
<tr>
<th>Time (min)</th>
<th>-125</th>
<th>-65</th>
<th>-20</th>
<th>0</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>240</th>
<th>300</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blood sample</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Immersion</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle temperature</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Muscle biopsy</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Drink</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Skin and core temperature</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Primed continuous L-[ring-\(2H_5\)]phenylalanine, L-[\(1^{13}\)C]leucine infusion
A

Skin temperature (°C)

-120 -60 0 60 120 180 240 300

Time (min)

* * *

CON

HWI

B

Muscle temperature (°C)

-120 -60 0 60 120 180 240 300

Time (min)

*
Exogenous $R_a$ (µmol phenylalanine·kg$^{-1}$·min$^{-1}$) vs Time (min)
L-[1-\textsuperscript{13}C]-phenylalanine

Myofibrillar protein-bound enrichment (MPE)

\( \text{CON} \)
\( \text{HWI} \)

Time (h)