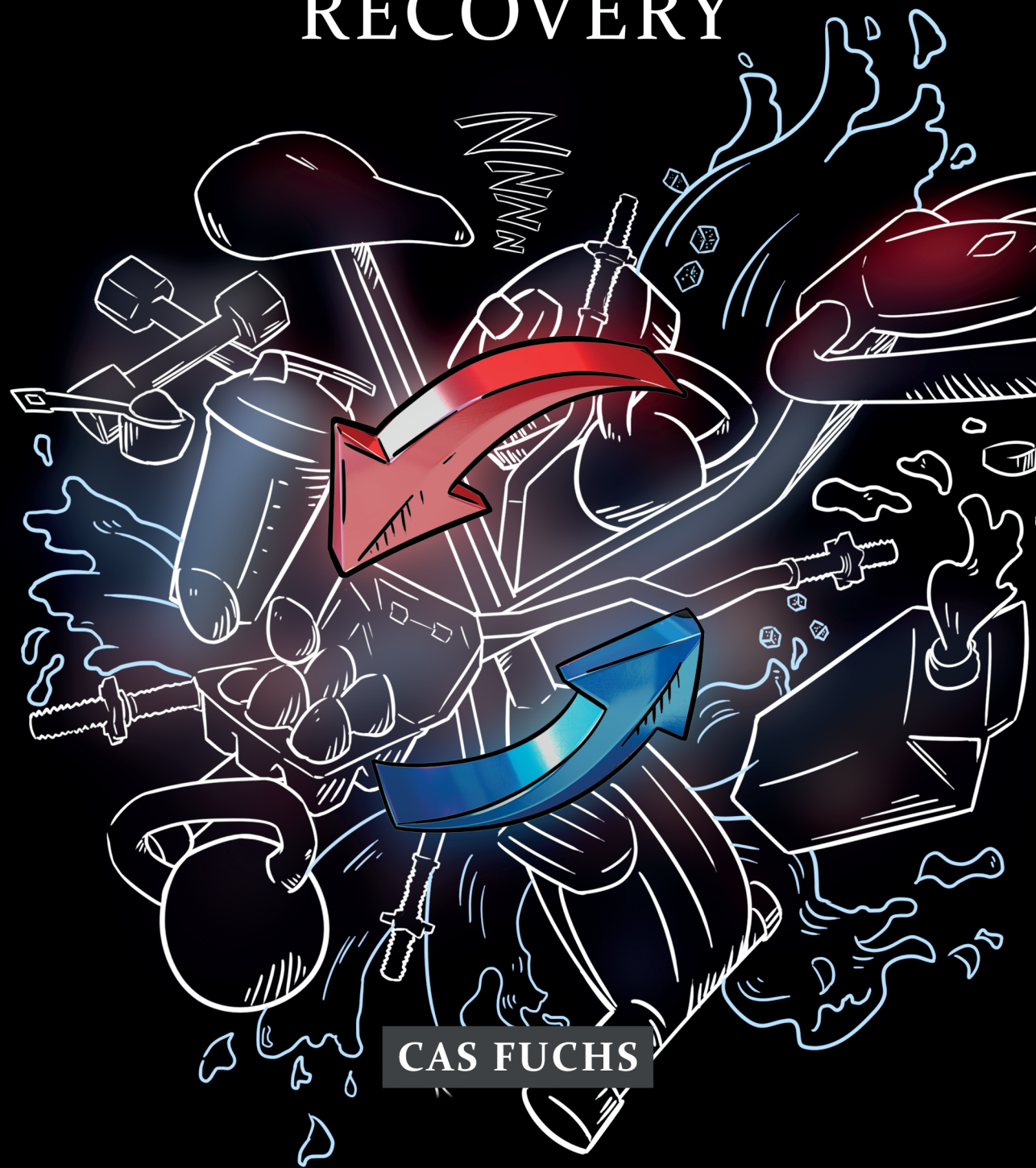
Strategies for Post-Exercise Recovery

STRATEGIES FOR POST-EXERCISE RECOVERY



CAS FUCHS

Strategies for post-exercise recovery

The studies presented in this thesis were performed within the NUTRIM, School of Nutrition and Translational Research in Metabolism at Maastricht University, at the department of Sport, Exercise, and Rehabilitation at Northumbria University, and the Newcastle Magnetic Resonance Centre, Newcastle upon Tyne, the United Kingdom.
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DISSERTATION

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CONTENTS

Chapter 1

General Introduction

Some parts of this chapter are based upon previous publications by the author:

(1) Fuchs CJ, Gonzalez JT, van Loon LJC. Fructose co-ingestion to increase carbohydrate availability in athletes. J Physiol. 2019;597(14):3549-60.

(2) Gonzalez JT, Fuchs CJ, Betts JA, van Loon LJ. Glucose Plus Fructose Ingestion for Post-Exercise Recovery-Greater than the Sum of Its Parts? Nutrients. 2017;9(4).

Exercise is important from both a health and performance perspective. Various nutritional and non-nutritional strategies exist to optimize exercise performance, accelerate post-exercise recovery, and improve exercise conditioning.
In this thesis, we further explored the potential of different nutritional and non-nutritional strategies to improve post-exercise recovery and/or the adaptive response to exercise and exercise training. In this introductory chapter, we provide an overview of the literature leading up to the studies described in this thesis.

Fuel selection during exercise
Fat and carbohydrate both contribute substantially as substrates for energy provision, with the contribution of fat being (moderately) higher than carbohydrates at rest (3, 4). Whereas protein can also contribute to energy expenditure, its contribution tends to be negligible under most conditions (5, 6). During exercise at an intensity of up to 55% of maximal workload capacity (W_{max}), fat and carbohydrate contribute approximately equally to energy expenditure. However, when exercise intensity increases (up to 75% W_{max}) the relative contribution of fat to total energy expenditure substantially decreases (to ~25%), whereas the relative contribution of carbohydrates for energy provision substantially increases (to ~75%) (4) (**Figure 1.1**).

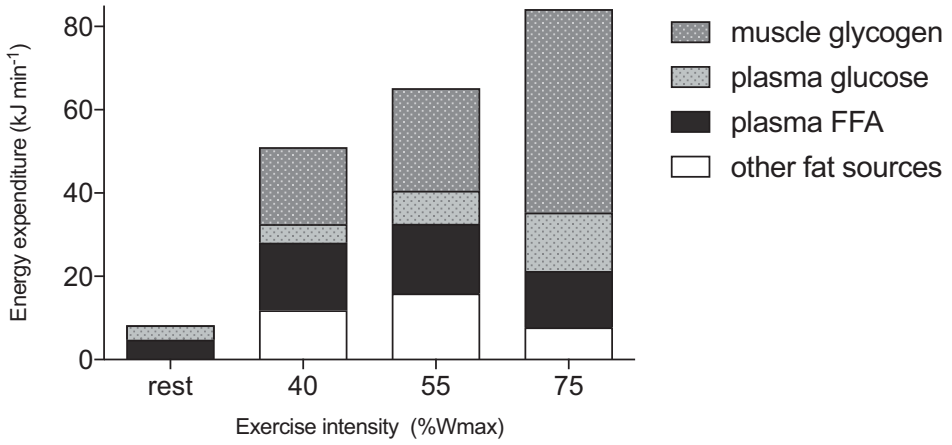

Figure 1.1. Energy expenditure and fuel selection as a function of exercise intensity. FFA, free fatty acids. Illustration adapted from van Loon et al. J Physiol., 2001 (4).

Therefore, during prolonged moderate- to high-intensity exercise, carbohydrates form the predominant fuel source. In the fasted state, carbohydrates will be derived predominantly from (hepatic) gluconeogenesis as well as liver and muscle glycogen stores (4). However, liver and muscle glycogen storage capacity are very limited and, as such, endogenous carbohydrate availability is often a limiting factor for performance during prolonged

moderate- to high-intensity exercise. Indeed, these glycogen stores can be depleted well within 90 min of moderate- to high-intensity exercise (7, 8), with the occurrence of fatigue strongly associated with the depletion of these endogenous carbohydrate stores (9-11). Therefore, it is paramount to optimize carbohydrate availability during such prolonged exercise sessions. As a result, (endurance) athletes often ingest simple carbohydrates (i.e. mono- and disaccharides) during prolonged exercise to increase carbohydrate availability and, as such, to maintain high carbohydrate oxidation rates by exercising muscle (12).

Carbohydrate ingestion during exercise

It has been well-established that, when ingesting glucose (polymers) during exercise, exogenous carbohydrate oxidation rates are limited to ~1.0-1.2 g/min (2, 12, 13). Interestingly, these exogenous carbohydrate oxidation rates can be further increased (up to ~1.75 g/min) by co-ingesting fructose (14). This is likely explained by the fact that with fructose co-ingestion even more carbohydrate can become available in the systemic circulation, as fructose is primarily absorbed across the apical membrane of the intestinal enterocytes by a different transport protein (i.e. GLUT5) than glucose (i.e. SGLT1) (15). Therefore, the co-ingestion of fructose with glucose (polymers) can further increase carbohydrate availability, allowing athletes to increase carbohydrate availability well beyond the limited endogenous stores.

There are several mechanisms by which increased carbohydrate availability can improve (endurance) exercise performance (**Figure 1.2**). Carbohydrate ingestion during exercise can maintain euglycemia, thereby allowing high rates of carbohydrate oxidation to be sustained for longer (11). Additionally, greater carbohydrate availability can increase endurance performance by reducing the oxygen cost of exercise as the energy yield per given volume of oxygen is higher from oxidizing carbohydrate when compared to the oxidation of fat-based fuels (16). In line with this, it has previously been observed in elite race walkers that both exercise economy and performance were negatively impacted following 3 weeks of a high-fat diet compared with a high-carbohydrate diet (17). Hence, greater carbohydrate availability could provide an advantage during exhaustive endurance exercise events where oxygen delivery can become a limiting factor.

Another proposed benefit of carbohydrate ingestion during exercise is that it may attenuate liver and muscle glycogen depletion. This would allow more carbohydrate to be available during the later stages of an endurance exercise event, which could help the athlete to increase workload intensity during the final stages of a race. In support, some studies have shown a sparing of muscle glycogen stores during exercise when carbohydrate was ingested. However, others have been unable to confirm the sparing of muscle glycogen stores with carbohydrate ingestion during exercise (for in-depth reviews see (18) and (19)). Though the exact reason for the inconsistency in the literature is not evident, it may be explained by differences in the type (e.g. cycling vs running), intensity, and duration of exercise that were

applied and the type, amount and timing of the carbohydrates ingested (18-20). Whether carbohydrate ingestion during exercise also lowers the use of liver glycogen remains even more speculative. However, Jeukendrup and colleagues showed that the ingestion of large amounts of glucose during cycling exercise completely suppresses endogenous glucose appearance rates (21), which implies liver glycogen sparing. However, the effects of carbohydrate ingestion on both liver and muscle glycogen depletion during exercise have never been assessed directly. In addition, it remains to be established whether higher carbohydrate availability following the combined ingestion of glucose (polymers) and fructose offers greater benefits to endogenous carbohydrate availability when compared to the ingestion of glucose (polymers) only.

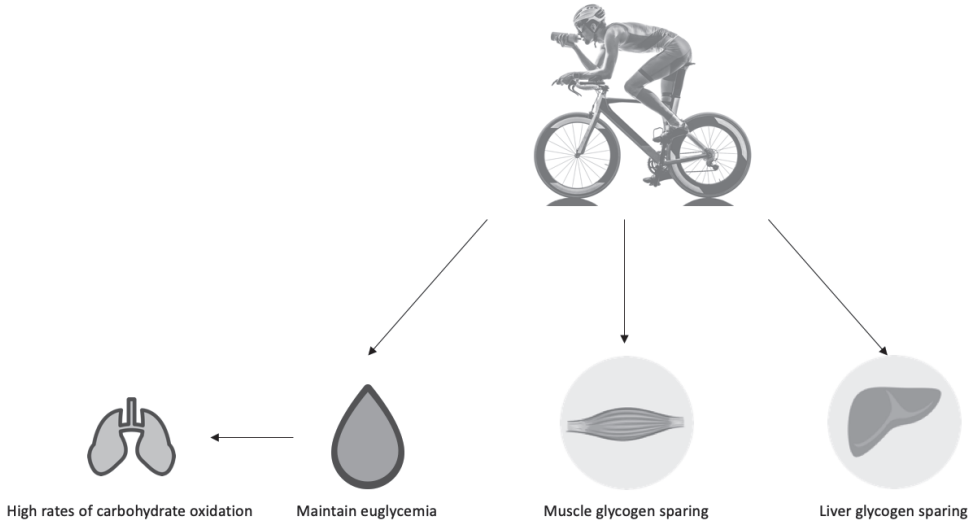

Figure 1.2. Proposed mechanisms of how carbohydrate administration during exercise can improve exercise performance and/or capacity.

Post-exercise liver and muscle glycogen repletion

Following prolonged moderate- to high-intensity exercise, endogenous carbohydrate stores will be substantially depleted (7, 8, 11, 22). To restore endogenous glycogen stores after exercise we need to ingest carbohydrates. Given that glycogen stores appear to be fully replenished within 24 h after exercise when sufficient carbohydrates are ingested (23, 24), further acceleration of glycogen repletion is only relevant when athletes have to maximize subsequent performance well within a 24 h time frame. This is, for example, the case for athletes during intensive training periods, tournament-style competitions, or in between stages in multiday races such as the Tour de France, the Vuelta a España, and the Giro d'Italia.

Current recommendations for maximizing post-exercise glycogen repletion rates are to ingest ample carbohydrates (~1.0-1.2 g/kg/h) with a moderate- to high glycemic index (e.g. glucose (polymers)) starting immediately after exercise and provided frequently with 15-30 min intervals (25, 26). Given that fructose co-ingestion with glucose (polymers) during exercise can increase total exogenous carbohydrate availability, it is tempting to speculate that this may also represent an effective strategy to accelerate muscle and liver glycogen repletion during post-exercise recovery. So far, fructose co-ingestion has failed to show an acceleration of post-exercise muscle glycogen repletion (27, 28). In contrast, fructose co-ingestion does appear to accelerate liver glycogen repletion when compared to the ingestion of glucose (polymers) only (29). Currently, no study has investigated what the effect of fructose co-ingestion is on both muscle and liver glycogen repletion when optimal amounts of carbohydrates are provided (≥1.2 g/kg/h). Furthermore, there is currently much interest in the application of co-ingestion of other monosaccharides (i.e. galactose) and their capacity to accelerate the replenishment of liver and muscle glycogen stores during acute post-exercise recovery (29). Many questions remain to be answered on the role of different types of carbohydrates and their capacity to accelerate muscle and liver glycogen repletion during recovery from exercise.

Post-exercise recovery and muscle reconditioning

Much of the interest on the impact of nutrition on post-exercise recovery has focused on restoring substrate (i.e. carbohydrate) availability for energy provision. However, over the last decades more research has been directed towards the impact of nutrition on the repair of muscle damage as well as the reconditioning of muscle tissue after exercise. One of the most important components facilitating skeletal muscle tissue repair and (re)conditioning, is muscle protein synthesis. After a single bout of resistance-type exercise both muscle protein synthesis and breakdown rates are elevated (albeit the latter to a lesser extent). Despite a marked increase in post-exercise muscle protein synthesis, net protein balance (i.e. muscle protein synthesis – muscle protein breakdown) remains negative in the absence of food intake (30, 31). Protein intake during recovery from exercise is important to further stimulate muscle protein synthesis, inhibit protein breakdown and, as such, achieve a positive net protein balance (32-35).

Two key factors that modulate the effects of protein intake immediately after exercise on muscle protein synthesis are the amount and type of protein. It has previously been observed that ingesting 20 g of a high-quality protein source (i.e. egg or whey protein) following lower body resistance exercise appears to maximize post-exercise muscle protein synthesis rates (36, 37). More recently, it was indicated that, when performing whole-body resistance exercise (and thereby recruiting more muscle tissue), ingesting 40 g of protein may further increase muscle protein synthesis rates when compared to 20 g (38). The most recent guideline for post-exercise protein intake suggests that protein intake should be normalized

to the body mass of an individual. Based on a re-analysis of previously published literature, Moore concluded that a protein dose of 0.31 g/kg body mass should be ingested after exercise and this does not appear to differ between gender or depend on the quantity of activated muscle mass (39).

With regards to the type of protein, studies have shown that dietary protein sources can differ in their capacity to stimulate muscle protein synthesis and that more rapidly digestible protein sources (e.g. whey) stimulate muscle protein synthesis to a greater extent than more slowly digestible protein sources (40, 41). In addition, it has been suggested that animal-based proteins may be more effective in stimulating muscle protein synthesis compared to plant-based protein sources. For example, it has been shown that milk protein ingestion stimulates post-exercise muscle protein synthesis rates to a greater extent when compared to the ingestion of soy protein (42). In general, plant-based protein sources have a lower essential amino acid content when compared to animal-based protein sources, which could result in a submaximal stimulation of muscle protein synthesis (43). Indeed, it has been well established that especially the essential amino acids are important for the post-prandial stimulation of muscle protein synthesis (34, 44, 45). Among the essential amino acids, the branched-chain amino acids, with leucine in particular, seem to be primarily responsible for driving the muscle protein synthetic response to feeding (46-50). It has been observed that leucine alone is already able to effectively stimulate skeletal muscle protein synthesis at rest in humans (51). In addition, leucine co-ingestion has also been observed to (further) increase post-prandial muscle protein synthesis both at rest and during recovery from exercise (when compared to protein intake) (47, 52-56). Taken together, athletes are currently advised to ingest ~20 g (or 0.31 g protein/kg body mass) of a high-quality dietary protein source (e.g. milk protein) that is relatively rich in leucine during acute post-exercise recovery.

Non-nutritional strategies to impact post-exercise recovery

Besides nutrition (carbohydrate and protein intake), there are also several non-nutritional factors that may modulate post-exercise recovery. This includes hydrotherapy with cooling (i.e. cold-water immersion) and heating (i.e. hot-water immersion), that are both being frequently applied by athletes in daily practice.

Post-exercise cooling is applied by many athletes with the main aim to support and accelerate post-exercise recovery and facilitate the skeletal muscle adaptive response to exercise (57, 58). In support, post-exercise cooling has been reported in several studies to reduce delayed onset muscle soreness (59-61) and muscle swelling (61-64), as well as to accelerate recovery of muscle performance (58). However, since post-exercise cooling lowers muscle temperature and limb blood flow (65-68), it could theoretically compromise muscle enzyme activity as well as nutrient supply to muscle tissue and, as such, impair muscle glycogen repletion and muscle protein synthesis. Indeed, a strong post-exercise cooling protocol has been reported to lower muscle glycogen repletion *in vitro* in isolated muscle fibers (69) as

well as *in vivo* in humans during recovery from glycogen depleting exercise (70). In contrast, a more practical (i.e. less severe) cooling protocol did not impact acute post-exercise glycogen repletion (65). Apart from glycogen repletion, it has been observed that acute anabolic signaling as well as ribosomal biogenesis appears to be blunted after post-exercise cooling (71-73). This suggests that post-exercise muscle protein synthesis may in fact be attenuated by post-exercise cooling. However, this has never been assessed directly and, therefore, more work is required to elucidate the effects of post-exercise cooling on muscle protein synthesis (**Figure 1.3**).

Another strategy that is currently receiving a lot of attention by many athletes is post-exercise heating to further increase post-exercise muscle temperature and blood flow (74). In contrast to post-exercise cooling, this could theoretically allow higher enzyme activity as well as more nutrient supply to muscle tissue. Therefore, it could be speculated that post-exercise heating may in fact accelerate muscle glycogen repletion and augment muscle protein synthesis. Whereas whole-body heating (in a heated chamber of 32.6°C for 4 h) during post-exercise recovery appears detrimental for muscle glycogen repletion (75), local heating may in fact be beneficial. There is currently one study that assessed the effects of local heating on muscle glycogen repletion, showing that post-exercise carbohydrate intake combined with local (i.e. thigh muscle) heat pack application induced a 22% greater increase in glycogen concentrations when compared to carbohydrate intake only during 4 hours of post-exercise recovery (76). Despite this promising study, more work is necessary to investigate the effects of other heating applications and strategies on muscle glycogen repletion. In addition, some studies have demonstrated that anabolic signaling is increased when heating is applied at rest in rat skeletal muscle (77) or before and during resistance-exercise in humans (78). The impact of post-exercise heating on muscle protein synthesis has not yet been assessed (**Figure 1.3**). In short, there is much to learn about the potential impact of heating as a strategy to improve post-exercise recovery.

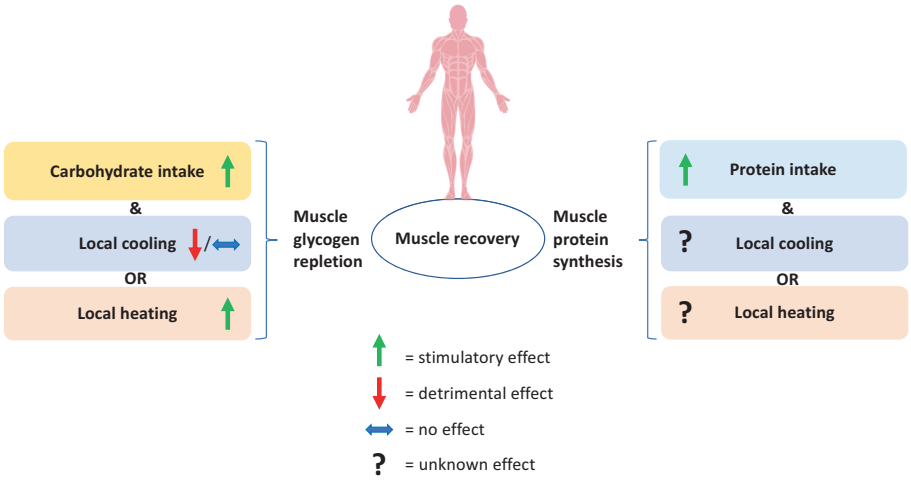

Figure 1.3. The (un)known impact of nutrition with (local) cooling and/or heating on post-exercise muscle glycogen repletion and muscle protein synthesis.

Outline of this thesis

This thesis describes a series of studies designed to investigate the impact of nutritional (carbohydrate, protein, amino acid) and non-nutritional (cooling and heating) strategies to improve post-exercise recovery and/or the adaptive response to exercise and exercise training. First, we evaluated how fructose co-ingestion during exercise can impact liver and muscle glycogen concentrations (**Chapter 2**). This was followed by a study investigating the effects of fructose co-ingestion on post-exercise liver and muscle glycogen repletion (**Chapter 3**). Based on this work we then provided an overview of what is currently known about the benefits of fructose co-ingestion with glucose (polymers) both during and after exercise (**Chapter 4**). In the second half of this thesis we evaluated the impact of protein, branched-chain amino acids, and branched-chain ketoacids on muscle protein synthesis (**Chapter 5**). This was followed by a more practical study, where we assessed the post-prandial increase in muscle protein synthesis following consumption of a number of raw vs boiled eggs during recovery from exercise (**Chapter 6**). Because there are suggestions that non-nutritional strategies may also impact post-exercise recovery and facilitate the skeletal muscle adaptive response to exercise, we evaluated the effects of cold (**Chapter 7**) and hot (**Chapter 8**) water immersion on post-prandial muscle protein synthesis rates during recovery from exercise. Finally, in **Chapter 9**, these studies are integrated in a broader perspective on non-nutritional strategies to optimize post-exercise muscle recovery and reconditioning and will provide many leads for future research.

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Chapter 2

Ingestion of Glucose or Sucrose Prevents Liver but not Muscle Glycogen Depletion During Prolonged Endurance-type Exercise in Trained Cyclists

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Abstract

Purpose: To define the effect of glucose ingestion compared to sucrose ingestion on liver and muscle glycogen depletion during prolonged endurance-type exercise.
Methods: Fourteen cyclists completed two 3-h bouts of cycling at 50% of peak power output while ingesting either glucose or sucrose at a rate of 1.7 g/min (102 g/h). Four cyclists performed an additional third test in which only water was consumed for reference. We employed ^{13}C magnetic resonance spectroscopy to determine liver and muscle glycogen concentrations before and after exercise. Expired breath was sampled during exercise to estimate whole-body substrate use.
Results: Following glucose and sucrose ingestion, liver glycogen levels did not show a significant decline following exercise (from 325±168 to 345±205 and 321±177 to 348±170 mmol/L, respectively; $P>0.05$) with no differences between treatments. Muscle glycogen concentrations declined (from 101±49 to 60±34 and 114±48 to 67±34 mmol/L, respectively; $P<0.05$), with no differences between treatments. Whole-body carbohydrate utilization was greater with sucrose (2.03±0.43 g/min) vs glucose ingestion (1.66±0.36 g/min; $P<0.05$). Both liver (from 454±33 to 283±82 mmol/L; $P<0.05$) and muscle (from 111±46 to 67±31 mmol/L; $P<0.01$) glycogen concentrations declined during exercise when only water was ingested.
Conclusion: Both glucose and sucrose ingestion prevent liver glycogen depletion during prolonged endurance-type exercise. Sucrose ingestion does not preserve liver glycogen concentrations more than glucose ingestion. However, sucrose ingestion does increase whole-body carbohydrate utilization compared to glucose ingestion.

Introduction

Carbohydrate and fat are the main substrates oxidized during moderate-intensity, endurance-type exercise (1). In the fasted state, muscle glycogen and plasma glucose are predominant sources of carbohydrate for oxidation (1), the latter continuously replenished by glycogenolysis and gluconeogenesis from the liver, with smaller contributions from the kidneys and intestine (2). Consequently, in the absence of carbohydrate consumption, liver and muscle glycogen concentrations decrease by 40-60% within 90 min of exercise at a workload of 70% of peak oxygen uptake ($\dot{V}O_{2peak}$) (3, 4). Given the importance of liver glycogen for metabolic regulation (5), and the close relationship between liver glycogen content and exercise tolerance (3), it is important to understand the impact of carbohydrate ingestion on liver glycogen depletion during exercise.

Carbohydrate feeding during prolonged (>2 h) moderate-to-high intensity, endurance-type exercise enhances endurance performance and capacity (6), attributed to the facilitation of high rates of carbohydrate oxidation, prevention of hypoglycaemia and (under certain conditions) sparing of muscle glycogen (7, 8). Though some support has been provided that carbohydrate ingestion can attenuate muscle glycogen depletion (9-11), others have failed to confirm these findings (12-15). Furthermore, prevention of liver glycogen depletion has been suggested (15-17), but this has never been experimentally assessed. We speculate that carbohydrate ingestion during exercise attenuates the decline in both liver as well as skeletal muscle glycogen contents.

To maximize carbohydrate availability during exercise, carbohydrate digestion and absorption should be optimized. Previous work suggests that exogenous glucose uptake by the gastrointestinal tract during exercise is restricted to ~1 g/min (18-20), attributed to saturation of the sodium-glucose luminal transporter-1 (SGLT-1). However, combined ingestion of glucose and fructose at ≥1.8 g/min has been shown to result in much higher exogenous carbohydrate oxidation rates (up to 1.75 g/min), compared to the ingestion of equal amount of glucose alone (18, 20). The greater uptake and oxidative capacity of glucose and fructose mixtures has been attributed to fructose being absorbed by the glucose transporter-5 (GLUT-5) in the intestine (21). As sucrose (commonly referred to as table sugar) combines glucose and fructose monomers, and sucrose hydrolysis is not rate limiting for intestinal absorption (22, 23), we hypothesize that sucrose ingestion at a rate exceeding 1 g/min will enhance exogenous carbohydrate availability when compared to the ingestion of an isoenergetic amount of glucose or glucose polymers. Moreover, since fructose appears to be preferentially directed to liver glycogen storage (relative to glucose) (24), sucrose may further prevent liver glycogen depletion during exercise.

The present study aimed to investigate the effect of high rates of glucose and sucrose ingestion on net changes in liver and muscle glycogen contents and intramyocellular lipid concentrations using magnetic resonance spectroscopy (MRS). We hypothesized that high-

rates of carbohydrate ingestion would spare liver glycogen during prolonged exercise, and that sucrose ingestion would better maintain liver glycogen relative to glucose ingestion.

Methods

Study design

Participants completed preliminary testing prior to 2 main trials, during which subjects either ingested glucose (GLU) or sucrose (SUC) in a randomized, double-blind, crossover design separated by 7-14 d. Trials were conducted at the Newcastle Magnetic Resonance Centre (Newcastle-upon-Tyne, UK) in accordance with the Second Declaration of Helsinki, and following approval from the Northumbria University Faculty of Health and Life Sciences Ethics Committee. Randomization was performed using online statistical software (http://www.randomizer.org/). Blinding and preparation of the test-drinks was performed by an assistant who was not involved in the exercise tests.

In addition to the two main trials, four participants completed an additional control trial (CON) as a reference to establish the change in liver glycogen concentration without carbohydrate ingestion. This was identical to the SUC and GLU trials, with the exception that only water was ingested during exercise (identical volume to GLU and SUC trials), and blood sampling was not performed.

Participants

Fifteen trained cyclists were recruited for the study. Inclusion criteria included healthy, endurance trained, male cyclists; $\dot{V}O_{2peak}$ ≥50 mL/min/kg/. Exclusion criteria included the use of medication that could influence substrate metabolism, smokers and any known metabolic disorders. One participant was unable to complete the full 3 h cycling protocol due to nausea on the GLU trial and was therefore excluded from the analysis. Consequently, 14 participants completed the two main trials.

Preliminary testing

An incremental cycling test was performed on an electromagnetically braked cycle ergometer (Velotron, RacerMate Inc., Seattle, WA, USA) to determine peak power output (W_{peak}) and peak oxygen uptake ($\dot{V}O_{2peak}$). Following a 5-min warm-up at 100 W, the workload began at 150 W and was increased by 50 W every 2.5 min to voluntary exhaustion (25). Expired gas was sampled continuously to determine oxygen uptake (Oxycon gas analyser, CareFusion corporation, San Diego, CA, USA).

Main trials

Participants arrived at Newcastle Magnetic Resonance Centre at 0700-0800 h following a 12 h fast. Strenuous exercise was prohibited for 24 h prior to trials, and participants were asked to record and replicate dietary intake for 24 h prior to trials. The final meal consumed on the evening before the main trials was provided by the investigators to participants to

standardize the macronutrient intake across participants for this meal (25 g protein, 51 g carbohydrate and 32 g fat; 2479 kJ; 592 kcal).

MRS was used to determine liver and muscle glycogen and intramyocellular lipid concentrations prior to and following 3 h of cycling. Following a 5 min warm-up at 100 W, power output was increased to 50% W_{peak} for the remainder of the 3 h. Immediately prior to exercise, participants were provided with 600 mL (86.4 g carbohydrate) of the test-drink, and then a further 150 mL (21.6 g carbohydrate) every 15 min during exercise. Four of the 14 cyclists did not manage to consume all of the carbohydrate on their first trial (which was a GLU trial for two participants, and a SUC trial for the other two participants) and therefore their carbohydrate intake was replicated for their second trial (the carbohydrate intakes for these four cyclists therefore ranged from 238-281 g, mean ± SD: 292 ± 101 g, compared to the 324 g prescribed). This lead to an average rate of carbohydrate intake for the entire group of 1.7 ± 0.2 g/min and 1.7 ± 0.2 g/min (102 ± 12 g/h and 102 ± 12 g/h) during GLU and SUC trials, respectively ($P > 0.05$; **Table 1**).

Table 1. Carbohydrate intake and physiological variables of trained cyclists during 3 h of cycling with ingestion of glucose, sucrose or water.

	GLU (n = 14)	SUC (n = 14)	CON (n = 4)
Carbohydrate intake (g/min)	1.7 ± 0.2	1.7 ± 0.2	0 ± 0
Fluid intake (L)	2.1 ± 0.2	2.2 ± 0.2	2.3 ± 0.0
Power output (W)	165 ± 17	165 ± 17	158 ± 7
Mean heart rate (beats/min)	145 ± 14	146 ± 12	122 ± 8

Data are expressed as means ± SEM. GLU, glucose; SUC, sucrose; CON, water control.

Carbohydrate drinks

Carbohydrate drinks were prepared by mixing 108 g of carbohydrate with 750 mL of water in an opaque bottle. This was replicated two more times to produce 3 bottles, each with 750 ml of a 14% carbohydrate solution; 324 g of carbohydrate in total. Both sources of carbohydrate were from plants that use C_3 carbon fixation to minimize differences in the natural abundance of ^{13}C (26). Accordingly, the glucose drink was produced with dextrose monohydrate obtained from wheat (Roquette, France) and the sucrose drink was produced with granulated sugar beet (AB Sugar, UK).

Blood sampling and analysis

Prior to exercise, an intravenous catheter was inserted into an antecubital vein for regular sampling. Blood samples were obtained prior to the exercise bout, and every 30 min during exercise. Briefly, 10 mL of blood was collected in EDTA-vacutainers and immediately centrifuged at 2000 g for 10 min at 4°C. Plasma was then aliquoted and stored at -80°C for

subsequent determination of insulin (IBL International, Hamburg, Germany) and non-esterified fatty acid (NEFA) concentrations (WAKO Diagnostics, Richmond, VA) in duplicate (intra- and inter-assay coefficients of variation all <10%). An additional 20 µL of whole blood was collected in a capillary tube and was used to determine glucose and lactate concentrations immediately (Biosen C_line, EKF Diagnostics, Magdeberg, Germany).

Expired gas analysis

Expired breath samples were taken every 30 min throughout exercise using the Douglas bag technique (27) accounting for variance in ambient oxygen and carbon dioxide concentrations (28). A mouthpiece connected to a two-way, non-rebreathing valve (model 2730, Hans Rudolph, Kansas City, Missouri), was used to collect gas samples (60 s sample after a 60 s stabilization phase), analysed for concentrations of oxygen and carbon dioxide using a paramagnetic and infrared transducers, respectively (Servomex 5200S, Crowborough, East Sussex, UK). Sensors were turned on 60 min prior to a two-point calibration (zero: 100% nitrogen; span: 20% oxygen and 8% carbon dioxide) using accuracy certified gas standards (BOC Industrial Gases, Linde AG, Munich, Germany).

Ambient temperature, humidity and barometric pressure using a Fortin barometer (F.D. and company, Watford, UK) were recorded, and expired gas samples were corrected to standard temperature and pressure (dry). Volume and temperature of expired gas samples were determined using a dry gas meter (Harvard Apparatus, Edenbridge, Kent, UK) and thermistor (model 810-080, ETI, Worthing, UK), respectively, during gas evacuation. Calibration of the dry gas meter was performed regularly with a 3-L syringe (Series 5530, Hans-Rudolph Inc, Kansas City, Missouri, USA).

Subjective ratings

Ratings of gut discomfort were assessed every 30 min during exercise using a 5-point scale, where 1 was anchored at "no discomfort' and 5 at "maximum discomfort". Ratings of perceived exertion (RPE) were assessed using the Borg scale (29).

Measurement of muscle and liver glycogen

Tissue glycogen concentration was determined from the magnitude of the natural abundance signal from the C-1 carbon of glycogen at a frequency of 100.3 ppm. A Philips 3 Tesla Achieva scanner (Philips Healthcare, Best, The Netherlands) was used with a 6 cm diameter ^{13}C surface coil with integral ^{1}H decoupling surface coil (PulseTeq, Worton under Edge, UK) to measure muscle glycogen concentration and an in-house built 12 cm $^{13}C/^{1}H$ surface coil used to measure liver glycogen concentration.

For muscle glycogen measurements, the surface coil was placed over the widest part of the *Vastus lateralis* and the leg was held in position with fabric straps to prevent movement. Pulse power was calibrated to a nominal value of 80° by observing the power dependent variation in signal from a fiducial marker located in the coil housing, containing a sample exhibiting ^{13}C signal with short T_1 (213 mM [2-^{13}C]-acetone and 25 mM $GdCl_3$ in water). Automated shimming was carried out to ensure that the magnetic field within the scanner was uniform over the active volume of the ^{13}C coil. The ^{13}C spectra were acquired over 15 min using a non-localized ^{1}H decoupled ^{13}C pulse-acquire sequence (TR 120 ms, spectral width 8 kHz, 7000 averages, WALTZ decoupling). ^{1}H decoupling was applied for 60% of the ^{13}C signal acquisition to allow a relatively fast TR of 120 ms to be used within the Specific Absorption Rate safety limitations.

For liver glycogen measurements the $^{13}C/^{1}H$ surface coil was placed over the right lobe of the liver. Spectra were acquired over 15 min using non-localized ^{1}H decoupled ^{13}C pulse acquisition sequences (TR 300 ms, spectral width 8 kHz, 2504 averages, WALTZ decoupling, nominal ^{13}C tip angle of 80°). Scout images were obtained at the start of each study to confirm optimal coil position relative to the liver.

Tissue glycogen concentrations were calculated from the amplitude of the C1-glycogen ^{13}C signal using Java Based Magnetic Resonance User Interface (jMRUI) version 3.0 and the AMARES algorithm [7] as described in detail previously (4, 30-32).

Measurement of intramyocellular lipid

Intramyocellular lipid content was determined routinely, as described in more detail previously (4). In short, a 12 cm ^{1}H transmitter/receiver coil was used to obtain ^{1}H spectra to measure intramyocellular lipid (IMCL) content in the widest part of the *gastrocnemius*. The PRESS (Point Resolved Spectroscopy) (33) sequence was used to acquire ^{1}H spectra from a 2×2×2 cm voxel, using an echo time of 25 ms, spectral resolution of 1 Hz and repetition time of 5000 ms with 32 acquisitions. Spectra were analyzed with JMRUI version 3.0 using the least square fitting AMARES algorithm (33, 34). The inter-observer bias was 0.09 mmol/L with a 95% limit of agreement of 0.8 mmol/L ($P > 0.05$).

Calculations and statistical analysis

Due to the lack of data regarding exercise-induced changes in liver glycogen concentrations with carbohydrate feeding, a sample size estimation was based on data from exogenous carbohydrate oxidation rates (as a surrogate for carbohydrate availability). Sucrose increases exogenous carbohydrate oxidation during cycling by ~30% (35). Using this figure, along with the 7% intra-individual coefficient of variation of hepatic glycogen content measured by ^{13}C

MRS (36), the study was designed to provide statistical power above 90% with an alpha level of 0.05 with a minimum sample size of $n = 7$ in a crossover design (37).

Whole-body rates of carbohydrate and lipid utilization were estimated using the following equations assuming negligible protein oxidation (38, 39):

$$\text{Net lipid utilization (g/min)} = \left(1.695 \times \dot{V}O_2\right) - (1.701 \times \dot{V}CO_2)$$
$$\text{Net carbohydrate utilization (g/min)} = \left(4.210 \times \dot{V}CO_2\right) - (2.962 \times \dot{V}O_2)$$

Units of $\dot{V}O_2$ and $\dot{V}CO_2$ are L/min

Unless otherwise stated, all data were expressed in the text as the mean ± standard deviation (SD) of the mean and the error bars presented in figures are 95% confidence intervals (CI). Data were checked for normal distribution and log-transformed if appropriate prior to statistical analysis.

Liver and muscle glycogen, and IMCL concentrations from the four participants who completed the CON trial were assessed by two-way (trial x time) repeated measures ANOVA with trial (GLU vs SUC vs CON) and time (pre- vs post-exercise) as within-subject factors. Rates of substrate utilization were assessed by a one-way repeated measures (GLU vs SUC vs CON) ANOVA. No further inferential statistics were performed on CON data since this was only a subgroup of the total sample and was only used as a reference for the change in liver glycogen concentration with 3 h of exercise in the absence of carbohydrate ingestion. Accordingly, all other comparisons were made between GLU and SUC only.

Blood, plasma and respiratory variables and subjective ratings were assessed by two-way (trial x time) repeated measures ANOVA with trial (GLU vs SUC) and time (all time points during exercise) as within-subject factors. Liver and muscle glycogen and intramyocellular lipid concentrations were also assessed by two-way (trial x time) repeated measures ANOVA with trial (GLU vs SUC) and time (pre- vs post-exercise) as within-subject factors. Mean exercise responses in GLU and SUC trials (carbohydrate intake, heart rate, fluid intake and power output) were assessed by paired t-tests. All P values are corrected for multiple comparisons (Holm-Sidak). A P value of ≤ 0.05 was used to determine statistical significance. All data were analyzed using Prism v5 (GraphPad Software, San Diego, CA).

Results

Participants

Participants' characteristics are provided in **Table 2**. No differences were observed for age, body mass, height, $\dot{V}O_{2peak}$, W_{peak}, body mass index, systolic or diastolic blood pressure between participants who completed the main trials (GLU and SUC) and the subgroup of participants who also completed the additional CON trial.

Table 2. Characteristics of trained cyclists who completed GLU, SUC and CON trials.

	GLU and SUC (n = 14)	CON (n = 4)
Age (y)	25 ± 5	26 ± 6
Body mass (kg)	73.1 ± 9.3	75.3 ± 10.7
Height (m)	1.78 ± 0.08	1.75 ± 0.09
$\dot{V}O_{2peak}$ (mL/min/kg)	58 ± 5	60± 7
W_{peak} (W)	330 ± 35	316 ± 27
BMI (kg/m^2)	23.0 ± 1.9	24.5 ± 1.8
Systolic blood pressure (mmHg)	133 ± 11	129 ± 6
Diastolic blood pressure (mmHg)	74 ± 8	71 ± 8

Data are expressed as means ± SD. GLU, glucose; SUC, sucrose; $\dot{V}O_{2peak}$, peak oxygen uptake; CON, water control; W_{peak}, peak power output.

Subjective ratings

RPE increased during exercise (time effect, $P < 0.001$), but to less of an extent during SUC when compared to GLU (interaction effect, $P < 0.05$; **Figure 1A**), becoming significantly different between trials from 150 min onwards ($P < 0.05$). Similarly, ratings of gut discomfort increased throughout exercise (time effect, $P < 0.001$) but to less of an extent during SUC when compared to GLU (interaction effect, $P < 0.01$), becoming significantly different at 180 min (Figure 1B, $P < 0.05$).

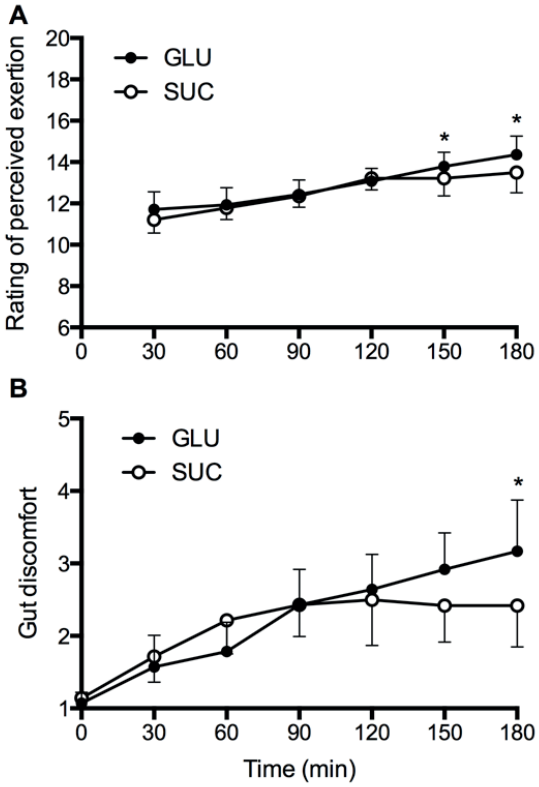

Figure 1. Ratings of perceived exertion (A) and gut discomfort (B) during 3 h of cycling with ingestion of glucose or sucrose in trained cyclists (n = 14). Data are expressed as means ± 95% CI. * $P < 0.05$, significantly different between GLU and SUC. GLU, glucose; SUC, sucrose.

Respiratory data and whole-body substrate utilization

$\dot{V}O_2$ and $\dot{V}CO_2$ remained stable during exercise (time effect, $P > 0.05$ for both) and were not different between GLU and SUC (both $P > 0.05$). Respiratory exchange ratio (RER) was higher with SUC vs GLU (trial effect, $P < 05$) for time points 90 min onwards (interaction effect, $P < 0.05$; **Figure 2C**). Whole-body carbohydrate utilization rates were higher during SUC (2.03 ± 0.43 g/min) when compared with GLU (1.66 ± 0.36 g/min; $P < 0.05$), at the expense of fat oxidation rates (SUC: 0.35 ± 0.15 vs GLU: 0.48 ± 0.12 g/min; $P < 0.05$), resulting in energy expenditure rates that did not differ between trials (SUC: 8.8 ± 1.2 vs GLU: 8.6 ± 0.9 MJ; $P > 0.05$; **Figure 3A**). In the subgroup who also completed the CON trial (n = 4), whole-body fat oxidation rates were lower during both GLU (0.42 ± 0.10 g/min) and SUC (0.33 ± 0.11 g/min), compared to CON (0.64 ± 0.19 g.min; $P < 0.05$), whilst carbohydrate oxidation rates (SUC: 2.04 ± 0.40 vs GLU: 1.79 ± 0.43 vs CON: 1.20 ± 0.44 MJ) did not significantly differ between trials ($P > 0.05$). Accordingly, energy expenditure (SUC: 8.7 ± 0.6 vs GLU: 8.6 ± 0.8 vs CON: 8.4 ± 0.4 MJ) also did not differ between trials ($P > 0.05$; Figure 3B).

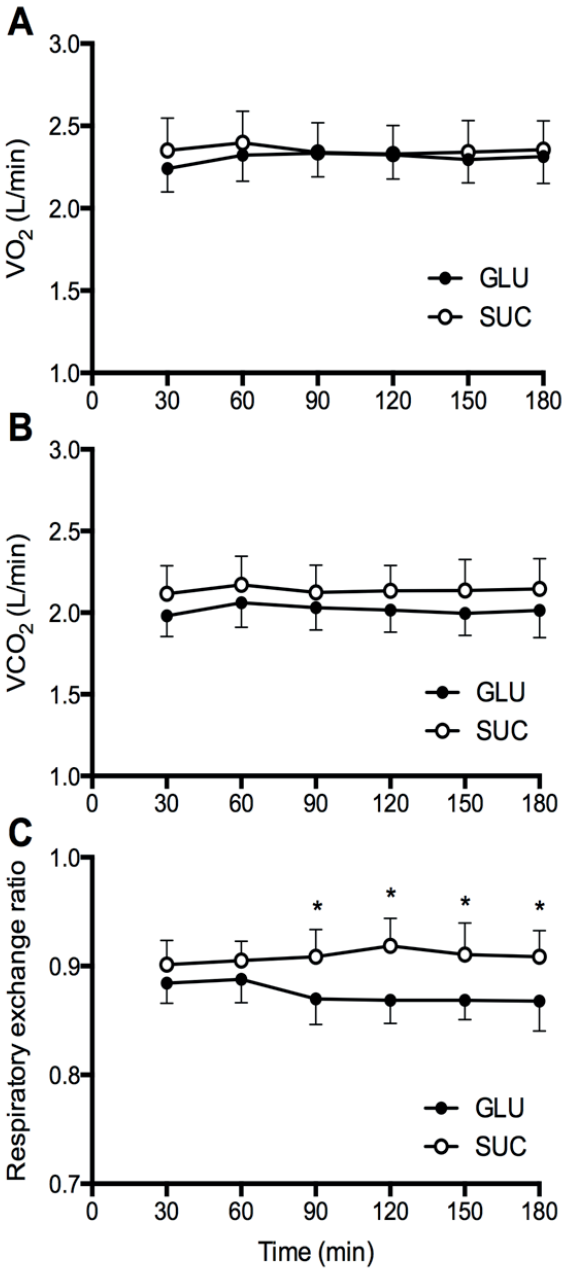

Figure 2. VO_2 (A), VCO_2 (B) and respiratory exchange ratio (C) during 3 h of cycling with ingestion of glucose or sucrose in trained cyclists (n = 14). Data are expressed as means ± 95% CI. * $P < 0.05$, significantly different between GLU and SUC. GLU, glucose; SUC, sucrose; VCO_2, rate of carbon dioxide production; VO_2, rate of oxygen consumption.

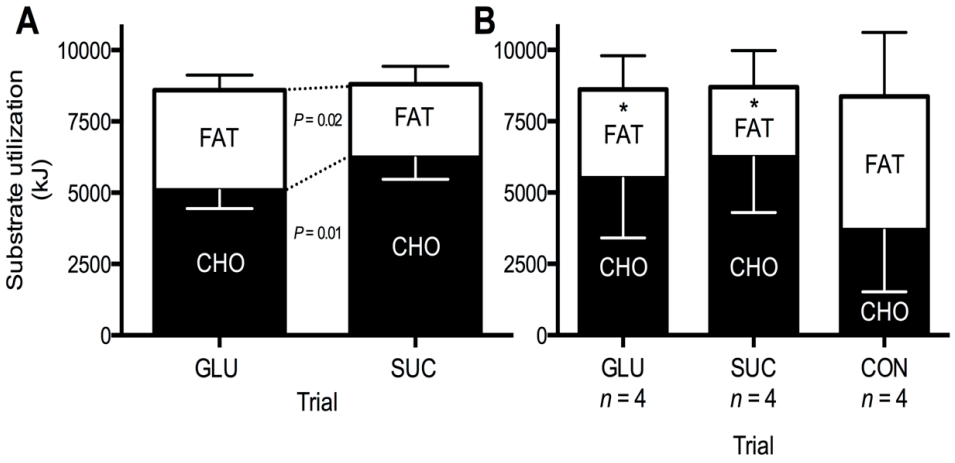

Figure 3. Substrate utilization during 3 h of cycling with ingestion of glucose or sucrose in trained cyclists (A; n = 14) and in the subgroup of trained cyclists (B; n = 4). Data are expressed as means ± 95% CI. * $P < 0.05$, significantly different from CON. CHO, carbohydrate; GLU, glucose; SUC, sucrose; CON, water control.

Circulating metabolite and insulin concentrations

Blood glucose and plasma insulin concentrations were not significantly different between trials (trial effect, $P > 0.05$; interaction effect, $P > 0.05$ for both variables; **Figure 4A**). In contrast, blood lactate concentrations were higher with SUC vs GLU (trial effect, $P < 0.01$), rising at the onset of exercise (time effect, $P < 0.001$) to a greater extent in SUC vs GLU until 120 min (interaction effect, $P < 0.01$; Figure 4B). Plasma NEFA concentrations fell from ~0.5 mmol/L to ~0.2 mmol/L during the first hour of exercise before rising again (time effect, $P < 0.001$), the latter of which occurred to a greater degree in GLU compared to SUC (interaction effect, $P < 0.01$; Figure 4D).

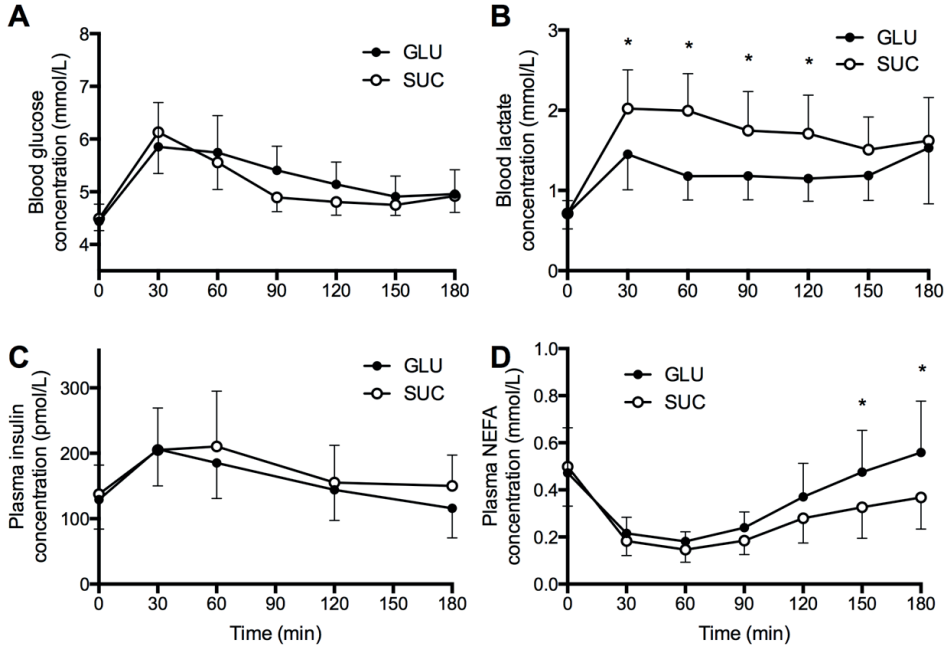

Figure 4. Blood glucose (A) and lactate (B), and plasma insulin (C) and NEFA (D) concentrations during 3 h of cycling with ingestion of glucose or sucrose in trained cyclists (n = 14). Data are expressed as means ± 95% CI. * $P < 0.05$, significantly different between GLU and SUC. GLU, glucose; NEFA, non-esterified fatty acid; SUC, sucrose.

Muscle and liver glycogen concentration

Muscle and liver glycogen concentrations are displayed in **Figures 5A**, 5B, 5C and 5D. Pre-exercise, no differences were observed in liver and muscle glycogen concentrations between trials $P > 0.05$ for both variables). The day-to-day coefficients of variation for pre-exercise liver and muscle glycogen concentrations were 12% and 20%, respectively. The between subject coefficient of variation for pre-exercise liver and muscle glycogen were 54% and 41%, respectively. In the subgroup who also completed the CON trial (n = 4), liver glycogen concentrations declined during exercise in CON, but not when either glucose or sucrose were ingested (interaction effect, $P < 0.05$; Figure 5B). In contrast to the liver, muscle glycogen concentrations declined during exercise regardless of trial (trial effect, $P > 0.05$; time effect, $P < 0.01$; interaction effect, $P > 0.05$; Figure 5D).

Post-exercise liver glycogen concentrations did not differ from pre-exercise values when either glucose or sucrose were ingested (time effect, $P > 0.05$; interaction effect, $P > 0.05$). The change in liver glycogen concentrations from pre- to post-exercise was positive with glucose (20 ± 55 mmol/L) and sucrose (27 ± 58 mmol/L; $P > 0.05$ GLU vs SUC) ingestion, but negative in the CON treatment (-171 ± 73 mmol/L).

Muscle glycogen concentrations were reduced following exercise (time effect, $P < 0.001$). The changes in muscle glycogen concentrations did not differ between trials (trial effect, $P > 0.05$; interaction effect, $P > 0.05$; Figures 5C and 4D). The pre- to post-exercise changes in muscle glycogen concentration did not differ between GLU (-40 ± 37 mmol/L) and SUC (-47 ± 36; $P > 0.05$).

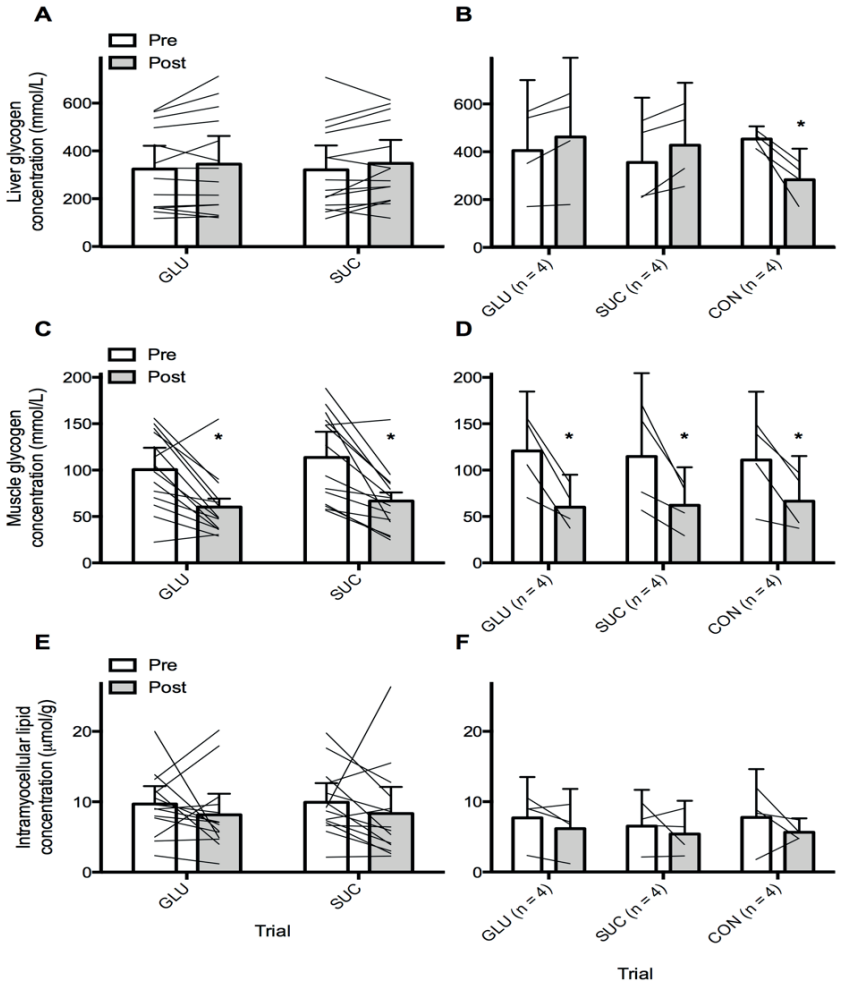

Figure 5. Liver glycogen (A), muscle glycogen (C) and intramyocellular lipid (E) concentrations prior to (Pre) and immediately following (Post) 3 h of cycling with ingestion of glucose or sucrose in the full sample of trained cyclists ($n = 14$), and in the subgroup of trained cyclists (B, D and F; $n = 4$). Data are expressed as means ± 95% CI. * $P <$ 0.05, significantly different when compared with pre-exercise values. GLU, glucose; SUC, sucrose; CON, water control.

Intramyocellular lipid concentration

No differences were observed in pre-exercise IMCL concentration ($P > 0.05$) between trials. The day-to-day coefficient of variation for pre-exercise IMCL concentration was 21%. The between-subject coefficient of variation for pre-exercise IMCL concentration was 47%. In the full sample ($n = 14$) exercise decreased IMCL concentrations (time effect $P < 0.01$) to a similar extent in both trials (trial effect, $P > 0.05$; interaction effect, $P > 0.05$; Figure 5E). The pre- to post-exercise changes in IMCL concentration did not differ between GLU (-1.5 ± 6.0 µmol/g) and SUC (-1.6 ± 6.4 µmol/g; $P > 0.05$).

In the subgroup who completed the CON trial ($n = 4$), post-exercise IMCL concentrations were not significantly different to pre-exercise values (time effect, $P > 0.05$), and the responses were not significantly different between trials (trial effect, $P > 0.05$; interaction effect, $P > 0.05$; Figure 5F).

Discussion

In the present study we provide novel data demonstrating that carbohydrate ingestion during endurance type exercise can prevent liver glycogen depletion, and that this effect is independent of the type of carbohydrate (glucose or sucrose) ingested. In contrast, neither glucose nor sucrose ingestion at 1.7 g/min (102 g/h) could attenuate the decline in muscle glycogen following exercise. Sucrose ingestion increased whole-body carbohydrate utilization when compared with glucose ingestion.

Muscle glycogen and plasma glucose are the main fuel sources during prolonged, moderate-intensity endurance type exercise (1). Plasma glucose is maintained during exercise by glycogenolysis and gluconeogenesis, primarily from the liver. Accordingly, continuous exercise lasting more than 60 min substantially depletes liver glycogen concentrations (4). Given that liver glycogen strongly associates with endurance capacity (3), maintaining liver glycogen concentrations is likely to benefit endurance performance. Previous research using glucose tracers has indicated that high rates of glucose ingestion can suppress endogenous glucose appearance (17), implying that carbohydrate ingestion during exercise may attenuate exercise induced liver glycogen depletion. Here we present the first quantitative evidence of liver glycogen maintenance following carbohydrate ingestion during exercise. We found that 3 h of cycling, in the absence of carbohydrate ingestion reduces liver glycogen concentrations by ~49%, which is consistent with previous findings (4). When ingesting ~1.7 g/min (~102 g/h) glucose or sucrose, liver glycogen concentrations are not lowered during prolonged exercise (Figures 5A and 5B).

Liver glycogen concentrations displayed a relatively high variability between subjects (coefficient of variation: 54%), compared to the day-to-day variability within subjects (coefficient of variation: 12%). This provides an explanation for the relatively higher baseline liver glycogen concentrations in the subgroup that completed the CON trial (n = 4; Figure 5B) compared to the entire sample (n = 14; Figure 5A).

Carbohydrate ingestion during exercise increases exogenous carbohydrate oxidation and has been shown to spare net muscle glycogen utilization under some conditions (11), although not typically during the latter stages of more prolonged (> 1 h), cycling exercise. These responses are thought to contribute to the performance benefits of carbohydrate ingestion during prolonged exercise (7). The present data demonstrate that neither the ingestion of glucose nor sucrose are able to attenuate net muscle glycogen utilization during prolonged moderate-intensity cycling, even when large quantities of multiple transportable carbohydrate (~1.7 g/min; 102 g/h) are ingested that augment exogenous carbohydrate availability. In contrast, whole body carbohydrate utilization rates were higher with sucrose vs glucose ingestion, with a concomitant reduction in fat use. Data from the subgroup also demonstrate that both glucose and sucrose ingestion suppress fat utilization relative to CON, although the numerical differences in carbohydrate utilization rates did not reach statistical

significance with the subgroup (n = 4; P = 0.07). At rest, fructose is preferentially stored as liver glycogen rather than muscle glycogen. This has led some to speculate that sucrose, when compared with glucose ingestion may be particularly effective at maintaining or increasing liver glycogen during exercise. In the present study, sucrose ingestion did not preserve liver glycogen concentrations to any greater extent than glucose ingestion. In line with previous observations of substantial declines in endogenous glucose production during exercise when glucose was ingested (17), our data seem to suggest that liver glycogen contents are maintained during exercise when ingesting large amounts (~1.7 g/min; ~102 g/h) of glucose or sucrose. The surplus carbohydrates are shunted towards oxidation rather than storage, at the expense of lipid oxidation.

The increase in whole-body carbohydrate utilization following sucrose vs glucose ingestion seems to confirm that sucrose ingestion increases exogenous carbohydrate availability and carbohydrate flux. This shift in metabolism is likely due to a number of coordinated factors, including the higher lactate concentrations observed following sucrose ingestion. Higher circulating lactate concentrations are very likely due to the fructose component of sucrose, the majority of which is converted to lactate and glucose upon bypassing the liver. Glucose-fructose co-ingestion during exercise has been shown to increase plasma lactate and glucose turnover and oxidation (40), with a minimal amount of fructose being directly oxidized (40). The greater whole-body carbohydrate utilization rate following sucrose ingestion is therefore likely attributed to a combination of (greater) plasma lactate, glucose and (to a lesser extent) fructose oxidation rates. Lactate also inhibits adipocyte lipolysis via the G-protein coupled receptor GPR81 (41). This is likely one of the factors responsible for the lower plasma NEFA concentrations following sucrose versus glucose ingestion in the presence of similar insulinemia. As there were no differences in muscle lipid content changes between treatments, the greater fat use in the glucose compared with the sucrose trial is likely entirely attributed to greater uptake and oxidation of plasma derived NEFA.

Lactate formation is associated with hydrogen ion production, which may displace CO_2 from bicarbonate stores with consequent implications for estimates of $\dot{V}CO_2$, RER and substrate utilization (38). The ~0.5 mmol/L increase in lactate concentration following SUC vs GLU however, would have a negligible (<0.07 mL/min) effect on CO_2 displacement (38). Therefore, values obtained from expiratory gas samples are likely to be a valid representation of net substrate utilization.

We observed a lower RPE towards the end of exercise following sucrose compared with glucose ingestion. This is in spite of the higher lactate concentrations following ingestion of sucrose compared to glucose, offering additional evidence of the disassociation between lactate concentrations and RPE (42). Exogenous carbohydrate oxidation rates have been shown to correlate with exercise performance during prolonged, moderate-to-high intensity exercise (43). Therefore, the lower RPE following sucrose versus glucose ingestion may be attributed to the greater exogenous carbohydrate uptake and subsequent oxidation rates

when co-ingesting fructose (18, 20). The lower RPE may of course also be directly attributed to the lesser occurrence of gastrointestinal discomfort when ingesting large amounts of multiple transportable carbohydrates versus glucose only (Figure 1).

In conclusion, ingestion of large amounts [~1.7 g/min (~102 g/h), relative to the ~1.5 g/min (90 g/h) recommended for exercise lasting >2.5 h] of glucose or sucrose during prolonged endurance type exercise prevent the exercise-induced decline in liver glycogen content without modulating muscle glycogen depletion.

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Chapter 3

Sucrose ingestion after exhaustive exercise accelerates liver, but not muscle glycogen repletion when compared to glucose ingestion in trained athletes

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Abstract

Purpose: To assess the effects of sucrose versus glucose ingestion on post-exercise liver and muscle glycogen repletion.
Methods: Fifteen well-trained male cyclists completed 2 test days. Each test day started with glycogen-depleting exercise, followed by 5 h of recovery, during which subjects ingested 1.5 g·kg^{-1}·h^{-1} sucrose or glucose. Blood was sampled frequently and ^{13}C magnetic resonance spectroscopy and imaging were employed 0, 120, and 300 min post-exercise to determine liver and muscle glycogen concentrations and liver volume.
Results: Post-exercise muscle glycogen concentrations increased significantly from 85±27 vs 86±35 mmol·L^{-1} to 140±23 vs 136±26 mmol·L^{-1} following sucrose and glucose ingestion, respectively (no differences between treatments: P=0.673). Post-exercise liver glycogen concentrations increased significantly from 183±47 vs 167±65 mmol·L^{-1} to 280±72 vs 234±81 mmol·L^{-1} following sucrose and glucose ingestion, respectively (time x treatment, P=0.051). Liver volume increased significantly over the 300 min period after sucrose ingestion only (time x treatment, P=0.001). As a result, total liver glycogen content increased during post-exercise recovery to a greater extent in the sucrose treatment (from 53.6±16.2 to 86.8±29.0 g) compared to the glucose treatment (49.3±25.5 to 65.7±27.1 g; time x treatment, P<0.001), equating to a 3.4 g·h^{-1} (95%CI: 1.6 to 5.1 g·h^{-1}) greater repletion rate with sucrose vs glucose ingestion.
Conclusion: Sucrose ingestion (1.5 g·kg^{-1}·h^{-1}) further accelerates post-exercise liver, but not muscle glycogen repletion when compared to glucose ingestion in trained athletes.

Introduction

Carbohydrates are a main substrate source used during prolonged moderate to high intensity exercise (35, 42). Both exogenous and endogenous carbohydrate stores can contribute to carbohydrate oxidation during exercise. Endogenous carbohydrate stores include liver and skeletal muscle glycogen, which can provide sufficient energy to sustain 45-60 min of high-intensity exercise (8, 10). However, at longer exercise durations (>60 min) endogenous glycogen stores may become depleted, causing early fatigue (1, 4-6, 9, 16, 20, 39). Due to the apparent relationship between glycogen depletion and exercise capacity (1, 4-6, 9, 12, 19, 20), the main factor determining the time needed to recover from exhaustive exercise is the rate of glycogen repletion. This is particularly relevant when exercise performance needs to be regained within 24 h, for example during tournament-style competitions or in between stages in races such as during the Tour de France.

Previous studies have shown that muscle glycogen repletion rates can reach maximal values when glucose (polymers) are ingested in an amount of 1.2 $g \cdot kg^{-1} \cdot h^{-1}$ (2, 43), with no further improvements at higher glucose ingestion rates (18). It has been speculated that post-exercise muscle glycogen synthesis rates may be further increased when ingesting multiple transportable carbohydrates (i.e., mix of glucose and fructose). Glucose and fructose are absorbed by several similar (GLUT2, GLUT8 and GLUT12) as well as different intestinal transporters (SGLT1 and GLUT5, respectively) (24, 37). Hence, the combined ingestion of both glucose and fructose may augment intestinal carbohydrate uptake and accelerate their subsequent delivery into the circulation (24, 37). To date, only one study investigated this hypothesis, showing no further improvements in post-exercise muscle glycogen repletion rates after the ingestion of ~1.2 $g \cdot kg^{-1} \cdot h^{-1}$ (or 90 $g \cdot h^{-1}$) of multiple transportable carbohydrates compared to an equivalent dose of glucose (44).

The use of multiple transportable carbohydrates is potentially more relevant for liver glycogen repletion, as fructose is preferentially metabolized and retained in the liver (30). Factors that contribute to this are the high first pass extraction of fructose by the liver and the high hepatic expression of fructokinase and triokinase, which are essential enzymes for the metabolism of fructose (30). Furthermore, it has been shown that intravenously administered fructose leads to greater increases in liver glycogen content when compared with intravenous glucose administration (33). Yet, few studies have tried to assess the effects of carbohydrate ingestion on post-exercise liver glycogen repletion (9, 14, 15, 31). This is mainly due to obvious methodological limitations, as liver biopsies are not considered appropriate for measuring liver glycogen concentrations for research purposes *in vivo* in humans (17). With the introduction of ^{13}C-Magnetic Resonance Spectroscopy (^{13}C-MRS), a non-invasive measurement to study changes in liver and muscle glycogen (40, 41), it has been demonstrated that post-exercise liver glycogen resynthesis is stimulated by carbohydrate ingestion (9, 14, 15). Only two studies assessed the effects of fructose ingestion on post-

exercise liver glycogen resynthesis rates. Décombaz *et al.* (14) reported elevated liver glycogen resynthesis rates when co-ingesting fructose with maltodextrin (~0.93 $g \cdot kg^{-1} \cdot h^{-1}$), whereas Casey *et al.* (9) reported no differences in post-exercise liver glycogen repletion following ingestion of ~0.25 $g \cdot kg^{-1} \cdot h^{-1}$ glucose versus sucrose (9). No study has assessed the impact of ingesting multiple transportable carbohydrates on both liver and muscle glycogen repletion when optimal amounts of carbohydrate are ingested during post-exercise recovery. We hypothesize that ingestion of large amounts of sucrose leads to higher liver and muscle glycogen repletion rates when compared to the ingestion of the same amount of glucose. To test this hypothesis, 15 well-trained cyclists completed glycogen depleting exercise, after which we applied ^{13}C MRS to compare liver and muscle glycogen repletion rates following the ingestion of 1.5 $g \cdot kg^{-1} \cdot h^{-1}$ sucrose or 1.5 $g \cdot kg^{-1} \cdot h^{-1}$ glucose during 5 hours of post-exercise recovery.

Methods

Subjects

Fifteen well-trained male cyclists participated in this study (age: 22±4 y, bodyweight: 74.4±7.5 kg, body mass index: 22.6±1.8 kg/m^2, maximal workload capacity (W_{max}): 350±30 W, peak oxygen uptake ($\dot{V}O_2$ peak): 61.5±5.2 mL·kg^{-1}·min^{-1}). Subjects were fully informed of the nature and possible risks of the experimental procedures, before written informed consent was obtained. Trials were conducted at the Newcastle Magnetic Resonance Centre (Newcastle-upon-Tyne, UK) in accordance with the Second Declaration of Helsinki, and following approval from the Northumbria University Faculty of Health and Life Sciences Ethics Committee.

Preliminary testing

All subjects participated in a screening session, which was performed ≥1 wk before the first experiment. Subjects performed an incremental cycling test on an electromagnetically braked cycle ergometer (Velotron, RacerMate Inc., Seattle, WA, USA) to determine maximal workload capacity (W_{max}) and peak oxygen uptake ($\dot{V}O_2$ peak). Following a 5 min warm-up at 100 W, the workload began at 150 W and was increased by 50 W every 2.5 min to exhaustion (27). Expired gas was sampled continuously to determine oxygen uptake (Oxycon gas analyser, CareFusion corporation, San Diego, CA, USA).

Diet and physical activity

All subjects received the same standardized dinner (2797 kJ; 666 kcal; providing 23.9 g fat, 83.7 g carbohydrate and 23.9 g protein) the evening before each test day. All volunteers refrained from exhaustive physical activity 24 h before each main trial and kept their diet as constant as possible 2 d before each experimental day. In addition, subjects filled in food intake and physical activity diaries for 2 d before the start of the first and second trial.

Study design

Participants performed 2 trials in a randomized, double-blind, crossover design separated by at least 7 d. During each trial, they were first subjected to a glycogen depletion protocol on a cycle ergometer. Thereafter, subjects were studied for 5 h while ingesting only glucose in the control trial (GLU) or sucrose in the SUC trial. During the 5 h post-exercise recovery period, subjects remained at rest in a supine position. Magnetic Resonance Spectroscopy (MRS) was performed immediately post-exercise and after 2 and 5 h of post-exercise recovery to determine liver and muscle glycogen concentrations. In addition, Magnetic Resonance Imaging (MRI) was performed immediately post-exercise and after 2 and 5 h of post-exercise recovery to determine liver volume.

Experimental protocol

Participants arrived at Newcastle Magnetic Resonance Centre at 0700-0730 h following a 12 h fast. Liver and muscle glycogen depletion was established by performing an intense exercise protocol on an electromagnetically braked cycle ergometer (26). The exercise protocol started with a 10 min warm-up at 50% W_{max}. Thereafter, subjects cycled for 2-min block periods at alternating workloads of 90% and 50% W_{max}, respectively. This was continued until subjects were no longer able to complete a 2 min, 90% W_{max} exercise period at a cycling cadence of 60 rpm. At this point, the high intensity blocks were reduced to 80% W_{max} after which the same regimen was continued. When subjects were no longer able to complete the 2 min blocks at 80% Wmax, the exercise intensity of the blocks was further reduced to 70%. Subjects were allowed to stop when pedaling speed could not be maintained at 70% W_{max}. Water was provided *ad libitum* during the exercise protocol. Two fans were placed 1 m from the subjects to provide cooling and air circulation during the exercise protocol. After cessation of exercise, gastrointestinal (GI) comfort was assessed using a visual analogue scale. Subsequently, the participants underwent a basal MRS and MRI measurement for approximately 45 min (Fig. 1). After this, they were allowed to take a brief (≤ 15 min) shower before the post-exercise recovery period started. While supine, a catheter was inserted into an antecubital vein of the forearm to allow frequent blood sampling. Following a resting blood sample (10 mL), subjects filled out another visual analogue scale for GI comfort before the first test drink was given (t=0 min). Participants were observed for the following 5 h during which they received a drink with a volume of 3.33 $mL \cdot kg^{-1}$ every 30 min until t=270 min. Blood samples were taken at 15 min intervals for the first 90 min of recovery and every 30 min thereafter until t=300 min. Further visual analogue scales for GI comfort were completed every 30 min until t=300 min. Due to time constraints of the MR measurement it was not possible to acquire a blood sample and collect a visual analogue scale at time point t=150 min. At t=120 and 300 min in the post-exercise recovery period another MR measurement was performed to assess liver and muscle glycogen concentrations as well as liver volume.

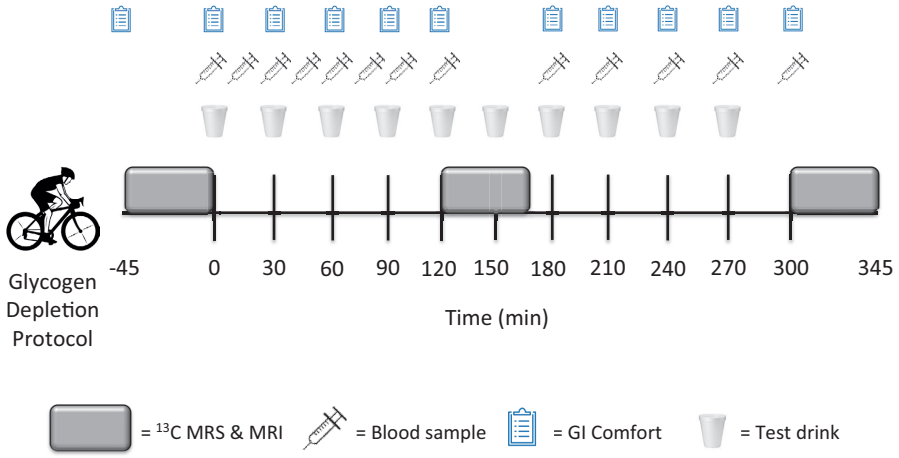

Figure 1. Schematic representation of the experiment. The initial glycogen depletion exercise protocol was followed by three ^{13}C MRS & MRI measurements at *t*=0, *t*=120 and *t*=300 min of post-exercise recovery. The test drink was ingested every 30 min from *t*=0 to *t*=270 min in the post-exercise recovery period as indicated in the figure. Blood samples were obtained every 15 min during the first 90 min of post-exercise recovery. Thereafter they were obtained every 30 min. Visual analogue scales of gastrointestinal (GI) comfort were obtained immediately post-exercise and every 30 min thereafter. At *t*=150 min, no blood sample and visual analogue scale were obtained due to MR scanning.

GI (dis)comfort

Subjects were asked to fill out computerized visual analogue scales to assess GI comfort. The visual analogue scales consisted of 16 questions. Each question started with "To what extent are you experiencing … right now?" and was answered by ticking a 100 mm line (0 mm = not at all, 100 mm = very, very much). The questions consisted of six questions related to upper GI symptoms (nausea, general stomach problems, belching, an urge to vomit, heartburn, stomach cramps), four questions related to lower GI symptoms (flatulence, an urge to defecate, intestinal cramps, diarrhea), and six questions related to central or other symptoms (dizziness, a headache, an urge to urinate, a bloated feeling, side aches (left), side aches (right)).

Drinks

Subjects received a drink volume of 3.33 mL·kg^{-1} every 30 min during recovery to ensure a given dose of 1.5 g·kg^{-1}·h^{-1} glucose (GLU) or 1.5 g·kg^{-1}·h^{-1} sucrose (SUC). To minimize differences in carbon isotope ratio between GLU and SUC, similar plant sources with low natural ^{13}C enrichments (i.e. wheat, potato and beet sugar, all of which use C3 metabolism) were selected for use in this study. The carbohydrates in the glucose drink (GLU) consisted of 60% dextrose monohydrate (Roquette, Lestrem, France) and 40% maltodextrin (MD14, AVEBE, Veendam, The Netherlands). The carbohydrate in the sucrose drink (SUC) consisted

of 100% sucrose derived from sugar beet (AB Sugar, Peterborough, United Kingdom). Both drinks contained 20 mmol·L^{-1} NaCl (Tesco, Cheshunt, United Kingdom).

Measurement of muscle and liver glycogen concentrations

Glycogen concentration was determined from the magnitude of the natural abundance signal from the C-1 carbon of glycogen at a frequency of 100.3 ppm. A Philips 3 Tesla Achieva scanner (Philips Healthcare, Best, The Netherlands) was used with a 6 cm diameter ^{13}C surface coil with integral ^{1}H decoupling surface coil (PulseTeq, Worton under Edge, UK) to measure muscle glycogen concentration and an in-house built 12 cm $^{13}C/^{1}H$ surface coil used to measure liver glycogen concentration. The intra-individual coefficient of variation of hepatic glycogen content measured by ^{13}C MRS has been shown to be 7% (36).

For muscle glycogen concentration measurements, the surface coil was placed over the widest part of the *vastus lateralis* muscle and was held in position with fabric straps to prevent movement. Pulse power was calibrated to a nominal value of 80° by observing the power dependent variation in signal from a fiducial marker located in the coil housing, containing a sample exhibiting ^{13}C signal with short T_1 (213 mM [2-^{13}C]-acetone and 25 mM $GdCl_3$ in water). Automated shimming was carried out to ensure that the magnetic field within the scanner was uniform over the active volume of the ^{13}C coil. The ^{13}C spectra were acquired over 15 min using a non-localized ^{1}H decoupled ^{13}C pulse-acquire sequence (TR 120 ms, spectral width 8 kHz, 7000 averages, WALTZ decoupling). ^{1}H decoupling was applied for 60% of the ^{13}C signal acquisition to allow a relatively fast TR of 120 ms to be used within Specific Absorption Rate safety limitations.

For liver glycogen measurements the $^{13}C/^{1}H$ surface coil was placed over the right lobe of the liver. Spectra were acquired over 15 min using non-localized ^{1}H decoupled ^{13}C pulse acquisition sequences (TR 300 ms, spectral width 8 kHz, 2504 averages, WALTZ decoupling, nominal ^{13}C tip angle of 80°). Scout images were obtained at the start of each study to confirm optimal coil position relative to the liver.

Tissue glycogen concentration was calculated from the amplitude of the C1-glycogen ^{13}C signal using Java Based Magnetic Resonance User Interface (jMRUI) version 3.0 and the AMARES algorithm [7]. For each subject the separation between RF coil and muscle / liver tissue was measured from ^{1}H images, and ^{13}C coil loading assessed from ^{13}C flip angle calibration data. Tissue glycogen concentration was determined by comparison of glycogen signal amplitude to spectra acquired from liver- and leg-shaped phantoms filled with aqueous solutions of glycogen (100 mM) and potassium chloride (70 mM). Phantom data were acquired at a range of flip angles and separation distances between coil and phantom. Quantification of each human ^{13}C spectrum employed a phantom dataset matched to body geometry and achieved flip angle so that account differences in coil sensitivity profile and loading were taken into account for each subject.

Measurement of liver volume

A turbo spin echo (TSE) sequence was used to obtain T_2-weighted axial images of the liver with a repetition time (TR) of 1687 msec. The matrix size was 188x152 mm, with a field of view of (303x240x375) mm. The body coil was used for both transmission and reception. Slice thickness was 10 mm with a 0 mm gap. Scans were obtained on expiration. The total number of liver slices used for volume analysis differed between subjects due to anatomical differences but numbered on average 20 slices. Liver volumes were measured in the open source Java image processing program ImageJ (38).

Calculation of liver glycogen content

Total liver glycogen content was calculated by multiplying liver volume with liver glycogen concentration. Subsequent conversion from mM to g was performed by using the molar mass of a glycosyl unit (i.e., 162 $g \cdot M^{-1}$).

Plasma analysis

Blood samples (10 mL) were collected in EDTA-containing tubes and immediately centrifuged at 3000 rpm for 10 min at 4°C. Plasma was then aliquoted and stored at -80°C for subsequent determination of glucose and lactate concentrations (Randox Daytona spectrophotometer, Randox, Ireland), insulin (IBL International, Hamburg, Germany) and non-esterified fatty acid concentrations (WAKO Diagnostics, Richmond, VA).

Statistics

Sample size estimation was based on previous data on liver glycogen content (14). Based on this, the expected effect size was calculated from the difference in post-exercise liver glycogen content after ingesting a mixture of maltodextrin with fructose vs glucose (polymer) (52±23 vs 23±9 g, respectively). A sample size of n=10 in a crossover design would provide statistical power above 90% with an α-level of 0.05. We therefore recruited 15 participants to ensure adequate power and ample data sets.

Unless otherwise stated, all data are expressed as mean±SD. Differences between primary outcomes in the text and the data in the figures are presented as mean±95% confidence interval (CI). All data were analyzed by two-way repeated measures ANOVA with treatment (GLU vs SUC) and time as within-subject factors. In case of a significant interaction, Bonferroni post hoc tests were applied to locate the differences. For non-time-dependent variables, a paired Student's t-test was used to compare differences between treatments. A P value <0.05 was used to determine statistical significance. All calculations were performed by using the SPSS 21.0.0.0 software package.

Results

Glycogen depletion protocol

Maximal workload capacity measured during preliminary testing averaged 350±30 W (4.75±0.6 W/kg). Consequently, average workload settings in the depletion protocol were 315±27, 280±24, 245±21, 175±15 W for the 90, 80, 70, and 50% W_{max} workload intensity respectively. On average, subjects cycled a total of 21±7 and 19±5 high-intensity blocks, which resulted in a total cycling time of 93±27 and 89±21 min in the SUC and GLU experiments, respectively. Total cycling time did not differ between trials (P=0.434).

Drink ingestion and gastrointestinal complaints

The total amount of drink ingested in both treatments was 2.48±0.25 L. The first drinks were ingested 75±7 min after cessation of exercise, due to timing of the MR measurements. Subjects reported upper GI issues following ingestion of the glucose drink only, and these issues included nausea, general stomach problems, belching and urge to vomit. These symptoms all displayed significant differences over time and between treatments (time x treatment, P<0.05; data not shown) and for every symptom the sucrose drink was better tolerated than the glucose drink.

Liver glycogen concentration

No significant differences in baseline liver glycogen concentrations were found between SUC and GLU (P=0.210; Table 1). Liver glycogen concentrations increased significantly over time during post-exercise recovery in both SUC and GLU (P<0.001). Liver glycogen repletion rates during 5 h of post-exercise recovery in SUC and GLU were 19±8 versus 14±12 mmol·L^{-1}·h^{-1}, respectively (P=0.052). Differences in liver glycogen repletion rates between SUC vs GLU were 5.8 mmol·L^{-1}·h^{-1} (95%CI: 0.4 to 11.2 mmol·L^{-1}·h^{-1}).

Liver volume

Liver volume data are shown in Table 1. Over the 5 h post-exercise recovery period, liver volume increased significantly in SUC (P=0.036), whereas no significant changes were observed in GLU (P=0.151). A significant time x treatment interaction was found between SUC and GLU (P=0.001).

Table 1. Liver glycogen concentration, liver volume and liver glycogen content

		Time (min)		
		0	120	300
Liver glycogen concentration ($mmol \cdot L^{-1}$)	GLU	167±65	191±66 #	234±81 #@
	SUC	183±47	219±63 #	280±72 #@
Liver volume (L)	GLU	1.79±0.28	1.70±0.24 #	1.72±0.24
	SUC	1.80±0.26	1.78±0.24 *	1.89±0.28 #@*

Values are mean±SD. Liver glycogen concentration ($mmol \cdot L^{-1}$) and Liver volume (L) at *t*=0, 120, and 300 min post-exercise, after ingesting 1.5 $g \cdot kg^{-1} \cdot h^{-1}$ glucose (*n*=15: GLU) or sucrose (*n*=15: SUC). Mean values were significantly different from baseline values: # $P<0.05$; 120 min: @ $P<0.05$; and significantly different from GLU: * $P<0.05$. GLU, glucose; SUC, sucrose.

Liver glycogen content

Liver glycogen content increased over time in both treatments ($P<0.01$; Fig. 2). Over time, liver glycogen content increased significantly more in the SUC compared to the GLU treatment (time x treatment interaction, $P<0.001$). Liver glycogen repletion rates during 5 h of post-exercise recovery in SUC and GLU were 6.6±3.3 versus 3.3±3.0 $g \cdot h^{-1}$, respectively ($P=0.002$). Differences in liver glycogen repletion rates between SUC vs GLU were 3.4 $g \cdot h^{-1}$ (95%CI: 1.6 to 5.1 $g \cdot h^{-1}$), leading to a 17 g difference (95%CI: 8 to 26 g) over the 5 h recovery period.

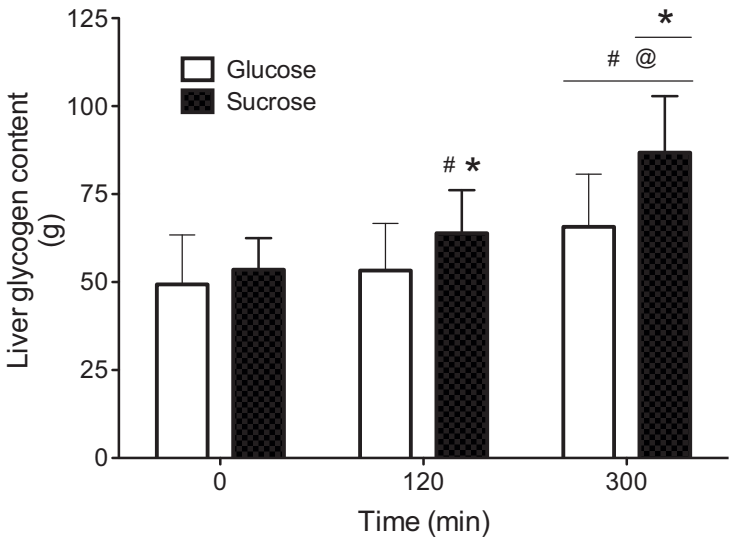

Figure 2. Liver glycogen contents during 5 h of post-exercise recovery while ingesting glucose or sucrose in well-trained cyclists (*n*=15). # $P<0.05$, significantly different when compared with baseline values; @ $P<0.05$, significantly different when compared to values at 120 min; * $P<0.05$, significantly different from the glucose treatment.

Muscle glycogen concentration

No significant differences in baseline muscle glycogen concentrations were observed between SUC and GLU (P=0.940; Fig. 3). Muscle glycogen concentrations increased significantly over the 5 h recovery period in both SUC and GLU (P<0.001). No significant differences were observed between treatments (time x treatment, P=0.673). Muscle glycogen repletion rates during 5 h of post-exercise recovery in SUC and GLU were 11±3 versus 10±5 mmol·L^{-1}·h^{-1}, respectively (P=0.558). Differences in muscle glycogen repletion rates between SUC vs GLU were 0.9 mmol·L^{-1}·h^{-1} (95%CI: -1.9 to 3.6 mmol·L^{-1}·h^{-1}).

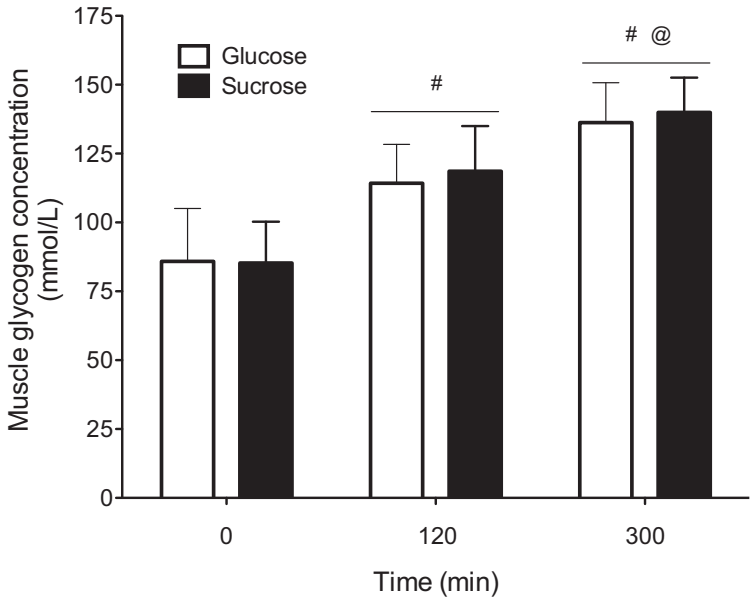

Figure 3. Muscle glycogen concentrations during 5 h of post-exercise recovery while ingesting glucose or sucrose in well-trained cyclists (n=15). # P<0.05, significantly different when compared with baseline values; @ P<0.05, significantly different when compared to values at 120 min. No significant differences between treatments (P=0.673).

Plasma analyses

In both experiments, plasma glucose concentration increased during the first 45 min of post-exercise recovery, after which concentrations gradually declined to baseline values (Fig. 4A). Plasma glucose concentrations were significantly higher at t=60, 75 and 90 min in the GLU compared to SUC treatment (P<0.05), whereas they were significantly higher in the sucrose treatment at time point 270 min (P<0.05). Plasma lactate concentrations increased significantly after 15 min in the SUC trial compared to GLU and remained significantly higher over the entire post-exercise recovery period (P<0.01; Fig. 4B). Plasma insulin concentrations

increased during the first 120 min of post-exercise recovery. Thereafter, plasma insulin concentrations decreased but remained elevated compared to baseline values during the entire post-exercise recovery period (Fig. 4C). Plasma insulin concentrations were significantly higher in the GLU compared with the SUC treatment at *t*=45, 75 and 90 min (*P*<0.05). Plasma NEFA concentrations decreased immediately after carbohydrate ingestion and remained low over the entire recovery period, with no differences between treatments (Fig. 4D).

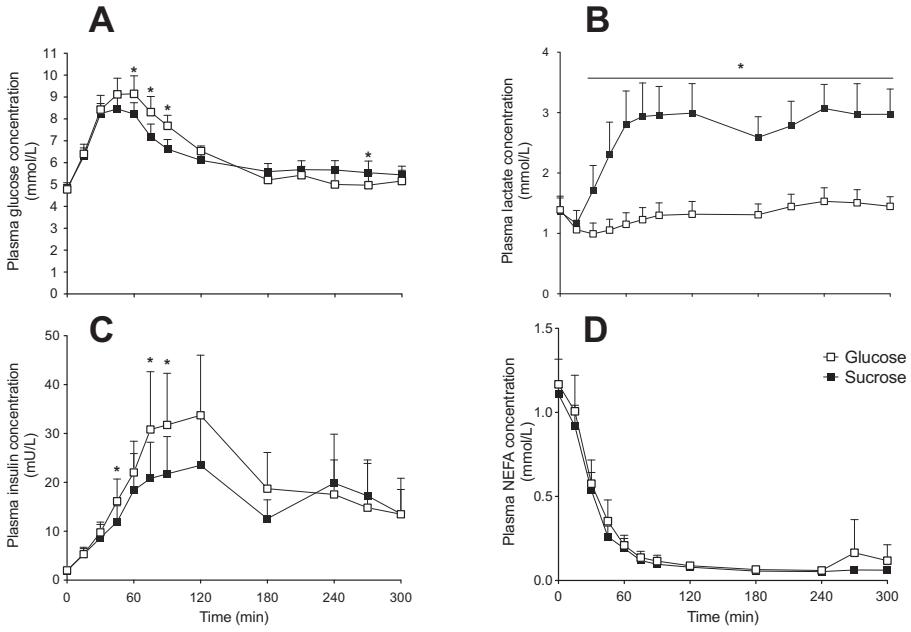

Figure 4. Plasma glucose (A), lactate (B), insulin (C) and NEFA (D) concentrations during 5 h of post-exercise recovery with ingestion of glucose or sucrose in well-trained cyclists (*n*=15). * *P*<0.05, significantly different between glucose and sucrose treatment. NEFA, non-esterified fatty acid.

Discussion

In this experiment we observed that sucrose ingestion (1.5 g·kg^{-1}·h^{-1}) during recovery from exhaustive exercise results in more rapid liver glycogen repletion, despite lower plasma insulin levels, when compared with the ingestion of glucose. Ingestion of sucrose or glucose did not result in differences in post-exercise muscle glycogen repletion rates.

Carbohydrate ingestion during 5 h of post-exercise recovery allowed substantial increases in muscle glycogen concentrations (Figure 3). This represents muscle glycogen repletion rates of 10±5 mmol·L^{-1}·h^{-1} after glucose ingestion and 11±3 mmol·L^{-1}·h^{-1} after sucrose ingestion. Assuming a skeletal muscle mass density of 1.112 g·cm^3 (46) and a wet-to-dry mass ratio of 4.28 (22), our muscle glycogen repletion rates assessed using ^{13}C MRS would translate to glycogen repletion rates of 39±20 and 42±11 mmol·kg^{-1} dw·h^{-1}, respectively. These values are in line with previously published data on post-exercise muscle glycogen resynthesis rates when ingesting ample amounts of carbohydrate (~1.2 g·kg^{-1}·h^{-1}), based upon muscle biopsy collection and concomitant muscle glycogen analyses, showing values ranging between 30-45 mmol·kg^{-1} dw·h^{-1} (3, 23, 43, 44). We did not observe differences in muscle glycogen repletion rates following ingestion of either sucrose or glucose (polymers) during the 5 h post-exercise recovery period (P=0.558). Hence, muscle glycogen resynthesis rates are not limited by exogenous carbohydrate availability when large amounts of glucose, glucose polymers and/or sucrose (≥1.2 g·kg^{-1}·h^{-1}) are consumed. This supports the contention that ingestion of ≥1.2 g carbohydrate·kg^{-1}·h^{-1} maximizes post-exercise muscle glycogen synthesis rates. This also implies that the limitation in exogenous carbohydrate oxidation rates residing in the rate of intestinal glucose absorption does not impose a restriction for post-exercise muscle glycogen synthesis in a post-exercise resting condition.

After exhaustive exercise, the ingestion of glucose and sucrose resulted in liver glycogen repletion rates of 14±12 and 19±8 mmol·L^{-1}·h^{-1}, respectively. These liver glycogen repletion rates together with our observed liver glycogen content values (Figure 2) are comparable to previous observations made by Décombaz and colleagues (14). However, we extend on previous work by showing a doubling of liver glycogen synthesis rates during recovery from exercise when sucrose as opposed to glucose (polymers) were ingested (6.6±3.3 versus 3.3±3.0 g·h^{-1}, respectively: P=0.002). When looking at the present data together with the results of Décombaz *et al*. (14), it can be concluded that ingestion of both submaximal (~0.93 g·kg^{-1}·h^{-1}) and maximal amounts (1.5 g·kg^{-1}·h^{-1}) of multiple transportable carbohydrates further accelerate post-exercise liver glycogen repletion compared to the ingestion of glucose (polymers) only. These observations can be attributed to the differential effects that glucose and fructose exert on hepatic carbohydrate metabolism. Glucose is a relatively poor substrate for hepatic glycogen synthesis (14, 32, 33) and much of it seems to be released into the systemic circulation to be either oxidized or stored as muscle glycogen (7, 10, 11). In contrast, fructose is primarily taken up by the liver where it can be phosphorylated and

converted to glycogen or metabolized to lactate and glucose (28, 29). Lactate will subsequently be released into the bloodstream for oxidation in extrahepatic tissues or can be used as substrate for muscle glycogen synthesis (via gluconeogenesis) (45). In agreement, we observed substantial differences in circulating plasma lactate concentrations between treatments (Figure 4B).

With liver glycogen contents returning to 66 and 87 g it seems that hepatic glycogen stores were not fully replenished within the 5 h recovery period, despite ingesting large amounts of glucose and sucrose. Liver glycogen content was significantly greater and closer to a normal liver glycogen content of ~100 g (21) following sucrose ingestion when compared to glucose ingestion. Since a significant relationship has been found between liver glycogen content at the end of post-exercise recovery and subsequent exercise time-to-exhaustion (9), sucrose as opposed to glucose ingestion may be of benefit for those athletes who need to maximize performance during a subsequent exercise task. To put this into perspective, the difference in liver glycogen content (15-20 g; 57-76 kJ assuming 22% efficiency) could provide enough energy to sustain an additional 3-5 minutes of exercise at 75% W_{max}. This difference is by no means negligible for trained cyclists as it represents a 7-14% difference in time to exhaustion (9). Future research should aim to prove the ergogenic benefit of accelerating liver glycogen repletion on subsequent performance in various (laboratory) exercise settings.

Besides the benefits of sucrose over glucose (polymer) ingestion to maximize liver glycogen repletion, we also observed much better tolerance to the ingestion of large amounts (1.5 $g \cdot kg^{-1} \cdot h^{-1}$) of sucrose when compared with glucose (polymers). In the present study we found considerably lower subjective ratings of upper gastro-intestinal complaints (including nausea, general stomach problems, urge to vomit and belching) after sucrose as opposed to glucose ingestion ($P<0.05$). These findings are not surprising, as after ingesting large amounts (≥1.2 g/kg/h) of a multiple transportable carbohydrate source (i.e., sucrose) more transporters in the gastrointestinal tract will be utilized, thereby decreasing water retention, enhancing absorption and subsequently causing less upper abdominal discomfort when compared to the ingestion of glucose (polymers) only (13). The form in which these carbohydrates are ingested may be of lesser importance, as previous work has shown no differences in post-exercise muscle glycogen repletion when ingesting carbohydrate in either liquid or solid form (25, 34).

In conclusion, post-exercise sucrose ingestion ($1.5\ g \cdot kg^{-1} \cdot h^{-1}$) accelerates liver, but not muscle glycogen repletion when compared with glucose (polymer) ingestion. Ingestion of large amounts of sucrose are better tolerated than glucose (polymers), making sucrose a more practical carbohydrate source to ingest during acute, post-exercise recovery.

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Chapter 4

Fructose co-ingestion to increase carbohydrate availability in athletes

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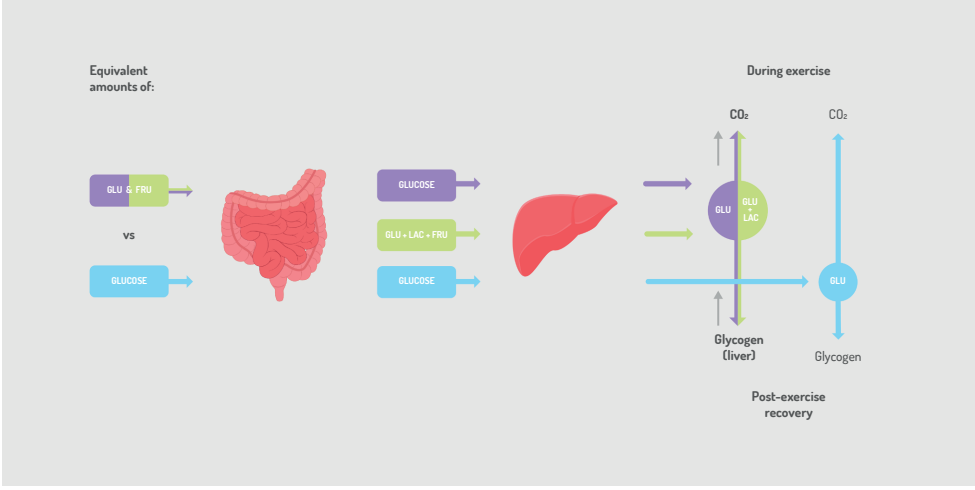
Abstract

Carbohydrate availability is important to maximize endurance performance during prolonged bouts of moderate- to high-intensity exercise as well as for acute post-exercise recovery. The primary form of carbohydrates that are typically ingested during and after exercise are glucose (polymers). However, intestinal glucose absorption can be limited by the capacity of the intestinal glucose transport system (SGLT1). Intestinal fructose uptake is not regulated by the same transport system, as it largely depends on GLUT5 as opposed to SGLT1 transporters. Combining the intake of glucose plus fructose can further increase total exogenous carbohydrate availability and, as such, allow higher exogenous carbohydrate oxidation rates. Ingesting a mixture of both glucose and fructose can improve endurance exercise performance when compared to equivalent amounts of glucose (polymers) only. Fructose co-ingestion can also accelerate post-exercise (liver) glycogen repletion rates, which may be relevant when rapid (<24 h) recovery is required. Furthermore, fructose co-ingestion can lower gastrointestinal distress when relatively large amounts of carbohydrate (>1.2 g/kg/h) are ingested during post-exercise recovery. In conclusion, combined ingestion of fructose with glucose may be preferred over the ingestion of glucose (polymers) only to help trained athletes maximize endurance performance during prolonged moderate- to high-intensity exercise sessions and accelerate post-exercise (liver) glycogen repletion.



Abstract Diagram Fructose co-ingestion appears to increase the total capacity to absorb carbohydrates. In addition, fructose can be converted within the intestine and the liver into glucose and lactate. This can be used as additional fuel source during exercise and also as a substrate for (liver) glycogen repletion during post-exercise recovery. Therefore, fructose co-ingestion may benefit athletes to maximize carbohydrate availability during exercise and during acute post-exercise recovery.

Introduction

Carbohydrates are a major substrate source during prolonged moderate- to high-intensity exercise (1, 2). In the fasted state, the main forms of carbohydrate utilised during exercise are skeletal muscle glycogen and plasma glucose (primarily derived from liver glycogen and gluconeogenesis) (2). However, these glycogen stores can be rapidly depleted (by ~40-60%) within 90 min of moderate to high-intensity exercise (3-5). Low endogenous glycogen stores can contribute to fatigue, thereby reducing endurance exercise capacity (6-9). Even when glycogen is not critically low (>100 mmol/kg ww), higher carbohydrate availability could increase endurance performance by reducing the oxygen cost of exercise as the energy yield per given volume of oxygen is higher from carbohydrate when compared to fat-based fuels (10). In line, it has previously been observed in elite race walkers that both exercise economy and performance were negatively impacted following 3 weeks of a high-fat diet when compared to high carbohydrate availability (11). Hence, high carbohydrate availability could provide an advantage during endurance exercise events where oxygen delivery can become a limiting factor. Therefore, nutritional strategies to complement or replace endogenous carbohydrate stores as a fuel source during exercise can be of importance for athletes trying to maximize endurance exercise performance. It is now well established that carbohydrate ingestion during exercise improves endurance performance and can delay fatigue in events requiring sustained moderate to high-intensity exercise for prolonged durations (i.e., more than ~45 min) (12, 13).

Due to the apparent relationship between glycogen depletion and endurance exercise capacity (3, 6), an important determinant of recovery time is the rate of glycogen repletion. This is particularly relevant when optimal performance needs to be regained well within 24 h, for example during intensive training periods, tournament-style competitions or in between stages in multiday races such as the Tour de France. In the hours following exercise, carbohydrate ingestion is a requirement for substantial repletion of liver and skeletal muscle glycogen stores (3, 14, 15).

Dietary carbohydrates come in many forms, with glucose (polymers) being the most ubiquitous carbohydrate in most people's diets (16). Glucose is also the primary cellular fuel source in most human tissues. As a result, glucose (polymers) have been recommended already for decades as the predominant source of carbohydrates to ingest around endurance exercise sessions for athletes (17). Fructose, on the other hand, has since long been considered as a suboptimal source of carbohydrate to ingest around exercise, as it seems less effective at increasing exogenous carbohydrate oxidation (when compared to glucose) and may cause gastrointestinal distress (18). Over more recent years, there has been an increasing appreciation of ingesting a combination of glucose and fructose both during and after exercise. Therefore, this review provides a brief overview of the potential benefits of

(co-)ingesting fructose with glucose (polymers) during exercise and acute post-exercise recovery.

Carbohydrate ingestion during exercise

During exercise, exogenous carbohydrate oxidation rates differ depending on the type of carbohydrate that is consumed (19). It has been well established that the maximal exogenous carbohydrate oxidation rate increases in a curvilinear fashion with carbohydrate ingestion rate, reaching peak exogenous oxidation rates of ~1.1 g/min when ingesting glucose (polymers) only during exercise (16, 20). Several factors may determine the rate at which exogenous carbohydrates are taken up and oxidized by the working muscles during exercise. These include the rate of gastric emptying, the rate of digestion and absorption, passage via the liver into the systemic blood supply, and the rate of glucose uptake and subsequent oxidation by the working muscle (21). The primary limitation of exogenous carbohydrate oxidation rates is unlikely caused by gastric emptying rates, as gastric emptying rates of glucose have been shown to exceed carbohydrate oxidation rates during prolonged exercise (22). In addition, the primary limitation of exogenous carbohydrate oxidation rates is also unlikely caused by glucose uptake and oxidation by the working muscle, as when glucose is directly infused (thereby bypassing the intestines and liver), peak exogenous carbohydrate oxidation rates of 1.8 g/min can be achieved (23). This implies that intestinal absorption and/or hepatic metabolism may be the primary factor limiting exogenous glucose oxidation rate during exercise (20, 24).

When (only) fructose is ingested, exogenous carbohydrate oxidation rates during exercise have been shown to be equivalent (25-27) or lower when compared to glucose ingestion (26, 28-33). Furthermore, ingestion of large amounts of fructose (alone) has been reported to cause gastrointestinal distress and also has a limited capacity for intestinal absorption when ingested alone (34, 35). Consequently, fructose has generally been considered of little interest for the athlete trying to optimize carbohydrate availability during exercise (19, 20). However, when fructose is co-ingested with glucose during exercise (at 50% W_{max}), exogenous carbohydrate oxidation rates can increase up to 1.75 g/min (36), which is substantially higher than what has been reported following ingestion of glucose (polymers) or fructose only. These exogenous carbohydrate oxidation rates do not appear to differ when fructose is co-ingested in the form of fructose or sucrose (37), which is in line with observations that rates of digestion and intestinal absorption of glucose and fructose do not differ when ingested as sucrose or as co-ingestion of free fructose with free glucose (38).

Fructose metabolism differs markedly from glucose metabolism. At rest, fructose is primarily absorbed across the apical membrane of the intestinal enterocytes by transport protein

GLUT5, whereas glucose is primarily absorbed across the apical membrane of the intestinal enterocytes by transport protein SGLT1 (please see Ferraris *et al*. for a comprehensive review on this topic (39)). After intestinal absorption, fructose appears to be actively metabolized in the splanchnic area (i.e., intestine, liver and kidneys), whereas glucose appears to be largely transmitted passively from the splanchnic area into the systemic circulation (16, 40-42) (**Figure 1**). Therefore, following fructose ingestion, plasma fructose concentrations remain relatively low (<0.5 mmol/L) (43, 44) as fructose is rapidly converted in the intestine and liver to glucose and lactate (**Figure 1**), which then enter the systemic circulation and are delivered to peripheral tissues (44) and/or contribute to liver glycogen synthesis (45). It should be noted that recent work has reported that ~15% of ingested fructose may escape first-pass extraction by the splanchnic organs (46) and, as such, may directly be metabolized in other tissues (40). However, direct oxidation of fructose in the muscle will unlikely play a significant quantitative role as fuel during exercise since fructose transport capacity over the muscle membrane is 8 times lower than glucose and this capacity is not further increased by exercise (47). In line, lower affinity of hexokinase for fructose when compared to glucose, further supports the notion that plasma fructose is not an important fuel source for exercising muscle (48). When fructose is co-ingested with glucose in large amounts (0.8 g/min and 1.2 g/min, respectively) during exercise, the systemic appearance of fructose-derived glucose and lactate is ~0.5 g/min (of which the contribution of glucose and lactate is equally split) (44). The oxidation of fructose-derived glucose and lactate by skeletal muscle can thus fully account for the higher exogenous carbohydrate oxidation rates observed following ingestion of glucose and fructose mixtures when compared to glucose only (16).

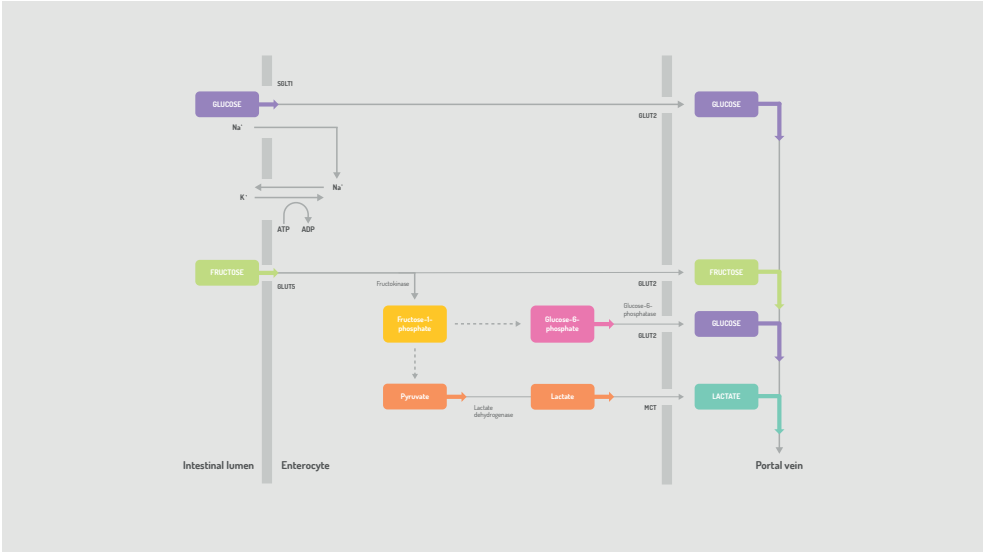


Figure 1A. Intestinal glucose and fructose absorption and fructose conversion into glucose and lactate within the enterocytes. Glucose is primarily absorbed via the sodium dependent glucose transporter 1 (SGLT1) and is largely transmitted passively into the portal vein. Fructose is primarily absorbed via glucose transporter 5 (GLUT5) and can be transmitted into the portal vein via glucose transporter 2 (GLUT2) or can be metabolized within the enterocyte. Via first conversion into fructose-1-phosphate (via fructokinase) glucose-6-phosphate and pyruvate can be formed. Glucose-6-phosphate can be converted into glucose (via glucose-6-phosphatase) and leave the enterocyte via GLUT2. Pyruvate can be converted into lactate (via lactate dehydrogenase) and leave the enterocyte via monocarboxylate transporter (MCT).

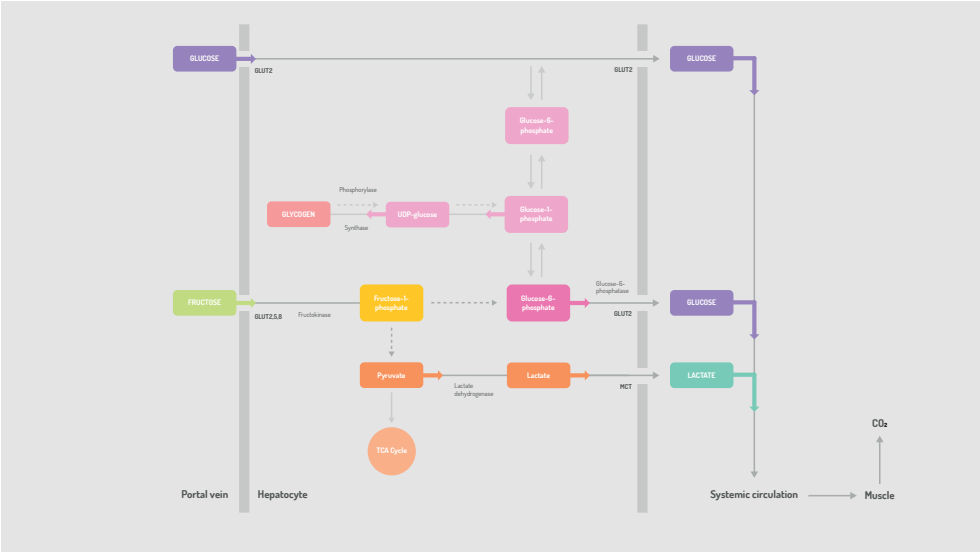


Figure 1B. Main pathways involved in hepatic glucose and fructose absorption and fructose conversion into glucose and lactate during exercise. Glucose is primarily taken up via GLUT2 and is largely transmitted passively into the systemic circulation. Fructose can be taken up via GLUT2, GLUT5 and/or GLUT8 and is largely metabolized into glucose and lactate during exercise. Via first conversion into fructose-1-phosphate (via fructokinase) glucose-6-phosphate and pyruvate can be formed. Glucose-6-phosphate can be converted into glucose (via glucose-6-phosphatase) and leave the hepatocyte via GLUT2. Glucose-6-phosphate can also be used as substrate for restoring liver glycogen during exercise (via first conversion into glucose-1-phosphate and subsequently UDP-glucose). However, fructose co-ingestion is not more effective in preventing liver glycogen depletion during exercise when compared to glucose ingestion only, suggesting that liver glycogen storage during exercise may not be a primary pathway. Pyruvate can be used as substrate to provide direct energy to the liver (TCA cycle) or can be converted into lactate (via lactate dehydrogenase) and leave the hepatocyte via monocarboxylate transporter (MCT). The additional glucose and lactate (derived from fructose) can be used as substrate for oxidation in the muscle during exercise. GLUT, glucose transporter; TCA Cycle, tricarboxylic acid cycle; UDP-glucose, uridine diphosphate glucose.

Next to providing fuel to the working muscle, ingesting carbohydrates during exercise has also been suggested to prevent the depletion of endogenous (muscle and liver) carbohydrate stores. Due to higher capacity for carbohydrate absorption and predominant hepatic metabolism of fructose (**Figure 1**), it could be speculated that the combined ingestion of glucose and fructose further prevents the lowering of endogenous carbohydrate stores during exercise, and particularly in the liver. We recently performed a study investigating the effects of ingesting either 1.7 g/min of glucose or 1.7 g/min of sucrose during 3 h of exercise (at 50% W_{peak}) on liver and muscle glycogen concentrations (4). We observed that neither type of carbohydrate was able to prevent the lowering of muscle glycogen concentrations during exercise. However, ingestion of either type of carbohydrate fully prevented liver glycogen depletion. This suggests (at least during submaximal exercise at 50% W_{peak}) that carbohydrate ingestion can prevent liver glycogen depletion during exercise, but that

fructose co-ingestion is not more effective than glucose (polymers) ingestion alone in preventing exercise-induced glycogen depletion. Whether the same holds true for exercise sessions performed at higher exercise intensities remains to be elucidated.
Increases in plasma glucose and insulin concentrations inhibit net hepatic glycogenolysis (49). We have observed that plasma glucose and insulin concentrations do not differ during moderate intensity exercise (at 50% W_{peak}) when fructose and glucose are co-ingested compared to ingesting glucose only (4, 37). These findings seem to be in line with the absence of differences in liver glycogen concentrations when either glucose or sucrose was ingested (4). When large amounts of carbohydrate are ingested during exercise, the co-ingestion of fructose lowers gastrointestinal distress when compared to ingesting equivalent amounts of glucose (polymers) alone (4, 37, 50, 51). Therefore, the main benefits of fructose co-ingestion (vs glucose (polymers) only) during exercise are due to increased exogenous (and total) carbohydrate oxidation rates and/or less gastrointestinal discomfort, rather than preventing muscle or liver glycogen depletion. By combining the ingestion of glucose (polymers) with fructose it has been observed that athletes can further improve endurance exercise performance when compared to ingesting glucose (polymers) only by ~8-9% (this was found when glucose-fructose mixtures (≥90 g/h) were compared to equivalent amounts of glucose as well as amounts of glucose (60 g/h) that are proposed to saturate intestinal absorption) (12, 52-54). It is important to note, however, that ingestion of large amounts (>1.2 g/min) of a mixture of glucose (polymers) and fructose is likely only of practical relevance to highly trained athletes that are able to sustain high-intensity exercise for a prolonged duration (i.e., >2.5 h) (55).

Carbohydrate ingestion after exercise

The suggestion that glucose-fructose co-ingestion will increase rates of carbohydrate absorption also raises the possibility to further accelerate the rate of endogenous (muscle and/or liver) carbohydrate stores during recovery from exercise. It has been hypothesized that the greater carbohydrate availability with the ingestion of large amounts of glucose and fructose (sucrose) mixtures could, therefore, augment post-exercise glycogen repletion rates.

Muscle glycogen

It has previously been demonstrated that net muscle glycogen (re)synthesis rates during 4 h of post-exercise recovery in the fasted state are in the range of ~2-12 mmol/kg dw/h (14, 15, 56), with no net muscle glycogen (re)synthesis observed beyond 4 h of recovery (56). In the first few hours (~4 h) after exercise, skeletal muscle glycogen (re)synthesis rates are enhanced due - at least in part - to an increase in insulin sensitivity (57). With sufficient carbohydrate intake immediately post-exercise, net muscle glycogen (re)synthesis rates have been

observed to increase up to 20-45 mmol/kg dw/h (58) and with frequent carbohydrate ingestion (alongside increased insulin availability) this can result in a full recovery of muscle glycogen levels within 24 h (59, 60). For more in-depth information on muscle glycogen repletion, the interested reader is referred to other reviews (e.g., Jentjens & Jeukendrup (61), Beelen *et al. (58)* and Burke *et al. (59)*). It has been well established that for optimal post-exercise net muscle glycogen (re)synthesis rates, athletes should ingest carbohydrates at a rate of ~1.2 g/kg/h immediately after cessation of exercise and in frequent intervals (i.e., 15-30 min) within the first ~4 h of the recovery period (59). With regards to the type of carbohydrate intake, it has previously been observed that glucose ingestion further increases post-exercise muscle glycogen repletion rates over fructose ingestion only (62, 63). However, it has been speculated that based on the metabolism of glucose and fructose during exercise (**Figure 1**), greater carbohydrate availability following ingestion of large amounts of glucose plus fructose could further increase post-exercise muscle glycogen repletion rates when compared to glucose ingestion only. Several studies have directly compared ingesting mixtures of glucose (polymers) with fructose and glucose (polymers) alone on post-exercise muscle glycogen repletion rates (3, 63-67). In these studies a wide range of carbohydrate ingestion rates have been employed ranging from 0.25 to 1.5 g/kg body mass/h over 2 to 6 h of recovery. Based on these studies it can be concluded that even with large and recommended carbohydrate ingestion rates (≥1.2 g/kg/h) provided at frequent intervals, there are no differences between the ingestion of glucose (polymers) and fructose (sucrose) mixtures vs glucose (polymers) alone on post-exercise muscle glycogen repletion rates (64-66). However, the ingestion of large amounts (≥1.2 g/kg/h) of glucose and fructose mixtures have been shown to induce lower gastrointestinal issues that can likely be explained by improved intestinal carbohydrate absorption (64, 65). This is a relevant finding as gastrointestinal distress could directly reduce the capacity to optimally perform in a subsequent bout of exercise.

Liver glycogen

In contrast to muscle, the liver plays a major role in fructose metabolism and is able to synthesize glucose from fructose in meaningful quantities. Over a 6-h period, up to ~50% of the ingested fructose can be found in the circulation as glucose, of which the conversion seems to occur primarily in the liver. In addition, there is some conversion of fructose into glucose that is subsequently stored directly as liver glycogen, which seems to account for at least >15% of fructose disposal at rest. (68, 69). Consequently, fructose co-ingestion may further accelerate liver glycogen repletion when compared to the ingestion of glucose (polymers) only.

Upon intestinal absorption, fructose can be metabolized within the small intestine (albeit most likely only in small amounts in humans, as saturation of intestinal fructose metabolism has been suggested to occur at ~5 g of fructose intake) (41, 42, 69, 70), or transported towards the liver via the portal vein (**Figure 1**). Fructose uptake in the liver is thought to be mainly operated by the glucose transporter GLUT2, however GLUT5 and GLUT8 may also contribute (71). Within the liver, fructose is largely extracted at first pass and rapidly phosphorylated into fructose-1-phosphate by the enzyme fructokinase (also known as ketohexokinase), which is highly specific for fructose. Fructose-1-phosphate is subsequently metabolized into glyceraldehyde and dihydroxyacetone phosphate (DHAP) via aldolase B. Subsequently, glyceraldehyde-3-phosphate can be formed (via the enzymes triose-phosphate-isomerase and triokinase) which can be further converted (first via fructose-1,6-biphosphate and fructose-6-phosphate) into glucose-6-phosphate (see **Figure 2** for schematic overview). Within the liver, glucose-6-phosphate can be converted into glucose (by glucose-6-phosphatase) and subsequently released into the systemic circulation (e.g. to maintain euglycemia) or stored (via first conversion into glucose-1-phosphate and subsequently UDP-glucose) as liver glycogen.

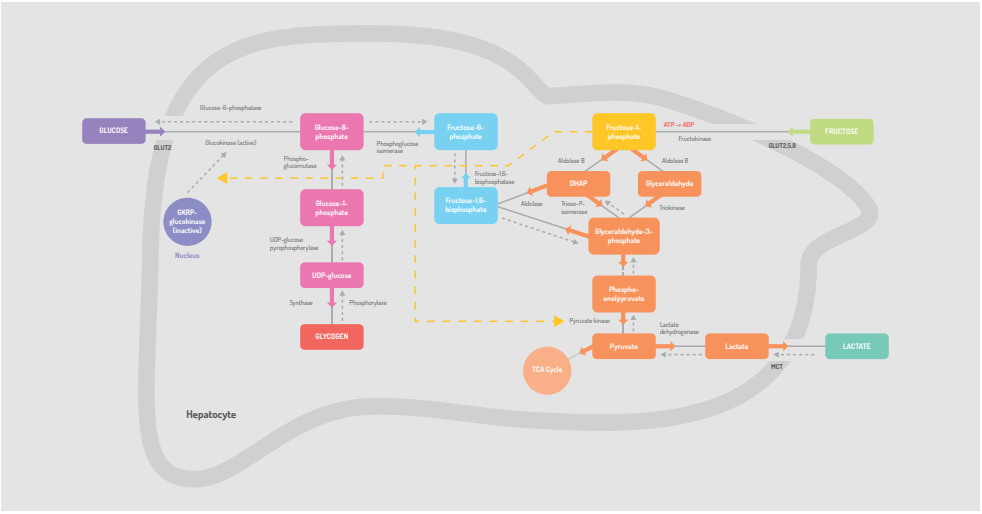


Figure 2. Proposed hepatic fructose metabolism into glucose, glycogen, and lactate after exercise. Upon entering hepatocytes, fructose is phosphorylated by fructokinase to fructose-1-phosphate. Fructose-1-phosphate is cleaved to DHAP and glyceraldehyde by aldolase B. DHAP and glyceraldehyde can be phosphorylated (by triose-P-isomerase and triokinase, respectively) into glyceraldehyde-3-phosphate. Both DHAP and glyceraldehyde-3-phosphate can enter the gluconeogenic and/or glycolytic metabolite pool, and can have several metabolic fates including conversion into glucose, glycogen, and lactate. Fructose-1-phosphate (shown in yellow) also regulates metabolic enzymes (yellow lines) involved in glycogen storage and lactate production. Responsible enzymes denoted in grey; responsible transporters denoted in black. DHAP, dihydroxyacetone phosphate; GKRP, glucokinase regulatory protein; GLUT, glucose transporter; MCT, monocarboxylate transporter; TCA cycle, tricarboxylic acid cycle; Triose-P-isomerase, Triose-phosphate-isomerase; UDP, uridine diphosphate.

In addition to providing indirect substrate for liver glycogen synthesis, fructose-1-phosphate can also act as a signalling molecule to further increase liver glycogen synthesis. Fructose-1-phosphate exerts a strong positive regulatory control on glucokinase by promoting its release from the inhibitory glucokinase regulatory protein (GKRP) (72-74). In the fasted state, GKRP sequesters glucokinase in the nucleus in an inactive state (73). Via activation of glucokinase, small amounts of fructose can promote hepatic glucose uptake and phosphorylation (into glucose-6-phosphate), leading to rapid glycogen synthesis (74-76) (**Figure 2**). Within the liver, elevated glucose-6-phosphate concentrations provoke the activation of glycogen synthase (77) and Petersen *et al.* demonstrated that low-dose fructose (~9 g) infusion during a 4 h hyperinsulinemic-euglycemic clamp further increased liver glycogen synthase flux (by ~2.5 fold) and subsequent net glycogen synthesis rates (by ~3 fold) in healthy young humans (75). It should be noted that insulin potentiates hepatic glycogen synthesis by activating glycogen synthase and inactivating glycogen phosphorylase (78, 79) and intravenous fructose administration during a hyperinsulinemic-euglycemic clamp is not necessarily reflective of oral fructose (co-)ingestion in the post-exercise state. Whilst previous studies have observed that post-exercise fructose-glucose co-ingestion (when ingested regularly in small boluses) can induce a robust increase in insulin concentrations that remain elevated during post-exercise recovery (64, 80). In fact, similar insulin concentrations can be observed when fructose is co-ingested (80) when compared to intravenous fructose administration during a hyperinsulinemic-euglycemic clamp (75). However, despite increased insulin concentrations (when compared to basal values) and a doubling of post-exercise liver glycogen repletion rates (when compared to ingesting glucose only), the increase in plasma insulin concentrations with fructose co-ingestion is typically still lower than those seen with ingestion of glucose only during post-exercise recovery (64). In addition, even in the absence of increased plasma insulin, the administration of small amounts of intravenous fructose (~2.2 g) with glucose markedly increase hepatic glycogen content when compared to glucose only in dogs (81). Thus, it appears that even small "catalytic" doses of fructose added to glucose can augment liver glucose uptake and glycogen synthesis, which cannot be attributed to changes in plasma insulin *per se*. The latter may be explained (at least in part) by the fact that the production of fructose-1-phosphate from fructose and activation of glucokinase by fructose-1-phosphate are not dependent on increased plasma insulin levels (72, 82). Fructose-1-phosphate has also been suggested to augment hepatic glycogen storage by inhibiting glycogen phosphorylase (83, 84). However, Petersen *et al.* did not show an inhibitory effect of fructose on glycogen phosphorylase flux in human subjects (75), suggesting that this pathway may not contribute to augmented liver glycogen storage when fructose is administered in humans.

Finally, fructose-1-phosphate can activate pyruvate kinase (85), thereby contributing to increased circulating levels of lactate following fructose (co-) ingestion (**Figure 2**). Indeed, we (64, 65) and others (43, 66) have previously observed greater post-exercise lactate

concentrations following fructose-glucose co-ingestion, compared to glucose (polymers) only. Lactate has been shown to serve as an additional substrate source, but can also be transported towards the muscle to directly stimulate muscle glycogen repletion (86-90). Lactate may also indirectly stimulate muscle glycogen repletion, via conversion within the liver (by gluconeogenesis) into glucose and subsequent transport towards the muscle. These effects of lactate could potentially explain (at least in part) equivalent muscle glycogen repletion with fructose co-ingestion (vs glucose alone) as fructose appears to retain some of the (exogenous) glucose within the hepatocytes. Finally, lactate can also indirectly contribute to hepatic glycogen synthesis (91).

Via these mechanisms, in addition to higher rates of carbohydrate absorption and availability, fructose co-ingestion could theoretically further accelerate liver glycogen synthesis rates over glucose ingestion only. Few studies have directly compared the effects of glucose and fructose co-ingestion vs glucose (polymer) ingestion only on post-exercise liver glycogen repletion rates (3, 64, 80, 92). Only two of these studies provided carbohydrates at relatively high ingestion rates (>0.9 g/kg body mass/h), which have been recommended for optimal post-exercise glycogen repletion in athletes (64, 80). Based on these studies, it can be concluded that when glucose is ingested alone, the rate of post-exercise liver glycogen repletion is ~3.5 g/h. When fructose is co-ingested with glucose (either as free glucose and fructose or as sucrose), the rate of liver glycogen repletion may increase two-fold (~7.4 g/h) (64, 80). Therefore, it can be concluded that the combined ingestion of glucose plus fructose accelerates liver glycogen repletion.

It has been suggested that fructose co-ingestion can increase endurance exercise capacity by an additional 3-5 min during a subsequent bout of cycling exercise (at 75% W_{max}) due to its capacity to accelerate liver glycogen repletion (64). In support, when fructose was co-ingested during the first 4 h of post-exercise recovery, endurance exercise capacity during a subsequent exercise bout (i.e., treadmill running to exhaustion at 70% VO_{2max}) was shown to be increased by ~32.4% when compared to ingesting glucose (polymers) only (93). This clearly shows the potential benefit of combining glucose with fructose in the first hours of post-exercise recovery when optimal performance during a subsequent endurance exercise session is key.
It should be noted that high dietary fructose intake has been proposed to induce adverse health effects and has been associated with the development of metabolic disease (40, 71). Therefore, more research in the area of fructose-rich diets and their potential adverse effects are warranted. However, exercise appears to be able to correct early markers of metabolic disease induced by high fructose intake, independent of energy balance (94, 95). In addition, elite athletes typically display exquisite metabolic health, as demonstrated by a three-fold higher insulin sensitivity than controls (96) despite consuming large amounts (>450 g per day)

of simple sugars during events such as the Tour de France (97). Therefore, given the ergogenic properties of fructose both during and after exercise, athletes may benefit from fructose co-ingestion.

Conclusions and Recommendations

The rate of appearance of ingested glucose in the circulation appears to be limited by the capacity of intestinal transporters. Since intestinal fructose absorption utilises a different transport mechanism, combining the ingestion of fructose and glucose takes advantage of both transport mechanisms, thereby increasing the total capacity for carbohydrate absorption. This can be beneficial during exercise to further increase exogenous carbohydrate oxidation rates and lower gastrointestinal discomfort when large amounts of carbohydrates are ingested, thereby improving endurance exercise performance during prolonged moderate-to-high intensity exercise. Consequently, when trying to maximize performance, well-trained athletes are advised to combine the ingestion of glucose and fructose at ingestion rates of 90 g/h during prolonged (>2.5 h) moderate-to-high intensity exercise. After exercise, rapid recovery of both muscle and liver glycogen stores are important determinants of the capacity to perform a subsequent bout of moderate-to-high intensity exercise. Muscle glycogen repletion rates cannot be further increased with fructose-glucose co-ingestion. However, due to higher absorption rates and/or predominant hepatic fructose metabolism, fructose co-ingestion has been observed to enhance post-exercise liver glycogen repletion rates without compromising muscle glycogen re-synthesis. In addition, when large amounts of carbohydrates are ingested after exercise, the combined ingestion of glucose plus fructose can result in less gastrointestinal distress. Therefore, when rapid recovery from prolonged exercise is a key objective, and maximal performance is required well within 24 hours, it is advised to consume more than 1 g carbohydrate/kg body mass/h, starting as soon as possible after exercise and at frequent intervals thereafter (i.e. every 15-30 min). In this context, fructose co-ingestion may be of benefit to lower gastrointestinal discomfort and accelerate liver glycogen synthesis rates.

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Chapter 5

Branched-chain amino acid and branched-chain ketoacid ingestion increase muscle protein synthesis rates *in vivo* in older adults: a double-blind, randomized trial

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Abstract

Background: Protein ingestion increases muscle protein synthesis rates. However, limited data are currently available on the effects of branched-chain amino acids (BCAA) and branched-chain ketoacids (BCKA) ingestion on postprandial muscle protein synthesis rates.
Objective: To compare the impact of ingesting 6 g BCAA, 6 g BCKA, and 30 g milk protein (MILK) on the postprandial rise in circulating amino acid concentrations and subsequent myofibrillar protein synthesis rates in older males.
Design: In a parallel design, forty-five older males (age: 71±1 y, BMI: 25.4±0.8 kg/m^2) were randomly assigned to ingest 6 g BCAA, 6 g BCKA, or 30 g MILK. Basal and postprandial myofibrillar protein synthesis rates were assessed by primed continuous L-[ring-$^{13}C_6$]phenylalanine infusions with the collection of blood samples and muscle biopsies.
Results: Plasma BCAA concentrations increased following test drink ingestion in all groups, with greater increases in BCAA and MILK vs BCKA (P<0.05). Plasma BCKA concentrations increased following test drink ingestion in all groups, with greater increases in BCKA vs BCAA and MILK (P<0.05). Ingestion of MILK, BCAA and BCKA significantly increased early myofibrillar protein synthesis rates (0-2 h) above basal rates (from 0.020±0.002%/h to 0.042±0.004%/h, 0.022±0.002%/h to 0.044±0.004%/h and 0.023±0.003%/h to 0.044±0.004%/h, respectively; P<0.001), with no differences between groups (P>0.05). Myofibrillar protein synthesis rates during the late postprandial phase (2-5 h) remained elevated in MILK (0.039±0.004%/h; P<0.001), but returned to baseline values following BCAA and BCKA ingestion (0.024±0.005%/h and 0.024±0.005%/h, respectively; P>0.05).
Conclusion: Ingestion of 6 g BCAA, 6 g BCKA, and 30 g MILK increases myofibrillar protein synthesis rates during the early postprandial phase (0-2 h) *in vivo* in healthy older males. The postprandial increase following the ingestion of 6 g BCAA and BCKA is short-lived, with higher myofibrillar protein synthesis rates only being maintained following the ingestion of an equivalent amount of intact milk protein.

Introduction

Protein ingestion strongly increases muscle protein synthesis rates (1, 2). The postprandial increase in muscle protein synthesis rate has been attributed to the rise in circulating amino acids (3, 4). Amino acids serve as precursors for *de novo* muscle protein synthesis and can act as strong signaling molecules activating translation initiation via the mechanistic/mammalian target of rapamycin complex-1 (mTORC1) pathway (5, 6). Several studies indicate that senescent muscle is less sensitive to these anabolic properties of amino acids (7, 8). Anabolic resistance to feeding has been reported in the elderly and in several patient populations suffering from chronic disease (9). As a consequence, older and/or more clinically compromised patient populations require greater amounts of protein to be consumed (7, 10-14) or may benefit from food fortification with BCAA (15-19) to augment postprandial muscle protein synthesis rates. Few data are available regarding the muscle protein synthetic response to ingesting BCAA (leucine, isoleucine and valine) only in humans. Recently, it was shown that BCAA ingestion increases myofibrillar protein synthesis rates during recovery from exercise in young males (20). However, whether BCAA ingestion can increase myofibrillar protein synthesis rates to a similar extent when compared to the ingestion of intact protein *in vivo* in older males remains to be assessed.

In many clinically compromised populations, simply increasing protein intake is not realistic and has been suggested to be unfavorable in patients with chronic kidney disease (CKD) because of potential renal injury (21). Hence (very) low protein diets are often prescribed in certain disease stages of patients with CKD, further compromising their capacity to preserve muscle mass (22-24). Supplementation with BCKA has been applied in these conditions as these keto-analogues do not provide nitrogen (N) and may help to lower N intake as BCKA can be transaminated into BCAA (25, 26). BCKA are readily available, safe for human consumption (27), and efficiently absorbed in the small intestine (28). However, intestinal absorption rates of BCKA appear to be moderately lower when compared to BCAA (29). In addition, oral administration of BCKA, but not BCAA, appears to induce substantial first-pass oxidation in splanchnic organs (30). Therefore, the nutritional efficiency, and thus the bioavailability of ingested BCKA may be considerably lower when compared to BCAA (26, 31). A lower bioavailability may suggest that BCKA do not stimulate muscle protein synthesis to a similar extent when compared to BCAA or intact protein. Furthermore, it has been observed that intravenous infusion of BCKA do not stimulate whole-body protein synthesis (32). However, it is important to note that whole-body protein synthesis is not necessarily reflective of muscle protein synthesis and evidence from animal work in fact does support a role for BCKA as a nutrient regulator of muscle protein synthesis (33, 34). To date there are no studies that have investigated the effects of ingesting BCKA on muscle protein synthesis in humans. We hypothesize that ingestion of intact protein, BCAA as well as BCKA stimulate myofibrillar protein synthesis *in vivo* in older males.

Methods

Subjects

Forty-five healthy (tracer naive) older men (age: 71 ± 1 y; BMI: 25.4 ± 0.8 kg/m^2) participated in this double-blind, parallel-group, randomized trial. The trial was conducted between January 2017 and May 2017 at Maastricht University Medical Centre+, in Maastricht, the Netherlands (for consort flow chart, please see **Supplementary Figure 1**). Subjects' characteristics are shown in **Table 1**. All subjects were informed about the purpose of the study, experimental procedures, and possible risks before providing written consent to participate. The procedures followed were in accordance with the ethical standards of the Medical Ethics Committee of Maastricht University Medical Centre+ on human experimentation and in accordance with the Helsinki Declaration of 1975 as revised in October 2013. This trial was registered at trialregister.nl as NTR6047.

Table 1. Subjects' characteristics

	BCAA	BCKA	MILK	*P*
Age, y	70±1	71±1	72±1	0.201
Body mass, kg	78.5±2.2	79.0±3.2	80.7±2.2	0.779
BMI, kg/m^2	25.9±0.9	25.4±0.6	24.9±0.8	0.568
Systolic BP, mmHg	138±3	134±4	131±3	0.345
Diastolic BP, mmHg	72±1	68±3	66±2	0.101
Fat, %	24.4±1.6	24.1±0.9	21.9±1.6	0.297
Appendicular lean mass, kg	25.3±0.8	26.1±0.9	26.5±0.9	0.558
Lean body mass, kg	57.2±1.5	57.6±2.2	60.7±1.5	0.246
Fasting glucose (OGTT), mmol/L	5.2±0.2	4.9±0.2	5.0±0.2	0.346
Two-hour glucose (OGTT), mmol/L	7.2±0.9	5.6±0.4	5.8±0.6	0.103
Fasting insulin (OGTT), mU/L	10.8±1.8	10.1±1.7	10.0±1.1	0.915
Two-hour insulin (OGTT), mU/L	74.0±20.9	59.1±19.1	39.9±10.0	0.307
HbA1c, %	5.5±0.2	5.5±0.1	5.5±0.1	0.986
HOMA2-IR	1.4±0.2	1.3±0.2	1.3±0.2	0.913
OGIS, mL/min/m^2	367±21	385±19	409±19	0.252

Values represent means±SEM, *n* = 15 per group. BCAA, 6 g branched-chain amino acids; BCKA, 6 g branched-chain ketoacids; BMI, body mass index; BP, blood pressure; HbA1c, glycated hemoglobin; HOMA2-IR, homeostatic model assessment of insulin resistance; MILK, 30 g milk protein; OGIS, oral glucose insulin sensitivity; OGTT, oral glucose tolerance test. Data were analyzed with a one-way ANOVA. No differences were detected between groups.

Pretesting

Volunteers between the age of 65 and 80 y and a BMI between 18.5 and 30.0 kg/m^2

underwent a medical screening to assess glycated hemoglobin, whole-body glucose tolerance (by a 2-h oral-glucose-tolerance test (35)), blood pressure, weight, height, and body composition (by Dual Energy X-Ray Absorptiometry (DXA); Discovery A; Hologic). Subjects were deemed healthy based on their responses to a medical questionnaire and their screening results.

Study design

Subjects were randomly assigned to consume 30 g MILK (ReFit MPI 85; Friesland Campina, Amersfoort, the Netherlands; *n* = 15), 6 g BCAA (Evonik Industries, Essen, Germany; *n* = 15), or 6 g BCKA (Myolution; Evonik Industries, Essen, Germany; *n* = 15). The 30 g MILK provides ~6 g BCAA with a total of 2.64 g leucine, which should theoretically induce a measurable increase in postprandial muscle protein synthesis rates (36). The ratio of (keto-)leucine, (keto-)isoleucine, and (keto-)valine in both BCAA and BCKA was 2:1:1. Hence, the BCAA and BCKA drink provided 3 g (keto-)leucine, 1.5 g (keto-)isoleucine, and 1.5 g (keto-)valine (total 6 g). Randomization was performed by using a computerized random-number generator. An independent person was responsible for random assignment and drink preparation.

Diet and physical activity

All subjects were instructed to refrain from any sort of strenuous physical activity 3 d prior to the infusion trial and to keep their diet as consistent as possible for 2 d prior to the experiment. On the evening before the experimental trial, all subjects consumed the same standardized meal (2061 kJ / 487 kcal) providing 31.8 g protein, 58.7 g carbohydrate, and 11.3 g fat at ~1800 h followed by an evening snack (985 kJ / 234 kcal) composed of 34.7 g protein, 18.5 g carbohydrate, and 0.0 g fat at 2200 h.

Infusion protocol

At 0800 h, after an overnight fast, subjects arrived at the laboratory by car or public transport. A catheter was inserted into an antecubital vein for stable isotope labeled amino acid infusion. A second catheter was inserted into a dorsal hand vein of the contralateral arm and placed in a hot box (60°C) for arterialized blood sampling (37). After obtaining a baseline blood sample, the plasma phenylalanine pools were primed with a single dose of L-[ring-$^{13}C_6$]-phenylalanine (2.25 μmol·kg^{-1}), after which a continuous L-[ring-$^{13}C_6$]-phenylalanine (0.05 μmol·kg^{-1}·min^{-1}) intravenous infusion was initiated (*t* = –180 min). Subsequently, the subjects rested in a supine position for 180 min during which 4 additional arterialized blood samples were drawn (*t* = –90, -60, -30 and 0 min). Subsequently, a muscle biopsy sample was collected from the *m. vastus lateralis* of a randomly chosen leg (*t* = 0 min). After collection of the first muscle biopsy sample, subjects consumed a drink containing 30 g MILK (*n* = 15), 6 g BCAA (*n* = 15), or 6 g BCKA (*n* = 15) at *t* = 0 min. A small amount of L-[ring-$^{13}C_6$]-phenylalanine (6 %) was added to the MILK beverage to prevent precursor pool dilution. Additional

arterialized blood samples were collected at *t* = 15, 30, 45, 60, 75, 90, 120, 150, 180, 210, 240, and 300 min. Second and third muscle biopsies were collected at *t* = 120 min and *t* = 300 min to determine postprandial myofibrillar protein synthesis rates. Blood samples were collected in EDTA-containing tubes and centrifuged at 1000 *g* for 10 min at 4 °C. Aliquots of plasma were snap frozen in liquid nitrogen and stored at -80 °C. Biopsy samples were collected from the middle region of the *m. vastus lateralis*, ~15 cm above the patella and 3 cm below entry through the fascia, by using the percutaneous needle biopsy technique (38). Muscle samples were dissected carefully, freed from any visible non-muscle material, immediately frozen in liquid nitrogen, and stored at -80 °C until further analysis. For a schematic representation of the infusion protocol, see **Figure 1**.

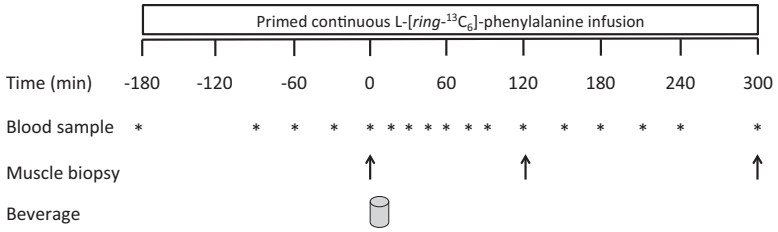

Figure 1. Schematic overview of the infusion protocol. Participants consumed either 30 g MILK, 6 g BCAA, or 6 g BCKA.

Plasma and muscle tissue analysis

Details of analysis related to the determination of plasma (glucose, insulin, ammonia, ketoacids, amino acids, HbA_{1c}, L-[ring-$^{13}C_6$]-phenylalanine, mixed plasma proteins) as well as muscle (myofibrillar protein L-[ring-$^{13}C_6$]-phenylalanine enrichments) data are presented in **Supplementary Methods**.

Calculations

Myofibrillar protein fractional synthetic rates (FSRs) were calculated by using the standard precursor-product equation, as follows (39):

$$FSR = \frac{\Delta E_p}{E_{\text{precursor}} \cdot t} \cdot 100$$

ΔE_p is the increment in myofibrillar protein–bound L-[ring-$^{13}C_6$]-phenylalanine enrichment after an incorporation period, $E_{\text{precursor}}$ is the weighted mean plasma L-[ring-$^{13}C_6$]-phenylalanine enrichment during that incorporation period, and *t* is the incorporation period

(h). Weighted mean plasma enrichments were calculated by taking the average enrichment between all consecutive time points and correcting for the time between these sampling time points. The weighted mean plasma precursor pool is preferred in this setting, because the more frequent sampling time points allow for a more accurate correction of the transient changes in precursor pool enrichments over time (40). For basal FSR, plasma protein samples at t = -180 min and muscle biopsy samples at t = 0 min were used; and for postprandial FSRs, muscle biopsy samples at t = 0, 120, and 300 min were used.

Statistical analysis

All data are expressed as means ± SEM. Baseline characteristics, incremental area under the curve (for postprandial plasma insulin and ammonia concentrations), and plasma amino acid enrichments were compared between treatment groups using one-way analysis of variance (ANOVA). For time-dependent variables, repeated-measures ANOVA with treatment as between-subjects factor and time as within-subjects factor was used (i.e., all time points for plasma concentrations and basal vs postprandial for muscle data). In case of significant interactions, separate analyses were performed within each treatment group, as well as between treatment groups for every time period separately (e.g., for FSR values, separate one-way ANOVA for basal, 0-2 h, 2-5 h, and 0-5 h). In case of significant treatment effects, Bonferroni post hoc analyses were performed to locate the effects. Significance was set at $P < 0.05$. Calculations were performed using SPSS (version 21.0, IBM Corp., Armonk, NY, USA).

Results

Plasma glucose, insulin and ammonia

Plasma glucose concentrations (**Figure 2**) slightly declined over time in BCKA and BCAA ($P<0.05$), whereas they slightly increased over time in MILK ($P<0.05$). Plasma glucose concentrations were significantly higher between t = 90 and 210 min in MILK vs BCAA ($P<0.05$). Plasma insulin concentrations (**Figure 3A**) showed a rapid and significant increase following beverage ingestion in BCAA and MILK ($P<0.05$), but not in BCKA. A greater increase in plasma insulin concentrations was observed in MILK compared with BCAA between t = 75 and 120 min ($P<0.05$). In line, the incremental area under the curve (iAUC) above fasting plasma insulin concentrations was significantly greater in MILK compared with BCKA and BCAA ($P<0.05$; **Figure 3B**). The iAUC of plasma ammonia (**Figure 4**) did not show significant differences between groups, despite the fact that plasma ammonia concentrations were substantially reduced during the postprandial period in BCKA.

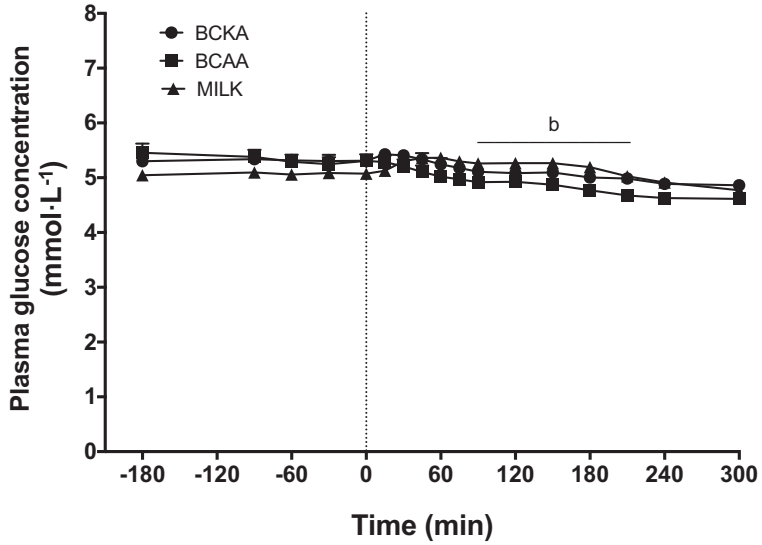

Figure 2. Plasma glucose concentrations over time after the ingestion of 30 g milk protein (MILK; n = 15), 6 g branched-chain amino acids (BCAA; n = 15), or 6 g branched-chain ketoacids (BCKA; n = 15) in healthy older males. The dotted line represents the ingestion of the drink. Values represent means+SEM. Data were analyzed with repeated measures (time x treatment group) ANOVA and separate analyses were performed when a significant interaction was detected. Bonferroni post hoc testing was used to detect differences between groups. Time x treatment interaction, $P<0.001$. b, MILK significantly different ($P<0.05$) from BCAA.

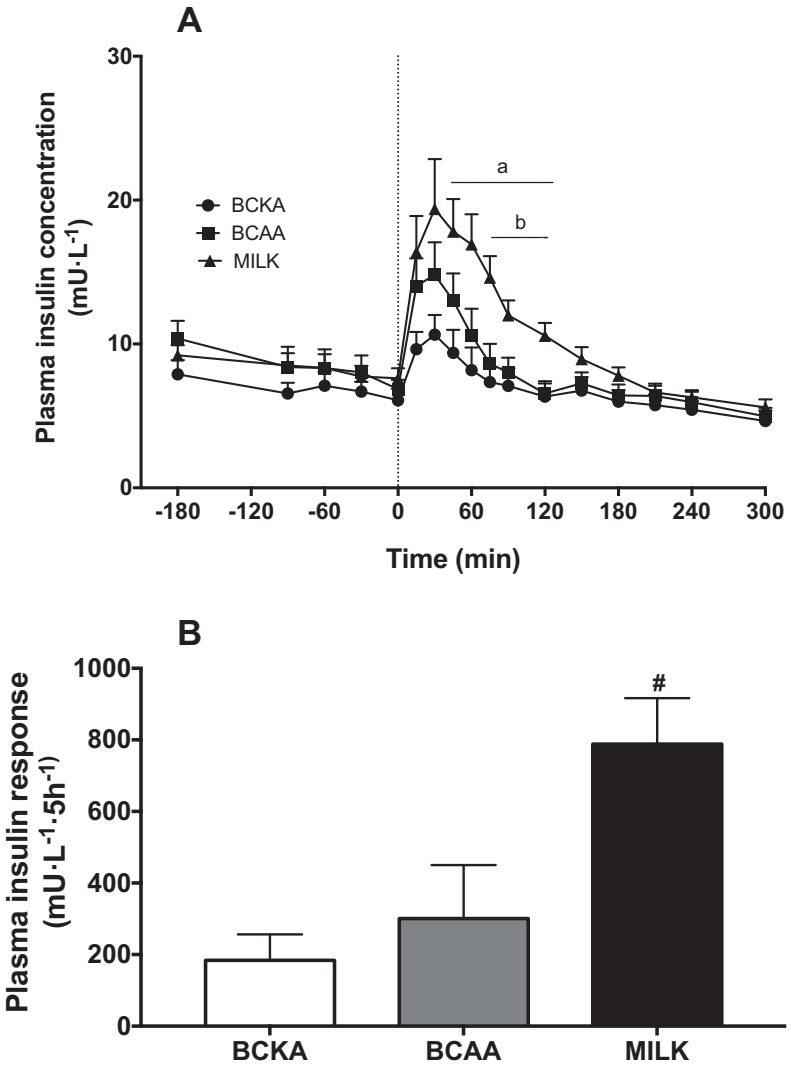

Figure 3. Plasma insulin concentrations over time (A) and total insulin responses, expressed as incremental area under the curve (B) after the ingestion of 30 g milk protein (MILK; *n* = 15), 6 g branched-chain amino acids (BCAA; *n* = 15), or 6 g branched-chain ketoacids (BCKA; *n* = 15) in healthy older males. The dotted line represents the ingestion of the drink. Values represent means+SEM. Data in panel A were analyzed with repeated measures (time x treatment group) ANOVA and separate analyses were performed when a significant interaction was detected. Bonferroni post hoc testing was used to detect differences between groups. Time x treatment interaction, $P<0.001$. a, MILK significantly different ($P<0.05$) from BCKA; b, MILK significantly different ($P<0.05$) from BCAA. Data in panel B were analyzed with a one-way ANOVA with Bonferroni correction. #Significantly different ($P<0.05$) from BCAA and BCKA.

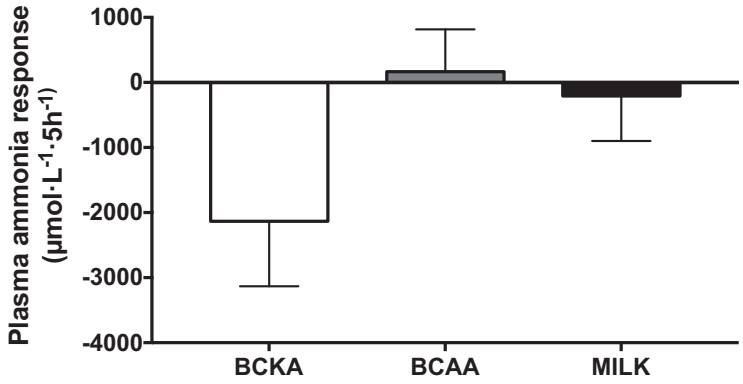

Figure 4. Plasma total ammonia concentrations, expressed as incremental area under the curve (iAUC) after the ingestion of 30 g milk protein (MILK; *n* = 15), 6 g branched-chain amino acids (BCAA; *n* = 15), or 6 g branched-chain ketoacids (BCKA; *n* = 15) in healthy older males. Values represent means±SEM. Data were analyzed with a one-way ANOVA. No significant differences between treatments ($P>0.05$).

Plasma amino acids

Plasma phenylalanine (**Figure 5A**), leucine (**Figure 5B**), isoleucine (**Figure 5C**), and valine (**Figure 5D**) concentrations over time are depicted in **Figure 5**. Significant time x treatment interactions were observed for plasma phenylalanine, leucine, isoleucine and valine concentrations ($P<0.001$). Following drink ingestion, a rapid increase in plasma phenylalanine concentrations was found in MILK ($P<0.05$), whereas plasma phenylalanine concentrations remained unchanged and decreased over time in BCKA and BCAA, respectively ($P<0.05$). Plasma leucine concentrations increased significantly after drink ingestion in all groups ($P<0.01$), with the highest peak plasma leucine concentrations measured in BCAA ($P<0.05$). Plasma isoleucine and valine concentrations increased significantly after drink ingestion and remained elevated for the entire postprandial period in BCAA and MILK ($P<0.01$), whereas no changes were observed in BCKA.

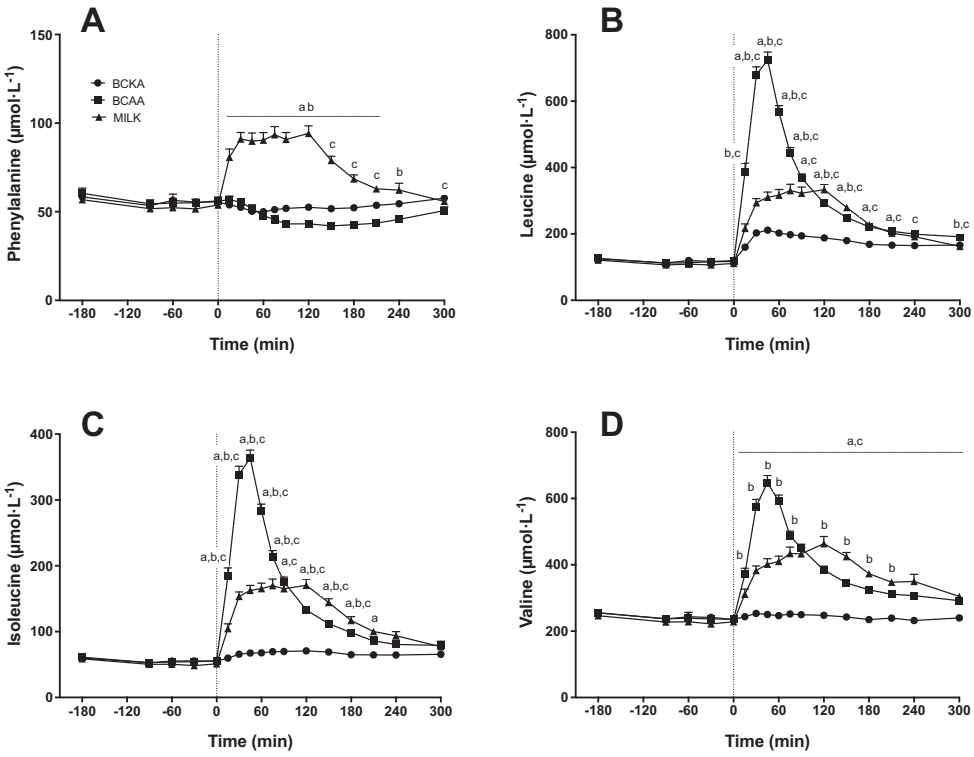

Figure 5. Plasma phenylalanine (A), leucine (B), isoleucine (C), valine (D) concentrations over time after the ingestion of 30 g milk protein (MILK; *n* = 15), 6 g branched-chain amino acids (BCAA; *n* = 15), or 6 g branched-chain ketoacids (BCKA; *n* = 15) in healthy older males. The dotted line represents the ingestion of the drink. Values represent means+SEM. Data were analyzed with repeated measures (time x treatment group) ANOVA and separate analyses were performed when a significant interaction was detected. Bonferroni post hoc testing was used to detect differences between groups. Time x treatment interaction, *P*<0.001. a, MILK significantly different (*P*<0.05) from BCKA; b, MILK significantly different (*P*<0.05) from BCAA; c, BCAA significantly different (*P*<0.05) from BCKA.

Plasma BCAA (**Figure 6A**), essential amino acid (EAA; **Figure 6B**), non-essential amino acid (NEAA-Glutamine; **Figure 6C**), and total amino acid (TAA; **Figure 6D**) concentrations over time are depicted in **Figure 6**. For BCAA, EAA, NEAA, and TAA concentrations, significant time x treatment interactions were observed (*P*<0.001). Plasma total BCAA concentrations increased significantly after drink ingestion and remained elevated for the entire postprandial period in BCAA and MILK (*P*<0.001). For BCKA, plasma total BCAA concentrations were only significantly higher than fasting values at *t* = 150 and 300 min (*P*<0.01). Plasma EAA concentrations increased significantly after drink ingestion and remained elevated for the entire postprandial period in BCAA (apart from *t* = 240 min; *P*>0.05) and MILK (*P*<0.05), whereas no increase was found after BCKA ingestion. Plasma total NEAA concentrations following drink ingestion significantly increased and remained elevated until *t* = 240 min after

MILK ingestion (P<0.001), did not change after BCKA ingestion and were significantly lower when compared to fasting values at t = 90, 180, 210, and 300 min in BCAA (P<0.05). Plasma TAA concentrations immediately increased after BCAA (until t = 120 min; P<0.001) and MILK (remained elevated for the entire postprandial period; P<0.01) ingestion. No significant changes were observed after BCKA ingestion.

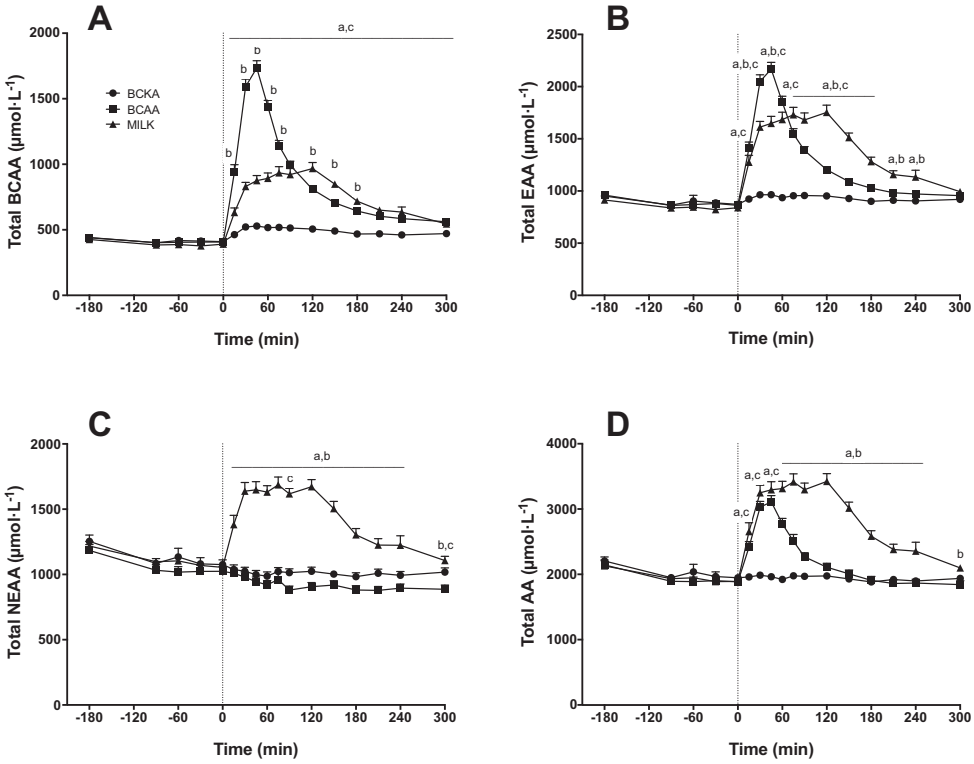

Figure 6. Plasma branched-chain amino acids (BCAA; A), essential amino acid (EAA; B), non-essential amino acid (NEAA-Glutamine; C) and total amino acid (TAA-Glutamine; D) concentrations over time after the ingestion of 30 g milk protein (MILK; n = 15), 6 g branched-chain amino acids (BCAA; n = 15), or 6 g branched-chain ketoacids (BCKA; n = 15) in healthy older males. The dotted line represents the ingestion of the drink. Values represent means+SEM. Data were analyzed with repeated measures (time x treatment group) ANOVA and separate analyses were performed when a significant interaction was detected. Bonferroni post hoc testing was used to detect differences between groups. Time x treatment interaction, P<0.001. a, MILK significantly different (P<0.05) from BCKA; b, MILK significantly different (P<0.05) from BCAA; c, BCAA significantly different (P<0.05) from BCKA.

Plasma **α**-*ketoacids*

Plasma **α**-ketoisocaproic acid (KIC: the **α**-ketoacid of leucine; **Figure 7A**), **α**-keto-**β**-methylvalerate (KMV: the **α**-ketoacid of isoleucine; **Figure 7B**), **α**-ketoisovalerate (KIV: the **α**-ketoacid of valine; **Figure 7C**), and total BCKA (**Figure 7D**) concentrations over time are depicted in **Figure 7**. Significant time x treatment interactions were observed for plasma KIC,

KMV, KIV, and total BCKA concentrations (P<0.001). Following drink ingestion, plasma KIC and KMV concentrations significantly increased and remained elevated for the entire postprandial period in BCKA (P<0.001, apart from t = 240 min for KMV) and BCAA (from t = 30 min; P<0.05). For MILK, KIC concentrations were only significantly higher than fasting values between t = 150-300 min and KMV concentrations were only significantly higher than fasting values between t = 75-210 min and at t = 300 min (P<0.05). Following drink ingestion, plasma KIV concentrations significantly increased in BCKA (from t =15 until t = 150 min; P<0.01), whereas no changes were observed in BCAA and MILK. Plasma total BCKA concentrations increased significantly after drink ingestion for the entire postprandial period in BCKA (P<0.001) and BCAA (P<0.001; apart from t = 15 min), whereas they were only significantly increased between t = 90-300 min in MILK (P<0.05).

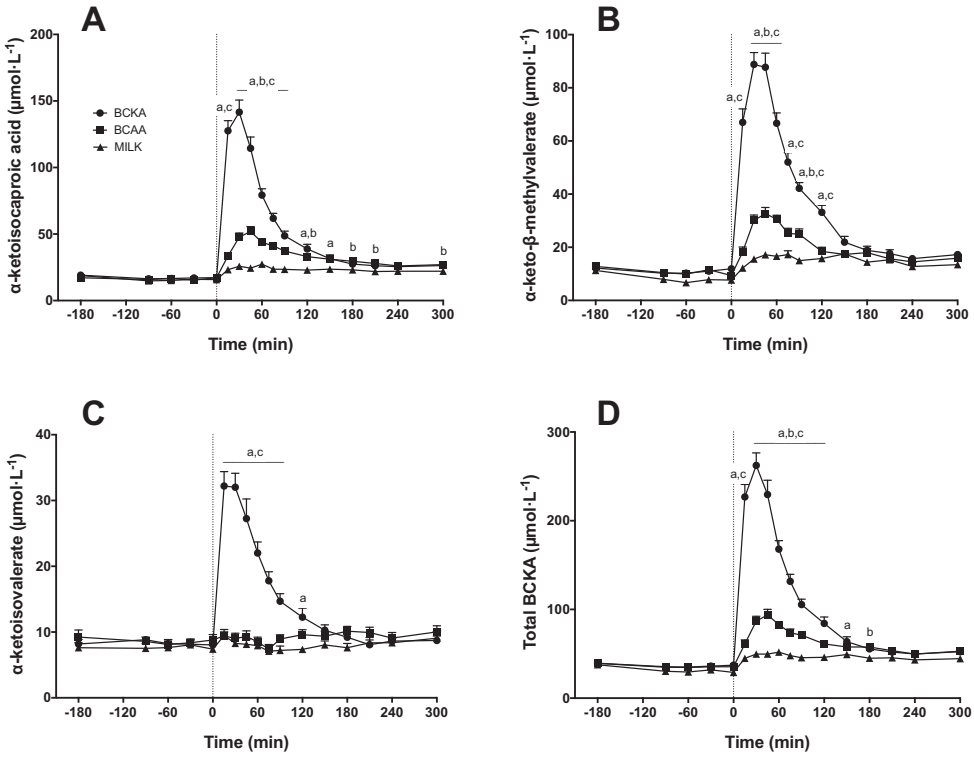

Figure 7. Plasma **α**-ketoisocaproic acid (KIC: the **α**-ketoacid of leucine; A), **α**-keto-**β**-methylvalerate (KMV: the **α**-ketoacid of isoleucine; B), **α**-ketoisovalerate (KIV: the **α**-ketoacid of valine; C) and BCKA (D) concentrations over time after the ingestion of 30 g milk protein (MILK; n = 15), 6 g branched-chain amino acids (BCAA; n = 15), or 6 g branched-chain ketoacids (BCKA; n = 15) in healthy older males. The dotted line represents the ingestion of the drink. Values represent means+SEM. Data were analyzed with repeated measures (time x treatment group) ANOVA and separate analyses were performed when a significant interaction was detected. Bonferroni post hoc testing was used to detect differences between groups. Time x treatment interaction, P<0.001. a, MILK significantly different (P<0.05) from BCKA; b, MILK significantly different (P<0.05) from BCAA; c, BCAA significantly different (P<0.05) from BCKA.

Isotope tracer analysis

Prior to BCAA, BCKA or MILK ingestion plasma L-[ring-$^{13}C_6$]phenylalanine enrichments averaged 7.5±0.3, 7.4±0.1, and 7.4±0.2 MPE, respectively, with no differences between treatments (*data not shown*). Plasma L-[ring-$^{13}C_6$]phenylalanine enrichments during the postprandial period averaged 8.7±0.3, 7.9±0.2, and 7.3±0.2 MPE for BCAA, BCKA, and MILK respectively, with significant differences between BCAA and BCKA (P<0.01) and BCAA and MILK (P<0.001), but not between BCKA and MILK (P=0.082).

Myofibrillar protein synthesis rates calculated based on the plasma precursor pool are depicted in **Figure 8**. No differences were observed in basal muscle protein synthesis rates between groups (P=0.624). Myofibrillar protein synthesis rates increased from basal to the early (0-2 h) postprandial period in all groups (from 0.020±0.002%/h to 0.042±0.004%/h in MILK, 0.022±0.002%/h to 0.044±0.004%/h in BCAA and 0.023±0.003%/h to 0.044±0.004%/h in BCKA; P<0.001), with no differences between treatment groups (time x treatment interaction: P=0.969, main treatment effect: P=0.732). A significant time x treatment interaction (P=0.002) showed that after ingestion of MILK, myofibrillar protein synthesis rates remained elevated over the 2-5 h postprandial period when compared to basal protein synthesis rates (from 0.020±0.002%/h to 0.039±0.004%/h; P<0.001). In BCKA and BCAA, myofibrillar protein synthesis rates decreased back to basal rates during the 2-5 h postprandial period (0.024±0.005%/h and 0.024±0.005%/h, respectively; P>0.05). During the late (2-5 h) postprandial period, myofibrillar protein synthesis rates were higher in MILK when compared to BCAA (P=0.023) and BCKA (P=0.023). A significant time x treatment interaction was also observed when comparing basal myofibrillar protein synthesis rates with the entire 5 h postprandial period (P=0.003). Over the entire 5 h postprandial period (0-5 h), myofibrillar protein synthesis rates were significantly elevated above basal levels for all treatments (P<0.005), but were significantly higher in MILK (0.040±0.002%/h) vs BCKA (0.032±0.003%/h; P=0.023) and BCAA (0.032±0.003%/h; P=0.019), with no significant differences between BCKA and BCAA (P>0.05).

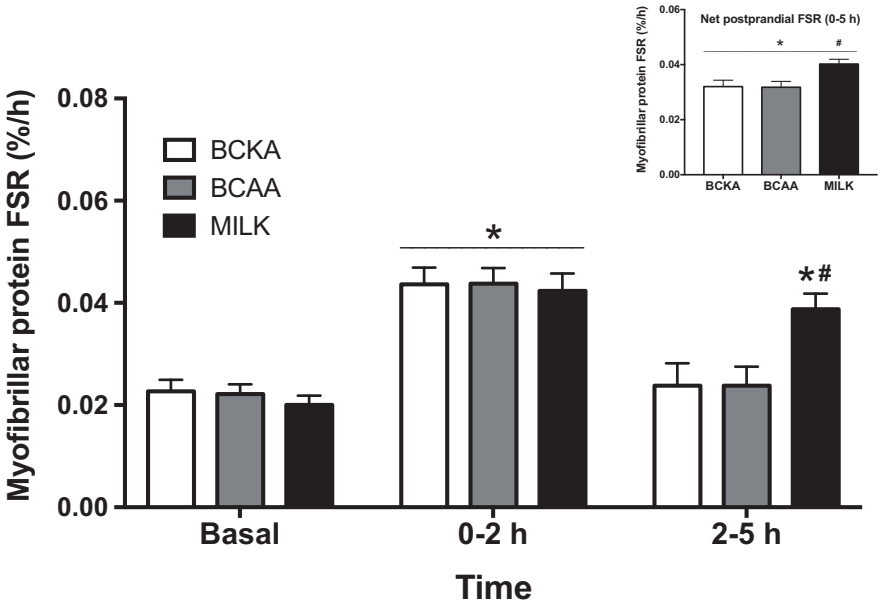

Figure 8. Myofibrillar protein fractional synthetic rates (FSR in %/h) during the fasting state (basal) and over the early (0-2 h), late (2-5 h) and entire (0-5 h) postprandial period after the ingestion of 30 g milk protein (MILK; *n* = 15), 6 g branched-chain amino acids (BCAA; *n* = 15), or 6 g branched-chain ketoacids (BCKA; *n* = 15) in healthy older males. Values represent means+SEM. Data were analyzed with repeated measures (time x treatment group) ANOVA and separate analyses were performed when a significant interaction was detected. Bonferroni post hoc testing was used to detect differences between groups. *Significantly different ($P<0.01$) from basal; #Significantly different ($P<0.05$) from BCAA and BCKA.

Discussion

The present study demonstrated that ingestion of BCAA, BCKA, and MILK (providing an equivalent amount of BCAA) significantly increased myofibrillar protein synthesis rates during the early (0-2 h) postprandial phase, with no differences observed between treatments. In the late (2-5 h) postprandial period myofibrillar protein synthesis rates remained elevated following MILK ingestion, with myofibrillar protein synthesis rates following BCAA and BCKA ingestion returning to baseline values.

It has been well-established that ingestion of dietary protein strongly increases muscle protein synthesis rates (1, 2, 41). The postprandial increase in muscle protein synthesis rates has been attributed to the postprandial release of (essential) amino acids (3, 4, 42, 43), with the rise in circulating BCAA and especially leucine being of particular relevance (16, 44-47). In line with previous work (10, 48, 49), we show a rapid postprandial increase in plasma (essential) amino acid concentrations following the ingestion of 30 g MILK, with plasma leucine concentrations reaching 294±12 μmol/L within 30 min (Figure 5B). The postprandial rise in plasma amino acid availability resulted in a 111% increase in myofibrillar protein synthesis rates within the first two hours after protein ingestion when compared to basal, postabsorptive myofibrillar protein synthesis rates (from 0.020±0.002 to 0.042±0.004%/h; Figure 8). In addition, postprandial myofibrillar protein synthesis rates assessed during the entire 5 h postprandial period were also higher following MILK ingestion when compared to baseline values (0.040±0.002 vs 0.020±0.002%/h, respectively).

As the postprandial increase in muscle protein synthesis has been attributed largely to the postprandial increase in plasma BCAA, we also assessed the impact of ingesting BCAA only. Following ingestion of 6 g BCAA, which equals the amount of BCAA present in 30 g MILK, we observed a rapid rise in circulating BCAA (Figure 6A), with plasma leucine concentrations reaching 2-3 fold higher concentrations when compared to MILK ingestion (Figure 5B). In line, we also observed a strong, significant increase in myofibrillar protein synthesis rates within the first two hours after BCAA ingestion (from 0.022±0.002 to 0.044±0.004%/h; Figure 8). This seems to be in line with previous work showing an increase in myofibrillar protein synthesis rate during a 2.5 h period after ingesting 3.4 g of free leucine (50). The rapid and marked increase in plasma BCAA availability after BCAA ingestion may have exceeded the maximal capacity to stimulate myofibrillar protein synthesis, so that during the 2 h postprandial period myofibrillar protein synthesis rates did not differ after ingestion of BCAA or MILK. Though this would be in line with the proposed muscle full effect (51, 52), these high myofibrillar protein synthesis rates could not be maintained following BCAA as opposed to MILK ingestion during the latter stages (2-5 h) of the postprandial period (Figure 8). These data suggest that besides the postprandial rise in plasma BCAA concentrations, other (essential) amino acids need to be provided to allow a more prolonged postprandial increase in muscle protein synthesis rate (53). Though the postprandial rise in muscle protein synthesis

rate following protein ingestion can be initiated by the increased BCAA availability, it may be maintained only when sufficient other (essential) amino acids are provided to serve as precursors for *de novo* muscle protein synthesis. Alternatively, it could be speculated that ingestion of greater amounts (>6 g) and/or a more sustained provision of BCAA are needed to prolong the elevation in muscle protein synthesis rates.

BCKA are ketoanalogues of the three BCAA and play an active role in branched-chain amino acid metabolism, due to their rapid *in vivo* interconvertibility (by transamination) (26). The transamination capacity is high in skeletal muscle (54). Hence, when BCKA are transaminated into BCAA, they may directly stimulate muscle protein synthesis. It has previously been shown that infusion with the BCKA α-ketoisocaproic acid stimulates muscle protein synthesis in neonatal pigs (33). However, the effect of BCKA ingestion on muscle protein synthesis rates in humans has never been assessed. Ingestion of 6 g BCKA, which was tolerated well in all subjects with no reported side effects, resulted in a strong postprandial increase in plasma BCKA concentrations (Figure 7). Concomitantly, we also observed a small but significant increase in plasma leucine concentrations (Figure 5B), suggesting that there is some, albeit limited, conversion of KIC to leucine *in vivo* in healthy humans. Despite only minimal changes in plasma BCAA or other amino acid concentrations following BCKA ingestion (Figure 6), we observed a rapid increase in myofibrillar protein synthesis rates during the early postprandial phase that did not differ from the early postprandial response following BCAA or MILK ingestion (Figure 8). However, similar to the postprandial response observed following BCAA ingestion, these elevated myofibrillar protein synthesis rates were not maintained during the latter postprandial phase (2-5 h). The observation that BCKA ingestion strongly increases (early) postprandial myofibrillar protein synthesis rates, despite a minimal rise in circulating plasma leucine concentrations, implies that either BCKA-derived BCAA (by transamination) are directly used within muscle to stimulate muscle protein synthesis or that alternative metabolites may be involved in the early postprandial stimulation of muscle protein synthesis. It has previously been shown that leucine metabolites, such as **β**-hydroxy-**β**-methylbutyrate can stimulate myofibrillar protein synthesis (50, 55). In contrast to the ketoanalogue of leucine, **β**-hydroxy-**β**-methylbutyrate cannot be converted back into leucine. In line with the minimal increase in plasma leucine concentrations following BCKA ingestion, our findings suggest that the stimulatory effect of BCKA ingestion on myofibrillar protein synthesis may work directly (KIC) and/or indirectly via conversion to **β**-hydroxy-**β**-methylbutyrate. Clearly, research is warranted to elucidate alternative metabolites and pathway(s) that could be (at least partially) responsible for the stimulatory effect of BCKA ingestion on muscle protein synthesis. This will also yield important information on whether the anabolic properties of BCKA and BCAA involve separate pathways and, as such, may be combined to further increase postprandial muscle protein synthesis rates. Ultimately, net muscle protein accretion is determined by the balance between muscle protein synthesis and breakdown rates and it has been suggested that BCKA, with KIC in particular, has a profound impact on muscle

protein breakdown. It appears that KIC, and not leucine *per se*, is primarily responsible for the inhibitory effect on muscle protein breakdown (56-59). This further supports the potential of BCKA as an anabolic agent to stimulate muscle protein accretion.

BCKA lack an amino group bound to the α-carbon in their molecular structure and, therefore, do not provide nitrogen. In support, transamination of the BCKA into their respective BCAA was likely responsible for the observed decline in plasma ammonia concentrations (Figure 4). The potential therapeutic value of BCKA has been studied in several diseases and disorders, but appear particularly relevant for patients with chronic kidney disease (25, 26). Patients with chronic kidney disease are advised to adhere to low protein diets during certain stages of their disease (60), restricting them in their capacity to maintain muscle mass. The observed stimulatory effect of BCKA ingestion on early postprandial myofibrillar protein synthesis rates can be of particular relevance for this patient group as it may support them in preventing or attenuating the progressive loss of muscle mass without increasing nitrogen intake (34, 61). Furthermore, there are suggestions that a low protein diet improves the nutritional efficiency of BCKA (26, 62). Therefore, future studies should look into the benefits of BCKA co-ingestion to stimulate postprandial muscle protein synthesis rates in patients with chronic kidney disease ingesting a diet relatively low in dietary protein content.

In conclusion, ingestion of 6 g BCAA, 6 g BCKA, and 30 g milk protein increases myofibrillar protein synthesis rates during the early postprandial phase (0-2 h) *in vivo* in healthy older males. The postprandial increase following the ingestion of 6 g BCAA and BCKA is short-lived, with higher myofibrillar protein synthesis rates only being maintained following the ingestion of an equivalent amount of intact milk protein.

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Supplementary Material

Supplementary Methods

Plasma and muscle tissue 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, St. Louis, MO, respectively). Plasma ammonia was measured enzymatically with glutamate dehydrogenase performed on a Cobas Fara automatic analyser (Roche, Basel, Switzerland) (1). Quantification of plasma keto- and amino acid concentrations was performed using ultra-performance liquid chromatograph mass spectrometry (UPLC-MS; ACQUITY UPLC H-Class with QDa; Waters, Saint-Quentin, France). 50 µl of blood plasma was deproteinized using 100 µL of 10 % SSA with 50 µM of MSK-A2 internal standard (Cambridge Isotope Laboratories, Massachusetts, USA). Subsequently, 50 µL of ultra-pure demineralized water was added and samples were centrifuged (15 min at 14000 rpm). After centrifugation, 10 µL of supernatant was added to 70 µL of Borate reaction buffer (Waters, Saint-Quentin, France). In addition, 20 µL of AccQ-Tag derivatizing reagent solution (Waters, Saint-Quentin, France) was added after which the solution was heated to 55 °C for 10 min. An aliquot of 1 µL was injected and measured using UPLC-MS. HbA_{1c} content was determined in venous blood samples by high-performance liquid chromatography (Bio-Rad Variant II, Munich, Germany). For plasma L-[ring-$^{13}C_6$]-phenylalanine enrichment measurements, plasma phenylalanine was derivatized to the tert-butyldimethylsilyl (TBDMS) derivative with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA), and the ^{13}C enrichments were determined by electron ionization gas chromatography-mass spectrometry (GC-MS; Agilent 7890A GC/5975C MSD; Agilent Technologies) using selected ion monitoring of masses 336 and 342 for unlabeled and labeled (ring-$^{13}C_6$) phenylalanine, respectively. We applied standard regression curves in all isotopic enrichment analyses to assess linearity of the mass spectrometer and to control for loss of tracer. Through the addition of an internal standard phenylalanine (m+10), concentrations of phenylalanine were determined in the same run. Phenylalanine enrichments were corrected for the natural level of ^{13}C isotopes. Mixed plasma proteins were isolated from blood samples by using perchloric acid (PCA) to a final concentration of 2 %. Samples were centrifuged at 1000 *g* at 4 °C for 10 min, and the supernatants were removed. The mixed plasma protein pellet was washed 3 times with 2 % PCA and dried. Amino acids were liberated by adding 6 M HCl and were heated at 120 °C for 15–18 h. Thereafter, the enrichments in hydrolyzed mixed plasma protein samples were assessed using the same procedures as the muscle protein-bound samples (described below).

Myofibrillar protein enriched fractions were isolated 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 800 *g* and 4 °C for 15 min. The pellet was washed with 500 µL ddH2O and centrifuged at 800 *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 PCA and spinning at 1000 *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 120 °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), washed 5 times with water and finally eluted with 2 M NH_4OH. To determine myofibrillar protein L-[ring-$^{13}C_6$]-phenylalanine enrichments, the purified amino acids were first converted into N-ethoxycarbonyl ethyl ester derivatives with ethyl chloroformate (ECF). The derivatives were then measured by GC-C-IRMS (Thermo Fisher Scientific Delta V, Bremen, Germany) using a DB-17MS-column (30 m x 0.25 mm x 0.5 µm; Agilent J+W scientific GC column, Santa Clara, CA, USA) and monitoring of ion masses 44, 45, and 46. Standard regression curves were applied to assess the linearity of the mass spectrum and to account for isotopic fractionation.

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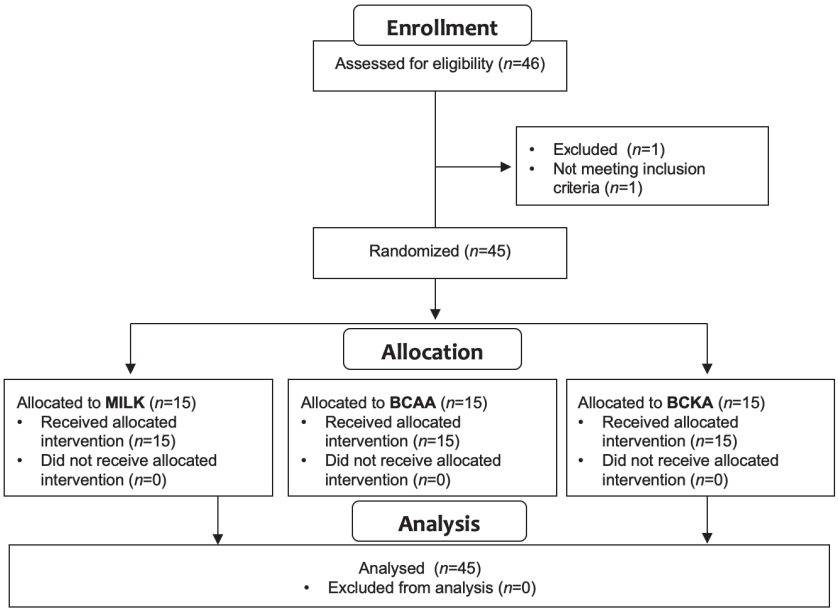
Supplementary Figure 1.



Chapter 6

Raw eggs to support post-exercise recovery in healthy young men: did Rocky get it right or wrong?

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Abstract

Background: Egg protein is ingested during recovery from exercise to facilitate the post-exercise increase in muscle protein synthesis rates and, as such, to support the skeletal muscle adaptive response to exercise training. The impact of cooking egg protein on post-exercise muscle protein synthesis is unknown.

Objective: To compare the impact of ingesting unboiled (raw) versus boiled eggs during post-exercise recovery on postprandial myofibrillar protein synthesis rates.

Design: In a parallel design, forty-five healthy, resistance trained young men (age: 24 (95% CI: 23 to 25) y) were randomly assigned to ingest 5 raw eggs (~30 g protein), 5 boiled eggs (~30 g protein), or a control breakfast (~5 g protein) during recovery from a single session of whole-body resistance type exercise. Primed continuous L-[ring-$^{13}C_6$]-phenylalanine infusions were applied, with frequent blood sampling. Muscle biopsies were collected immediately after cessation of whole-body resistance exercise and at 2 and 5 h into the post-exercise recovery period.

Results: Ingestion of eggs strongly increased plasma essential amino acid concentrations, with greater increases following ingestion of boiled eggs compared with raw eggs ($P<0.001$). Myofibrillar protein synthesis rates were significantly increased during the post-exercise period when compared to basal, post-absorptive values in all groups ($P<0.001$). Postprandial myofibrillar protein synthesis rates were 20% higher after ingesting raw eggs (effect size (Cohen's *d*): 0.63), and 18% higher after ingesting boiled eggs (effect size (Cohen's *d*): 0.69) when compared to the control breakfast, with no significant differences between groups (time X group interaction: $P=0.077$).

Conclusions: The ingestion of raw, unboiled eggs, as opposed to boiled eggs, attenuates the postprandial rise in circulating essential amino acid concentrations. However, post-exercise muscle protein synthesis rates do not differ after ingestion of 5 raw versus 5 boiled eggs.

Introduction

"*… Rocky's alarm clock goes off at exactly 4 a.m. Not accustomed to rising this early, with great difficulty Rocky staggers to his feet and wavers to the bathroom. He turns the light on and roaches scatter. At the top of the mirror hang the telegrams. Rocky fills the basin and submerges his face in cold water. Rocky sways to the icebox and removes a dozen eggs. He cracks five raw eggs into a glass and downs it in one swill... his body quivers. Rocky steps outside. He is dressed in a well-worn sweat suit with a hood, gloves and sneakers. It is pitch dark and his steaming breath attests to the cold. He begins running down the center of the deserted street…*" (1). This iconic scene of the 1976 movie 'Rocky' has been imprinted in the brain of many young and older athletes and has been an early sign of the recognition of dietary protein consumption as an important factor in supporting the anabolic response to exercise. However, how much evidence is there for the use of ingesting raw eggs to optimize recovery from training? Scientific evidence has previously shown that raw eggs do not show the same digestion and absorption kinetics when compared to cooked eggs (2, 3). In fact, raw eggs have been reported to show merely ~51% protein digestion and amino acid absorption, whereas cooked eggs show ~91% protein digestion and amino acid absorption (2). This clearly demonstrates that food processing (such as heat treatment) strongly influences protein digestibility. Indeed, previous work has shown that heat treatment is an important factor determining protein digestion, amino acid absorption, and subsequent postprandial plasma amino acid availability (4, 5).

It is well known that protein digestion and amino acid absorption and the splanchnic extraction of dietary protein-derived amino acids are of key importance in driving the postprandial skeletal muscle adaptive response. Specifically, rapid protein digestion rates facilitate a strong increase in postprandial plasma amino acid availability to stimulate muscle protein synthesis (6-10). Hence, protein ingestion during recovery from exercise helps athletes to further augment muscle protein synthesis rates and, as such, is frequently applied to facilitate the conditioning process following exercise training.

The purpose of this study was to investigate the effectiveness of eating raw eggs, as depicted in Rocky's iconic cinematic scene, to support skeletal muscle conditioning. We hypothesize that the postprandial rise in circulating amino acids will be attenuated after the ingestion of raw eggs when compared with boiled eggs, thereby lowering the postprandial stimulation of muscle protein synthesis. If true, this would suggest that Rocky could have done better by boiling his eggs. However, to date, no study has compared the impact of ingesting raw, unboiled eggs versus boiled eggs on postprandial plasma amino acid concentrations and muscle protein synthesis rates during recovery from exercise. In the present study, we recruited 45 healthy young men to assess the postprandial increase in plasma amino acid concentrations and the subsequent increase in skeletal muscle protein synthesis rates

following ingestion of 5 raw eggs, 5 boiled eggs, or a control breakfast during recovery from exercise.

Methods

Subjects

Forty-five healthy, resistance trained young men (age 24 (95% CI: 23 to 25) y) participated in this randomized controlled intervention study. This study was conducted between November 2017 and March 2019 at Maastricht University Medical Centre+, in Maastricht, The Netherlands (for consort flow chart, please see **Supplementary Figure 1**). 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 were eligible to participate if they were males between 18-35 y old, had a BMI between 18.5 and 30 kg/m^2, were nonsmokers, were deemed to be healthy (i.e. free of cardiovascular, musculoskeletal, or metabolic conditions), were not taking any medication, did not have any food allergies to eggs, and were familiar with performing resistance type exercise training (≥2 times per week). This study was approved by the Medical Ethics Committee of Maastricht University Medical Centre+ (METC 173030) and conforms to the principles outlined in the declaration of Helsinki (of 1975 as revised in October 2013) for use of human subjects and tissue. This trial was registered at www.trailregister.nl as with the number of NL6506.

Table 1. Subjects' characteristics

	Control breakfast	Raw eggs	Boiled eggs	*P*
Age, y	23 (22 to 25)	24 (22 to 26)	24 (22 to 26)	0.587
Body mass, kg	75.2 (71.1 to 79.3)	79.8 (75.6 to 83.9)	76.0 (71.9 to 80.1)	0.211
Height, m	1.80 (1.76 to 1.85)	1.84 (1.80 to 1.88)	1.81 (1.76 to 1.86)	0.463
BMI, kg/m^2	23.1 (22.1 to 24.3)	23.6 (22.7 to 24.4)	23.3 (22.4 to 24.1)	0.775
Fat, %	19.0 (16.2 to 21.7)	18.0 (15.4 to 20.6)	19.9 (18.0 to 21.9)	0.506
Appendicular lean mass, kg	27.6 (25.8 to 29.3)	30.0 (27.5 to 32.5)	27.7 (25.7 to 29.6)	0.141
Lean body mass, kg	58.2 (55.0 to 61.4)	63.1 (58.9 to 67.3)	59.2 (55.5 to 63.0)	0.116
Leg press 1RM, kg	308 (282 to 334)	297 (262 to 331)	297 (254 to 339)	0.843
Leg extension 1RM, kg	114 (100 to 129)	127 (109 to 144)	123 (104 to 143)	0.536
Chest press 1RM, kg	103 (91 to 114)	104 (93 to 116)	104 (91 to 117)	0.969
Horizontal row 1RM, kg	80 (75 to 86)	87 (77 to 96)	79 (72 to 86)	0.274
Vertical pull-down 1RM, kg	80 (72 to 88)	85 (75 to 96)	78 (71 to 84)	0.365
Shoulder press 1RM, kg	111 (97 to 125)	104 (91 to 118)	104 (88 to 121)	0.734

Values represent means and 95% CI, *n*=15 per group. Data were analyzed using a one-way ANOVA. 1RM, one repetition maximum; BMI, body mass index.

Pretesting

All subjects participated in a screening session, which was performed at least 1 week prior to the experiment. First, subjects' body mass and height were measured as well as body composition by dual-energy X-ray absorptiometry (DXA, Discovery A; Hologic, Bedford, MA, USA). 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. 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, leg extension, chest press, horizontal row, vertical pull-down and shoulder press as described previously (11) (**Table 1**). The subjects were deemed healthy on the basis of their responses to a medical questionnaire.

Diet and activity prior to the experiment

All subjects received a standardized dinner of the same composition (1900 kJ, providing 24.6 g protein, 69.3 g carbohydrate, and 19.1 g fat) the evening prior to the test day at the internal facility (Maastricht University Medical Centre+). Thereafter, all participants stayed overnight and slept at the internal facility. All volunteers refrained from alcohol and any sort of exhaustive physical labor and/or exercise 3 days prior to the experimental day. In addition, subjects were asked to report their dietary intake for 2 days prior to the experimental day. Energy and macronutrient intakes were calculated with the use of the Dutch Nutrients Database (NEVO-online version 2019/6.0; https://nevo-online.rivm.nl/).

Study design

Each subject was randomly assigned to one of three experiments, in which the effect of post-exercise ingestion of a low protein control breakfast vs raw eggs vs boiled eggs on myofibrillar protein synthesis was studied. At the start of the experiment primed continuous L-[*ring*-$^{13}C_6$]-phenylalanine infusion was applied together with repeated blood sampling during the experimental day. After 2.5 h of rest, participants performed 15 min of cycling followed by 45 min of whole-body resistance type exercise training. Thereafter a biopsy from the *M. vastus lateralis* was taken, before ingesting a low protein control breakfast, 5 raw eggs, or 5 boiled eggs. In the subsequent 5 h recovery period, biopsies were taken from the *M. vastus lateralis* at t= 2 and 5 h. Randomization was performed by using a computerized random-number generator.

Experimental protocol

The experimental protocol is outlined in **Figure 1**. Each subject participated in one experiment. At the start of the experimental day at 8:00 AM, following an overnight fast at our facilities, a Teflon catheter was inserted into an antecubital vein for intravenous stable isotope tracer infusion. 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= -210 min), the plasma phenylalanine pool was primed with a single intravenous dose of L-[*ring*-$^{13}C_6$]-phenylalanine (2.25 μmol·kg^{-1}). Subsequently, an intravenous infusion of L-[*ring*-$^{13}C_6$]-phenylalanine (infusion rate of 0.05 μmol·kg^{-1}·min^{-1}) was initiated and maintained until the end of the trial using a calibrated IVAC 598 pump (San Diego, USA). During 2.5 h of supine rest, 2 additional arterialized blood samples (t= -120 and -60 min) were obtained. Subsequently, participants performed a whole-body resistance type exercise session. After a 15 min warm-up on a cycle ergometer at self-selected intensity (88 (95% CI: 84 to 91) W), subjects performed 4 sets of ~8-10 repetitions (at 80% 1RM) on both the leg press and leg extension exercise followed by 2 sets of ~8-10 repetitions (at 80% 1RM) on the chest press, horizontal row, vertical pull-down and shoulder press. After completion of the exercise bout (t= 0 min), another arterialized blood sample was obtained together with a biopsy from the *M. vastus lateralis*. Immediately afterwards, subjects ingested a control breakfast (protein content of ~5 g), 5 raw eggs, or 5 boiled eggs (both a protein content of ~30 g) at t= 0 min. Thereafter, repeated blood samples (t= 15, 30, 45, 60, 75, 90, 120, 150, 180, 240, and 300 min) were obtained together with biopsies from the *M. vastus lateralis* at t= 120 and t= 300 min. The biopsies were collected from the middle region of the *M. vastus lateralis* (~15 cm above the patella) with a Bergström needle under local anesthesia (12). The first two biopsies (at t= 0 and t= 120 min) were taken from the same leg. The last biopsy (t= 300 min) was collected from the contralateral leg. 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.

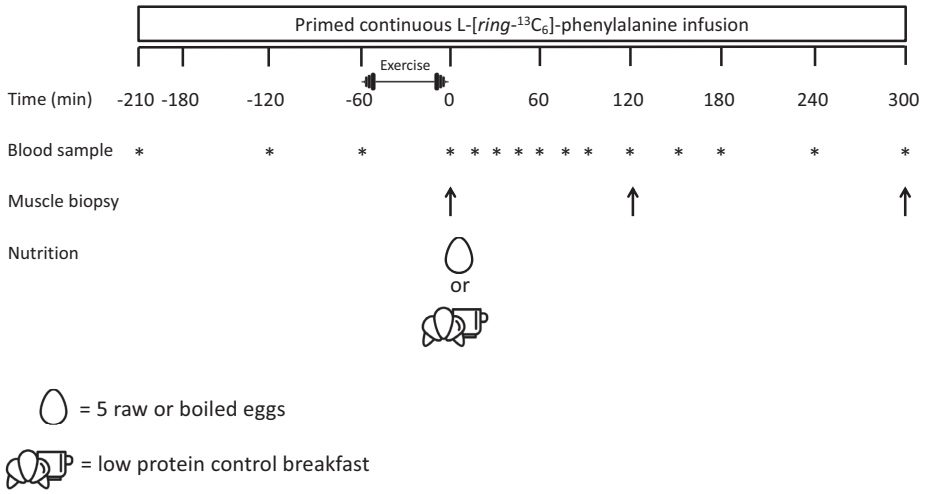

Figure 1. Schematic overview of the experimental protocol. Participants performed whole-body resistance exercise and consumed either a low protein control breakfast, 5 raw eggs, or 5 boiled eggs.

Nutrition

Subjects ingested a low protein control breakfast, 5 raw, or 5 boiled eggs (Albert Heijn, Zaandam, the Netherlands) during the experimental infusion trial. The control breakfast consisted of a croissant (AH roomboter croissants, Albert Heijn, Zaandam, the Netherlands), 10 g butter (Albert Heijn, Zaandam, the Netherlands) and 350 mL orange juice (Jumbo, Veghel, the Netherlands). The control breakfast provided 1650 kJ, with 47 g carbohydrate, 20 g fat, and 5 g protein). Eggs were carefully weighed to ensure that every participant received a similar amount of boiled or raw eggs. The (hard) boiled eggs were steamed for ~13 min with 65 mL water in an egg steamer. A total of 5 (un)boiled eggs provided 1400 kJ, with 0 g carbohydrate, 23 g fat, and 30 g protein. Participants were allowed to drink 300 mL of water with their eggs.

Preparation of tracer

The stable isotope tracer L-[*ring*-$^{13}C_6$]-phenylalanine was purchased from Cambridge Isotopes (Andover, MA) and dissolved in 0.9 % saline before infusion (Apotheek A15, Gorinchem, the Netherlands).

Plasma and muscle tissue 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 (GLUC3, Roche, Ref: 05168791 190, and Immunologic, Roche, Ref: 12017547 122, respectively). Amino acid concentrations were determined by using ultraperformance liquid chromatography mass spectrometry, as described previously (13). Plasma amino acid enrichments were determined by gas chromatography-mass spectrometry analysis (GC-MS; Agilent 7890A GC/5975C; MSD, Agilent Technologies). Myofibrillar protein-bound L-[*ring*-$^{13}C_6$]-phenylalanine enrichments were determined by gas chromatography-combustion-isotope ratio mass spectrometer analysis (GC-C-IRMS; Thermo Fisher Scientific Delta V, Bremen, Germany) as described in our previous work (13). For western blot analysis, a portion of each muscle sample frozen for biochemical analyses was taken and analyzed as described previously (14).

Calculations

Myofibrillar protein fractional synthetic rates (FSRs) were calculated by using the standard precursor-product equation, as follows:

$$FSR = \frac{\Delta E_p}{E_{\text{precursor}} \cdot t} \cdot 100$$

ΔEp is the increment in myofibrillar protein–bound L-[*ring*-$^{13}C_6$]-phenylalanine enrichment after an incorporation period, $E_{precursor}$ is the weighted mean plasma L-[*ring*-$^{13}C_6$]-phenylalanine enrichment during that incorporation period, and *t* is the incorporation period (h). Weighted mean plasma enrichments were calculated by taking the average enrichment between all consecutive time points and correcting for the time between these sampling time points. The weighted mean plasma precursor pool is preferred in this setting, because the more frequent sampling time points allow for a more accurate correction of the transient changes in precursor pool enrichments over time (15). For basal (post-absorptive) FSR, plasma protein samples at t= -210 min and muscle biopsy samples at t= 0 min were used; and for postprandial FSRs, muscle biopsy samples at t= 0, 120, and 300 min were used.

Statistical Analysis

All results are expressed as mean ± 95% confidence interval (CI). Baseline characteristics, dietary intake, and incremental area under the curve (iAUC) for integrated postprandial plasma amino acid concentrations above and below baseline (t= 0 min) were compared between groups using a one-way analysis of variance (ANOVA). For time-dependent variables, a two-factor (group x time) repeated-measures ANOVA with group as a between-subjects factor and time as a within-subjects factor was used (i.e., all time points for plasma concentrations, plasma amino acid enrichment, anabolic signaling, and basal compared with postprandial muscle data). In case of significant interactions, separate analyses were performed within each treatment group, as well as between treatment groups for every time period separately. In the case of significant group or time effects (when appropriate), Bonferroni post hoc analysis were performed to locate the effects. Cohen's effect size (*d*) of the differences between raw eggs, control breakfast, and boiled eggs were calculated for the primary outcome (i.e. FSR) data. Effect sizes of 0.2 are considered small, 0.5 are considered medium, and 0.8 are considered large. Statistical significance was set at $P \leq 0.05$. All calculations were performed using SPSS (version 26.0, IBM Corp., Armonk, NY, USA).

Results

Dietary intake

Habitual dietary intakes are presented in **Table 2**. Analysis of the 2-d dietary intake records (collected in the 2 days prior to the experimental protocol) showed no differences in habitual food intake between groups. Daily protein intake in these subjects averaged 1.6 g/kg body mass/day in all three groups.

Table 2. Habitual dietary intake

	Control breakfast	Raw eggs	Boiled eggs	*P*
Energy (MJ/day)	10.8 (8.5 to 13.1)	12.0 (10.0 to 13.9)	11.1 (9.5 to 12.7)	0.664
Energy (kcal/day)	2589 (2041 to 3138)	2860 (2388 to 3333)	2655 (2276 to 3034)	0.664
Energy (kcal/kg BM/day)	34.8 (27.1 to 42.5)	35.8 (30.5 to 41.0)	35.5 (29.4 to 41.7)	0.973
Carbohydrate (g/day)	279 (236 to 321)	346 (281 to 411)	295 (242 to 347)	0.160
Carbohydrate (En%/day)	47 (41 to 53)	50 (47 to 53)	46 (43 to 50)	0.383
Fat (g/day)	101 (59 to 143)	97 (80 to 115)	102 (86 to 119)	0.962
Fat (En%/day)	33 (28 to 38)	31 (28 to 34)	35 (31 to 38)	0.312
Protein (g/day)	123 (94 to 151)	128 (103 to 152)	120 (99 to 142)	0.908
Protein (En%/day)	20 (17 to 24)	19 (16 to 22)	19 (17 to 22)	0.747
Protein (g/kg BM/day)	1.6 (1.3 to 2.0)	1.6 (1.3 to 1.9)	1.6 (1.3 to 1.9)	0.960

Values represent means and 95% CI, *n*=15 per group. Participants' habitual energy, carbohydrate, fat, and protein intakes were calculated from a 2-d dietary record. Data were analyzed using a one-way ANOVA. En%, percentage of energy; kg BM, kilogram body mass; MJ, Megajoule.

Plasma glucose and insulin

For both plasma glucose and insulin concentrations a main effect of time, a main effect of group, and a time X group interaction were observed (all $P<0.01$). No significant differences were observed in fasting plasma glucose concentrations between groups (**Figure 2A**). Following food ingestion, a significant increase in plasma glucose concentrations was observed in the control breakfast group only (up to t= 45 min; $P<0.005$), with higher plasma glucose concentrations (up to t= 75 min) in the control breakfast group compared to both the boiled eggs and the raw eggs group ($P<0.005$).

Plasma insulin concentrations (**Figure 2B**) were significantly increased immediately after exercise in all three groups ($P<0.005$), with no significant differences being observed in plasma insulin concentrations between groups prior to food ingestion. Postprandial plasma insulin concentrations were significantly increased in the control breakfast group only (up to

t= 60 min; $P<0.05$), with higher plasma insulin concentrations in the control breakfast group compared to both the boiled eggs and the raw eggs group ($P<0.05$).

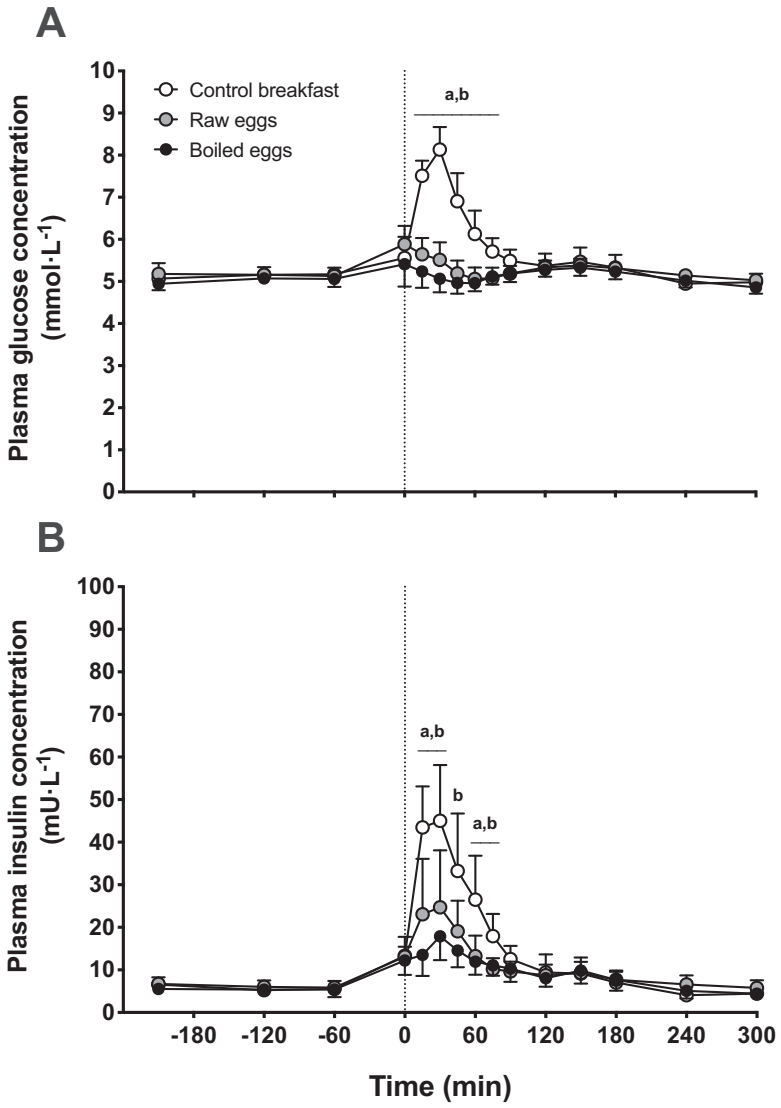

Figure 2. Plasma glucose (**A**) and insulin (**B**) concentrations over time after the ingestion of a low protein control breakfast (n = 15), 5 raw eggs (n = 15), or 5 boiled eggs (n = 15) in healthy young males. Exercise was performed between t= -60 and 0 min. The dotted line represents the time of food ingestion. Values represent means and 95% CI. Data were analyzed with repeated measures (time X treatment group) ANOVA and separate analyses were performed when a significant interaction was detected. Bonferroni post hoc testing was used to detect differences between groups. Time X group interaction, $P<0.01$. a, Raw eggs significantly different ($P<0.05$) from control breakfast; b, Boiled eggs significantly different ($P<0.05$) from control breakfast.

Plasma amino acids

Plasma leucine and total branched-chain amino acid (BCAA) concentrations are depicted in **Figure 3**. Significant time X group interactions were observed for both plasma leucine and total BCAA concentrations ($P<0.001$). In addition, a significant group effect was observed for the iAUC for both leucine and total BCAA concentrations ($P<0.001$). Postprandial plasma leucine (**Figure 3A**) and BCAA (**Figure 3B**) concentrations significantly increased from baseline values (t= 0 min) onwards and remained elevated throughout the entire postprandial period in both the raw eggs and boiled eggs group ($P<0.005$). As a result, postprandial plasma leucine and BCAA concentrations were significantly higher in both the raw eggs and boiled eggs group compared to the control breakfast group ($P<0.05$). Postprandial plasma leucine and BCAA concentrations showed a greater increase following boiled eggs vs raw eggs ingestion, with significantly higher values between t= 45-180 min in the boiled eggs versus raw eggs group ($P<0.05$). In agreement, the postprandial plasma leucine (Figure 3A insert) and BCAA (Figure 3B insert) iAUC were significantly greater in the raw eggs and boiled eggs group compared to the control breakfast group ($P<0.001$), with higher values following ingestion of boiled versus raw eggs ($P<0.001$).

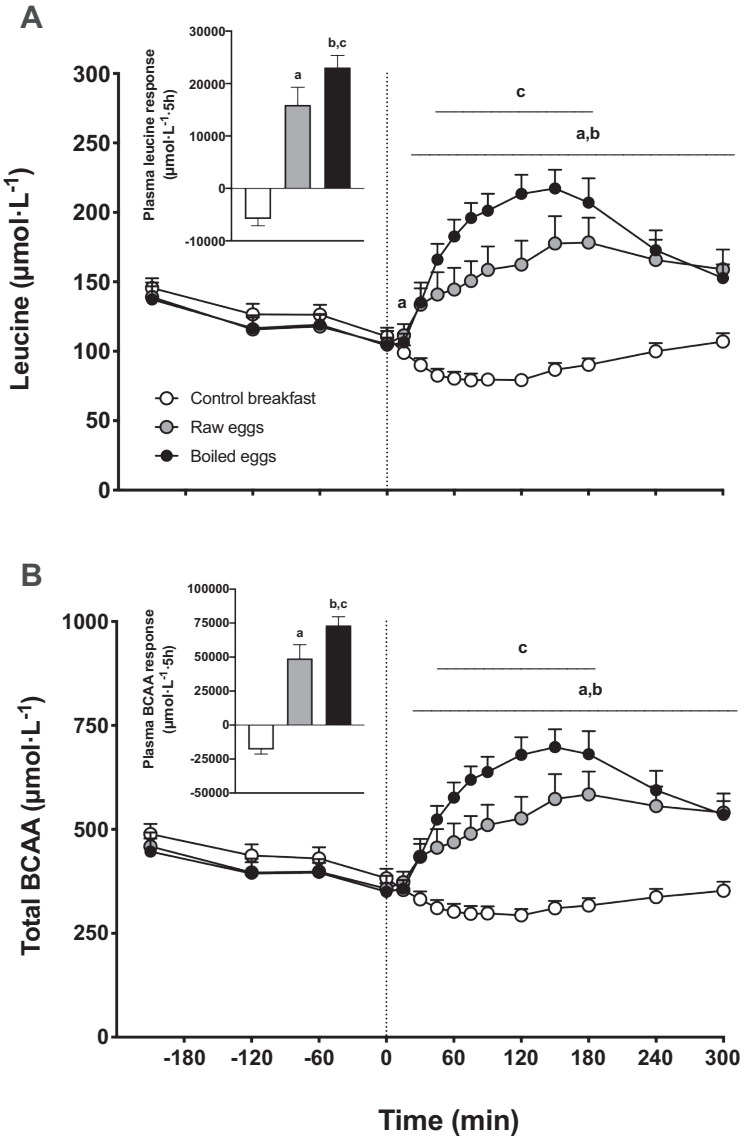

Figure 3. Plasma leucine (**A**), and total BCAA (**B**) concentrations over time and total leucine (panel A insert) and BCAA (panel B insert) responses, expressed as iAUC, following the ingestion of a low protein control breakfast (*n* = 15), 5 raw eggs (*n* = 15), or 5 boiled eggs (*n* = 15) in healthy young males. Exercise was performed between t= -60 and 0 min. The dotted line represents the time of food ingestion. Values represent means and 95% CI. For plasma concentrations over time, data were analyzed with repeated measures (time X treatment group) ANOVA and separate analyses were performed when a significant interaction was detected. The iAUC data was analyzed with a one-way ANOVA. Bonferroni post hoc testing was used to detect differences between groups. For plasma concentrations over time, a time X group interaction was observed, $P<0.001$; For iAUC data, a group effect was observed, $P<0.001$. a, Raw eggs significantly different ($P<0.05$) from control breakfast; b, Boiled eggs significantly different ($P<0.05$) from control breakfast; c, Boiled eggs significantly different ($P<0.05$) from raw eggs.

Plasma total essential amino acid (EAA), total nonessential amino acid (NEAA-glutamine), and total amino acid (AA-glutamine) concentrations are depicted in **Figure 4**. Significant time X group interactions were observed for plasma total EAA, total NEAA, and total AA concentrations ($P<0.001$). In addition, a significant group effect was observed for the iAUC for total EAA, total NEAA, and total AA concentrations ($P<0.001$). Postprandial plasma EAA, NEAA, and total AA concentrations decreased in the control breakfast group, with significantly lower values compared to baseline values throughout the entire postprandial period ($P<0.05$). In both the raw eggs and boiled eggs group, postprandial plasma EAA concentrations were significantly higher and postprandial plasma NEAA concentrations were significantly lower compared to baseline values ($P<0.05$). For total plasma AA concentrations, only a significant increase was observed in the boiled eggs group ($P<0.001$). Postprandial plasma EAA and total AA concentrations were significantly higher in both the raw eggs and boiled eggs group compared to the control breakfast group ($P<0.05$), with the boiled eggs group showing significantly higher postprandial plasma EAA and total AA concentrations compared to the raw eggs group ($P<0.05$). In agreement, the iAUC of postprandial plasma EAA (Figure 4A insert) and total AA (Figure 4C insert) concentrations were significantly greater in the raw eggs and boiled eggs group compared to the control breakfast group ($P<0.001$) and higher in the boiled eggs group compared to the raw eggs group ($P<0.05$). Postprandial plasma NEAA concentrations were significantly higher in the boiled eggs group compared to the control breakfast group ($P<0.001$), with no differences between the control breakfast and the raw eggs group. Postprandial plasma NEAA concentrations were significantly higher in the boiled eggs group compared to the raw eggs group at t= 75 and 120 min ($P<0.05$). The iAUC of postprandial plasma NEAA concentrations (Figure 4B insert) were significantly different in the raw eggs and boiled eggs group compared to the control breakfast group (both $P<0.005$), with no differences observed between the boiled eggs and raw eggs group ($P>0.05$).

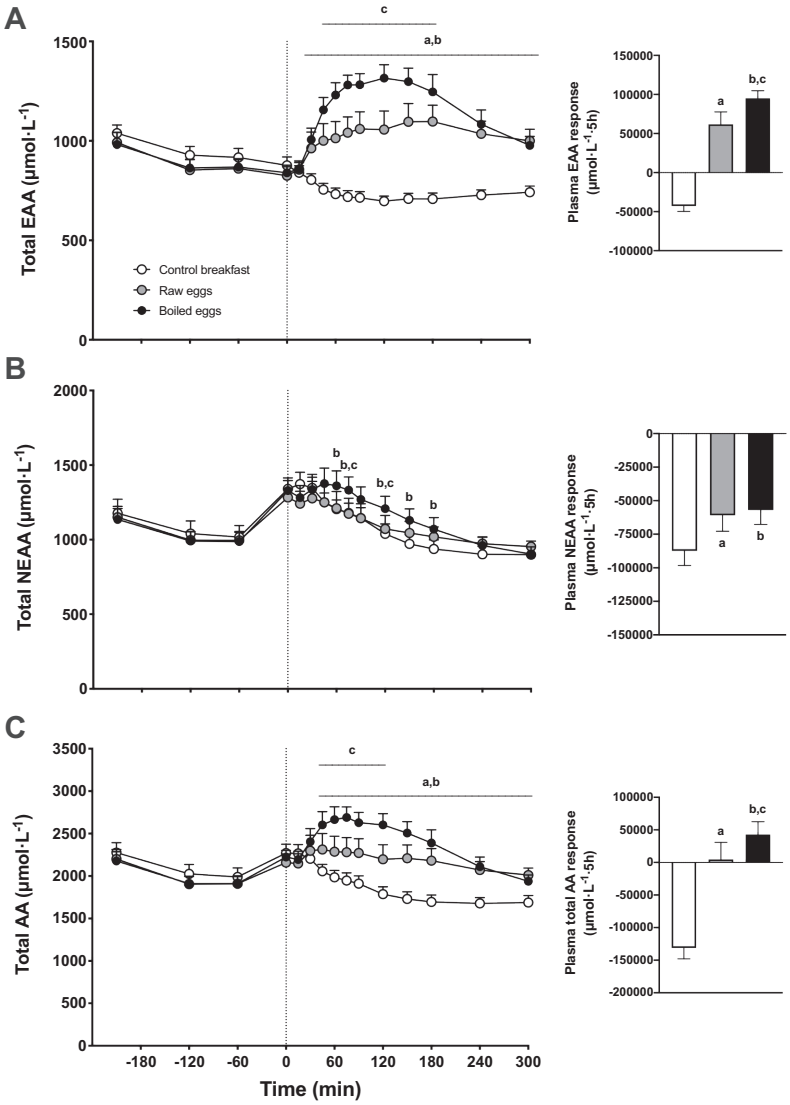

Figure 4. Plasma total EAA (**A**), NEAA (**B**), and AA (**C**) concentrations over time and total EAA (panel A insert), NEAA (panel B insert), and AA (panel C insert) responses, expressed as iAUC, after the ingestion of a low protein control breakfast (*n* = 15), 5 raw eggs (*n* = 15), or 5 boiled eggs (*n* = 15) in healthy young males. Exercise was performed between t= -60 and 0 min. The dotted line represents the time of food ingestion. Values represent means and 95% CI. For plasma concentrations over time, data were analyzed with repeated measures (time X treatment group) ANOVA and separate analyses were performed when a significant interaction was detected. The iAUC data was analyzed with a one-way ANOVA. Bonferroni post hoc testing was used to detect differences between groups. For plasma concentrations over time, a time X group interaction was observed, $P<0.001$; For iAUC data, a group effect was observed, $P<0.001$. a, Raw eggs significantly different ($P<0.05$) from control breakfast; b, Boiled eggs significantly different ($P<0.05$) from control breakfast; c, Boiled eggs significantly different ($P<0.05$) from raw eggs.

Isotope tracer analysis

A significant time X group interaction was observed for plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments (P<0.001). Prior to food ingestion, plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments averaged 6.8 (95% CI: 6.5 to 7.1), 6.5 (95% CI: 6.2 to 6.9), and 6.8 (95% CI: 6.5 to 7.1) mole % excess (MPE) in the control breakfast, raw eggs, and boiled eggs group, respectively, with no differences over time or between groups (**Figure 5**). Postprandial plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments (compared to baseline (t= 0 min)) were significantly lower in the raw and boiled eggs groups during the postprandial period (P<0.05). Plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments during the entire postprandial period averaged 7.1 (95% CI: 6.8 to 7.4), 5.7 (95% CI: 5.5 to 6.0), and 5.7 (95% CI: 5.4 to 6.0) MPE for the control breakfast, raw eggs, and boiled eggs group, respectively, with significantly lower values in the raw and boiled eggs groups compared with the control breakfast group (P<0.05).

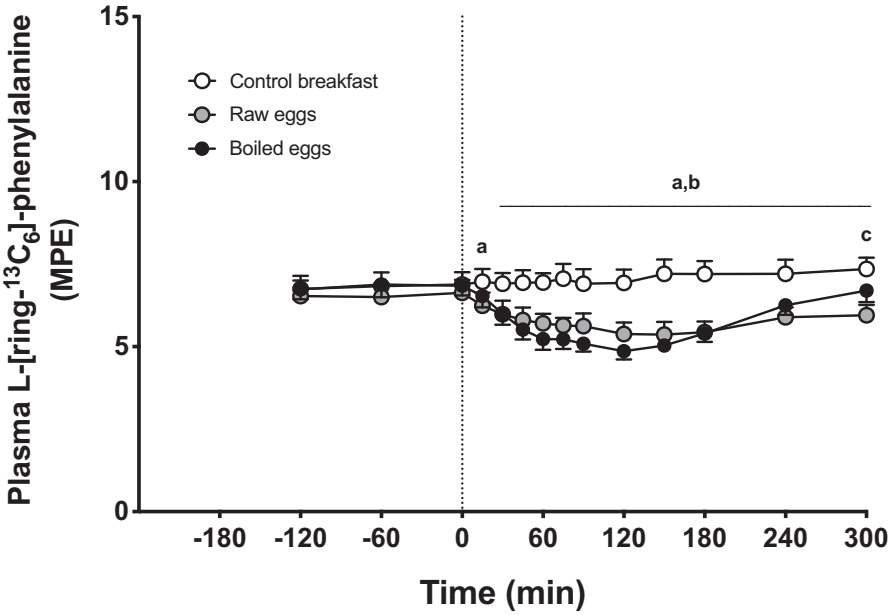

Figure 5. Plasma L-[ring-$^{13}C_6$]-phenylalanine enrichments over time after the ingestion of a low protein control breakfast (n = 15), 5 raw eggs (n = 15), or 5 boiled eggs (n = 15) in healthy young males. Exercise was performed between t= -60 and 0 min. The dotted line represents the time of food ingestion. Values represent means and 95% CI. Data were analyzed with repeated measures (time X treatment group) ANOVA and separate analyses were performed when a significant interaction was detected. Bonferroni post hoc testing was used to detect differences between groups. Time X group interaction, P<0.001. a, Raw eggs significantly different (P<0.05) from control breakfast; b, Boiled eggs significantly different (P<0.05) from control breakfast. c, Boiled eggs significantly different (P<0.05) from raw eggs.

Myofibrillar protein synthesis rates calculated based on the plasma precursor pool are depicted in **Figure 6** and **7**. Myofibrillar protein synthesis rates increased following food ingestion in all groups. From basal fasting values to the early 0-2 h postprandial period, myofibrillar protein synthesis rates increased from 0.017 (95% CI: 0.012 to 0.023) to 0.059 %/h (95% CI: 0.047 to 0.071), from 0.018 (95% CI: 0.014 to 0.022) to 0.064 %/h (95% CI: 0.055 to 0.074), and from 0.013 (95% CI: 0.010 to 0.017) to 0.066 %/h (95% CI: 0.056 to 0.076), in the control breakfast, raw eggs, and boiled eggs group, respectively (P<0.001; Figure 6). Myofibrillar protein synthesis rates remained elevated during the late (2-5 h) postprandial period in all groups with values of 0.053 (95% CI: 0.041 to 0.065), 0.069 (95% CI: 0.052 to 0.086), and 0.066 (95% CI: 0.052 to 0.080) %/h in the control breakfast, raw eggs, and boiled eggs group, respectively (P<0.001). Throughout the entire 0-5 h postprandial period, myofibrillar protein synthesis rates were elevated above basal values (main effect of time: P <0.001; Figure 7) and were ~20% higher following ingestion of the raw eggs (0.067 %/h (95% CI: 0.056 to 0.077)) and boiled eggs (0.065 %/h (95% CI: 0.058 to 0.073)) compared to the control breakfast group (0.056 %/h (95% CI: 0.048 to 0.063)). Although this difference did not reach statistical significance (time X group interaction: P =0.077), meaningful effect sizes were observed between the egg groups and the control breakfast group (d for raw eggs vs control breakfast: 0.63; d for boiled eggs vs control breakfast: 0.69), but not between both egg groups (d: 0.07).

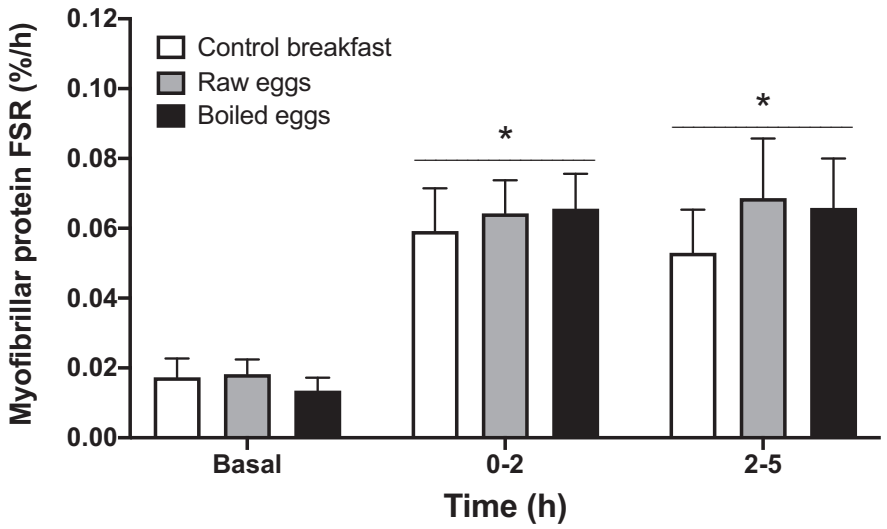

Figure 6. Myofibrillar protein fractional synthetic rates (FSR; in %/h) during the fasted state (basal) and over the early (0-2 h), and late (2-5 h) post-exercise and postprandial period after the ingestion of a low protein control breakfast (n = 15), 5 raw eggs (n = 15), or 5 boiled eggs (n = 15) in healthy young males. Values represent means and 95% CI. Data were analyzed with repeated measures (time X treatment group) ANOVA. *, significant time effect (P<0.001).

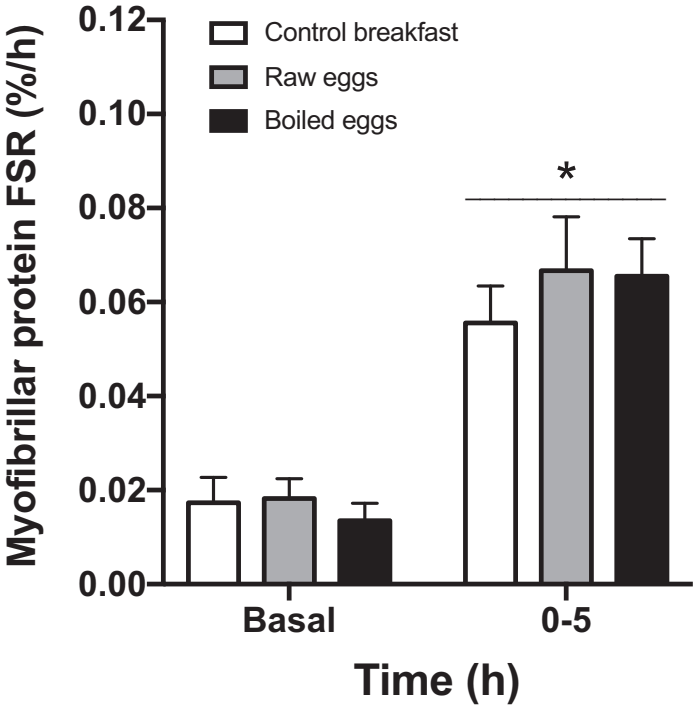

Figure 7. Myofibrillar protein fractional synthetic rates (FSR; in %/h) during the fasted state (basal) and over the entire (0-5 h) post-exercise and postprandial period after the ingestion of a low protein control breakfast (*n* = 15), 5 raw eggs (*n* = 15), or 5 boiled eggs (*n* = 15) in healthy young males. Values represent means and 95% CI. Data were analyzed with repeated measures (time X treatment group) ANOVA. *, significant time effect (*P*<0.001); There was a trend for a significant interaction effect (*P*=0.077).

Anabolic signaling

The phosphorylation status (ratio of phosphorylated to total protein) of key proteins involved in the initiation of muscle protein synthesis are presented in **Figure 8 (A-F)**. No significant time effect, group effect, or time X group interactions were observed for muscle mTOR (Ser2448) (**Figure 8A**) and p70S6K (Thr389) (**Figure 8B**) phosphorylation status. A significant time effect was observed for muscle p70S6K (Thr421/Ser424) (**Figure 8C**), rpS6 (Ser235/236) (**Figure 8E**), and 4E-BP1 (Thr37/46) (**Figure 8F**) phosphorylation status (*P*<0.05), with no significant group effects or time X group interactions observed. For muscle p70S6K (Thr421/Ser424), time points t= 2 and 5 h were significantly lower compared to t= 0 h (*P*<0.001; Figure 8C). For muscle rpS6 (Ser235/236), time point t= 2 h was significantly lower compared to t= 0 h (*P*=0.033; Figure 8E) and for muscle 4E-BP1 (Thr37/46) t=5 h was significantly higher compared to t=2 h (*P*=0.036; Figure 8F). A significant group effect was

observed for muscle rpS6 (Ser240/244) phosphorylation status (P=0.012; **Figure 8D**) with boiled eggs being higher compared to raw eggs (P=0.010), with no significant time effect or time X group interaction observed.

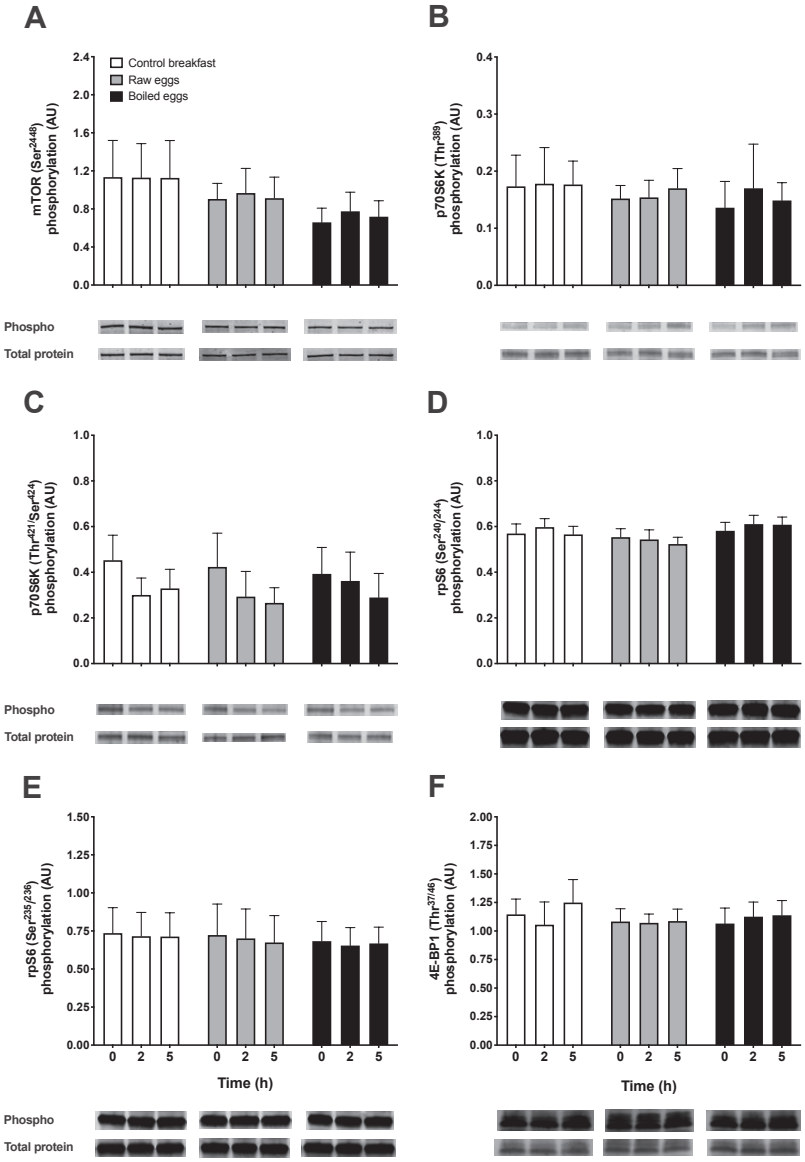

Figure 8. Skeletal muscle phosphorylation status (ratio of phosphorylated to total protein) of mTOR (Ser2448) (**A**), p70S6K (Thr389) (**B**), p70S6K (Thr421/Ser424) (**C**), rpS6 (Ser240/244) (**D**), rpS6 (Ser235/236) (**E**), and 4E-BP1 (Thr37/46) (**F**) immediately after exercise (t= 0 h) and at t= 2 and 5 h after the ingestion of a low protein control breakfast (n = 15), 5 raw eggs (n = 15), or 5 boiled eggs (n = 15) in healthy young males. Values represent means and 95% CI. Data were analyzed with repeated measures (time X treatment group) ANOVA. No significant interaction was detected.

Discussion

In the present study we observed that ingestion of 30 g protein provided as 5 eggs strongly increased circulating plasma essential amino acid concentrations, with a greater rise in circulating amino acids following the ingestion of 5 boiled compared with 5 unboiled, raw eggs. Despite the more pronounced rise in postprandial plasma amino acid responses after the ingestion of boiled versus raw eggs, we observed no significant differences in postprandial myofibrillar protein synthesis rates throughout 5 hours of post-exercise recovery.

Eggs represent a high-quality protein source based upon a high and well-balanced essential amino acid content (well above the WHO/FAO/UNU requirements for adults) and a high digestible indispensable amino acid score (DIAAS) (16, 17). Not surprisingly, ingestion of egg protein has been shown to strongly increase post-exercise muscle protein synthesis rates (18, 19) with maximal muscle protein synthesis rates observed after ingesting 20-40 g protein (19). When consumed as whole-foods, eggs are generally cooked or boiled prior to consumption. This heat treatment may strongly impact postprandial protein digestion and subsequent amino acid absorption kinetics (2, 3). Here, we compared the postprandial rise in circulating amino acids following ingestion of 5 boiled compared with 5 unboiled, raw eggs in healthy young males recovering from a bout of exercise (Figures 3 and 4). Following egg ingestion, we demonstrated an increase in plasma leucine, BCAA, and EAA concentrations, with greater increases after ingesting boiled when compared with raw eggs. These results are in line with previous pioneering work by Evenepoel *et al.* (2, 3), who showed that cooked eggs have a much higher digestibility (~91%) when compared to raw eggs (~51%). The greater digestibility of cooked eggs has been attributed to the denaturation of the protein, resulting in structural changes of the different egg proteins (e.g. ovalbumin, ovotransferrin) that facilitate the hydrolytic action of digestive enzymes (20). Furthermore, heating may also (partly) inactivate the effect of different trypsin inhibitors present within the egg (21). Consequently, given its attenuated protein digestion, amino acid absorption, and subsequent postprandial rise in circulating amino acids, it could be speculated that ingesting raw (vs boiled) eggs would compromise the postprandial stimulation of muscle protein synthesis.

In the present study we observed that myofibrillar protein synthesis rates were significantly increased during the early (0-2 h), late (2-5 h), and total (0-5 h) post-exercise period when compared to basal, resting values (Figure 6 and 7). These data confirm numerous observations showing increases in muscle protein synthesis rates after a bout of exercise (22-27). No significant differences were observed between groups during 5 hours of post-exercise recovery (P=0.077), however, we did observe ~20% higher postprandial myofibrillar protein synthesis rates following the ingestion of boiled or raw eggs when compared to the control breakfast (Figure 7). In addition, meaningful effect sizes of 0.6-0.7 were observed

between the egg groups and the control breakfast group. Taken together, our results support the contention that adequate (≥20 g) high-quality protein intake further increases post-exercise muscle protein synthesis rates (19, 28-31). However, in contrast to our hypothesis, differences in postprandial amino acid responses after the ingestion of 5 boiled versus unboiled, raw eggs did not translate into differences in post-exercise muscle protein synthesis rates.

We can only speculate on the factors responsible for the absence of differences in post-exercise muscle protein synthesis rates after the ingestion of boiled vs raw eggs, in spite of the obvious differences in postprandial plasma amino acid availability. However, it seems evident that the provision of 5 eggs already provided more protein (30 g) than the amount previously reported to be required to maximize post-exercise myofibrillar protein synthesis rates (19, 31, 32). In support, our results are consistent with recent work showing that higher plasma amino acid availability does not further stimulate muscle protein synthesis when ample amounts of protein are ingested during recovery from resistance type exercise (33). Furthermore, several other studies have failed to show differences in post-exercise muscle protein synthesis when ingesting ample amounts of different protein sources (despite substantial differences in the magnitude of postprandial aminoacidemia induced) (34-41). Accordingly, the postprandial rise in circulating amino acids is not a key determinant to modulate muscle protein synthesis rates when ample protein is ingested during recovery from exercise. Clearly, this also applies to the anabolic response after the ingestion of as much as 5 raw or boiled eggs.

As resistance type exercise and dietary protein intake activate intramuscular signaling proteins that regulate protein translation-initiation, we also assessed several targets of the mTOR pathway (i.e., mTOR, p70S6 kinase, rpS6, and 4E-BP1) in order to assess potential differences between treatment groups. In the current study we did not observe any significant differences between groups over time (Figure 8). This is not necessarily surprising as we only collected muscle tissue biopsies during recovery from exercise and, as such, most of the signaling responses were likely already strongly activated in the time period leading up to the collection of the first biopsy (18). The fact that no differences were observed in anabolic signaling between the different groups during post-exercise recovery is in line with recent findings showing that differences in aminoacidemia do not necessarily appear to influence anabolic signaling when assessed during recovery from exhaustive exercise (33). Overall, the current findings in anabolic signaling agree with the absence of differences in postprandial muscle protein synthesis rates between groups.

As post-exercise muscle protein synthesis rates did not differ following the ingestion of 5 boiled versus raw eggs, it is evident that Rocky did not compromise his skeletal muscle adaptive response to exercise by not boiling his eggs. However, it should be noted that there may still be other reasons why people want to consider boiling eggs prior to consumption. One obvious reason may be for general taste and texture preferences. Another good reason

may be to eliminate the risk of salmonella infection. Even though it appears that there is only a small (<0.06%) percentage of eggs that are contaminated with salmonella and thus, a low infection risk when eating raw eggs (42), this risk will be fully eliminated when the eggs are properly boiled prior to consumption (43).

In conclusion, ingestion of 5 raw, unboiled eggs as opposed to 5 boiled eggs attenuates the postprandial rise in circulating essential amino acid concentrations, but does not compromise the postprandial increase in myofibrillar protein synthesis rates during recovery from exercise. Though Rocky was not aware of the benefits of boiling eggs to postprandial protein handling, he did not compromise post-exercise muscle conditioning by consuming his eggs unboiled.

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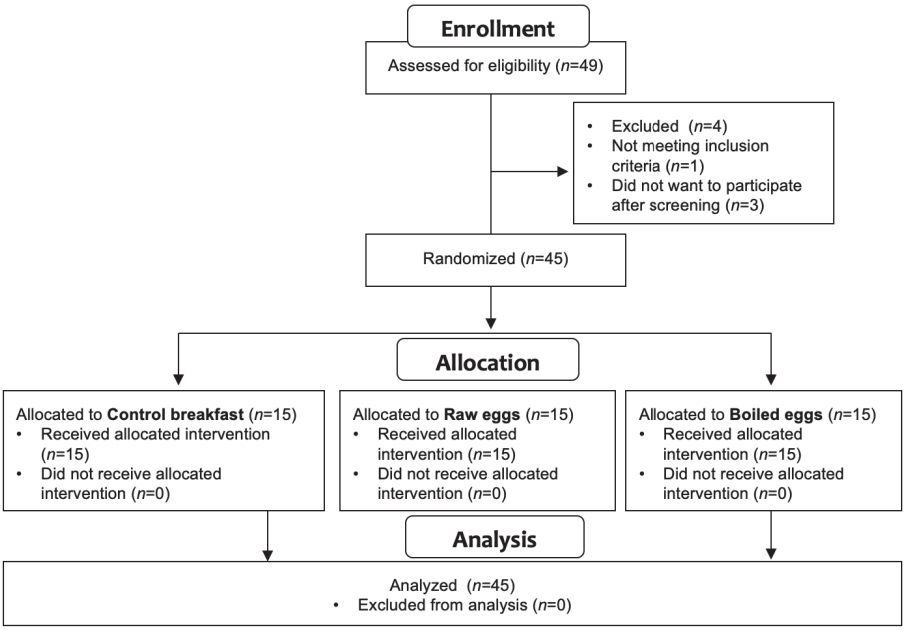
Supplementary Material

Supplementary Figure 1.



Chapter 7

Postexercise cooling impairs muscle protein synthesis rates in recreational athletes

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Abstract

We measured the impact of postexercise cooling on acute postprandial (hourly) as well as prolonged (daily) myofibrillar protein synthesis rates during adaptation to resistance-type exercise over a 2-week period. Twelve healthy males (age: 21±2 y) performed a single resistance-type exercise session followed by water immersion of both legs for 20 min. One leg was immersed in cold water (8°C: CWI) 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) labeled 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. In addition, deuterated water ($^{2}H_2O$) was applied with the collection of saliva, blood, and muscle biopsies over 2 weeks to assess the effects of postexercise cooling with protein intake on myofibrillar protein synthesis rates during more prolonged resistance-type exercise training (thereby reflecting short-term training adaptation). Incorporation of dietary protein-derived L-[1-^{13}C]-phenylalanine into myofibrillar protein was significantly lower in CWI compared with CON (0.016±0.006 vs 0.021±0.007 MPE; P=0.016). Postexercise myofibrillar protein synthesis rates were lower in CWI compared with CON based upon L-[1-^{13}C]-leucine (0.058±0.011 vs 0.072±0.017%·h^{-1}, respectively; P=0.024) and L-[*ring*-$^{2}H_5$]-phenylalanine (0.042±0.009 vs 0.053±0.013%·h^{-1}, respectively; P=0.025). Daily myofibrillar protein synthesis rates assessed over 2 weeks were significantly lower in CWI compared with CON (1.48±0.17 vs 1.67±0.36%·d^{-1}, respectively; P=0.042). Cold-water immersion during recovery from resistance-type exercise reduces myofibrillar protein synthesis rates and, as such, likely impairs muscle conditioning.

Introduction

Resistance-type exercise training is an effective strategy to increase skeletal muscle mass and strength. A single session of resistance-type exercise stimulates both muscle protein synthesis and breakdown rates, albeit the latter to a lesser extent (~2-4 fold lower increase). Thereby, resistance-type exercise improves net muscle protein balance, yet the balance remains negative in the absence of protein ingestion (1, 2). Protein ingestion after exercise augments the increase in muscle protein synthesis rates and inhibits muscle protein breakdown rates, resulting in a positive net muscle protein balance during the acute stages of postexercise recovery (3, 4). Therefore, postexercise protein ingestion is widely applied by athletes as a strategy to increase postexercise muscle protein synthesis rates and, as such, to facilitate skeletal muscle conditioning. Current guidelines for protein intake after lower body resistance-type exercise recommend the ingestion of 20 g (or 0.3 g/kg body mass) of a high quality protein source after exercise in healthy, young males (5-7).

Another strategy frequently applied by athletes to support postexercise skeletal muscle conditioning is cold-water immersion (CWI). CWI, primarily due to its ability to decrease tissue temperature and blood flow (8-13), has been suggested to reduce delayed onset muscle soreness (14-16) and muscle edema/swelling (15, 17-19), improve recovery of muscle function/performance (15, 19-21), and increase gene expression of molecular markers of endurance exercise adaptation (22-24). However, it is important to note that not all studies show beneficial effects of CWI on markers of postexercise recovery (20, 25-27). A recent meta-analysis reported that the proposed beneficial effects of CWI on postexercise recovery seem to be based more upon subjective rather than objective (blood) markers (16). Therefore, beneficial effects of CWI on postexercise recovery appear to be context specific and several factors such as body composition, sex, and training status should also be taken into account (16, 28-30).

So far, few studies have investigated the interaction between postexercise protein intake and cooling. Previously, Roberts *et al.* have observed that combining postexercise cooling (CWI at ~10°C for 10 min) with protein intake lowers anabolic signaling when compared to combining active recovery (cycling at ~37 W for 10 min) with protein intake in physically active, young men (31). To date, no studies have assessed the effects of combining postexercise cooling with protein intake on the incorporation of dietary protein-derived amino acids into skeletal muscle tissue and subsequent muscle protein synthesis rates. We hypothesized that postexercise cooling lowers postprandial muscle protein accretion and subsequent muscle protein synthesis rates during recovery in healthy, young men.

As it could be speculated that potential negative effects of postexercise cooling on muscle protein synthesis are only transient and would not translate into a negative impact on muscle protein synthesis when assessed over a more prolonged period, we investigated the combined effects of more prolonged application of postexercise cooling and protein intake

on muscle protein synthesis rates. In line with our first hypothesis, we hypothesized that postexercise cooling lowers myofibrillar protein synthesis rates during 2 weeks of resistance-type exercise training in healthy, young men.

In the present study, we combined contemporary stable isotope tracer methodology with the ingestion of specifically produced intrinsically (L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine) labeled milk protein to assess the acute effects of postexercise cooling on the incorporation of dietary protein-derived amino acids into muscle protein and postprandial myofibrillar protein synthesis rates during 5 h of postexercise recovery. In addition, we applied oral deuterated water-dosing methodology to determine the impact of combining postexercise cooling with protein intake on myofibrillar protein synthesis rates assessed over a 2-week exercise-training period.

Methods

Ethical approval

This study was approved by the Medical Ethics Committee of the Maastricht University Medical Centre+ (METC 15-3-038) and conformed to the principles outlined in the declaration of Helsinki for use of human subjects and tissue. Subjects were fully informed of the nature and possible risks of the experimental procedures before their written informed consent was obtained. This study was registered at clinicaltrials.gov as NCT02596542.

Subjects

Twelve healthy young men (age 21±2 y) participated in this study. All participants were considered recreationally active (exercising ~3 times per week for a total duration of ~4.5 h) and were familiar with resistance-type exercise (but none were participating in structured resistance-type exercise training). Subjects' characteristics are presented in **Table 1**. Participants had no prior history of participating in stable isotope amino acid tracer experiments and were deemed healthy based on their responses to a medical questionnaire.

Table 1. Subjects' characteristics

	Subjects (*n*=12)
Age (y)	21 ± 2
Body mass (kg)	76.2 ± 10.4
Height (cm)	184 ± 10
BMI (kg/m^2)	22.6 ± 1.9
LBM (kg)	61.9 ± 7.3
CON leg lean mass (kg)	10.3 ± 1.5
CWI leg lean mass (kg)	10.2 ± 1.4
Whole body fat mass (kg)	12.7 ± 3.8
CON leg fat mass (kg)	2.5 ± 0.8
CWI leg fat mass (kg)	2.5 ± 0.7
Whole body fat mass (%)	16.1 ± 3.2
Leg press 1RM (kg)	274 ± 59
Leg extension 1RM (kg)	138 ± 29

Values are expressed as means ± SD. BMI, body mass index; LBM, lean body mass; 1RM, one repetition maximum. CWI, Cold water immersion (8°C) leg. CON, Thermoneutral water immersion (30°C) leg.

General study design

Each subject participated in one infusion day (**Figure 1**) and a subsequent 2-week exercise-training program (**Figure 2**). During the infusion day, the effect of postexercise cooling on postprandial muscle protein synthesis was studied following the ingestion of 20 g milk protein with 45 g of carbohydrates. At the start of the infusion day, primed continuous L-

[*ring*-$^{2}H_5$]-phenylalanine and L-[1-^{13}C]-leucine intravenous infusions were applied together with repeated blood sampling. After 1 h of rest, participants performed ~45 min of resistance-type exercise, after which they immersed both legs in water for 20 min (1 leg at 8 °C and 1 leg at 30 °C). Thereafter, skeletal muscle biopsies from both legs were taken, before ingesting 20 g intrinsically (L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine) labeled milk protein with 45 g glucose (polymers). In the subsequent 5 h recovery period, additional muscle biopsies were taken from the *m. vastus lateralis* of both legs at t= 2 and 5 h after drink ingestion (Figure 1). During the 2-week exercise-training program, subjects performed progressive lower-body resistance-type exercise training (total of 7 training sessions). Immediately after each exercise session, one leg was randomly assigned (the same leg as during the infusion day) to undergo 20 min of cold-water immersion (8 °C; CWI), while the contralateral leg was immersed in thermoneutral water (30 °C; CON). Deuterium-oxide was ingested daily and muscle biopsy samples were obtained at the start (day 0) and at the end of the 2-week training period (day 14) to assess myofibrillar protein synthesis rates. More specifically, to calculate myofibrillar protein synthesis rates over the 2-week exercise-training period, biopsies from both legs were used from the first training (infusion) day, 2 h after postexercise water immersion and the final biopsies from both legs were taken after the last training session, again 2 h after postexercise water immersion.

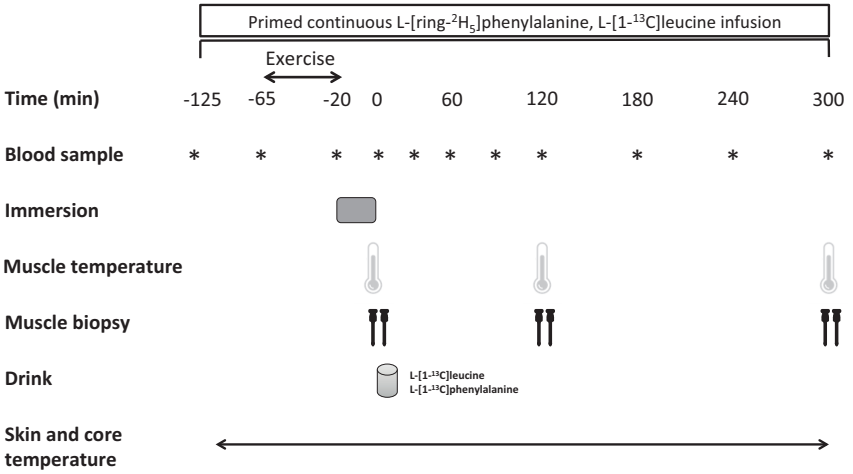

Figure 1. Graphical representation of the infusion day (day 0 of 2-week training program; see figure 2). Participants performed leg press and leg extension exercise followed by water immersion of both legs (1 leg was immersed in 8°C; the other leg was immersed in 30°C) for a total duration of 20 min. 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 postexercise 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.

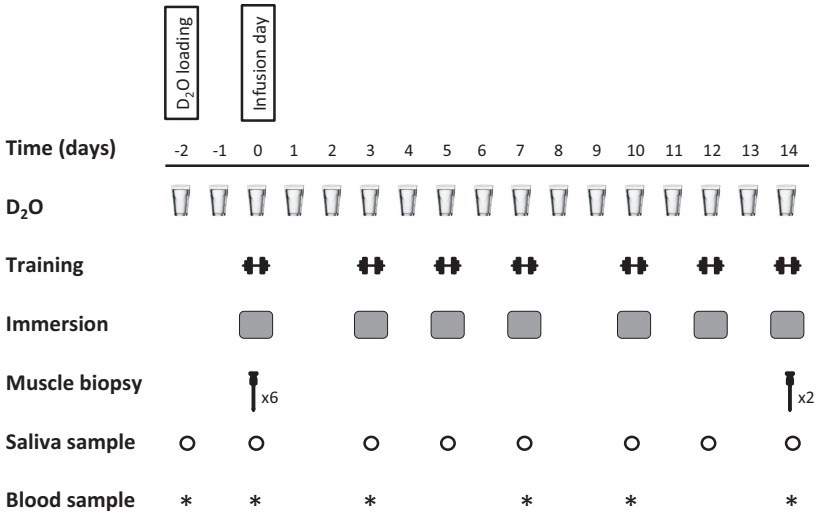

Figure 2. Graphical representation of the experimental protocol of the 2-week training program. 2 days before the start of the first training session (infusion day; see Figure 1), participants performed the D_2O loading day. Thereafter, D_2O was provided every day during the 2-week training period. The 2-week training program consisted of seven lower body resistance exercise sessions (leg press and extension machine). Every exercise session was followed by water immersion of both legs (1 leg was immersed in 8°C; the other leg was immersed in 30°C) for a duration of 20 min. Bilateral muscle biopsies were collected after the first training session (infusion day; day 0) and after the last training session (day 14). Blood and saliva samples were collected throughout the 2-week training program.

Pretesting

All subjects participated in a screening session, which was performed at least 1 week prior to the start of the experiment. First, subjects' body mass and height were measured as well as body composition by dual-energy X-ray absorptiometry (DEXA, Discovery A; Hologic, Bedford, MA, USA). 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 participant's *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 using the multiple repetitions testing procedure (32) (**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 cold water (8 °C: CWI) 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 cooled (CWI) was randomized between subjects' dominant and nondominant leg and the same limb was cooled during the infusion day and after every exercise session during the 2-week training period. The contralateral leg was immersed in thermoneutral water after every exercise session. 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 water and/or ice from the top and remove water from below the tank. During water immersion, temperature was monitored and kept constant and still at 8 °C in CWI and 30 °C in CON. The same setup and procedures were applied for every water immersion session in the current study.

Deuterated water-dosing protocol (D_2O loading)

Two days prior to initiation of the infusion day and thus the 2-week training period, subjects reported to the university for a deuterium oxide (D_2O) dosing day. During this day, subjects ingested 8 x 50 mL boluses of 70% D_2O (Cambridge Isotopes Laboratories, Andover, MA, USA) spaced evenly over the day. For the remainder of the 2-week experiment, subjects ingested 50 mL of deuterium oxide each day upon waking to maintain the enrichment. At the start of the dosing day, a basal saliva and blood sample was obtained together with saliva sample collection prior to all training sessions to monitor body water D_2O enrichment during the 2-week exercise-training program.

Diet and activity prior to the infusion trial and last exercise session

All subjects received the same standardized dinner (1710 kJ, consisting of 20.25 g protein, 51.75 g carbohydrate and 11.25 g fat) the evening prior to the first (infusion day) and last day of the 2-week exercise-training program. All volunteers refrained from alcohol and any sort of additional exhaustive physical labor and/or exercise 2 days prior to the start (infusion day) and throughout the 2-week training program. In order to assess compliance, subjects filled in food intake and physical activity questionnaires for 2 days prior to the start of the first (infusion day) and last day of the 2-week training program.

Experimental protocol of infusion day (first day of 2-week training program)

The experimental protocol for the infusion day is outlined in **Figure 1**. At the start of the

infusion 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' venous 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*-$^{2}H_{5}$]-phenylalanine (2.000 μmol·kg^{-1}) and L-[1-^{13}C]-leucine (3.991 μmol·kg^{-1}), respectively. Subsequently, an intravenous infusion of L-[*ring*-$^{2}H_{5}$]-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 (~129 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. 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 a recovery beverage (see description in next section) 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 min and t= 300 min. Muscle biopsies were collected from the middle region of the *m. vastus lateralis* (~15 cm above the patella) with a Bergström needle under local anesthesia (33). 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, and 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-^{13}C]-phenylalanine and L-[1-^{13}C]-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 up to a total volume of 400 mL with water.

Preparation of intravenous tracers

The stable isotope tracers L-[*ring*-$^{2}H_5$]-phenylalanine and L-[1-^{13}C]-leucine were purchased from Cambridge Isotopes (Andover, MA) and dissolved in 0.9 % saline before infusion (A15 Pharmacy, Gorinchem, the Netherlands).

Experimental protocol of 2-week training program

The experimental protocol for the 2-week training program is outlined in **Figure 2**. Seven supervised training sessions (bilateral exercise) were performed on non-consecutive days during two weeks. During every exercise day, first a saliva sample was collected (followed by blood sample collection at day 0, 3, 7, 10, and 14). After collection of body fluids, the exercise session started with a 5-min warm-up on a cycle ergometer. After the warm-up, the training session consisted of 4 sets of 8-10 repetitions on both leg press and knee extension machines (Technogym, Rotterdam, the Netherlands). Initially, the workload was set at 80% of 1RM. When all 10 repetitions could be performed in 4 sets during an exercise session, workload was increased for the subsequent exercise session. Within 5 min of completing every exercise session, the recovery protocol (20 min water immersion in either 8 °C (CWI) or 30 °C (CON) water) was initiated. Immediately after water immersion, subjects ingested a drink containing 20 g of milk protein (MPC80, FrieslandCampina, Amersfoort, the Netherlands) and 45 g of a vanilla flavored proprietary carbohydrate blend consisting of dextrose and maltodextrin (PepsiCo, Purchase, NY, USA). Subjects then remained in the laboratory and rested in the supine position for 2 h after completion of the recovery protocol to prevent reheating from showering or physical activity. For the last exercise day (day 14), subjects reported to the laboratory again at ~7:30 AM, following an overnight fast. First, basal saliva and blood samples were collected before the exercise session started followed by water immersion and drink ingestion. Two hours after drink ingestion, bilateral percutaneous needle muscle biopsy samples were taken from the *m. vastus lateralis*, approximately 15 cm above the patella. Both muscle biopsy samples were freed from any visible adipose tissue and blood, and immediately frozen in liquid nitrogen. Muscle samples were subsequently stored at -80 °C until further analyses. All experimental sessions were conducted within the same temperature controlled laboratory (temperature: 21.1±0.7 °C and humidity: 41.9±2.3 %).

Plasma and saliva 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*-butyldimethylsilyl (*tert*-BDMS) derivatives with N-tert-butyldimethylsilyl-N-methyltrifluoroacetamide (MTBSTFA) before analysis by GC-MS. The amino acid concentrations 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) leucine, respectively; m/z 336, 337, and 341 for unlabeled and labeled (1-^{13}C and *ring*-$^{2}H_5$) phenylalanine, respectively. The plasma free alanine mass isotopomers (M and M + 1) were determined using selective ion monitoring at *m/z* 232 and 233. 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.

Body water enrichment was analyzed using the saliva samples collected throughout the 2-week training program. Saliva samples were collected at least 30 min after meal and drink ingestion. To collect saliva, subjects lightly chewed on a cotton swab (Celluron, Hartmann, Germany) for enough time to saturate the cotton swab with saliva. The swab was then removed and depressed using a syringe to extract the saliva into a sample tube. After collection, saliva was frozen in liquid nitrogen and stored at -80 °C. All samples were centrifuged at 10,000 *g* to remove any debris. Following centrifugation, all samples were diluted 70-fold with ddH_2O to achieve deuterium enrichments within the detection limits of the isotope ratio mass spectrometer (IRMS). After dilution, samples were prepared for analysis on IRMS using a modified protocol of Scrimgeour *et al.* (34). Briefly, platinum rod (1091831, Thermo Fisher Scientific, Bremen, Germany) was placed inside 12-ml glass vials (Labco Exetainer; Labco, Lampeter, UK). Three-hundred microliters of diluted saliva sample were then transferred into the vials. The glass vials were sealed using rubber septums and a screw cap. Air in each vial was simultaneously evacuated and replaced by 2% H_2 in helium gas. The prepared vials were left at 21 °C for a minimum of 48 h for deuterium equilibration to occur between the hydrogen gas and the saliva samples. The deuterium enrichment of the

hydrogen gas was then measured in duplicate on a IRMS (Delta V Advantage with a gasbench II, Thermo Fisher Scientific, Bremen, Germany). 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.

Muscle analysis

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 ddH2O 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 and the supernatant containing the myofibrillar proteins was collected. The remaining pellet was washed with 1 mL of 0.3 M NaOH and then centrifuged at 9500 *g* and 4 °C for 5 min. Subsequently, the supernatant was collected and added to the first supernatant. The remaining 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 NH_4OH. The purified amino acids (L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine enrichments) were analysed by GC-C-IRMS analysis. To determine myofibrillar protein L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-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*-$^{2}H_5$]-phenylalanine and [^{2}H]alanine enrichment in the myofibrillar protein pools, the eluate was dried, and the purified amino acids were derivatized to their N(O,S)-ethoxycarbonyl ethyl esters. 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 60-m DB-17MS column and 5-m precolumn (No. 122–4762; Agilent) and GC-Isolink. Ion masses 1 and 2 were monitored to determine the $^{2}H/^{1}H$ ratios of muscle protein bound alanine. Standard regression curves were applied to assess the linearity of the mass spectrometer and to account for isotopic fractionation.

Western blotting

Western blot analysis was performed on muscle tissue (t= 0, 2, and 5 h) from the infusion day (**Figure 1**). 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-pyrosphospate, 100 mM NaF, 2 mM Na3VO4, 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 100 °C and immediately placed on ice. Immediately before analyses, the muscle extraction sample was warmed to 50 °C 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 4 °C 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 and anti-4E-BP1 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). Ponceau S staining was used to standardize for the amount of protein loaded. Phosphorylation status as a proxy of activation of the signalling proteins was expressed relative to the total amount of each protein.

mRNA analyses

mRNA analysis was performed on muscle tissue (t= 0, 2, and 5 h) from the infusion day (**Figure 1**). Total RNA was isolated from 10-20 mg of frozen muscle tissue using TRIzol® Reagent (Life Technologies, Invitrogen), according to the manufacturer's protocol. Total RNA quantification was carried out spectrophotometrically at 260 nm (NanoDrop ND-1000 Spectrophotometer, Thermo Fisher Scientific, USA), and RNA purity was determined as the ratio of readings at 260/280 nm. Thereafter, first strand cDNA was synthesized from 1 µg RNA sample using iScript™ cDNA synthesis kit (BioRad; cat. 170-8891). Taqman PCR was carried out using a 7300 Real Time PCR System (AppliedBiosystems, USA), with 2 µL of cDNA, 12.5 µl Taqman™ master mix, 1.25 µl Taqman™ probe and 9.25 µl H_2O in a 25 µL final well volume. Each sample was run in duplicate, together with a serial dilution standard curve. The housekeeping gene 18S was used as an internal control. Taqman primer/probe sets were obtained from Applied Biosystems (Foster City, USA): FOXO1 (Hs 01054576_m1), MuRF1 (Hs 00261590_m1), MAFbx (Hs 01041408_m1), mTOR (Hs 00234508_m1), p70S6K (Hs 00177357_m1), GLUT4 (Hs 00168966_m1), TNF-**α** (Hs 01113624_g1), IL-6 (Hs 00985639_m1), LAT1/SLC (Hs 00185826_m1), PAT1 (Hs 01092773_m1), SNAT2 (Hs 01089954_m1), CD98 (Hs 00374243_m1) and 18S (Hs 03003631_g1). The thermal cycling conditions used were: 2 min at 50 °C, 10 min at 95 °C, followed by 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Ct values of the target genes were normalized to Ct values of the internal control and final results were calculated as relative expression against the standard curve.

Calculations

Ingestion of L-[1-^{13}C]-phenylalanine labeled protein, intravenous infusion of L-[*ring*-$^{2}H_5$]-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 the total amount of phenylalanine that was ingested (Phe_{plasma}). For these calculations modified Steele's equations (in non-steady state conditions) were used (35, 36).

These parameters were calculated as follows:

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

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

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

where F_{iv} is the intravenous tracer infusion rate (μmol·kg^{-1}·min^{-1}), pV (0.125 L·kg^{-1}) is the distribution volume for phenylalanine (35). $C(t)$ is the mean plasma phenylalanine concentration between 2 consecutive time points. dE_{iv}/dt represents the time-dependent variation 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-^{13}C]-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-^{13}C]-leucine, L-[*ring*-^{2}H$_5$]-phenylalanine, or [^{2}H]-alanine, by the enrichment of the respective precursor enrichments (i.e., plasma free amino acids or mean body water deuterium enrichment corrected by a factor of 3.7 (37), respectively). Weighted mean plasma L-[*ring*-^{2}H$_5$]-phenylalanine and L-[1-^{13}C]-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-^{13}C]-leucine tracers. Consequently, myofibrillar *FSR* was calculated as follows:

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

where $E_{m2} - E_{m1}$ represents muscle protein-bound L-[*ring*-^{2}H$_5$]-phenylalanine, L-[1-^{13}C]-leucine or [^{2}H]-alanine. $E_{precursor}$ represents the average plasma L-[*ring*-^{2}H$_5$]-phenylalanine or L-[1-^{13}C]-leucine enrichment (for the infusion day) or the corrected mean body water deuterium enrichment (for the 2-week training program) during the tracer incorporation period. t indicates the time interval (h or d) between biopsies.

Statistical analysis

Unless otherwise stated, all data are expressed as mean ± SD. Changes in body water deuterium enrichments, 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}C]-phenylalanine myofibrillar protein-bound enrichments, skeletal muscle and skin temperature,

anabolic signaling, and gene expression. A Student's paired *t* test was performed for the analysis of FSR values. In case of a significant main effect of time or time X treatment interaction, Bonferroni corrected pairwise comparisons were performed where appropriate. Cohen's effect size (d) and 95% confidence intervals (CI) of the differences between CWI and CON were calculated for the primary outcomes variables. In addition, Cohen's effect sizes (d) were also calculated when significant differences between CWI and CON were observed for markers of anabolic signaling and gene expression. Effect sizes of 0.2 are considered small, 0.5 considered medium, and 0.8 are considered large (38). 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 and thigh skin temperature over the entire experiment are shown in **Figure 3A**. Core temperature slightly increased after water immersion (time effect, $P<0.01$) but did not change significantly throughout the remainder of the experiment. A significant time X treatment interaction was observed for thigh skin temperature ($P<0.001$). Thigh skin temperature was significantly higher after exercise in both the CWI and CON leg ($P<0.001$), after which it decreased back to baseline values following water immersion and did not change throughout the remainder of the experiment in the CON leg. For the CWI leg, thigh skin temperature was significantly reduced immediately after water immersion by ~20.5°C ($P<0.001$) after which it returned towards baseline values over the remainder of the experiment. Thigh skin temperature was significantly different between the CWI and CON legs immediately after water immersion up to 3 h after ingestion of the recovery beverage ($P<0.001$).

Muscle temperature of both legs after water immersion is shown in **Figure 3B**. Considering a ~0.5 cm skinfold thickness, the muscle temperature probe (5 cm) was inserted in the muscle at a depth of ~4.5 cm. A significant time X treatment interaction was observed for muscle temperature ($P<0.001$). Muscle temperature did not change over time in the CON leg, but was significantly different between 0 and 120, 120 and 300, and 0 and 300 min in the CWI leg ($P<0.01$). After water-immersion (t= 0 min), muscle temperature in the CWI leg was significantly lower (~5°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.

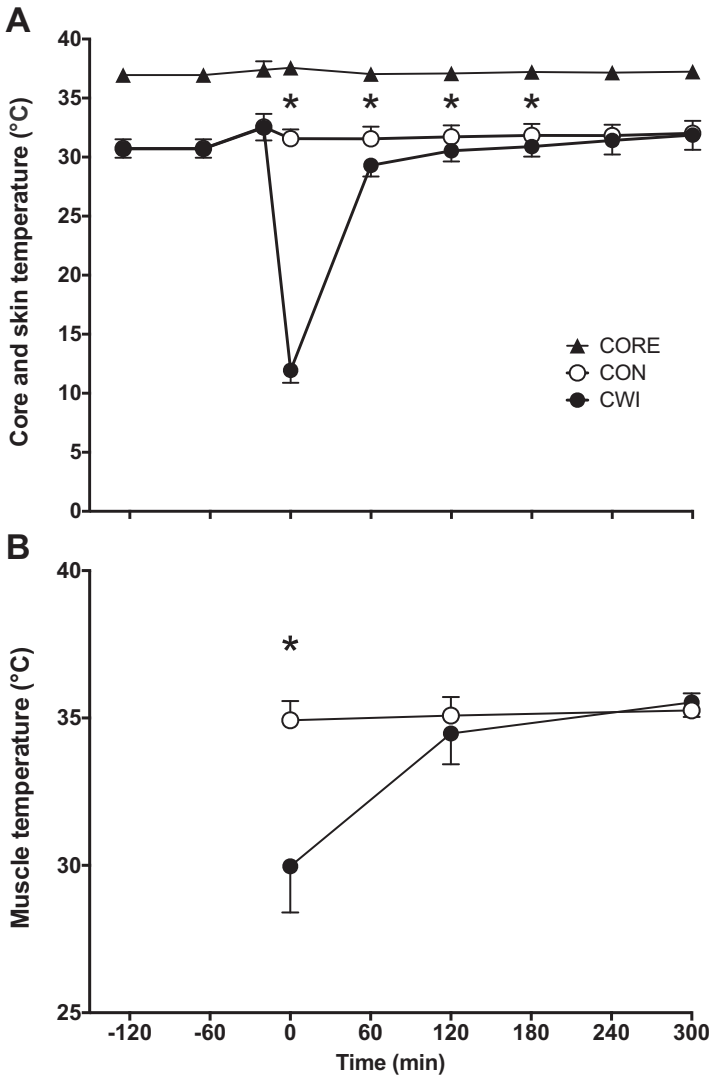

Figure 3. Core and skin temperature (A) during the entire experimental protocol and muscle temperature (B) immediately after water immersion (*t*= 0) and *t*= 120 and 300 min after drink ingestion in CON and CWI in healthy young men (*n*=12). Values represent means±SD. *, significantly different (*P*<0.001) from CON. CWI, Cold water immersion (8°C) leg. CON, Thermoneutral water immersion (30°C) leg. CORE, Core temperature.

Plasma and saliva analyses

Plasma glucose concentrations significantly increased from t= 0 to t= 30 min (from ~4.9 to ~7.1 mmol/L; time effect, *P*<0.001). At other time points no significant differences were observed when compared to baseline (t= 0 min) (*data not shown*). Plasma insulin concentrations were significantly increased from t= 0 (~8.5 mU/L) to t= 30 (~68.4 mU/L) and

60 min (~24.9 mU/L) (time effect, $P<0.05$). At other time points after drink ingestion, no significant differences were observed when compared to baseline (t= 0 min) (*data not shown*). Both plasma phenylalanine and leucine concentrations increased following drink ingestion (time effect, $P<0.001$) and remained above basal levels for 120 min (*data not shown*).

During the postabsorptive period, plasma L-[ring-$^{2}H_5$]-phenylalanine and L-[1-^{13}C]-leucine remained in a steady state at ~6.5-7.0 and ~5.0-5.5 MPE, respectively (*data not shown*). Following drink ingestion, plasma L-[ring-$^{2}H_5$]-phenylalanine enrichments were significantly lower for 60 min before returning to fasting steady-state levels (time effect, $P<0.001$), whereas plasma L-[1-^{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 postprandial period. Following drink ingestion, plasma L-[1-^{13}C]-phenylalanine enrichments increased rapidly (time effect, $P<0.001$) from ~0.0 to ~13.0 MPE after 30 min and began to decline thereafter, remaining elevated above fasting levels for the remainder of the postprandial period (*data not shown*).

Ingestion of the 20 g intrinsically labeled milk protein resulted in a rapid rise in exogenous phenylalanine appearance rates (time effect, $P<0.001$; **Figure 4**) and this remained significantly elevated when compared to baseline (t= 0 min) throughout the 5-h postprandial period. Over the entire 5 h period, 14.1±1.2 g (71±6%) of the ingested protein derived amino acids had been released into the circulation.

Body water deuterium enrichment (*data not shown*) over the 2-week exercise-training period slightly increased (time effect, $P<0.001$) over the duration of the experiment and averaged 0.72±0.10%.

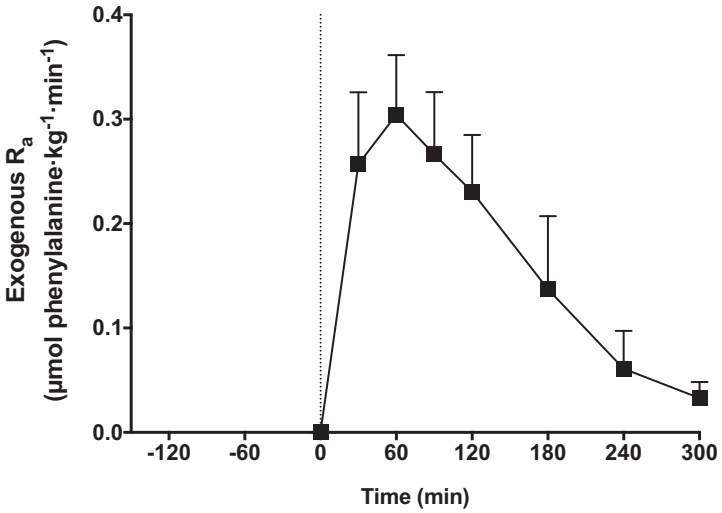

Figure 4. Exogenous phenylalanine R_a in healthy young men (n=12). The dotted line represents the ingestion of 20 g intrinsically labeled milk protein with 45 g carbohydrate (at t= 0 min). Values represent means±SD.

Muscle analysis

Myofibrillar L-[1-^{13}C]-phenylalanine enrichments are displayed in **Figure 5**. A significant time X treatment interaction was observed for myofibrillar L-[1-^{13}C]-phenylalanine enrichments (P=0.021). Myofibrillar L-[1-^{13}C]-phenylalanine enrichments in both the CWI and CON leg were significantly increased from t= 0 to 2 h (0.011±0.005 and 0.013±0.005 MPE, respectively; P<0.001) and 5 h (0.016±0.006 and 0.021±0.007 MPE, respectively; P<0.001). Myofibrillar L-[1-^{13}C]-phenylalanine enrichments did not differ between the CWI and CON leg at 2 h (P=0.132; d=0.46; 95% CI: -0.007 to 0.001 MPE). However, myofibrillar L-[1-^{13}C]-phenylalanine enrichments were lower in the CWI leg when compared to the CON leg at 5 h (P=0.016; d=0.83; 95% CI: -0.010 to -0.001 MPE).

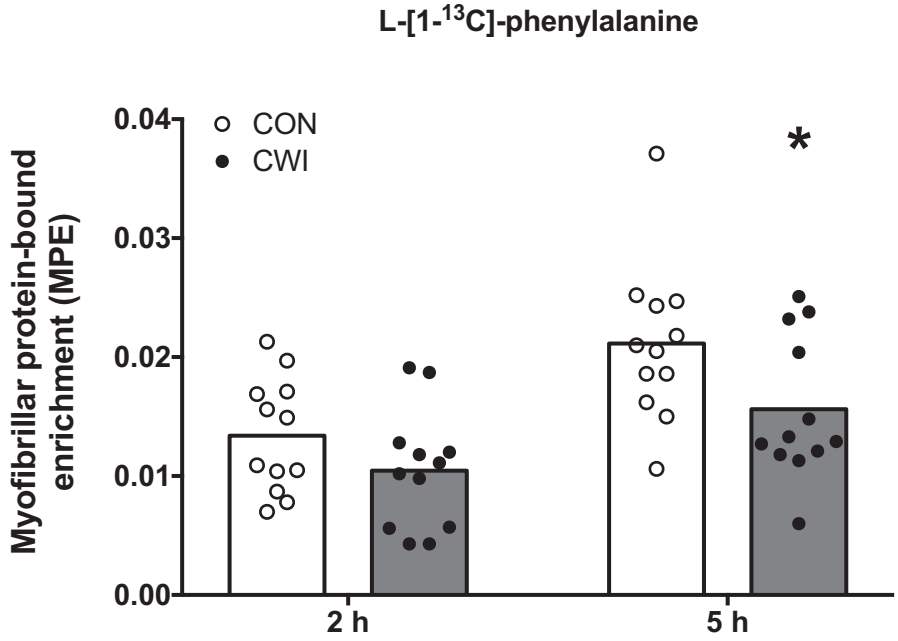

Figure 5. L-[1-^{13}C]-phenylalanine incorporation into myofibrillar protein after drink ingestion with intrinsically labeled L-[1-^{13}C]-phenylalanine in healthy young men (n=12). Bars are means and dots represent individual values. *, significantly different (P=0.016) from CON. MPE, mole percent excess. CWI, Cold water immersion (8°C) leg. CON, Thermoneutral water immersion (30°C) leg.

Myofibrillar protein FSR based on L-[*ring*-$^{2}H_5$]-phenylalanine infusion with plasma L-[*ring*-$^{2}H_5$]-phenylalanine enrichments as precursor (**Figure 6A**) or using L-[1-^{13}C]-leucine infusion and ingestion with plasma L-[1-^{13}C]-leucine enrichments as precursor (**Figure 6B**) are displayed in **Figure 6**. Myofibrillar protein FSR as calculated over 0-2 h did not differ between the CWI and CON leg based on the L-[*ring*-$^{2}H_5$]-phenylalanine tracer (0.052±0.016 vs 0.064±0.015%·h^{-1}, respectively; P=0.085; d=0.55; 95% CI: -0.027 to 0.002%·h^{-1}) and the L-[1-^{13}C]-leucine tracer

(0.074±0.021 vs 0.079±0.022%·h^{-1}, respectively; *P*=0.455; *d*=0.16; 95% CI: -0.019 to 0.009%·h^{-1}). Myofibrillar protein FSR as calculated over 0-5 h was significantly lower in the CWI when compared to the CON leg based on the L-[*ring*-$^{2}H_5$]-phenylalanine tracer (0.042±0.009 vs 0.053±0.013%·h^{-1}, respectively; *P*=0.025; *d*=0.75; 95% CI: -0.020 to -0.002%·h^{-1}) as well as the L-[1-^{13}C]-leucine tracer (0.058±0.011 vs 0.072±0.017%·h^{-1}, respectively; *P*=0.024; *d*=0.75; 95% CI: -0.026 to -0.002%·h^{-1}). Myofibrillar protein FSR (in %/day) over the 2-week training period was calculated using mean body water deuterium enrichment corrected by a factor of 3.7 as precursor and is displayed in **Figure 7**. In accordance with the acute myofibrillar protein FSR measurements, myofibrillar protein synthesis rates over 2 weeks were significantly lower in the CWI when compared to the CON leg (1.48±0.17 vs 1.67±0.36%·day^{-1}, respectively; *P*=0.042; *d*=0.67; 95% CI: -0.38 to -0.01%·h^{-1}).

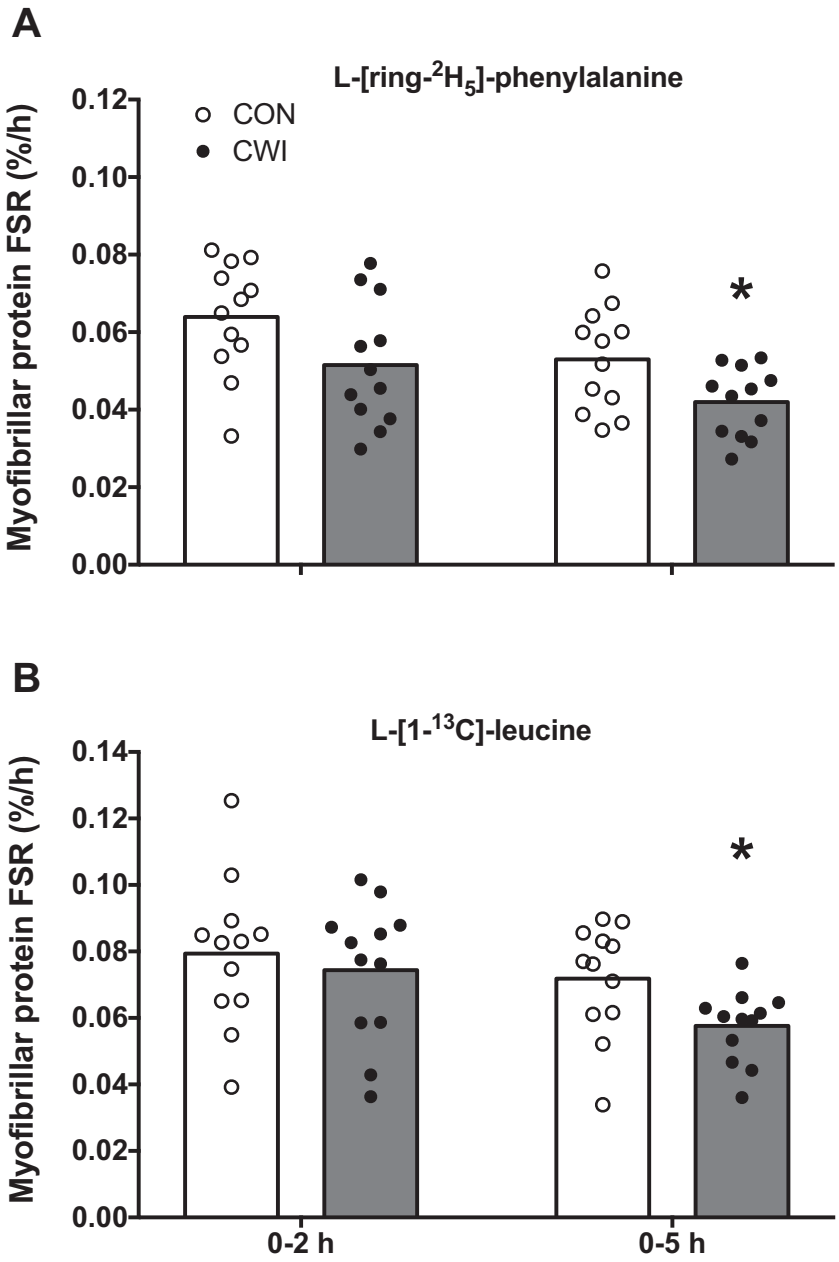

Figure 6. Myofibrillar protein FSRs as calculated with L-[ring-$^{2}H_5$]-phenylalanine (A) or L-[1-^{13}C]-leucine (B) as tracer during 5 h of postexercise recovery with the ingestion of 20 g intrinsically labeled milk protein with 45 g carbohydrate in healthy young men (*n*=12). Bars are means and dots represent individual values. *, significantly different ($P<0.05$) from CON. FSR, fractional synthetic rate. CWI, Cold water immersion (8°C) leg. CON, Thermoneutral water immersion (30°C) leg.

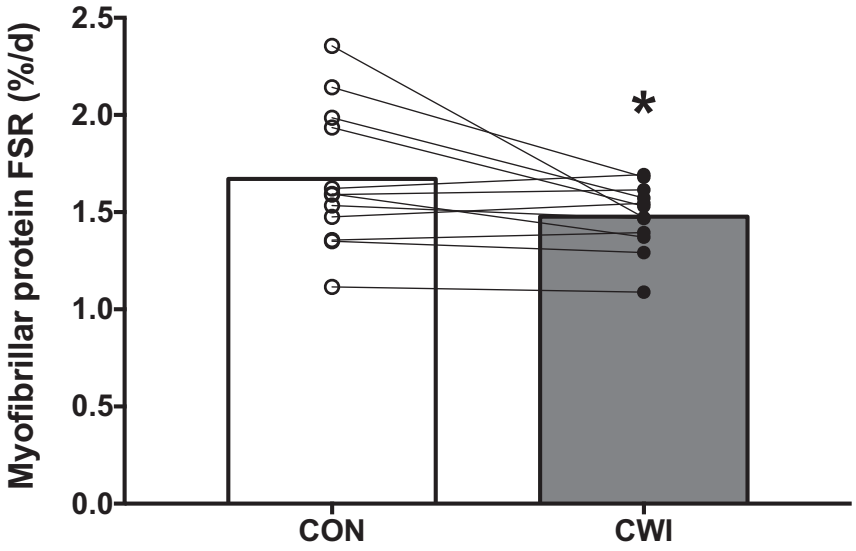

Figure 7. Myofibrillar protein FSR as calculated using body water deuterium as precursor during 2 weeks of resistance training with or without CWI in healthy young men (*n*=12). Bars are means and dots represent individual values. *, significantly different (*P*=0.042) from CON. FSR, fractional synthetic rate. CWI, Cold water immersion (8°C) leg. CON, Thermoneutral water immersion (30°C) leg.

Anabolic signaling & gene expression

The phosphorylation status (ratio of phosphorylated to total protein) of key proteins involved in the initiation of muscle protein synthesis are presented in **Figure 8 (A-F)**. No significant differences were observed for muscle mTOR (Ser2448) phosphorylation status (**Figure 8A**). A significant time X treatment interaction was observed for muscle p70S6K (Thr389) phosphorylation status (*P*<0.05; **Figure 8B**). Muscle p70S6K (Thr389) phosphorylation status was significantly decreased from 2 to 5 h (*P*=0.021) in the CWI leg and significantly increased from 0 to 2 h (*P*=0.001) in the CON leg, with a significantly higher phosphorylation status at t= 0 h in the CWI when compared to the CON leg (*P*=0.016; *d*=0.86). Muscle p70S6K (Thr421/Ser424) phosphorylation status (**Figure 8C**) was significantly decreased from 2 to 5 h (*P*=0.003) and from 0 to 5 h (*P*=0.006) in both the CWI and CON leg, with no significant treatment effect or time X treatment interaction observed. Muscle rpS6 (Ser240/244) phosphorylation status (**Figure 8D**) was significantly increased at both the 2 h (*P*=0.004) and 5 h (*P*=0.033) time point when compared with 0 h for both the CWI and CON leg, with no significant treatment effect or time X treatment interaction observed. Muscle rpS6 (Ser235/236) phosphorylation status (**Figure 8E**) was significantly increased at both the 2 h (*P*=0.017) and 5 h (*P*=0.004) time point when compared with 0 h for both the CWI and CON leg, 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 8F**).

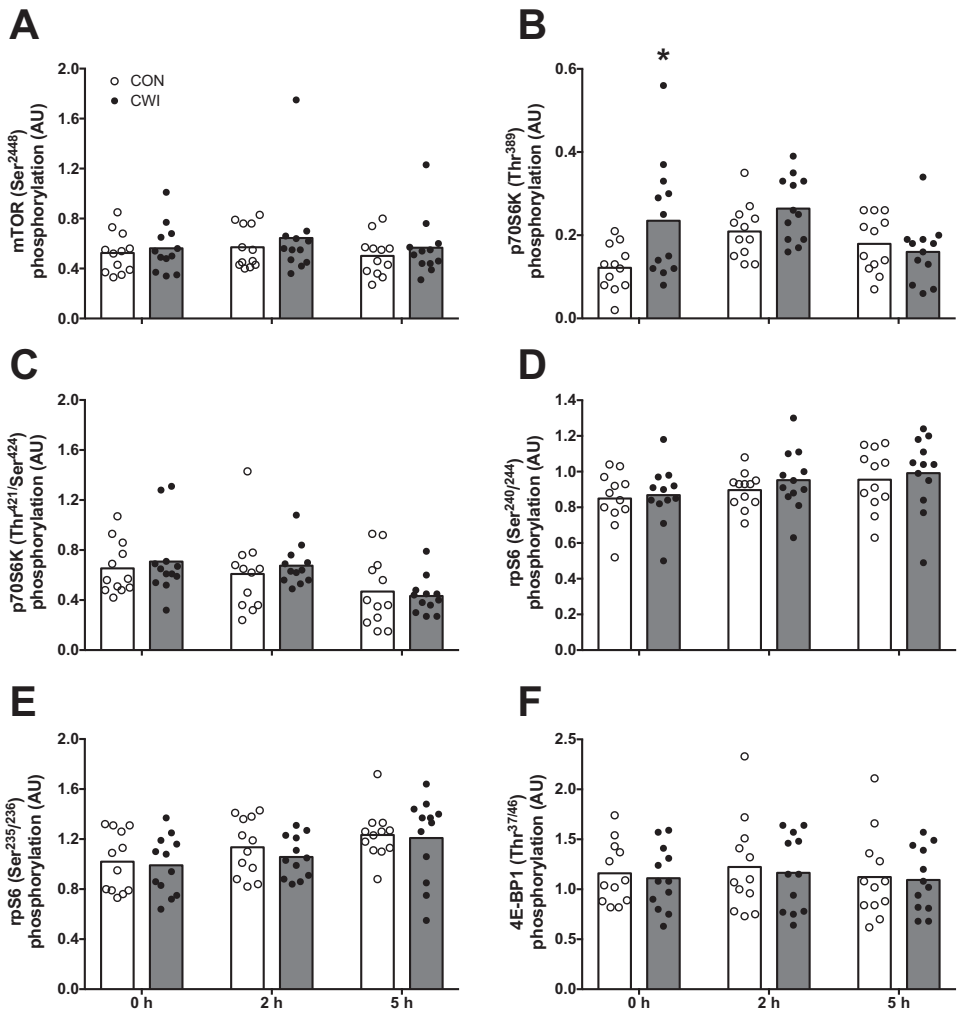

Figure 8. Skeletal muscle phosphorylation status (ratio of phosphorylated to total protein) of mTOR (Ser2448) (A), p70S6K (Thr389) (B), p70S6K (Thr421/Ser424) (C), rpS6 (Ser240/244) (D), rpS6 (Ser235/236) (E) and 4E-BP1 (Thr37/46) (F) immediately after postexercise water immersion (*t*= 0 h) and after 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. *, significantly different ($P<0.05$) from CON. CWI, Cold water immersion (8°C) leg. CON, Thermoneutral water immersion (30°C) leg.

Skeletal muscle mRNA expression for selected genes implicated in the regulation of muscle mass, intracellular amino acid and glucose transport and inflammation are displayed in **Figure 9 (A-L)**. No treatment effects and time X treatment interactions were observed for muscle

FOXO1 (**Figure 9A**), MuRF1 (**Figure 9B**), MAFbx (**Figure 9C**), mTOR (**Figure 9D**), P70S6K (**Figure 9E**), GLUT4 (**Figure 9F**), IL-6 (**Figure 9H**), and PAT1 (**Figure 9J**) mRNA expression. Over time, muscle FOXO1 (2 h: P=0.034; 5 h: P=0.007) and PAT1 (2 h: P=0.010; 5 h: P=0.005) mRNA expression were significantly increased at both the 2 h and 5 h time point (**Figure 9A** and **9J**, respectively), while muscle MAFbx (2 h: P=0.003; 5 h: P=0.033) and GLUT4 (2 h: P=0.019; 5 h: P=0.037) mRNA expression (**Figure 9C** and **9F**, respectively) were significantly decreased at both the 2 h and 5 h time point compared with 0 h for both the CWI and CON leg. Muscle IL-6 mRNA expression (**Figure 9H**) was significantly higher at 5 h when compared to 0 h (P<0.001) and 2 h (P<0.001) for both the CWI and CON leg. Muscle LAT1/SLC mRNA expression (**Figure 9I**) was significantly higher at 5 h compared to 0 h (P=0.016), with a significant treatment effect (P=0.009) but no time X treatment interaction observed. A significant time X treatment interaction was observed for muscle TNF-**α** (P=0.001; **Figure 9G**), SNAT2 (P=0.018; **Figure 9K**) and CD98 (P=0.015; **Figure 9L**) mRNA expression. Muscle TNF-**α** mRNA expression was significantly decreased at time point 2 h (P=0.002) and 5 h (P=0.025) compared to 0 h and was significantly higher at 5 h compared to 2 h (P=0.019) in the CWI leg. In addition, muscle TNF-**α** mRNA expression was significantly higher at 5 h compared to 2 h (P=0.033) in the CON leg. Muscle TNF-**α** mRNA expression was significantly higher at t= 0 h in the CWI leg compared to the CON leg (P=0.004; d=1.04). Muscle SNAT2 mRNA expression was significantly lower at 5 h compared to 0 h in both the CWI (P=0.031) and the CON (P<0.001) leg, and at 5 h compared to 2 h (P=0.002) in the CON leg only. Muscle SNAT2 mRNA expression was significantly lower at t= 0 h in the CWI leg compared to the CON leg (P=0.037; d=0.68). Muscle CD98 mRNA expression was significantly higher at 5 h compared to 0 h (P=0.024) and 2 h (P=0.010) in the CWI leg. For the CON leg, muscle CD98 mRNA expression was significantly higher at 2 h compared to 0 h (P=0.018) and at 5 h compared to both 0 h (P=0.001) and 2 h (P=0.011). Muscle CD98 mRNA expression was significantly lower at t= 2 (P=0.012; d=0.79) and 5 h (P=0.007; d=1.03) in the CWI leg compared to the CON leg.

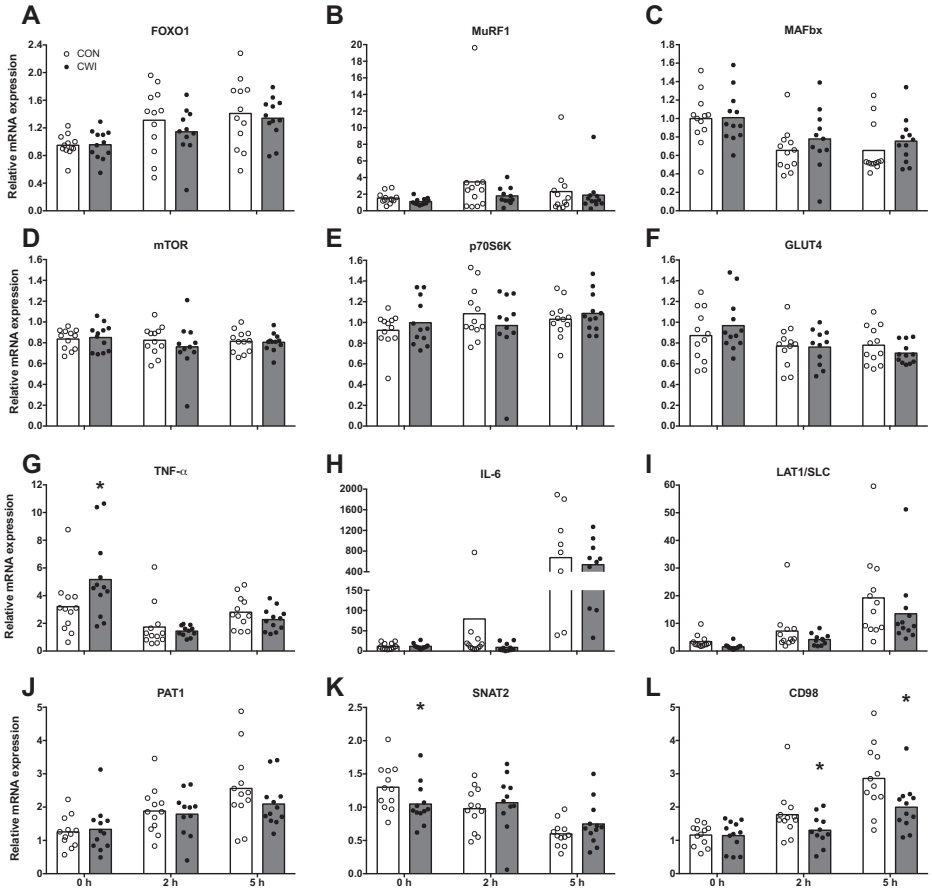

Figure 9. Skeletal muscle mRNA expression of FOXO1 (A), MuRF1 (B), MAFbx (C), mTOR (D), p70S6K (E), GLUT4 (F), TNF-**α** (G), IL-6 (H), LAT1/SLC (I), PAT1 (J), SNAT2 (K) and CD98 (L) immediately after postexercise water immersion (*t*= 0 h) and after ingestion of 20 g intrinsically labeled milk protein with 45 g of carbohydrate (*t*= 2 and 5 h) in healthy young men (*n*=12). Bars are means and dots represent individual values. *, significantly different (*P*<0.05) from CON. CWI, Cold water immersion (8°C) leg. CON, Thermoneutral water immersion (30°C) leg.

Discussion

In the present study, we assessed the impact of postexercise cooling on postprandial myofibrillar protein synthesis rates during recovery from resistance-type exercise. Cooling lowered skin and muscle temperature and blunted the postexercise increase in myofibrillar protein synthesis rate, with less dietary protein-derived amino acids being taken up and used for *de novo* myofibrillar protein synthesis. Furthermore, when applied over a 2-week period, postexercise cooling also resulted in significantly lower daily myofibrillar protein synthesis rates.

In the present study, we provided a bolus of 20 grams intrinsically labeled milk protein (together with 45 g of carbohydrates) after resistance-type exercise. In line with previous research (39-41), we showed that the dietary protein-derived amino acids are effectively being taken up and released in the systemic circulation (Figure 4), thereby providing precursors for *de novo* myofibrillar protein synthesis (Figure 5). In total, more than 70% of the ingested protein derived amino acids were released in the circulation, thereby strongly increasing plasma amino acid concentrations.

In addition to dietary protein ingestion, postexercise cooling is a strategy applied by many athletes to support postexercise recovery, with cold-water immersion (CWI) being a popular form of cooling (42, 43). However, there is now evidence to suggest that postexercise CWI blunts important molecular pathways involved in the regulation of myofibrillar protein synthesis, such as anabolic signaling and ribosomal biogenesis (31, 44, 45). This would suggest that postexercise cooling may lower postprandial myofibrillar protein synthesis rates. However, this has never been assessed. Therefore, we extend on previous work by investigating the impact of postexercise cooling on the incorporation of dietary protein-derived amino acids within skeletal muscle and subsequent myofibrillar protein synthesis rates. In the present study, we showed that postexercise cooling lowered skin and muscle temperature (when compared to the CON leg) immediately after cold-water immersion (by ~20.5 and ~5°C, respectively) (Figure 3). We also observed that postexercise cooling lowers the incorporation of dietary protein-derived amino acids into myofibrillar protein by as much as 26% (Figure 5) and postprandial myofibrillar protein synthesis rates by nearly 20% over the entire 5-h postexercise recovery period (Figure 6).

In order to gain more insight into potential mechanisms that may underlie the observed acute effects of postexercise cooling on postprandial myofibrillar protein synthesis, we assessed the phosphorylation status of several molecular markers that are important in the regulation of myofibrillar protein synthesis (Figure 8). Apart from a significantly higher phosphorylation of p70S6K^{Thr389} immediately after water immersion in the CWI leg when compared to the CON leg, we observed no significant differences between both legs in intramuscular signaling proteins that regulate protein translation-initiation (i.e., mTOR, p70S6K$^{Thr421/Ser424}$, rpS6, and 4E-BP1). Therefore, these findings in anabolic signaling could not explain the differences we

found in myofibrillar protein synthesis between the CWI and CON leg. This could potentially be explained by the fact that our biopsy timing, which was chosen to detect differences in myofibrillar protein synthesis, may not have allowed us to assess transient changes in phosphorylation status of these signaling proteins. Alternatively, it is possible that other factors were responsible for the postexercise CWI induced lowering of postprandial myofibrillar protein synthesis rates, such as a blunting of ribosomal biogenesis (44). In agreement with the absence in differences in anabolic signaling, we also did not observe differences in mRNA expression of mTOR and p70S6K between the CWI and CON leg (Figure 9D-E). Also, when assessing markers of muscle protein breakdown we did not detect any significant differences between mRNA expression of FOXO1, MuRF1, and MAFbx between the CWI and CON leg (Figure 9A-C). This is in line with a recent study, showing that prolonged muscle cooling around resistance exercise does not change gene expression of these markers of muscle protein breakdown compared with muscle heating (46). In addition, it was recently shown that there were no clear effects of postexercise CWI on markers of muscle protein breakdown both before and after seven weeks of resistance-type exercise training (45). Together, these findings suggest that muscle protein breakdown may not be elevated following postexercise muscle cooling in humans, despite indications in rodents that 24 h of cold-exposure will increase markers of muscle protein breakdown (47). We also assessed mRNA expression of intramuscular transporters involved in glucose (i.e., GLUT4) and amino acid absorption (Figure 9F and 9I-L, respectively). We observed no significant differences in GLUT4 mRNA expression between the CWI and CON leg, however, we did observe that some markers (i.e., SNAT2 and CD98) of amino acid transport were significantly lower in the CWI leg compared with the CON leg. This may be either a cause of a lower amino acid uptake in the CWI leg, or simply a consequence of lower blood supply to the cooled muscle tissue, both of which would compromise amino acid uptake, explaining (at least in part) the lower dietary protein-derived amino acid incorporation in myofibrillar protein in the CWI leg (Figure 5). Finally, we assessed whether we could detect differences in inflammatory markers (i.e., TNF-**α** and IL-6) between the CWI and CON leg (Figure 9G-H), as cooling has been proposed to reduce postexercise inflammation. For mRNA expression of both IL-6 and TNF-**α**, we did not observe any significant reductions in the CWI leg compared with the CON leg, suggesting that postexercise cooling does not reduce (local) intramuscular inflammation. In fact, we observed that TNF-**α** mRNA expression was significantly higher immediately after water immersion in the CWI leg compared with the CON leg. In line with recent observations by Peake *et al.*, these findings further challenge the notion that postexercise cooling reduces local intramuscular inflammation in humans (48). In the present study we show that cooling impairs the capacity of protein feeding to increase myofibrillar protein synthesis rates during acute recovery from exercise. It could be speculated that this merely represents a transient response and that cooling does not have a negative impact on myofibrillar protein synthesis rates when assessed over a more

prolonged period. Therefore, in the present study we applied postexercise cooling over a 2-week period, during which seven consecutive resistance exercise sessions were performed. To assess myofibrillar protein synthesis rates over this entire 2-week period, we applied D_2O tracer methodology (Figure 2). This allows us to measure the incorporation of ^2H-labeled alanine into muscle tissue over the entire 2 weeks (49). We (37) and others (50, 51) have previously applied D_2O tracer methodology to show that resistance-type exercise training increases daily muscle protein synthesis rates. We now extend on those as well as on our acute findings, by showing that the repetitive application of postexercise cooling after every exercise session lowers daily myofibrillar protein synthesis rate by ~12% during a 2-week training period (Figure 7). The fact that daily myofibrillar protein synthesis is substantially reduced throughout 2 weeks of exercise training would suggest that postexercise cooling negatively affects the skeletal muscle adaptive response to prolonged exercise training. Though some studies have not been able to detect (clear) detrimental effects of postexercise cooling on gains in muscle mass and strength (45, 52, 53), others have reported attenuated gains in muscle mass and strength due to postexercise cooling during prolonged resistance exercise training (31, 54, 55).

It could be speculated whether our data would also apply to more elite level athletes. Differences in body composition between recreational and more elite level athletes may modulate the impact of postexercise cooling on muscle temperature (29, 30). In addition, differences in training status may impact postexercise muscle protein synthesis (56). However, considering that cooling lowered muscle protein synthesis rates by nearly 20% during 5 h of postexercise recovery, our findings are more than likely to translate to more elite level athletes. In support, a long-term study by Roberts *et al*. observed attenuated gains in muscle mass and strength when postexercise CWI was applied during 12 weeks of resistance-type exercise training in well-trained males (31).

Our data suggest that postexercise cooling reduces the delivery and/or uptake of dietary protein derived amino acids during acute postexercise recovery. However, more work will be required to confirm this by directly measuring muscle perfusion and/or amino acid uptake in skeletal muscle tissue. Furthermore, the increase in muscle protein synthesis rate during acute postexercise recovery is shown to be severely blunted following cooling. When postexercise cooling is applied consistently during more prolonged exercise training, muscle protein synthesis rates remain lower throughout the entire training period. These data indicate that postexercise cooling attenuates the skeletal muscle adaptive response to exercise training and may, therefore, compromise exercise-training efficacy. Individuals aiming to improve skeletal muscle conditioning should, therefore, reconsider applying cooling as a part of their postexercise recovery strategy.

In conclusion, cold-water immersion during recovery from resistance-type exercise lowers the capacity of the muscle to take up and direct dietary protein-derived amino acids towards *de novo* myofibrillar protein accretion in healthy, recreationally active males. In addition, when

applied consistently cold-water immersion during recovery from resistance-type exercise lowers myofibrillar protein synthesis rates during more prolonged resistance-type exercise training and, as such, may attenuate skeletal muscle conditioning.

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Chapter 8

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

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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-^{13}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.

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 (1, 2). 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 (3, 4), 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 (5-7). HWI has been reported to increase (muscle) tissue temperature and stimulate limb blood flow during recovery from exercise (8, 9). HWI during post-exercise recovery has also been reported to improve performance recovery, such as attenuating the decrease in jump power (10) and enhancing the recovery of isometric squat force (6). 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 (5). 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 (11). 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 (12). 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-^{13}C]-phenylalanine and L-[1-^{13}C]-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 *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.

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.

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-^{13}C]-phenylalanine and L-[1-^{13}C]-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*-^{2}H$_5$]-phenylalanine and L-[1-^{13}C]-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-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine labeled milk protein with 45 g of glucose (polymers). Subsequent muscle biopsies were taken from the *M. vastus lateralis* of both legs after 2 and 5 hours of post-exercise recovery.

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' *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 (**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 between 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*-$^{2}H_5$]-phenylalanine (2 μmol·kg^{-1}) and L-[1-^{13}C]-leucine (4 μmol·kg^{-1}), respectively. Subsequently, an intravenous infusion of L-[*ring*-$^{2}H_5$]-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 *M. vastus lateralis* (~15 cm above the patella) with a Bergström needle under local anesthesia (13). 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.

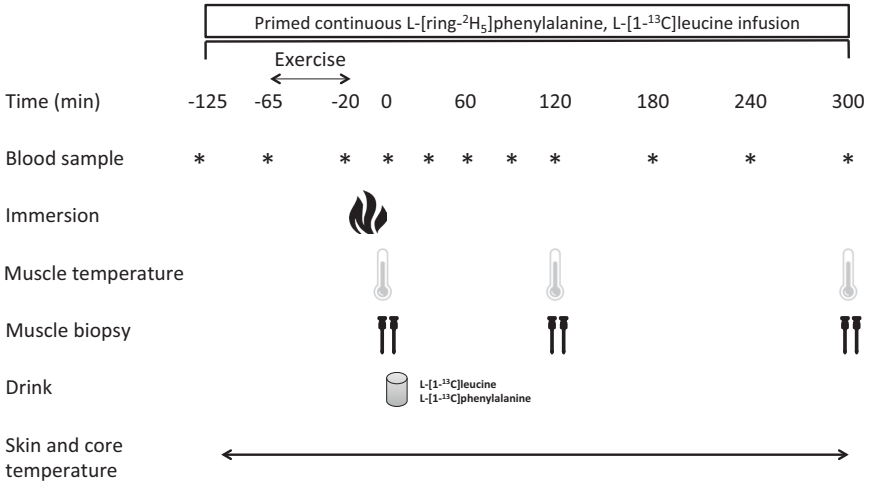

Figure 1. Schematic representation of the experimental design. Participants performed leg press and leg extension exercise followed by water immersion of both legs (1 leg was immersed in 46°C; the other leg was immersed in thermoneutral water at 30°C) for a total duration of 20 min. After muscle temperature 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.

Beverage

Subjects received a total beverage volume of 400 mL. The beverage contained 20 g intrinsically L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-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*-^{2}H$_5$]-phenylalanine and L-[1-^{13}C]-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*-butyldimethylsilyl (*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) leucine, respectively; m/z 336, 337, and 341 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 ddH2O 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 NH_4OH. The purified amino acids (L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-leucine enrichments) were analyzed by GC-C-IRMS analysis. To determine myofibrillar protein L-[1-^{13}C]-phenylalanine and L-[1-^{13}C]-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*-$^{2}H_5$]-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-pyrosphospate, 100 mM NaF, 2 mM Na3VO4, 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 100°C and immediately placed on ice. Immediately before analyses, the muscle extraction sample was warmed to 50°C 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 4°C 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-[*ring*-$^{2}H_5$]-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_{plasma}). For these calculations modified Steele's equations (in non-steady state conditions) were used (14, 15). These parameters were calculated as follows:

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

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

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

where F_{iv} is the intravenous tracer infusion rate (μmol·kg^{-1}·min^{-1}), pV (0.125 L·kg^{-1}) is the distribution volume for phenylalanine (14). $C(t)$ is the mean plasma phenylalanine concentration between 2 consecutive time points. 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-^{13}C]-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-^{13}C]-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-^{13}C]-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-^{13}C]-leucine tracers. Consequently, myofibrillar *FSR* was calculated as follows:

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

where $E_{m2} - E_{m1}$ represents muscle protein bound L-[*ring*-$^{2}H_5$]-phenylalanine or L-[1-^{13}C]-leucine. $E_{precursor}$ represent the average plasma L-[*ring*-$^{2}H_5$]-phenylalanine or L-[1-^{13}C]-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}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 ~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 ~0.5 cm skinfold thickness, the muscle temperature probe (5 cm) was inserted in the muscle at a depth of ~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 (~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.

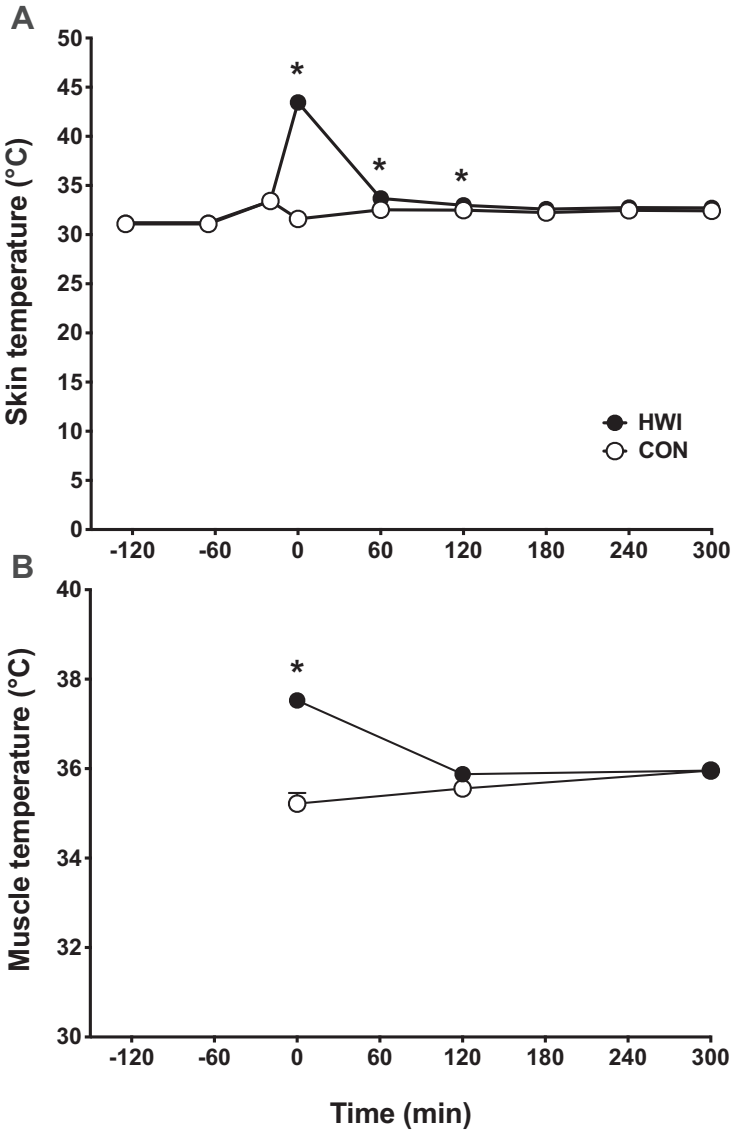

Figure 2. Skin temperature (A) during the entire experimental protocol and muscle temperature (B) immediately after water immersion (t = 0 min) and t = 120 and 300 min after drink ingestion in CON and HWI in healthy, young men (*n*=12). Values represent means±SEM. Data for skin and muscle temperature 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.

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) (*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) (*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 (**Figure 3A**) and L-[1-^{13}C]-leucine (**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-[1-^{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-[1-^{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 (**Figure 3C**).

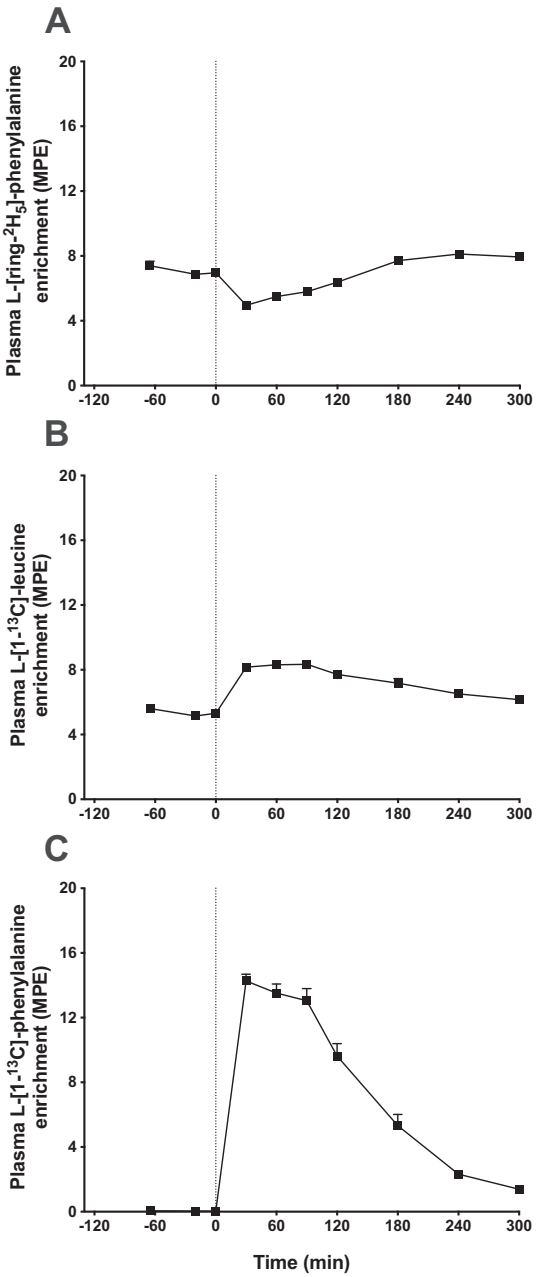

Figure 3. Plasma L-[ring-$^{2}H_5$]-phenylalanine (A), L-[1-^{13}C]-leucine (B), and L-[1-^{13}C]-phenylalanine (C) enrichments in MPE in healthy, young men (*n*=12). The dotted line represents the ingestion of 20 g intrinsically labeled milk protein with 45 g carbohydrate (at t = 0 min). Values represent 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, $P<0.001$.

Ingestion of the 20 g intrinsically labeled milk protein resulted in a rapid rise in exogenous phenylalanine appearance rates (time effect, $P<0.001$; **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.

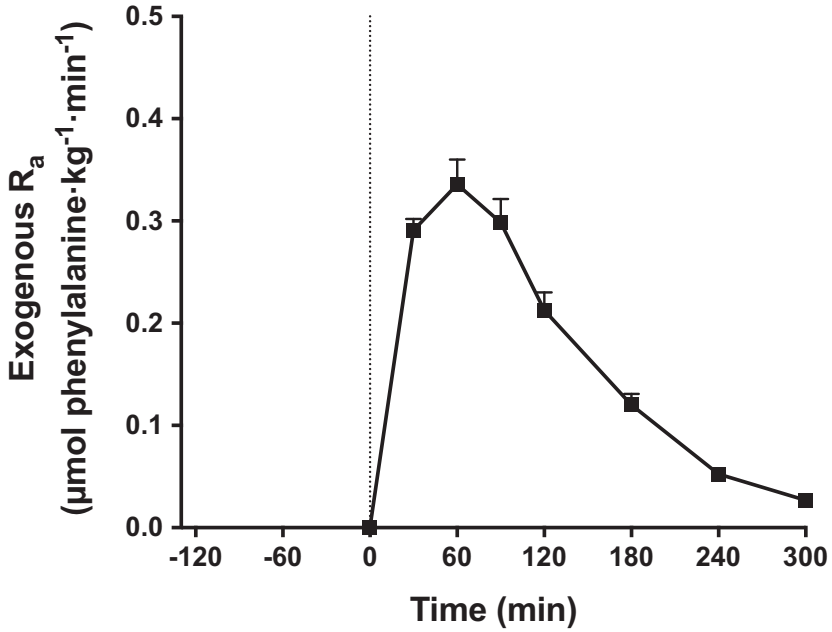

Figure 4. Exogenous phenylalanine rate of appearance (R_a) in healthy, young men (n=12). The dotted line represents the ingestion of 20 g intrinsically labeled milk protein with 45 g carbohydrate (at t = 0 min). Values represent means±SEM. Data were analyzed with a one-way repeated measures ANOVA with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

Muscle tracer analysis

Myofibrillar protein FSR based on intravenous L-[*ring*-$^{2}H_5$]-phenylalanine infusion with plasma L-[*ring*-$^{2}H_5$]-phenylalanine enrichments as precursor pool (**Figure 5A**) or intravenous L-[1-^{13}C]-leucine infusion combined with intrinsically L-[1-^{13}C]-leucine labeled protein ingestion with plasma L-[1-^{13}C]-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*-$^{2}H_5$]-phenylalanine tracer (0.065±0.002 vs 0.066±0.004%·h^{-1}, respectively; P=0.704) and the L-[1-^{13}C]-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*-

$^{2}H_5$]-phenylalanine tracer (0.048±0.002 vs 0.047±0.003%·h^{-1}, respectively; *P*=0.877) as well as the L-[1-^{13}C]-leucine tracer (0.050±0.005 vs 0.049±0.002%·h^{-1}, respectively; *P*=0.815).

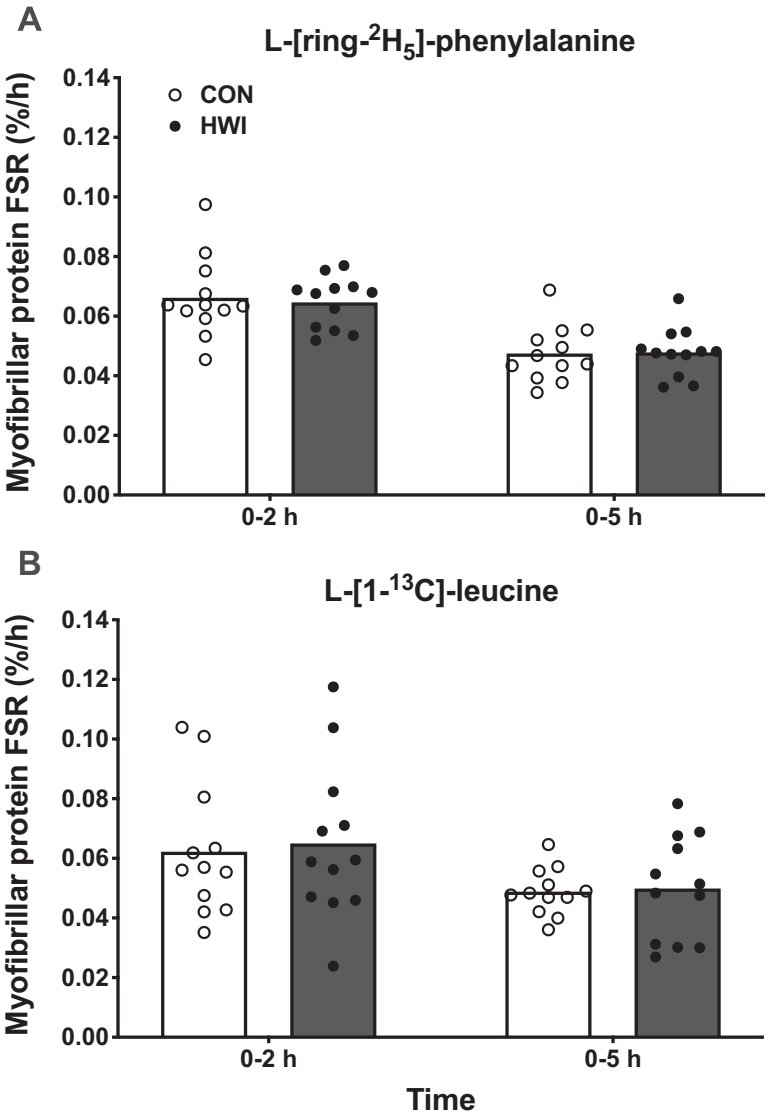

Figure 5. Myofibrillar protein FSRs as calculated with L-[ring-$^{2}H_5$]-phenylalanine (A) or L-[1-^{13}C]-leucine (B) as tracer during 5 h of post-exercise recovery with the ingestion of 20 g intrinsically labeled milk protein with 45 g carbohydrate in healthy, young men (*n*=12). Bars are means and dots represent individual values. Data were 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.

Myofibrillar L-[1-^{13}C]-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-^{13}C]-phenylalanine enrichments at 5 h vs 2 h for both the CON and HWI leg, with no differences between legs.

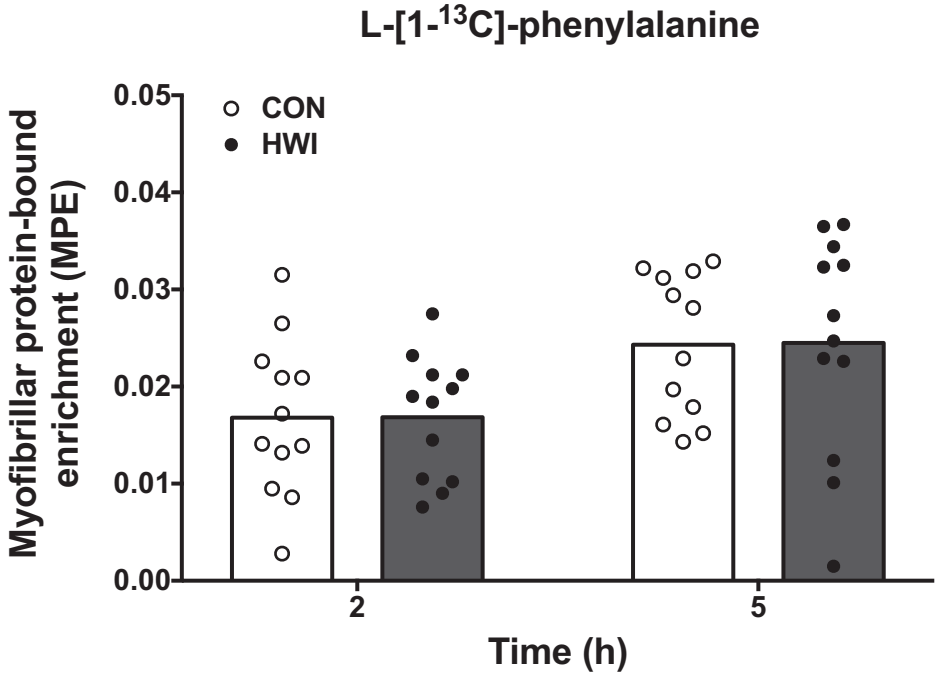

Figure 6. L-[1-^{13}C]-phenylalanine incorporation into myofibrillar protein after drink ingestion with intrinsically labeled L-[1-^{13}C]-phenylalanine in healthy, young men (n=12). Bars are means and dots represent individual values. Data were analyzed with a two-way repeated measures ANOVA (time X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

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.

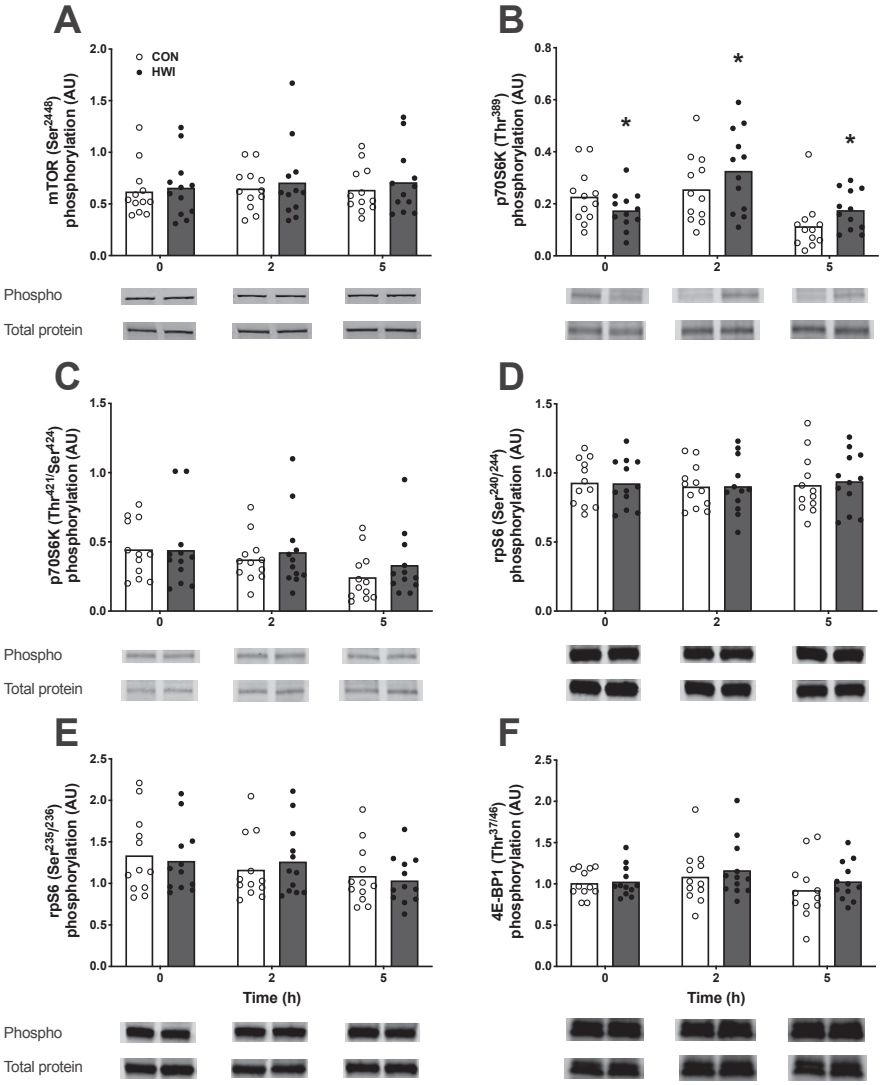

Figure 7. Skeletal muscle phosphorylation status (ratio of phosphorylated protein to total protein) of mTOR (Ser2448) (A), p70S6K (Thr389) (B), p70S6K (Thr421/Ser424) (C), rpS6 (Ser240/244) (D), rpS6 (Ser235/236) (E) and 4E-BP1 (Thr37/46) (F) immediately after post-exercise water immersion (t = 0 h) 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.

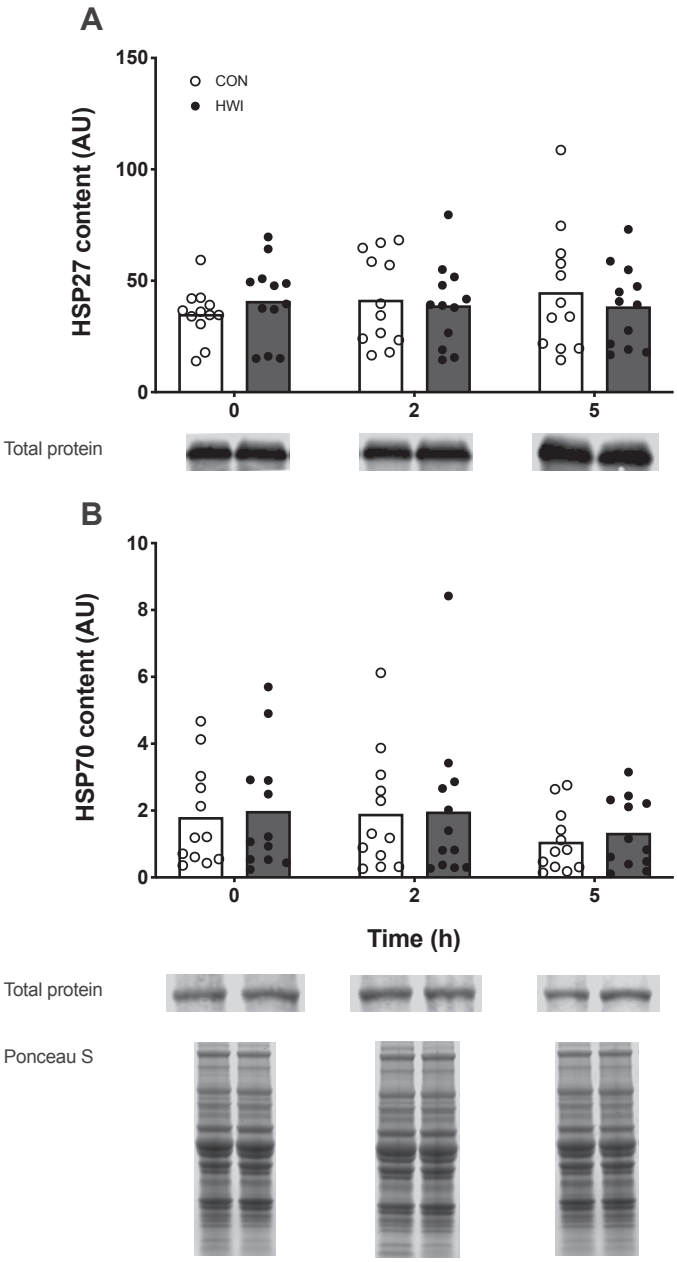

Figure 8. Skeletal muscle heat shock protein content (expressed relative to ponceau S staining) of HSP27 (A) and HSP70 (B) immediately after post-exercise water immersion (t = 0 h) and after ingestion of 20 g intrinsically labeled milk protein with 45 g of carbohydrate (t = 2 and 5 h) in healthy, young 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.

Discussion

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 (3, 16-18), 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 (3, 4). 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 (19-23), 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 (7). Two popular hydrotherapy strategies are cold-water immersion (CWI) and hot-water immersion (HWI) (7). 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 (6, 7, 24-26). However, CWI has been shown to lower tissue temperature and reduce blood flow (27, 28), 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% (22). 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 (7) 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 (11, 12). 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) (11). In line, Kakigi *et al.* (12) 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 (22). 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 (5), 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 (29). In contrast, two studies by Goto *et al.* showed that heating applied

with (30) or even without (31) exercise training over a period of 10 weeks increased gains in muscle cross-sectional area and strength (30, 31). 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) (29), 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) (30) or did not perform exercise at all (31) 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 (32), 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 (33-35). Muscle temperature may be further increased (up to ~40°C) by applying diathermy (36, 37). 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 (38). 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 (36, 37, 39).

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.

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Chapter 9

General Discussion

In this thesis we provided an overview of studies looking at strategies to promote post-exercise recovery and facilitate skeletal muscle conditioning. It has been well-established that post-exercise food intake (carbohydrate and protein) stimulates muscle glycogen repletion and increases muscle protein synthesis rates, thereby accelerating recovery and supporting skeletal muscle conditioning. Many athletes are presently looking for other, non-nutritional, strategies to improve post-exercise recovery and support muscle conditioning. In Chapter 7 and 8, we already discussed the effects of cold- and hot-water immersion as strategies to modulate post-exercise recovery. In this chapter, we will focus on several (other) non-nutritional strategies that are commonly applied by athletes to improve recovery and facilitate the skeletal muscle adaptive response to exercise, with a primary focus on muscle glycogen repletion and muscle protein synthesis (**Figure 1**). We will also define future research aims to better understand the proposed effects of several strategies on post-exercise muscle recovery and conditioning.

Cooling

Post-exercise cooling (also referred to as cold therapy or cryotherapy) is a popular and widely applied recovery strategy among athletes. Post-exercise cooling is purported to facilitate post-exercise muscle recovery by decreasing tissue temperature (1), thereby reducing blood flow, tissue perfusion, and tissue metabolic rate (2-4). In addition, cold-induced decrements in tissue temperature may lower acetylcholine production and nerve conduction velocity (5, 6), thereby exerting an analgesic (pain reducing) effect, especially when skin temperature is below 13°C (7, 8). Overall, these mechanisms explain, at least partly, the reported benefits of post-exercise cooling on reducing the sense of fatigue, delayed onset muscle soreness (DOMS), oedema/swelling, and (secondary) exercise induced muscle damage (9-15). Likely due to alleviating feelings of discomfort, several studies have shown that post-exercise cooling can improve recovery of muscle function/performance (11, 12, 16-18). Furthermore, it has been suggested that post-exercise cooling may support muscle conditioning by stimulating mitochondrial biogenesis and angiogenesis (19-21). However, it is important to note that many other studies have failed to observe benefits of cooling on various aspects of post-exercise recovery (22-27), with previously reported benefits likely being attributed to a placebo effect (26-28). The traditional belief that cooling is an effective strategy to lower inflammation after regular exercise has recently been challenged (29), with evidence indicating no impact of post-exercise cooling on inflammatory markers (30). Moreover, a decline in muscle temperature and its associated reductions in metabolic activity and/or blood flow may compromise (other) key aspects of post-exercise recovery, by attenuating the post-exercise increase in muscle glycogen repletion (31-33) and muscle protein synthesis (34-36) rates.

The most commonly applied cooling strategy by athletes is cold-water immersion, during which athletes submerge the limbs or body (without the head) in water of ~5-15°C for a

duration of ~5-20 min (17). In Chapter 7 we discussed the effects of post-exercise cold-water immersion on postprandial muscle protein synthesis rates. We observed that post-exercise cold-water immersion for 20 min at 8°C effectively lowered both skin (from ~32.5 to ~12°C) and muscle (from ~35 to ~30°C) temperature. We also observed that cold-water immersion lowered postprandial muscle protein synthesis rates during recovery from a bout of resistance-type exercise, with less dietary protein-derived amino acids being taken up and incorporated into muscle protein. In addition, we reported that regular post-exercise cold-water immersion reduced daily muscle protein synthesis rates when assessed throughout 2 weeks of resistance-type exercise training. These findings imply that post-exercise cooling attenuates skeletal muscle recovery and compromises skeletal muscle tissue conditioning, thereby extending on previous studies that showed post-exercise cold-water immersion to attenuate the rise in anabolic signaling and impair ribosomal biogenesis during recovery from a bout of exercise (37-39). Our findings also explain why the routine application of post-exercise cold-water immersion attenuates gains in muscle mass and strength during prolonged resistance-type exercise training (38, 40). In short, evidence has been accumulating to show that post-exercise cold-water immersion compromises the skeletal muscle adaptive response to (resistance) exercise training (30, 41). Furthermore, cold-water immersion does not accelerate the process of post-exercise muscle glycogen repletion (42). Consequently, though cold-water immersion may be effective in improving some aspects of post-exercise recovery (i.e. reducing pain and swelling), at present there are ample data to indicate that post-exercise cold-water immersion should not (always) be recommended when aiming to accelerate muscle recovery and support post-exercise muscle conditioning.

Ice (e.g. ice-pack or crushed ice) application is another traditional cooling strategy that has been applied for decades with the primary aim to 1) treat acute musculoskeletal injury (often combined with rest, compression, and elevation) (43) and/or 2) accelerate post-exercise recovery. Evidence for this method to speed recovery from musculoskeletal injury is lacking and several authors (including the ones that originally proposed this strategy) are nowadays recommending against this practice (44, 45). For post-exercise recovery, ice application can (under some circumstances) be more advantageous compared to cold-water immersion given that it is more practical and can be applied locally. Though dependent on the applied protocol and whether skin insulation is applied or not, ice application can effectively lower both skin and muscle temperature to levels well below those observed following cold-water immersion. This is attributed to the fact that the thermal conductivity of ice is ~4 fold higher compared to water and because it undergoes phase change from solid into liquid (8, 46, 47). Therefore, analgesic effects can be achieved faster (i.e. within 5 min) with ice packs compared with other cooling modalities such as cold-water immersion (8). However, the stronger reduction in (local) tissue temperature may actually be detrimental for other aspects of post-exercise recovery. Previous work has shown that prolonged (i.e. total of 2.5 h intermittent) ice application, reducing muscle temperature to ~24°C, impairs muscle glycogen repletion

during recovery from exercise (47). This strong reduction in tissue temperature likely reduces muscle perfusion and tissue metabolism (48), thereby also lowering muscle tissue protein synthesis rates. Therefore, whereas ice (packs) may be applied effectively to quickly reduce (local) pain and swelling, it should not be recommended when trying to accelerate muscle glycogen repletion and support skeletal muscle conditioning.

Apart from the more traditional cooling strategies, a relatively new cooling strategy is whole-body or partial-body cryotherapy. Although initially intended to treat patients with conditions such as rheumatoid arthritis and multiple sclerosis, it has become popular among athletes as a means to accelerate post-exercise recovery. This strategy can be performed either in a cryochamber (i.e. whole-body cryotherapy, where individuals are exposed to cold air in a closed chamber system) or cryosauna (i.e. partial-body cryotherapy, where individuals are exposed to vaporized liquid nitrogen in a head-free cabin system). During these cryotherapy sessions athletes may be exposed to very low temperatures (below -110°C) for a relatively short (2-5 min) duration (46, 49, 50). Given the extremely low temperatures, there is a supposition that this strategy offers enhanced cooling compared to the more traditional cooling strategies and, consequently, may accelerate recovery (51). However, it is important to note that the thermal conductivity of air is ~24 times lower compared to water and even ~90 times lower compared to ice. Therefore, despite the extremely low temperatures, given that air is not the best medium to transfer heat, it cannot necessarily be considered a superior cooling and/or recovery strategy. Indeed, whereas whole/partial-body cryotherapy as a cooling modality may also be applied effectively to reduce pain and improve some aspects of post-exercise recovery (51, 52), it is not better (26, 27, 53, 54) and may even be worse (26, 55) compared to cold-water immersion for improving several markers of post-exercise recovery, such as reducing DOMS and improving muscle function recovery. Furthermore, studies that have assessed the impact of whole/partial-body cryotherapy on reductions in tissue temperature and blood flow did not observe greater reductions compared to cold-water immersion (56, 57). In fact, greater reductions in tissue temperature and blood flow have been observed when an ecologically valid cold-water immersion (8°C for 10 min), as opposed to whole-body cryotherapy (-110°C for 2 min), protocol was applied during recovery from exercise (56). Therefore, whole/partial-body cryotherapy may have less impact on post-exercise muscle protein synthesis and glycogen repletion rates when compared with other cooling strategies. However, there are no apparent favorable effects of whole-body or partial-body cryotherapy on post-exercise muscle protein synthesis and/or glycogen repletion.

Heating

Heating, also referred to as heat therapy or thermotherapy, mostly in the form of hot baths and sauna, has been used since ancient times for medicinal purposes, such as the treatment of muscle disorders (58, 59). Despite its long-term application and its widespread popularity,

until recently the potential benefits of heating on muscle tissue have remained largely unknown. There is now evidence to suggest that heating may promote capillary growth, induce hypertrophy, and improve mitochondrial content and function, as well as glucose metabolism and insulin signaling (60). Additionally, there is an increasing interest in heating as a means to accelerate post-exercise muscle recovery and tissue conditioning (61-63). In contrast to cooling, heating elevates skeletal muscle temperature and, as such, may increase muscle blood flow/perfusion and intramuscular metabolism (64-66). The increase in tissue perfusion may increase delivery and uptake of nutrients (e.g. glucose and/or amino acids) in muscle tissue. Indeed, there are suggestions that heating facilitates glucose transport (possibly by promoting translocation of GLUT4 transporters to the cell membrane) as well as increases anabolic signaling in skeletal muscle (60, 61). Consequently, heating may form an effective strategy to accelerate muscle glycogen repletion and/or increase tissue protein synthesis rates.

Of all heating modalities, hot-water immersion is a generally accessible and commonly applied strategy by athletes. In Chapter 8 we discussed the effects of post-exercise hot-water immersion (at 46°C for 20 min) on postprandial muscle protein synthesis rates. Despite a clear increase in muscle temperature (from 35.2 to 37.5°C) immediately after post-exercise hot-water immersion, we did not observe an increase in postprandial muscle protein synthesis rates during the early stages of recovery from a single bout of resistance-type exercise. So far, there are no data available on the impact of post-exercise hot-water immersion on muscle glycogen repletion. Consequently, albeit limited, our data suggest that post-exercise hot-water immersion does not promote muscle recovery. It could be speculated that post-exercise muscle temperature is already increased to such an extent that it fully supports muscle recovery after exercise or that a more potent heating strategy may be necessary to further augment muscle recovery.

Another heating strategy commonly applied by athletes is the use of local heat (e.g. with hot/heat packs or pads). Prolonged (i.e. total of 3 h intermittent) local heat pack application has been shown to increase local muscle temperature to ~37.5°C, which may persist for a more prolonged duration compared with hot-water immersion (67). More prolonged heating may increase the impact on local muscle metabolism. In support, it has been shown that local heat pack use can accelerate postprandial muscle glycogen repletion rates during 4 hours of recovery from a single bout of endurance exercise (67). Whether such a strong increase in local muscle temperature may also further augment muscle protein synthesis rates during the acute stages of post-exercise recovery remains to be established. However, it is interesting to note that a recent study did not show any upregulation of anabolic signaling responses following 60 min of local heat therapy at rest (68), suggesting no benefit of local heating on muscle anabolism. Whereas some studies reported benefits of local heating (69, 70), other studies failed to detect any benefits (71-73) on muscle hypertrophy during a timeframe of 6-12 weeks of habitual activity or a period of resistance-type exercise training. More research

is warranted to assess whether the application of prolonged (local) heating can stimulate post-exercise recovery and improve muscle conditioning.

Another common and well-known heating strategy is whole-body heating and/or sauna. In contrast to local heating, 60 min of whole-body heating (between 44-50°C with 50% humidity) has been reported to stimulate anabolic signaling in muscle (68). This may simply be attributed to the greater increase in muscle temperature following whole-body when compared to local heating (68). Alternatively, it is possible that the increase in core temperature following whole-body compared with local heating is mainly responsible for the discrepant findings. Regardless of the potential mechanism(s) and despite the limited evidence available, it could be hypothesized that whole-body heating represents an effective strategy to increase muscle protein synthesis rates. However, previous work has shown that whole-body heating impairs, rather than stimulates, muscle glycogen (re)synthesis during recovery from exercise (74). This may be explained by the fact that during whole-body heating, the body will increase skin blood flow in order to maintain core temperature and avoid overheating (by sweating) which, consequently, may impair blood flow in skeletal muscle tissue. This will likely lower the provision of nutrients to support muscle glycogen (and potentially also protein) synthesis rates. As a result, more research is necessary before whole-body heating strategies such as sauna can be proposed as a strategy to support post-exercise muscle recovery.

Given the apparent equivocal effects of whole-body heating on important aspects of muscle recovery, it may be relevant to first focus on the impact of further increasing local muscle temperature without (substantially) increasing core temperature, which may potentially offer most benefits for general muscle recovery, i.e. increased muscle protein synthesis as well as glycogen repletion. A heating strategy that can increase local muscle temperature to higher levels than the previously mentioned approaches, is (microwave and/or pulsed shortwave) diathermy (62, 75, 76), which is a device that uses high-frequency electromagnetic waves and is used to heat deep tissue, without heating the skin to uncomfortable levels. Consequently, the application of diathermy can provide us with proof-of-principle whether a further increase in muscle temperature during acute recovery from exercise can augment post-exercise recovery as well as the skeletal muscle adaptive response. With diathermy, intramuscular temperatures of 40°C or higher can be reached (75, 76). These higher temperatures may be relevant as it appears that, apart from increases in local muscle metabolism, muscle perfusion is also increased at higher intramuscular temperatures (61), which may (further) increase the delivery and uptake of nutrients within muscle tissue. Therefore, it is worth investigating what the effect of (pulsed shortwave) diathermy during recovery from exercise is on muscle protein synthesis and/or glycogen repletion.

Contrast (water) therapy

Contrast therapy is a treatment modality where (parts of) the body are exposed to a contrast of temperatures, with alterations between cold and hot temperatures. It can be performed in a variety of ways, including alterations between cold and hot packs, cold and hot showers, and also cold and hot water baths. Contrast water therapy is a popular form of hydrotherapy used by athletes to improve post-exercise recovery. With contrast water therapy, athletes typically alternate continuously (e.g. every min) between cold and hot water for up to approximately 15 min (17). Given that cooling restricts and heating opens the vascular bed within muscle tissue, it has been suggested that alternations in cold and hot (water) may induce a so called "pump" effect, which could subsequently stimulate blood flow in the applied area. This could theoretically improve post-exercise recovery by accelerating the removal of metabolic by-products as well as increasing the supply of nutrients. However, it is important to note that previous work has, at best, only shown small increases in arterial blood flow following contrast water therapy application (77, 78). Furthermore, previous studies have not shown any substantial changes in muscle temperature after contrast therapy (79-81). Overall, this would question the physiological benefit of contrast therapy. Nonetheless, it has been reported that contrast water therapy may be a beneficial post-exercise recovery strategy by reducing delayed onset muscle soreness (DOMS), muscle damage markers (i.e. creatine kinase), swelling, and enhancing performance recovery (11, 17, 82, 83). The impact of contrast (water) therapy on skeletal muscle tissue perfusion, post-exercise muscle protein synthesis and/or glycogen repletion remains to be assessed. However, given its limited impact on blood flow and muscle temperature it could be speculated that contrast (water) therapy will not further stimulate muscle protein synthesis and/or glycogen repletion during recovery from exercise.

Massage

Massage is common practice for many athletes during recovery from exercise. Massage can be defined as "a mechanical manipulation of body tissues with rhythmical pressure and stroking for the purpose of promoting health and well-being" (84). Massage has been shown to effectively alleviate DOMS and fatigue, and to improve muscle performance (82, 85). The benefits of massage on reducing DOMS and fatigue appear to be greater compared to other commonly applied recovery strategies (82). Several physiological mechanisms have been proposed by which massage may induce its beneficial effects for promoting (post-exercise) muscle recovery, including increases in muscle temperature and blood flow, and more recently mechanotransduction (86, 87). Studies investigating the impact of massage on muscle temperature have observed that massage (likely through tissue friction) only increases muscle temperature superficially when applied at rest (88), but not after exercise (89). Therefore, potential benefits of massage on muscle recovery are unlikely attributed to local increases in muscle temperature. Studies have also investigated the impact of massage on

arterial blood flow, and despite suggestions of an increase in some studies (90, 91), most studies do not show an increase in arterial blood flow after massage application (89, 92, 93). In fact, a study by Wiltshire *et al.* (94) showed that massage impaired blood flow during recovery from isometric handgrip exercise. Taken together, massage does not appear effective to stimulate blood flow during recovery from exercise. Given that there do not seem to be major benefits of massage in increasing muscle temperature and/or blood flow, massage may induce its potential physiological benefits through mechanical effects on skeletal muscle, which subsequently induces intracellular regulatory cascades (87). Indeed, a study by Crane *et al.* (95) observed that phosphorylation of signaling proteins (i.e. focal adhesion kinase (FAK) and extra-cellular signal-regulated kinase 1/2 (ERK1/2)) that mediate mechanotransduction was greater immediately after post-exercise massage. Interestingly, a recent study by Miller *et al.* (96) also demonstrated that massage during recovery from muscle disuse in rats changed the muscle mechanotransduction machinery, with elevated FAK phosphorylation and increased levels of integrin-α7 mRNA. Therefore, via this mechanism massage may potentially increase muscle protein synthesis and, as such, modulate (post-exercise) muscle conditioning.

Currently there is only limited evidence on the effects of post-exercise massage on muscle protein synthesis. Indirect markers of muscle protein synthesis, such as Akt and mTOR phosphorylation, were not further increased when massage therapy was applied after exhaustive endurance exercise (95). In a rat model, massage (when applied on 4 non-consecutive days during reloading) did show increased rates of myofibrillar and cytosolic protein synthesis and greater muscle fiber size during 7 days of recovery from muscle disuse (96). However, other studies in rats do not support an (obvious) anabolic effect of massage during normal, weight-bearing and/or muscle disuse conditions (97, 98). Whether massage may benefit muscle anabolism in humans during periods of resistance-type exercise (training) remains to be elucidated.

The potential effects of post-exercise massage on muscle glycogen repletion are also not well studied. A study by Crane *et al.* (95) had recreationally active males performing ~70 min of exhaustive endurance-type exercise followed by 10 min of unilateral massage. The authors failed to detect differences in muscle glycogen concentrations (i.e. proglycogen, macroglycogen, and total glycogen) 2.5 hours after massage between the leg that was given a massage and the control leg. As no carbohydrates were provided to the athletes in that study, it remains to be established if post-exercise massage can further accelerate muscle glycogen repletion when sufficient carbohydrates are provided during post-exercise recovery. So far, many questions still remain to be answered around the physiological effects of massage on muscle metabolism and how it may potentially support post-exercise recovery. In addition, much remains unknown regarding the massage type and/or technique, intensity, duration, and timing that would be required to optimize post-exercise recovery.

Sleep

Sleep is of key importance in many physiological and cognitive functions, and adults are generally recommended to obtain between 7 and 9 h of sleep every night (99-102). Athletes may potentially require even more sleep to recover from the stress of training and competition and for consolidation of what has been learnt from training (103, 104). The importance of sleep for athletic performance can be demonstrated through extension of sleep duration as well as under conditions of (partial) sleep deprivation. Whereas sleep extension has been shown to improve exercise performance, sleep deprivation has been shown to negatively impact both physiological and psychological well-being, with adverse effects being observed on mood, cognition (e.g. attention, memory, and reasoning), and exercise performance (105). Apart from the detrimental impact of sleep restriction from a psychological and exercise performance perspective, (partial) sleep deprivation may also negatively impact important physiological aspects related to post-exercise recovery. Previous work has reported that sleep deprivation and/or sleep restriction can substantially decrease skeletal muscle enzyme activity (106) and alter hormonal secretion patterns, resulting in a more catabolic environment (107). Prior work has suggested that testosterone levels may be decreased during periods of sleep restriction (108, 109) and work from our laboratory has shown a relation between overnight testosterone levels and muscle protein synthesis rates (110). Overall, data seem to indicate sleep inadequacy can negatively impact (post-exercise) skeletal muscle glycogen repletion and lower protein synthesis rates. It has been shown that one night of sleep deprivation reduces post-exercise muscle glycogen concentrations (111). In addition, a more recent study showed that 5 days of sleep restriction (~4 h each night) lowers daily muscle protein synthesis rates (112). A decline in muscle protein synthesis rate may explain why previous studies in humans and rodents have observed reductions in fat free and/or muscle mass and muscle fiber cross-sectional areas during periods of sleep deprivation (113-115). Together, these data highlight the importance of sufficient sleep to maintain normal rates of muscle protein synthesis and, as such, skeletal muscle mass over a more prolonged period of time. However, given the limited available evidence, more work is necessary to understand the impact of (in)sufficient sleep on skeletal muscle protein and glycogen metabolism during recovery from exercise. More work will be required to define strategies that will optimize exercise, nutrition, rest and/or sleep to maximize skeletal muscle recovery and conditioning.

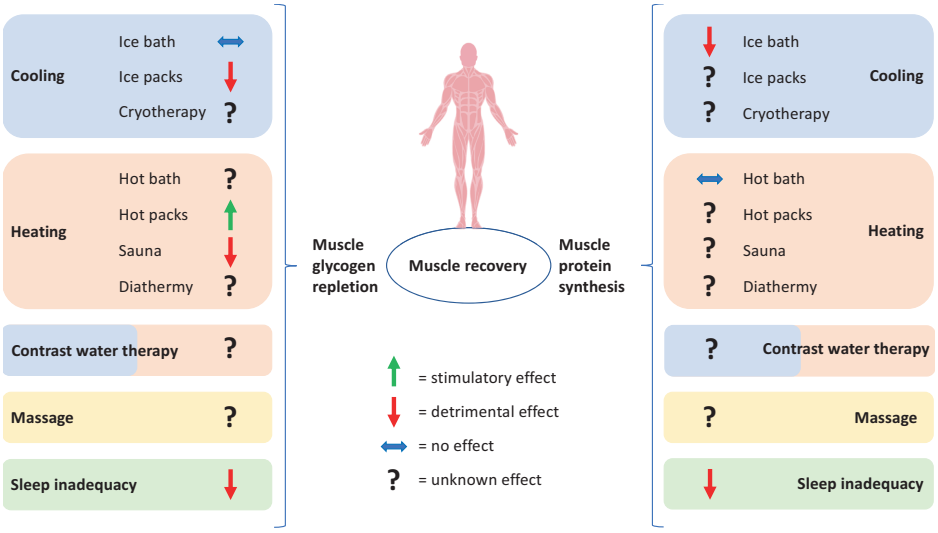

Figure 1. Current understanding of how common (non-nutritional) post-exercise recovery strategies may impact muscle glycogen repletion and/or muscle protein synthesis.

Conclusions

Whereas the role of nutrition on accelerating post-exercise recovery and facilitating skeletal muscle conditioning has been well-established, the effects of many non-nutritional strategies to support post-exercise recovery are less evident. Overall, it appears that (vigorous) cooling strategies may attenuate muscle glycogen repletion rates and compromise muscle conditioning during recovery from exercise. In contrast, heating strategies do not appear to be detrimental and may even offer small benefits for glycogen repletion (when applied locally) during recovery from exercise. Based on the marginal impact on blood flow and muscle temperature, it is unlikely that contrast (water) therapy will prove effective as a means to accelerate muscle glycogen repletion and/or increase muscle protein synthesis rates during the acute stages of post-exercise recovery. Despite its theoretical potential, it remains to be established whether post-exercise massage can contribute to a more efficient training response. Sleep is important and there is an increasing amount of data to suggest that insufficient sleep will compromise recovery after exercise. It will be challenging to define the amount and quality of sleep that is required to optimize post-exercise recovery and to develop strategies to achieve such targets. With athletes reaching limits in their training load, the speed of post-exercise recovery will become a key target for intervention. Accelerating post-exercise recovery and facilitating tissue conditioning will improve exercise training efficiency and may contribute to greater health and performance. Consequently, ongoing research will be targeted to accelerate recovery and augment the skeletal muscle tissue adaptive response to exercise training.

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Summary

Recovery after exercise is an essential element of the training-adaptation cycle. As a result, many professional and recreational athletes apply recovery strategies after their exercise sessions and/or competitive events. One frequently applied recovery strategy is nutrient intake, mainly carbohydrates and protein. Carbohydrates are an important fuel source used by our skeletal muscles allowing maximal performance during intense exercise. Our body is able to store only a limited amount of these carbohydrates in, mainly, our muscles and liver. Therefore, lots of carbohydrates are often ingested to quickly replenish carbohydrate (i.e. glycogen) stores in the muscles and liver during and/or after exercise. However, the absorption of carbohydrates in the intestine appears to be a rate-limiting factor. Therefore, it is relevant to investigate whether intestinal carbohydrate absorption can be improved. The monosaccharides glucose and fructose are absorbed via different pathways within our intestines, which means that the combined ingestion of glucose and fructose can further accelerate carbohydrate absorption rates when compared with the ingestion of only glucose or fructose. Given that table sugar (sucrose) contains both glucose and fructose in a 1:1 ratio, it may be an interesting carbohydrate source to ingest both during and after exhaustive exercise.

In **Chapter 2** we investigated whether the ingestion of sucrose during exercise is preferred to attenuate glycogen depletion in the liver and muscles when compared to the ingestion of only glucose or water. As expected, we observed a clear reduction in both liver and muscle glycogen concentrations when the athletes ingested only water during exercise. However, when the athletes ingested sucrose or glucose we observed that liver, but not muscle, glycogen concentrations were completely preserved during exercise. These results suggest that the ingestion of carbohydrate during exercise will spare liver, but not muscle, glycogen concentrations, independent of the source of carbohydrate.

In addition to the impact of sucrose intake during exercise, we investigated whether sucrose forms an effective strategy to accelerate the recovery of liver and muscle glycogen when ingested after exercise. In **Chapter 3** we describe a study where we investigated whether sucrose ingestion during recovery from exercise accelerates liver and muscle glycogen repletion when compared to glucose ingestion. Sucrose intake substantially accelerated liver, but not muscle, glycogen repletion compared to glucose intake. Therefore, it is concluded that the intake of sucrose will be more beneficial compared to glucose when glycogen stores in the liver need to be replenished rapidly during recovery from a bout of prolonged, exhaustive exercise.

In **Chapter 4** we provided an overview of what we currently know about the benefits of combining fructose and glucose ingestion during and after exercise. In this chapter we focus on explaining the benefits of fructose plus glucose ingestion for maximal performance during prolonged intense exercise as well as to accelerate post-exercise recovery.

As a second macronutrient of interest, protein and/or its constituent amino acids are frequently ingested after exercise in order to provide the body with building blocks for

muscle recovery and growth. In particular, the branched-chain amino acids (BCAA's) are known to effectively stimulate muscle protein synthesis, which is an essential process for muscle recovery and growth. Within our muscles, BCAA's can be converted into their respective ketoacids (BCKA's) and these ketoacids can in turn also be converted back into BCAA's. There is still a lot unknown about the ingestion of these ketoacids and whether they can have a positive effect on muscle protein synthesis. In **Chapter 5**, we assessed the impact of BCAA and BCKA ingestion to stimulate muscle protein synthesis and compared this with the ingestion of a high-quality protein source (milk protein). We observed that BCAA, BCKA, and milk protein are all equally effective as a means to stimulate muscle protein synthesis during the first 2 hours after ingestion. However, after 2 hours only the ingestion of milk protein continued to stimulate this process. These findings suggest that consuming a high-quality protein source is a better option to stimulate muscle protein synthesis when compared to the ingestion of only the equivalent BCAA or BCKA.

Another high-quality protein source to ingest after exercise are eggs. Previous research has shown that when eggs are processed (i.e. cooked) prior to consumption, protein digestion and amino acid absorption will be faster and more effective. Consequently, more amino acids will become available in the circulation that can be used to stimulate muscle protein synthesis. In **Chapter 6** we investigated whether the consumption of boiled eggs will indeed lead to a stronger increase in muscle protein synthesis compared to the ingestion of raw, unboiled eggs. Despite amino acid availability being greater after ingesting boiled vs raw eggs, we did not find a difference in postprandial muscle protein synthesis rates during recovery from exercise.

Apart from nutritional strategies, athletes also apply other strategies to improve recovery after exercise. One of the frequently applied recovery methods after exercise is cold-water immersion. However, the impact of cold-water immersion on stimulating muscle protein synthesis remained unknown. In **Chapter 7** we showed that cold-water immersion after exercise lowers muscle temperature and attenuates the postprandial rise in muscle protein synthesis rates. In addition, we observed that muscle cooling lowers the capacity of the muscle to take up and incorporate amino acids coming from a milk protein drink into muscle protein. We can conclude that cold-water immersion does not always represent a good strategy to accelerate recovery after exercise and may actually compromise muscle conditioning during prolonged exercise training.

Given that a decline in muscle temperature attenuates the post-exercise increase in muscle protein synthesis rates, we subsequently investigated whether heating the leg muscles may increase muscle protein synthesis rates. In **Chapter 8** we investigated whether hot-water immersion during recovery from resistance exercise increases muscle protein synthesis rates. Heating skeletal muscle tissue did not further increase muscle protein synthesis rates during recovery from exercise and, as such, does not seem to be a proper strategy to improve muscle conditioning.

Besides nutritional strategies there are various other strategies that are being applied frequently by athletes with the aim to improve recovery after exercise. In the final **Chapter 9** we provided an overview of the known effects of different commonly applied post-exercise recovery strategies on accelerating glycogen repletion or stimulating muscle protein synthesis. With research on these topics being scarce, we specifically identify some interesting topics for future research in the area of post-exercise recovery and muscle conditioning.

Samenvatting

Herstel na inspanning is een essentieel onderdeel van fysieke training en trainingsadaptatie. Zodoende gebruiken veel professionele als ook recreatieve sporters herstel strategieën na hun trainingssessies en/of wedstrijden. Een veel gebruikte methode is de inname van voeding, met name in de vorm van koolhydraten en eiwitten. Koolhydraten vormen een belangrijke brandstof die gebruikt wordt door de spieren tijdens intensieve inspanning om zodoende optimaal te kunnen presteren. Ons lichaam is in staat om een beperkte hoeveelheid van deze koolhydraten op te slaan in, met name, onze spieren en de lever. Koolhydraat inname is een veelgebruikte strategie om de koolhydraat (lees glycogeen) voorraden in de spieren en lever snel aan te vullen tijdens en/of na inspanning. Echter, de opname van koolhydraten in de darmen lijkt de beperkende factor bij het versnellen van dit proces. Het is daarom interessant om te kijken of de opname verbeterd kan worden. De enkelvoudige koolhydraten glucose en fructose worden op verschillende manieren opgenomen vanuit de darmen, wat ertoe leidt dat de gecombineerde inname van glucose en fructose kan zorgen voor een hogere snelheid van koolhydraat opname vergeleken met de inname van enkel glucose of fructose. Gezien dat tafelsuiker (sucrose) voor 50% uit glucose en 50% uit fructose bestaat, is dit potentieel een interessante koolhydraat bron om in te nemen tijdens en na zware inspanning. In **hoofdstuk 2** hebben we onderzocht of de inname van sucrose tijdens inspanning zorgt voor het sparen van de glycogeenvoorraden in de lever en de spieren ten opzichte van enkel de inname van glucose of water. Wanneer de atleten enkel water dronken tijdens inspanning, zagen wij een duidelijke daling van de glycogeenvoorraden in de lever en de spieren. Echter, wanneer de atleten sucrose of glucose consumeerden, vonden wij dat de glycogeenvoorraden in de lever, maar niet in de spieren, gespaard bleven tijdens inspanning. Deze resultaten suggereren dat de inname van suikers tijdens inspanning, onafhankelijk van de vorm, de glycogeenvoorraden in de lever, maar niet in de spieren, helpt behouden.

Naast de inname tijdens inspanning hebben wij vervolgens gekeken of sucrose effectiever is ten opzichte van glucose om het herstel van de glycogeenvoorraden na inspanning te versnellen. In **hoofdstuk 3** hebben wij onderzocht of sucrose zorgt voor een sneller herstel van de glycogeenvoorraden in de lever en de spieren na intensieve inspanning. Sucrose inname leidde tot een aanzienlijke versnelling van het herstel van de glycogeenvoorraden in de lever vergeleken met glucose inname, maar dit verschil was niet evident in de spieren. Inname van sucrose biedt dus een meerwaarde over glucose wanneer de glycogeenvoorraden in de lever snel aangevuld moeten worden na intensieve inspanning.

In **hoofdstuk 4** hebben wij tot slot een beknopt overzicht gegeven over wat we momenteel weten over de voordelen van gecombineerde fructose en glucose inname tijdens en na inspanning. Hier gaan we dieper in op het voordeel van fructose plus glucose inname om maximaal te kunnen presteren tijdens langdurige intensieve inspanning als ook om het herstel na inspanning te versnellen.

Eiwitten en/of aminozuren worden veelvuldig ingenomen na inspanning om zodoende het lichaam te voorzien van bouwstenen voor spierherstel en groei. Met name de vertakte keten aminozuren (BCAA's) staan bekend om hun effectiviteit om nieuwe spiereiwitten aan te maken die nodig zijn voor herstel en groei. BCAA's kunnen in onze spieren worden omgezet in ketozuren (BCKA's) en deze ketozuren kunnen op hun beurt ook weer terug worden omgezet in BCAA's. Er is nog heel weinig bekend of de inname van deze ketozuren ook een positief effect kan hebben op spiereiwit aanmaak. In **hoofdstuk 5** hebben wij het vermogen van BCAA en BCKA inname vergeleken met de inname van een hoogkwalitatieve eiwitbron (melkeiwit) om de spiereiwit aanmaak te stimuleren. Wij vonden dat de BCAA, BCKA en melkeiwit even effectief waren om de spiereiwit aanmaak te stimuleren in de eerste 2 uur na inname. Echter, na 2 uur was enkel de inname van melkeiwit nog steeds effectief in het stimuleren van dit proces. Deze bevindingen suggereren dat het consumeren van een hoogkwalitatieve eiwitbron een betere optie is om de spiereiwit aanmaak langdurig te stimuleren dan enkel de inname van BCAA of BCKA.

Een andere hoogkwalitatieve bron van eiwitten om in te nemen na inspanning zijn eieren. Eerder onderzoek heeft aangetoond dat wanneer men eieren kookt voor ze worden ingenomen, de eiwitvertering en opname van aminozuren sneller en meer effectief verloopt. Zodoende komen er meer aminozuren beschikbaar in het bloed die vervolgens kunnen worden gebruikt voor de aanmaak van spiereiwit. In **hoofdstuk 6** hebben wij gekeken of het consumeren van gekookte eieren inderdaad leidt tot een sterkere verhoging van de spiereiwit aanmaak in vergelijking met de inname van rauwe, ongekookte eieren. Ondanks dat de aminozuur beschikbaarheid groter was na de inname van gekookte versus rauwe eieren, vonden we geen verschil in de spiereiwit aanmaak tijdens herstel na inspanning tussen gekookte en rauwe eieren.

Los van voeding passen atleten ook andere strategieën toe om spierherstel te bevorderen na inspanning. Een veelgebruikte herstel methode is om het lichaam na inspanning in koud water onder te dompelen. Echter het effect van een dergelijk koud water bad op het proces van spiereiwit aanmaak was nog niet bekend. In **hoofdstuk 7** laten we zien dat een koud water bad na inspanning zorgt voor een verlaging van de spier temperatuur en daarmee de spiereiwit aanmaak remt. Ook vonden wij dat na inname van melkeiwit de opname en inbouw van de hieruit afkomstige aminozuren verminderd is in de spieren die gekoeld zijn. Zodoende wordt geconcludeerd dat een koud water bad geen goede strategie vormt om het herstel na inspanning te bespoedigen.

Gezien dat een koude spiertemperatuur zorgt voor een remming van de spiereiwit aanmaak, wilden we ook onderzoeken of het verwarmen van de spieren na inspanning kan zorgen voor een verhoging van de spiereiwit aanmaak. In **hoofdstuk 8** hebben wij daarom gekeken of een warm water bad na krachtinspanning kan zorgen voor een hogere spiereiwit aanmaak. Wij vonden echter dat het verwarmen van de spieren niet zorgt voor een hogere spiereiwit

aanmaak na inspanning. Zodoende, is er dus geen reden om de spieren te verwarmen na inspanning als men de spiereiwit aanmaak wil stimuleren.
Omdat er nog veel andere strategieën worden toegepast door atleten om het herstel te bevorderen na inspanning, hebben wij in **hoofdstuk 9** wat meer inzicht gegeven in de mogelijke effecten van verschillende strategieën op het herstel van de koolhydraat voorraden in het lichaam en het bevorderen van de spiereiwit aanmaak. Ondanks dat er nog maar beperkt wetenschappelijk onderzoek is gedaan naar deze verschillende strategieën, lijkt met name voldoende slaap van essentieel belang te zijn voor een vlot herstel van intensieve inspanning.

Impact

The main objective of this dissertation was to investigate the effect of several nutritional and non-nutritional strategies to improve post-exercise recovery in healthy adults. This thesis supports previous work that both carbohydrate and protein are important nutrients to support muscle recovery and facilitate skeletal muscle conditioning, by accelerating the replenishment of the body's energy stores (i.e., glycogen repletion), and by stimulating the recovery and growth of the structural components within muscle tissue (i.e., muscle protein synthesis). More specifically, in this thesis we presented the first quantitative evidence that ingesting ample amounts of carbohydrate during prolonged cycling exercise can fully prevent the lowering of glycogen stores in the liver. Furthermore, the work in this thesis supports the combined ingestion of fructose plus glucose as a means to further accelerate post-exercise liver glycogen repletion during recovery from endurance-type exercise. This thesis also showed that the ingestion of branched-chain amino acids as well as branched-chain keto acids both represent an effective means to stimulate muscle protein synthesis, but that the response is relatively short-lived when compared to the ingestion of a high-quality *intact* protein source. With respect to protein metabolism, we also reported that pre-heating of dietary protein (in this case eggs) before ingestion does not substantially impact the subsequent increase in muscle protein synthesis rates during recovery from exercise. Finally, the findings in this thesis do not support the common belief that either cooling or heating your muscles following a session of intense exercise will facilitate post-exercise recovery and/or the skeletal muscle conditioning in response to exercise. Whereas heating does not seem to impact this response at all, cooling is in fact *detrimental* for post-exercise muscle protein synthesis.

Post-exercise recovery is a fundamental principle of exercise training and adaptation and, as such, has gained increasing attention over the last few decades. As a result, many professional as well as recreational athletes are using several post-exercise recovery strategies after their workouts, despite a clear lack of knowledge on the effectiveness of many of these strategies. Hence, it is of importance to better understand the physiological mechanisms underlying such strategies and to determine their efficacy to facilitate post-exercise recovery. The (potential) impact of this dissertation in terms of scientific relevance, the target population and societal relevance, as well as the implications for translation into practice will be discussed below.

Scientific relevance

This thesis broadens our understanding on the physiology behind several commonly applied post-exercise recovery strategies. With this work, we have made substantial contributions to the scientific field in order to better understand the physiological impact of several of these strategies and their beneficial and/or detrimental effects on post-exercise recovery in healthy individuals. In particular, in contrast to common belief, we have shown that post-exercise

cooling may in fact be detrimental for myofibrillar protein synthesis, rather than beneficial, when applied during recovery from exercise.

The work in this thesis has opened up many new hypotheses and research questions that will hopefully be answered in the (near) future. It would for example be relevant to investigate whether the negative effects of cooling after strength exercise on myofibrillar protein synthesis rates would also translate into negative effects on mitochondrial protein synthesis rates when cooling is applied after endurance exercise. This is especially relevant given that previous work suggests that cooling after endurance exercise improves markers of mitochondrial biogenesis. Another topic of interest would be whether the application of heating strategies that elicit a higher temperature (compared to hot-water immersion) for a more prolonged duration may be beneficial during post-exercise recovery. With respect to nutritional strategies, it is relevant to investigate the dose of carbohydrates that is necessary to prevent liver glycogen depletion during exercise as well as whether carbohydrate ingestion can also prevent liver glycogen depletion at higher exercise intensities (as commonly performed by professional athletes). In addition, it would be relevant to further explore the exact mechanisms by which branched-chain keto acid ingestion can stimulate muscle protein synthesis as well as its potential relevance both in an athletic and in a more clinically compromised population. Taken together, there are many more questions based on this thesis that require more research.

All the work in this thesis has been presented at well-known international conferences, such as the American College of Sports Medicine (ACSM) and the European College of Sport Science (ECSS). Therefore, all results have been shared within the sports science and medicine field. All the presented data has been really well received by the different audiences, as also demonstrated by various prize nominations and prestigious awards (please refer to CV).

Target groups and societal relevance

All studies presented in this thesis were performed in healthy male adults in order to investigate the physiological impact in healthy volunteers. More specifically, the carbohydrate studies were performed in endurance trained young men, given that carbohydrate intake following exercise is primarily relevant for endurance trained athletes who need to recover fast after an exercise session. For the protein studies, the target groups were somewhat broader (i.e. healthy, recreationally active young and older individuals) given that protein intake is not only relevant as a recovery strategy for competitive athletes, but also for the general population to aid, for example, skeletal muscle conditioning and support muscle mass maintenance over the lifespan. The work on cooling and heating was primarily targeted to healthy young (recreationally active) volunteers, given that such strategies are most commonly applied by (recreational) athletes after exercise. Overall, the work from this

thesis is thus relevant for a broad spectrum of people, especially those that are active and aim to maintain a healthy lifestyle. Apart from (athletic) individuals, the results from this thesis are also highly relevant for sports coaches and sports organizations to improve their knowledge on post-exercise recovery strategies, as well as for the food industry to develop evidence-based food products that are relevant for sports performance and recovery.
Obviously, these target groups are not automatically reached through the standard academic routes of publishing and presenting at scientific conferences. Therefore, we have actively been involved in translating the findings to the generic public in various ways. The data presented in this thesis have received a lot of public and social media attention, as demonstrated by high Altmetric attention scores (e.g. a score of 643 for one of the papers). In addition, it led to several invitations for interviews (with newspapers and television programs) and podcasts. As an example, several well-known public newspapers (e.g. Reuters, Men's Health, de Volkskrant etc.) as well as national television programs (e.g. NPO and NOS) have been sharing results from this work. Overall, this clearly shows the popularity of the presented work for the wider public, alongside our ability to convey a 'practical' message to different audiences.

Translation into practice

Despite the scientific rigor, the accompanying practicality of the work in the present thesis, allows a relatively easy translation and application into practice. Most of the strategies that were investigated in this thesis are already applied in practice by many athletes, exercise enthusiasts and/or the general public. Our work has improved the physiological knowledge surrounding these strategies, while also opening up many new questions that we will continue trying to answer as to further broaden our understanding of post-exercise recovery. At the same time, we will continue informing the general public on the use and beneficial and/or detrimental effects of post-exercise recovery strategies that are, according to this author, often too quickly applied by individuals and sports organizations without proper insight into their actual effectiveness. In doing so, we hope to contribute to a better evidence-base and eventually enable a better-informed decision by many athletes and sports enthusiasts on the use of the various post-exercise recovery strategies.

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Curriculum Vitae

Caspar Johannes Wilhelmus Maria Fuchs was born on March 27, 1990 in Asten, the Netherlands. In 2009, he completed secondary school (Atheneum) at the Varendonck College in Asten. Cas obtained his Bachelor of Science degree in Health Sciences (track: Human Movement Sciences) in 2012 and his Master of Science degree in Health Food Innovation Management in 2014.

Between 2014 and 2015, Cas worked as a research assistant at Northumbria University and Newcastle University in Newcastle upon Tyne, the United Kingdom. During this period, Cas investigated the impact of sugar intake during and after exercise on liver and muscle glycogen metabolism by using non-invasive Magnetic Resonance Spectroscopy. In 2015, Cas started his PhD at the department of Human Biology at Maastricht University within NUTRIM School of Nutrition and Translational Research in Metabolism under the supervision of Professor Luc van Loon and Dr. Lex Verdijk. Here, Cas primarily conducted research in the area of protein metabolism and postexercise recovery. More specifically, he focused on the impact of protein and amino acid intake on muscle protein accretion in rest and during recovery from exercise. He also investigated the role of cooling and heating the muscles during postexercise recovery on muscle protein accretion.

During his PhD, Cas attended several national and international conferences and was awarded several prestigious awards, including the GSSI Sport Nutrition and Young Investigator awards at the European College of Sports Science (ECSS) and the International Student and Young Investigator in Sports Nutrition awards at the American College of Sports Medicine (ACSM).

Currently, Cas Fuchs continues to work at the department of Human Biology at Maastricht University as a Postdoctoral researcher under the supervision of Professor Luc van Loon.

LIST OF PUBLICATIONS

1. **Fuchs CJ**, Hermans WJH, Smeets JSJ, Senden JM, van Kranenburg J, Gorissen SHM, Burd NA, Verdijk LB, van Loon LJC (2022). Raw eggs to support post-exercise recovery in healthy young men: did Rocky get it right or wrong? Accepted in *J Nutr*, 2022.

2. Hermans WJH, **Fuchs CJ**, Hendriks FK, Houben LHP, Senden JM, Verdijk LB, van Loon LJC (2022). Cheese ingestion increases muscle protein synthesis rates both at rest and during recovery from exercise in healthy, young males: a randomized parallel-group trial. *J Nutr* 152 (4): 1022-1030, 2022.

3. Hermans WJH, Senden JM, Churchward-Venne TA, Paulussen KJM, **Fuchs CJ**, Smeets JSJ, van Loon JJA, Verdijk LB, van Loon LJC (2021). Insects are a viable protein source for human consumption: from insect protein digestion to post-prandial muscle protein synthesis *in vivo* in humans: a double-blind randomized trial. *AJCN* 114 (3): 934-944, 2021.

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5. **Fuchs CJ**, Smeets JSJ, Senden JM, Zorenc AH, Goessens JPB, van Marken Lichtenbelt WD, Verdijk LB, van Loon LJC (2020). Hot-water immersion does not increase post-prandial muscle protein synthesis rates during recovery from resistance-type exercise in healthy, young males. *J Appl Physiol* 128: 1012–1022, 2020.

6. 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 JMJM, de Groot LCPGM, Verdijk LB, van Loon LJC (2020). Protein Type, Protein Dose, and Age Modulate Dietary Protein Digestion and Phenylalanine Absorption Kinetics and Plasma Phenylalanine Availability in Humans. *J Nutr* 150 (8): 2041-2050, 2020.

7. **Fuchs CJ**, Kouw IWK, Churchward-Venne TA, Smeets JSJ, Senden JM, van Marken Lichtenbelt WD, Verdijk LB, van Loon LJC (2020). Postexercise cooling impairs muscle protein synthesis rates in recreational athletes. *J Physiol* 598 (4): 755-772, 2020.

8. **Fuchs CJ**, Hermans WJH, Holwerda AM, Smeets JSJ, Senden JM, van Kranenburg J, Gijsen AP, Wodzig WKHW, Schierbeek H, Verdijk LB, van Loon LJC (2019). Branched-chain amino acid and branched-chain ketoacid ingestion increase muscle protein synthesis rates *in vivo* in older adults: a double-blind, randomized trial. *AJCN* 110: 862-872, 2019.

9. **Fuchs CJ**, Gonzalez JT, van Loon LJC (2019). Fructose co-ingestion to increase carbohydrate availability in athletes. *J Physiol* 597 (14): 3549-3560, 2019.

10. Nyakayiru J, **Fuchs CJ**, Trommelen J, Smeets JSJ, Senden JM, Gijsen AP, Zorenc AH, van Loon LJC, Verdijk LB (2019). Blood Flow Restriction Only Increases Myofibrillar Protein Synthesis With Exercise. *Medicine & Science in Sports & Exercise* 2019 Jun; 51(6): 1137-1145.

11. Backx EMP, Horstman AMH, Marzuca-Nassr GN, van Kranenburg J, Smeets JS, **Fuchs CJ**, Janssen AAW, de Groot LCPGM, Snijders T, Verdijk LB, van Loon LJC (2018). Leucine supplementation does not attenuate skeletal muscle loss during leg immobilization in healthy, young men. *Nutrients* 2018, 10(5), 635.

12. Gonzalez JT, **Fuchs CJ**, Betts JA, van Loon LJC (2017). Glucose plus Fructose Ingestion for Post-Exercise Recovery – Greater than the Sum of its Parts? *Nutrients* 2017, 9(4), 344.

13. Trommelen J, **Fuchs CJ**, Beelen M, Lenaerts K, Jeukendrup AE, Cermak NM, van Loon LJC (2017). Fructose and Sucrose Intake Increase Exogenous Carbohydrate Oxidation during Exercise. *Nutrients* 2017, 9(2), 167.

14. Gonzalez JT, **Fuchs CJ**, Betts JA, van Loon LJC (2016). Liver glycogen metabolism during and after prolonged endurance-type exercise. *Am J Physiol Endocrinol Metab* 311: E543–E553, 2016.

15. **Fuchs CJ**, Gonzalez JT, Beelen M, Cermak NM, Smith FE, Thelwall PE, Taylor R, Trenell MI, Stevenson EJ, van Loon LJC (2016). Sucrose ingestion accelerates post-exercise liver, but not muscle glycogen repletion when compared to glucose ingestion in trained athletes. *J Appl Physiol* 120: 1328–1334, 2016.

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trained cyclists. *Am J Physiol Endocrinol Metab* 309: E1032–E1039, 2015.

17. Veasey RC, Haskell-Ramsay CF, Kennedy DO, Wishart K, Maggini S, **Fuchs CJ**, Stevenson EJ (2015). The Effects of Supplementation with a Vitamin and Mineral Complex with Guaraná Prior to Fasted Exercise on Affect, Exertion, Cognitive Performance, and Substrate Metabolism: A Randomized Controlled Trial. *Nutrients* 2015, 7, 6109-6127.

ORAL PRESENTATIONS

- ECSS (European College of Sport Science) 2021 (Virtual conference): Ingesting raw eggs to support muscle reconditioning: did Rocky get it right or wrong?

- ECSS (European College of Sport Science) 2020 (Virtual conference): Daily blood flow restriction does not attenuate muscle mass and strength loss during 2 weeks of bed rest.

- ACSM (American College of Sports Medicine) 2020 (Virtual conference): Hot-water immersion does not increase post-exercise muscle protein synthesis rates.

- NUTRIM symposium 2019 (Maastricht, the Netherlands): Branched-chain amino acid and branched-chain ketoacid ingestion increase muscle protein synthesis rates *in vivo* in older adults (**Invited lecture**).

- NUTRIM symposium 2018 (Maastricht, the Netherlands): Post-exercise cooling impairs muscle protein synthesis rates in healthy young males (**Invited lecture**).

- Europhysiology 2018 (London, United Kingdom): Fructose for endurance exercise performance and recovery (**Invited lecture** in Symposium "Fructose in physiology: friend or foe?").

- ECSS (European College of Sport Science) 2018 (Dublin, Ireland): Post-exercise cooling impairs the postprandial muscle protein synthetic response to protein ingestion.

- Evonik Industries 2018 (Darmstadt, Germany): Anabolic properties of BCKA and BCAA *in vivo* in older men.

- AMRA/Scannexus symposium 2017 (Maastricht, the Netherlands): Sharing experiences about MRI scanning for body composition research (**Invited lecture**).

- ECSS (European College of Sport Science) 2017 (MetropolisRuhr Essen, Germany): Fructose and sucrose ingestion increase exogenous carbohydrate oxidation rates during exercise in trained cyclists.

- ACSM (American College of Sports Medicine) 2016 (Boston, MA, USA): Sucrose ingestion accelerates post-exercise liver, but not muscle glycogen repletion when compared to glucose ingestion in trained athletes.

POSTER PRESENTATIONS

- ACSM (American College of Sports Medicine) 2021 (Virtual conference): Ingesting raw eggs to support muscle reconditioning: did Rocky get it right or wrong?

- NUTRIM symposium 2019 (Maastricht, the Netherlands): Branched-chain amino acid and branched-chain ketoacid ingestion increase muscle protein synthesis rates *in vivo* in older adults.

- ESPEN (European society for clinical nutrition and metabolism) 2019 (Krakow, Poland): Branched-chain amino acid and branched-chain ketoacid ingestion increase muscle protein synthesis rates *in vivo* in older adults.

- ECSS (European College of Sport Science) 2019 (Prague, Czech Republic): Post-exercise cooling impairs daily muscle protein synthesis rates during 2 weeks of resistance-type exercise training in healthy young males.

- ACSM (American College of Sports Medicine) 2019 (Orlando, FL, USA): Post-exercise cooling impairs muscle protein synthesis rates in healthy young males.

- NUTRIM symposium 2018 (Maastricht, the Netherlands): Post-exercise cooling impairs muscle protein synthesis rates in healthy young males.

- VvBN (Vereniging voor Bewegingswetenschappen Nederland) PhD-day 2016 (Maastricht, the Netherlands): Sucrose ingestion accelerates post-exercise liver, but not muscle glycogen repletion when compared to glucose ingestion in trained athletes.

- NUTRIM symposium 2015 (Maastricht, the Netherlands): Sucrose ingestion accelerates post-exercise liver, but not muscle glycogen repletion when compared to glucose ingestion in trained athletes.

AWARDS

- 2020 – Winner of the GSSI-ACSM Young Investigator in Sports Nutrition Award at the American College of Sports Medicine (Virtual session).

- 2019 – Winner of the Young Investigator Award (YIA) at the European College of Sports Science (Prague, Czech Republic).

- 2019 – ACSM International Student Award at the American College of Sports Medicine (Orlando, FL, USA).

- 2019 - Gail E. Butterfield Nutrition Travel Award at the American College of Sports Medicine (Orlando, FL, USA).

- 2018 - First place NUTRIM Poster Award 2018 (Maastricht, the Netherlands).

- 2018 - First place GSSI Sport Nutrition Award at the European College of Sports Science (Dublin, Ireland).

- 2016 - Michael L. Pollock Student Scholarship at the American College of Sports Medicine (Boston, MA, USA).

- 2016 - Best Poster Price at the VvBN PhD Symposium (Maastricht, the Netherlands).

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CAS FUCHS

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