# WHOLE BODY AND MUSCLE RESPONSE TO PROTEIN AND BRANCHED CHAIN AMINO ACID FEEDING FOLLOWING INTENSE EXERCISE

**By**

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A thesis submitted to
The School of Sport and Exercise Sciences
The University of Birmingham
For the degree of
DOCTOR OF PHILOSOPHY

Exercise Metabolism Research Group
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University of Birmingham
September 2011

# Abstract

The purpose of this thesis was to examine the role of orally ingested protein and branched chain amino acids (BCAA) in exercise recovery. The use of nutritional interventions to aid recovery from multiple lengthening contractions has received a considerable amount of attention. Specifically, investigations have focussed on the efficacy of protein and amino acid ingestion on exercise recovery, although the results of these studies are conflicting. Equivocal findings may be attributed to several discrepancies in study design and lack of adequate dietary control. Two studies included in this thesis were designed to examine the effect of ingesting amino acids following an intense bout of multiple lengthening contractions of the quadriceps muscle in untrained males. Ingestion of BCAA (chapter 2) resulted in decreased muscle soreness. Moreover, ingestion of whey protein isolate (chapter 3) resulted in reduced muscle soreness and a reduction in the decrement of superimposed electrically stimulated maximal force production. The exercise protocol was replicated in both studies to facilitate comparison between whey protein and BCAA ingestion. Furthermore, strict dietary control was implemented as an integral part of the study design. Our results suggest that ingestion of all amino acids are required to reduce decrements of maximal force production associated with intense lengthening contractions, whereas ingestion of only the BCAA results in decreased muscle soreness.

The importance of protein feeding for maximising the anabolic effect of resistance exercise is well established. Ingestion of essential amino acids or intact protein sources during exercise recovery further stimulates muscle protein synthesis. Signalling proteins within the muscle influence the translation of messenger RNA and therefore muscle protein synthesis. It has been demonstrated in rodents and humans that ingestion of only BCAA leads to an altered phosphorylation status of signalling proteins. However, the effect on muscle protein synthesis

of ingesting only BCAA has not been investigated. This thesis demonstrates that following an acute bout of resistance exercise in the fed state, ingestion of BCAA resulted in a 22% increase in muscle protein synthesis (chapter 4). These results suggest that only the ingestion of BCAA is required to augment the response of muscle protein synthesis to resistance exercise.

# Acknowledgements

First and foremost, thanks to my supervisors Professors Kevin Tipton and Asker Jeukendrup. I would like to thank you for giving me the opportunity to research protein nutrition under your guidance. Kevin, thank you for being my inspiration and helping me develop my passion for scientific research. Your expertise and teaching has given me a sound base from which I have developed my research skills. Thank you for your patience, especially with the writing, for always being there, for your time and unfailing support. You have always encouraged, trusted and believed in me. Asker, your advice and assistance has always been greatly appreciated and has helped me develop my scientific skills. You have shown me how important dedication and commitment is in a scientific career.

Thanks to the other members of the original Team Tipton – Olly Witard and Leigh Breen. Olly, thanks for the help you have given me throughout my time at Birmingham. Becca Randell and Adrian Hodgson– I thank you for the help with all the biopsies, without your help it would have been a nightmare! I have been lucky enough to spend some time in other laboratories, Drs Keith Baar and Kenny Smith I thank you for your teaching of new analytical techniques that have helped me develop my skills.

To my housemates, James and Mark thanks for being there and understanding the ups and downs of the PhD – it has definitely made things easier. Thanks for putting up with me when I got up at silly hours of the morning!! My fellow PhD students Becca Randell, Jules Clark, and Amy Scarfe, thanks for making me smile and being around to chat to. Carly and Kate thanks for helping me to remember there is more to life than a PhD. Finally to my family, Mum, Dad, Cat and Tim, thank you for always being there, both on the end of the phone and, when needed, in person. I would not be the person I am today without your love, support and encouragement. I am sincerely grateful for everything that you have done for me.

# Publications

## Published

- Witard OC, Turner JE, Jackman SR, Tipton, KD, Jeukendrup AE, Kies, AK and Bosch, JA High-intensity training reduces CD8+ T Cell Redistribution in Response to Exercise. *Med Sci Sports Exerc* In press.
- Breen LB, Philp A, Witard OC, **Jackman SR**, Selby A, Smith K, Baar K and Tipton KD. The influence of carbohydrate-protein co-ingestion following endurance exercise on myofibrillar and mitochondrial protein synthesis. *J Physiol* 589: 4011-4025, 2011.
- Witard OC, **Jackman SR**, Kies AK, Jeukendrup AE, and Tipton KD. Effect of increased dietary protein on tolerance to intensified training. *Med Sci Sports Exerc* 43: 598-607, 2011.
- **Jackman SR**, Witard OC, Jeukendrup AE, and Tipton KD. Branched-chain amino acid ingestion can ameliorate soreness from eccentric exercise. *Med Sci Sports Exerc* 42: 962-970, 2010.

## In Preparation

- **Jackman SR**, Witard OC, Jeukendrup AE, and Tipton KD. Whey protien ingestion can ameliorate soreness from eccentric exercise.
- **Jackman SR**, Witard OC, Philp A, Ranasinghe AM, Wallis GA, Barr, K and Tipton KD. Amino acid feeding and recovery from resistance exercise.
- **Jackman SR**, Witard OC, Philp A, Ranasinghe AM, Wallis GA, Barr, K and Tipton KD. Amino acid and carbohydrate feeding and recovery from resistance exercise.

## Poster presentations

- BCAA ingestion may ameliorate muscle soreness from intense eccentric exercise at European College of Sports Science (ECSS), Estoril, Portugal, 2008

## Oral Presentations

- Whey protein ingestion can impact muscle soreness from eccentric exercise at European College of Sports Science (ECSS), Antalya, Turkey, 2010 .
- The influence of whey protein dose on muscle protein synthesis following leg resistance exercise at European College of Sports Science (ECSS), Liverpool, UK, 2011 Third place Young Investigator Award – awarded ECSS-JSPFSM delegate.
- The influence of whey protein dose on muscle protein synthesis following leg resistance exercise at Japanese Society for Physical Fitness and Sports Medicine (JSPFSM) Meeting, Yamaguchi, Japan, 2011.

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# List of Abbreviations

| | |
|---|---|
| ATP | Adenosine triphosphate |
| BCAA | Branched chain amino acid |
| CHO | Carbohydrate |
| CHO-P | Carbohydrate and protein |
| CK | Creatine kinase |
| DNA | Deoxyribonucleic acid |
| EAA | Essential amino acids |
| EGTA | Ethylene glycol tetraacetic acid |
| EDTA | Ethylenediaminetetraacetic acid |
| 4E-BP1 | Eukaryotic initiaion factor 4 E binding protein |
| eEF2 | Eurkaryotic elongation factor 2 |
| eIF | Eurkaryotic initiation factor |
| FSR | Fractional synthetic rate |
| IL-6 | Interleukin 6 |
| $Ca_i$ | Intracellular calcium ion |
| LDH | Lactate dehydrpgenase |
| LH | lithium heparin |
| mTOR | Mammalian target of rapamycin |
| MIS | Maximal isometric strength |
| MVC | Maximal voluntary contraction |
| mRNA | Messenger ribonucleic acid |
| MPB | Muscle protein breakdown |
| MPS | Muscle protein synthesis |
| Mb | Myoglobin |
| TBDMS | N-tert-butyldimethyl-silyl-N-methyltrifluoracetamide |
| NBAL | Net muscle protein balance |
| NEAA | Non-essential amino acids |
| 1RM | One repetition maximum |
| S6K1 | P70 S6 protein kinase |
| PT | Peak torque |
| PRAS40 | Proline rich AKT substrate |
| AKT | Protein kinase B |
| ROM | Range of motion |
| RPE | Rating of perceived exertion |
| RPS | Rating of perceived soreness |
| RPS6 | Ribosomal protein S6 |
| SER | Serine |
| NaVO4 | Sodium orthovandate |
| THR | Threonine |

| | |
|---|---|
| TW | Total work |
| tRNA | Transfer ribonucleic acid |
| TBS | Tris-buffered saline |
| VAS | Visual analogue scale |

# Chapter 1: General Introduction

Muscle protein metabolism during and after exercise and feeding is a popular topic in which there has been a plethora of research. Muscle contains ~40 % of the total protein in an adult male body and accounts for 25-30% of whole body protein turnover. Protein consists of individual amino acids joined by peptide bonds. Amino acids can be subdivided into essential amino acids (EAA) or indispensible amino acids, and non essential amino acids (NEAA) or dispensable amino acids. Unlike NEAA, ESS cannot be synthesised by the human body, and must, therefore, be consumed in the diet. The branched chain amino acids (BCAA), leucine, isoleucine and valine are three EAA that can be oxidised within skeletal muscle. Currently it is the BCAA that are thought to be the most important for muscle protein synthesis and in exercise recovery.

## 1.1 Muscle damage

### 1.1.1 Mechanisms proposed to cause lengthening contraction induced muscle damage

Skeletal muscle consists of both contractile and structural proteins, either of which may become damaged during unaccustomed lengthening contractions. Although the mechanisms behind lengthening induced muscle damage are yet to be fully elucidated, it is purported that there are 3 separate phases (8). It has been suggested that following lengthening contractions normal sarcomeres become disrupted due to overstretching leading to membrane damage, resulting in local contracture and then fibre death (115).

*1.1.1.1 Initial phase of lengthening contraction induced muscle damage*

During exercise, the mechanical stress from lengthening contractions stretch the myofibrils and sarcomeres within each muscle fibre (98). This leads to z line streaming, regional disorganisation of myofibrils, damage to t-tubules and loss of membrane integrity (115, 124).

The popping sarcomere hypothesis (103) suggests that the non-uniform stretching of sarcomeres results in the muscle being stretched beyond optimum length. The overstretched sarcomeres become progressively weaker until they reach their yield point, when they lengthen rapidly until there is little or no overlap between the myofilaments (51). This overstretching of sarcomeres occurs at random and is therefore distributed across the muscle fibre. Such non-uniform lengthening promotes shearing of the myofibrils, which may results in exposure of internal cell membranes, especially t-tubules and a disturbance in the intracellular calcium ion ($Ca_i$) homeostasis. When the muscle relaxes the majority of the overstretched sarcomeres re-interdigitate enabling normal function (127). However, a few myofilaments will not re-interdigitate and so become permanently disrupted.

Disruption to sarcomeres can occur during the muscular contraction or relaxation, which influences the localisation of muscle damage. If the muscle is overstretched when actin and myosin are bound together, these contractile proteins become damaged and an increase in plasma concentrations of other proteins associated with actin (e.g troponin I) (120) and myosin (myosin heavy chain) (121) are observed. If the overstretching of sarcomeres occurs when actin and myosin are disassociated, tension of the overstretched sarcomeres may be transferred to cyoskeletal proteins (e.g desmin and titin) causing disruption to the structure of the myfibrils (127). Damage to titin, the elastic filament that secures the thick myosin filament to the Z line, may cause the next sarcomere to overstretch, thus propagating damage longitudinally (1). Desmin may be damaged by excessive forces early in exercise (90) and may cause the damage to spread laterally. Damage to the sarcoplasmic reticulum, transverse tubules or sarcolemma can occur if the longitudinal and lateral damage spreads to the adjacent areas of the muscle (1).

Damage to the sarcomeres can lead to problems with excitation-contraction coupling, thereby impairing the generation of force. If the cell membrane is disturbed there is leaking of some proteins into the extracellular space and eventually the circulation. Consequently, increased levels of these proteins in the blood indicate increased permeability of the muscle cell membrane (55). Mechanical damage also may promote an inflammatory response that involves an increase in fluid, plasma proteins, and inflammatory cytokines at the site of damage (95).

*1.1.1.2 Autogenic phase of lengthening contraction induced muscle damage*

The autogenic phase of muscle damage is caused by elevations in intracellular concentrations of $Ca_i$ (43). Whether the cause of the initial damage is metabolic or mechanical, there is diffusion of $Ca_i$ ions into the sarcomeres (93). An early study demonstrated a degeneration of myofibrils when intracellular concentration of $Ca_i$ was artificially increased through treatment with calcium ionophore (44). It was later established that, if the increase in intracellular $Ca_i$ did not occur, muscle fibre damage was prevented (77). In a rodent model it was observed that exercise consisting of downhill walking resulted in increased $Ca_i$ that accumulated within the mitochondria (43), and that this increase can alter the function of the sarcoplasmic reticulum as demonstrated in rodents (25).

Failure of the sarcoplasmic reticulum to re-sequester $Ca_i$ may lead to elevated intracellular $Ca_i$ concentrations (8). It has been demonstrated that in single mouse muscle fibres, isolated muscles and rodents repeated stretches increase intracellular resting $Ca_i$ (11, 74, 94). Increased concentration of intracellular $Ca_i$ activates phospholipase $A_{2,}$ ubiquitin-proteosome and calpain-mediated proteases that primarily mediate degradation of the cell membrane and proteins, both structural and contractile (12, 147). Phospholipase $A_{2,}$ activation promotes the production of arachidonic acid, which acts as a detergent to the cell membranes and results in

increased prostoglandins, leukotrienes and thromboxanes (8, 45). Treatment of muscle cells with cyclooxygenase inhibitors to reduce the formation of prostaglandins, leukotrienes and thromboxanes from arichidonic acid results in the prevention of leakage of muscle enzymes. Thus, it seems that enzyme leakage is not equivalent to physical damage to the plasma membrane (45, 76). Thus demonstrating that enzyme leakage is not caused by physical damage Acyl-CoA binding protein concentration can increase following sarcolemmal disruptions and can decrease the $Ca_i$ concentration required for m-calpain activation from 300 uM to around 10uM (99). Calpain $Ca_i$–dependant proteases degrade the z-disks, further disrupting the myofibrils (8). Additionally, stimulation of lysosomal proteases through calcium dependant mechanisms may play a role in the autogenic phase of muscle damage (8). Enhanced $Ca_i$-activated proteolytic activity results in further degradation of muscle proteins and lipid structures (56), which is evident in biopsy samples taken 2-5 days after exercise characterised high proportion of lengthening contractions that display more cellular disruption than those taken immediately after (49). Elevated resting intracellular $Ca_i$ levels may lead to a greater number of cross-bridge formations, thus reducing the relaxation phase of contraction. This reduction in the relaxation phase of muscle contraction may be the cause of reduced range of motion of the associated joint and increased muscle stiffness following damaging exercise (89). Furthermore, uncontrollable contraction of the muscle fibre may result in a depletion of local ATP stores, eliciting further structural damage due to both mechanical and metabolic stress (8, 26). However, it should be noted that the increases in $Ca_i$ concentration have not been shown in humans and future studies are required to establish this.

*1.1.1.3 Phagocytic phase of lengthening contraction induced muscle damage*

The phagocytic stage results in increased mobilisation of immune cells that are directed towards the injured tissue and is prevalent from 4-6 h to 2-4 d after the onset of damage (7). The tissue injury triggers the accumulation of bradykinin, histamine and prostaglandins that attract monocytes/macrophages and neutrophils. Neutrophils release chemo-attractants to promote the migration of other immune cells to the site of damage, however they are also a source of free radicals. The inflammatory cytokines, tumour necrosis factor-α (TNF- α), interleukin-1β (IL-1β) and interleukin-6 (IL-6)) are secreted by the immune cells (110). However, IL-6 also is released by exercising muscle (111). The infiltration of immune cells to the injured tissue is followed by a fluid influx and activation of type IV nerve endings, developing gradually over 3-4 days (115).

Although resulting events from lengthening contractions are not completely understood, it is likely that there is mechanical stress that occurs immediately during the exercise resulting in metabolic homeostatic imbalance in the recovery period. There are many symptoms associated with unaccustomed lengthening contractions that include decreased muscle function (both reduced muscle force production and low frequency fatigue), increased muscle soreness, decreased range of motion and increased plasma concentrations of myofibre proteins.

### 1.1.2 Methods to measure muscle damage

It is difficult to directly measure muscle damage, however there are a wide variety of methods with which to assess it. Methods that have been used in previous studies include muscle function (usually measured by maximal voluntary contraction), immunohistochemistry (investigating membrane disruption), blood parameters (especially myofibre protein concentrations) and subjective measures of muscle soreness, such as a visual

analogue scale (139). The quantification of the observed magnitude and duration of damage can vary between the different methodologies (139).

*1.1.2.1 Muscle function*

A decrease in voluntary maximal force generation commonly occurs following unaccustomed exercise where there is a large number of lengthening contractions. It is generally the decrements in force production that cause reductions in athletic performance. Many studies have demonstrated significant reductions in power and strength following lengthening-based exercise protocols (29). Downhill running protocols produce damage and typically reduce maximal force production by 10-30% immediately following exercise (47, 48). High-force, repeated lengthening contractions result in the greatest maximal force loss (35 – 50%) (105, 106, 117). Force loss resulting from intense exercise which has a high number lengthening contractions can last up to 2 wk (105).

The force generation capacity of the muscle can be measured using maximal voluntary contraction (MVC). However, the subject's motivation may have an influence on the outcome of an MVC measurement. Superimposition of an electrical stimulation to the muscle during the MVC will result in the production of maximal force by the muscle regardless of motivation. Thus, electrical stimulation during the MVC provides a indication of the impact of peripheral, rather than central, factors on muscle force production. However, even when this technique is adopted a reduction in force production following intense unaccustomed exercise still occurs (75). Therefore, it is likely that peripheral mechanisms (within the muscle) are the primary cause of the decline in muscle function following unaccustomed exercise involving a high frequency of lengthening contractions.

The force frequency relationship of the muscle is another measure of muscle function. Comparison of force production at high and low frequencies indicates the force frequency

relationship of the muscle. A reduction in force at low frequencies indicates that there is an impairment of excitation-contraction coupling (141). This reduction is observed following intense exercise that has a high component of lengthening contractions. Muscle function is one of the most valid and reliable indirect measures of muscle damage in humans (139). Therefore, studies aiming to investigate injury from lengthening contractions should use muscle function as a marker of exercise-induced damage. We will implement two measures of muscle function, MVC with superimposed electrical twitch stimulation and low frequency fatigue in chapters 2 and 3.

*1.1.2.2 Muscle biopsies and myofibre proteins*

Many human studies have used both qualitative and quantitative histology analysis of muscle biopsies in an attempt to investigate disruption within the muscle tissue (34, 52-54, 57-59, 65, 108, 123, 143). Within the biopsies the state of the fibres can be analysed to determine the amount of fibre degeneration and subsequently, the amount of regeneration. Furthermore, levels of enzyme activity and cellular membrane damage can be classified. It was recently demonstrated that the biopsy technique and / or the processing may lead to changes that can be mistakenly attributed to exercise-induced damage (96). Additionally the biopsy technique may lead to increases in myofibre proteins in the blood due to damage to muscle cell membranes, thus confounding other measurements (72). Furthermore, the muscle biopsy is small when compared to the whole muscle and quantification of damage of the whole muscle from a small sample is problematic, potentially under or over estimating damage (139). Often both the time-course and magnitude of disruption observed in muscle tissue biopsies correlate poorly with functional measurements of the muscle (139), so the practical meaning is difficult to interpret as people are often more concerned with functional

measures. However, the muscle biopsy technique is the only direct method in which to determine muscle structure disruption.

Plasma concentrations or activity of myofibre proteins are often used as markers of muscle damage. These proteins include creatine kinase (CK), myoglobin and lactate dehydrogenase. CK is the most commonly measured of the myofibre proteins, although most of the proteins respond in a similar way. There is much debate surrounding myofibre proteins, in particular CK, as an indirect marker of muscle damage. It was suggested that CK appeared in the blood if there was muscle damage, however when measured there was no correlation between loss of muscle force production and the concentration of CK (27, 28, 105). Furthermore, the concentration of the myofibre proteins in the circulation is minimal in comparison to the total amount in the muscle and the permeability of the cellular membranes is mediated by arachidonic acid metabolites. Therefore it is not the physical breakdown of the membrane that is indicated by increased myofibre proteins in plasma, but if anything they may suggest increased membrane permeability (76). Nevertheless, changes in the plasma levels of these proteins are commonly used as an indicator of muscle damage.

The greatest maximal force loss is generally seen immediately after the exercise bout, whereas CK levels have a biphasic response peaking at 24-48 h and then 96-120 h post exercise (139). Additionally, when repeated bouts of lengthening contractions are performed the maximum force decrement is less after each consecutive bout. However, the increase in CK concentrations remains large. Thus, there is no correlation between force decrement and CK concentrations following repeated eccentric exercise bouts. Furthermore, the response of CK to lengthening contractions is highly individualised (28). A difference in peak values for individuals has been shown to differ ~358% in response to the same relative intensity eccentric exercise bout (75). Therefore, comparisons between interventions are often

problematic, as it is difficult to decipher the effect of the intervention from that of individual responders and non-responders. Additionally, a one off measure of myofibre proteins in the blood does not only reflect their release into the blood stream but also their removal. CK is often measured to allow comparisons to previous studies, however it is not reflective of functional measures.

*1.1.2.3 Inflammation*

After muscle injury there is likely to be an inflammatory response. Magnetic resonance imaging can be used to determine swelling through measurements of the limb cross-sectional area. Alternatively, oedema within the limb can be calculated using T2 weighted contrast images or diffusion weighted images. Calculation of limb volume by measurement of the length and circumference of leg segments to calculate the volume of a cone (146) also may give an indication of swelling that has occurred to the limb. However, this measurement lacks resolution, thus small differences are unlikely to be detected. Due to the expense of the equipment required in chapters 2 and 3 we did not take any measures of limb swelling or oedema.

Elevated levels of inflammatory cytokines, C-reactive protein and white blood cell counts in the blood are also consistent in an inflammatory response. Current evidence as to whether the response of cytokines in the blood is proportional to the damage is equivocal (97, 119). Therefore, studies should not only rely on the use of an inflammatory response to suggest damage. In chapters 2 and 3 we will measure interleukin 6 as an indicator of inflammatory cytokines in the plasma. However, this measurement may not be the most appropriate as IL-6 is released from muscle in response to exercise and therefore is not be indicative of muscle damage.

*1.1.2.4 Muscle Soreness*

Muscle soreness is associated with unaccustomed lengthening contractions and is the sensation of pain that usually peaks 2-3 d after the exercise bout. Soreness is frequently used as a measure of injury severity (139), and can be measured by application of pressure using a force transducer or by pain scales. The use of force transducers requires subjects to distinguish between pressure and pain, which is often difficult. Thus, there is a poor correlation between soreness values obtained from force transducers and soreness values for other measurement methods. Pain scales include rating of perceived soreness (RPS) and visual analogue scale (VAS). The RPS is a scale from 1 (not sore) to 10 (very very sore) in which subjects provide a value which best represents their perception of pain. The VAS can be either a 100 mm or 50 mm line anchored by ‘no soreness’ and ‘extremely sore’. Subjects mark the point along this line that best represents the soreness they feel. Scores are recorded by measuring the distance from the left hand anchor point (no soreness) to the marked point. It should be noted that whilst soreness scores are highly subjective, it has been shown that 90% of pain ratings can be repeated within 9mm using a 10cm VAS (13). Thus, the VAS is considered effective in monitoring changes in perceived pain. In chapters 2 and 3 we will use a 10cm VAS to measure subjects pain. No numbers are recorded and therefore subjects are unlikely to be influenced by numbers they stated at the previous measuring point.

### 1.1.3 Interventions to reduce muscle damage

Completing unaccustomed lengthening contractions can lead to a decrease in the ability to complete everyday activities. A premature return to strenuous activity, without recovery, may increase the risk of further injury (26). Therefore, it is important to restore maximal function as rapidly as possible. A range of treatments that may help alleviate symptoms associated with intense unaccustomed exercise have been suggested. These

treatments include the administration of nonsteroidal anti-inflammatory drugs, compression garments, cryotherapy, and stretching (26). However, the efficacy of most treatment strategies across a range of populations (e.g. untrained, aging, athletes returning from injury) remains equivocal. Recently, studies have been performed in an attempt to investigate the potential impact of nutritional strategies in aiding the recovery from exercise-induced muscle damage. Specifically, the role of protein supplementation in attenuating muscle damage or speeding up the regeneration of new proteins to replace the damaged ones has been studied. However, these investigations provide equivocal results.

*1.1.3.1 Protein and amino acid sources*

Recent studies have focused on nutritional interventions during post exercise recovery in an attempt to alleviate muscle soreness and impairments in muscle function. An overview of studies that implemented a single bout of intense lengthening contraction-based resistance exercise with ingestion of protein and other amino acid sources is shown in Table 1.1. Although there are several studies investigating the efficacy of amino acids as a recovery tool, the results appear somewhat inconsistent. The effect of the ingestion of amino acid sources on muscle function, muscle soreness and blood parameters is equivocal.

There are several reasons that may contribute to the differing results, including inconsistencies in exercise models, which prevent direct comparisons between studies. Exercise protocols previously implemented include arm curls (107), knee flexor/extensor exercise (24, 30, 31, 142), mixed lower limb exercise (33) and squats (81, 118). Furthermore, there are different types of supplements, including mixed amino acids (107, 125), carbohydrate with protein (30, 31, 142), milk (30, 31), single amino acids (81, 118), and different forms of whey protein, both isolate (24) and hydrolysate (24, 33). Additionally the controls vary from isoenergetic controls to flavoured water controls. The supplementation

pattern also has differed from a single bolus (31, 118) to 14 d of supplementation (33). Additionally, some studies measure muscle function with and without electrical stimulation. The type of subjects also varies between studies. Only two studies (30, 31) have investigated the effect of protein nutrition in trained subjects and only one study (118) has studied females. Lack of dietary control may help explain discrepancies between studies, as no study has strictly controlled diet of the subjects. Changes in energy and/or protein intake may have increased the variability in the response to the eccentric exercise that may have obscured any response to the interventions. Therefore it is difficult to determine whether discrepancies between studies are caused by the supplementation or variations in habitual energy and protein intake, thereby precluding the direct comparisons of inferences.

1

**Table 1.1a:Studies investigating protein/amino acid interventions to reduce the negative indices associated with a single bout of intense exercise**

| Study | Subjects / Study design | Exercise protocol | Dietary control | Supplement | Muscle function | Muscle soreness | Blood parameters | Other Measures |
|---|---|---|---|---|---|---|---|---|
| Sugita et al. (2003) (125) | 22 males Crossover | 24 arm curl | No control | 12 amino acids 2×/d for 10 d | Isometric elbow extensor ✔ (AA>PLA) Isometric elbow flexor ✖ Concentric/eccentric elbow extensor/flexor ✖ | Not measured | Not measured | n/a |
| Nosaka et al. (2006) Experiment 1 (107) | 14 males Sedentary Crossover | Arm curl with wrist weight | Requested to eat same food | 9 EAA, 3 NEAA PLA 30min pre EX Immediately post EX | MVC ✖ | VAS ✖ | Mb ✖ CK ✖ Aldolase ✖ | Circumference ✖ ROM Extension ✖ ROM Flexion ✖ |
| Nosaka et al. (2006) Experiment 2 (107) | 24 males Sedentary Crossover | Arm curl with wrist weight | Requested to eat same food | 9 EAA, 3 NEAA PLA 30min pre EX Immediately post EX 2×/d next 3 d | MVC ✖ | VAS ✔ (AA<PLA) | Mb ✔ (AA < PLA) CK ✔ (AA < PLA) Aldolase ✔ (AA < PLA) | Circumference ✖ ROM Extension ✖ ROM Flexion ✖ |
| Cockburn et al. (2008) (30) | 24 males Team sports Parallel | 60 eccentric – concentric knee flexor exercise | Asked to maintain habitual diet | CHO-P Milk (M) CHO Water (CON) Immediately post EX 2 h post EX | PT ✔ (CHO-P >CHO/CON) (M > CHO) TW ✔ (CHO-P>CHO/CON) (M > CHO/CON) | VAS ✖ | CK ✔ (CHO-P< CHO) (M >CHO) Mb ✔ (CHO-P >CHO) | n/a |
| White et al. (2008) (142) | 27 males Untrained Parallel | 50 eccentric quadriceps contractions | No control | CHO-P Pre EX CHO-P Post EX Control | MVC ✖ | VAS ✖ | CK ✖ | n/a |

EAA = Essential amino acids, NEAA = non-essential amino acids, CHO = carbohydrate, CHO-P = carbohydrate and protein, BCAA = branched chain amino acid MVC = maximal voluntary contraction, PT = peak torque, TW = total work, VAS = visual analogue scale, CK = creatine kinase, Mb = myoglobin, LDH lactate dehydrogenase, ROM = range of motion, ✔ =positive effect of intervention, ✖ = no effect of intervention

**Table 1.1b:Studies investigating protein/amino acid interventions to reduce the negative indices associated with a single bout of intense exercise**

| Study | Subjects / Study design | Exercise protocol | Dietary control | Supplement | Muscle function | Muscle soreness | Blood parameters | Other Measures |
|---|---|---|---|---|---|---|---|---|
| Buckley et al. (2008) (24) | 28mMales Sedentary Parallel | 100 eccentric contractions of knee extensor | No control | WPH<br>WPI<br>Water (PLA)<br>Immediately post EX<br>2, 6, 24 h post Ex | PT ✔<br>(WPH>WPI)<br>(WPH>PLA) | VAS ✖ | CK ✖<br>TNF α ✖ | n/a |
| Shimomura et al. (2010) (118) | 12 females Untrained Crossover | 140 squats | No control | BCAA<br>CHO<br>Pre | MVC ✔<br>(BCAA>CHO) | VAS ✔<br>(BCAA>CHO) | Mb ✔<br>(BCAA<CHO) | Elastase ✔<br>(BCAA<CHO) |
| Cockburn et al. (2010)<br>(72 h period only) (31) | 32 males Active Parallel | 60 eccentric – concentric knee flexor exercise | Asked to maintain habitual diet | CHO-P (Pre only)<br>CHO-P (post only)<br>CHO-P (24 h only)<br>Water (CON) | PT ✔<br>(Pre >CON)<br>(POST>CON)<br>(24 h >CON)<br>(24 h >POST) | VAS ✔<br>(Pre<CON) | CK ✔<br>(PRE<CON)<br>POST<CON) | Reactive strength index<br>(CON>PRE)<br>(24 h > CON)<br>(POST > PRE)<br>(24 > PRE) |
| Cooke et al. (2010) (33) | 17 males Untrained Parallel | 40 × 120% MVC<br>Leg press, Leg extension, Leg flexion | Asked to maintain habitual diet | WPH<br>CHO<br>1.5g/kgBM/d × 14 d | Isometric knee extension strength ✔<br>(WPH > CHO) | Not measured | CK ✖<br>LDH ✖ | n/a |
| Kirby et al. (2011) (81) | 27 males Untrained | 100 depth jumps<br>60 eccentric only leg press | Asked to keep a diet diary | Leucine (Leu)<br>Placebo (PLA)<br>30 min pre EX<br>Immediately post EX<br>Morning × 4 d | Isometric peak force ✔<br>(Leu > PLA)<br><br>Static jump height ✖ | VAS ✔<br>(Leu < PLA) | CK activity ✖<br>Mb ✖ | n/a |

EAA = Essential amino acids, NEAA = non-essential amino acids, CHO = carbohydrate, CHO-P = carbohydrate and protein, BCAA = branched chain amino acid MVC = maximal voluntary contraction, PT = peak torque, TW = total work, VAS = visual analogue scale, CK = creatine kinase, Mb = myoglobin, LDH lactate dehydrogenase, ROM = range of motion, ✔ =positive effect of intervention, ✖ = no effect of intervention

### 1.1.4 Summary of muscle damage and nutrition

Although exact mechanisms are not fully understood, unaccustomed lengthening contractions will induce muscle damage. Symptoms of muscle damage include increased soreness, loss of muscle force production, reduced LFF, inflammation, decreased range of motion and increased levels of myofibre proteins in the blood. These methods have their own limitations and the functional measures have been suggested as the most ecologically valid (139). Supplementation of protein and / or amino acids prior to and during recovery from eccentric exercise may speed up recovery. However dietary intake has not been sufficiently controlled in this area of research.

Chapters 2 and 3 describe studies in which the effects of ingesting BCAA and whey protein on the detrimental effects associated with lengthening contractions were investigated. We measured muscle soreness using a VAS, maximal isometric strength with electrical stimulation, force frequency, plasma concentrations of CK and myoglobin and serum IL6 concentration as an indirect marker of inflammation. Furthermore, we strictly controlled subjects' diets. Resistance based exercise protocols were implemented as they produce the greatest decrements in force production and therefore maximise the chance of observing an effect of nutritional intervention.

## 1.2 Muscle protein metabolism

Muscle protein turnover refers to the constant and concurrent synthesis and degradation of muscle protein. Net muscle protein balance (NBAL) is the term used to describe the algebraic difference between muscle protein synthesis (MPS) and muscle protein breakdown (MPB) (136). A positive NBAL over a given time period is required to build muscle proteins. To achieve a positive NBAL the rate of MPS must exceed MPB. Conversely, if MPB exceeds MPS muscle protein loss occurs.

### 1.2.1 Control of muscle protein synthesis

#### *1.2.1.1 Anabolic signalling*

Protein synthesis involves translation of a messenger RNA (mRNA) into a sequence of amino acids to form a polypeptide chain and then a functioning protein. Control of gene transcription and translation collectively regulate the synthesis rate of specific proteins. Gene transcription is the copying of DNA through complementary base pairing to form mRNA. This mRNA molecule is then transported out of the nucleus into the cytoplasm of the cell where translation of this mRNA molecule occurs.

Translation is a process in which the mRNA is used as the template to form a polypeptide chain. Translation can be subdivided into three steps, initiation, elongation and termination. The purpose of initiation is for a translationally competent ribosome and the start codon (AUG) near the 5' end of the mRNA to become joined together (109). Translation initiation can be categorised into three stages (80). Firstly, the specific inhibitor methionyl-tRNA has to bind to the small 40S ribosomal subunit forming the 43S preinitiation complex. The 43S preinitiation complex combines with the mRNA which is capped with eukaryotic initiation factor (eIF) 4F forming the 48S preinitiation complex. The combining of eIF4E, eIF4A and eIF4G into eIF4F is partially regulated by the reversible association of eIF4E with binding proteins, e.g. eIF4E binding protein (4E-BP1). The larger 60S ribosomal subunit then attaches to the 48S preinitiation complex creating the 80S active initiation complex. The second stage of mRNA translation is elongation. Elongation involves reading the mRNA base sequence while building the polypeptide chain. The final stage of mRNA translation is termination. Termination occurs when the ribosome reaches the stop codon. Usually, post-translational modifications occur to the polypeptide chain to form a functioning protein.

Translation initiation and elongation are the main regulatory steps (138) and are controlled through signalling proteins. Mammalian target of rapamycin (mTOR) is controlled by the phosphorylation of a series of upstream proteins including phosphoinositol-dependent kinase 1 (PDK1) and protein kinase B (Akt). During basal conditions mTOR and PDK1 are inactive, causing downstream molecules including p70-S6 protein kinase (S6K1) to be in their inactive form. Exercise and nutrition both have effect on the phosphorylation status of proteins associated with translation initiation. Similar to initiation, translation elongation is controlled by phosphorylation of proteins called eukaryotic elongation factors (eEF). eEF1A and eEF1B are active when they are phosphorylated and are involved in recruitment of the amino acyl-tRNAs to the ribosome. The translocation of the ribosome along the mRNA is inhibited if eEF2 is phosphorylated (23). By measuring the phosphorylation status of signalling proteins, simultaneously with MPS, mechanisms of MPS in response to exercise and nutrition can be investigated.

### 1.2.2 Measurement of muscle protein synthesis

There are several methods that are used in order to measure muscle protein metabolism. Using a continuous infusion of an amino acid tracer is one of the more commonly applied methods to measure MPS. The tracer amino acid that is infused enriches the amino acid pools and proteins. The enrichments are then measured on a mass spectrometer. The rate at which amino acids are converted into proteins is calculated by the change in the enrichment of the tracer in the bound muscle over a given time period. Recently, the majority of studies implementing the precursor-product method have used labelled leucine or phenylalanine as the tracer.

When using the continuous infusion precursor-product method measurement of MPS can be completed using the infusion of a tracer over several hours. Usually this method involves priming of the amino acid pool to achieve a steady state plateau of the infusion more quickly. Percutaneous muscle biopsies are obtained at two time points. The difference between the enrichment in the two biopsies over a period of time and the measurement of a precursor allows calculation of fractional synthetic rate (FSR). FSR is an expression of MPS and is calculated using the following formula:

$$FSR\left(\% \cdot h^{-1}\right) = \left(\frac{\Delta\, E_m}{E_P \cdot t}\right) \cdot 100$$

where $E_m$ is the bound protein enrichment in the muscle and $E_p$ is the precursor enrichment.

The advantage of the precursor-product method is that it allows simultaneous measurement of whole body and muscle protein turnover. Furthermore, the synthesis rate of specific types of protein (e.g sarcoplasmic, myofibrillar) within the muscle can be calculated. The measurement of different protein fractions within the muscle is important as the turnover rates are different and the responses to exercise and nutrition differ (22, 102). In chapter 4 we will measure FSR using a primed continuous infusion of $^{13}C_6$ phenylalanine. This method will allow us to measure myofibrillar protein synthesis. We will measure the tracer enrichment in the intracellular and extracellular fluid over the incorporation period to ensure that over the period of FSR measurement (i.e. the time between the biopsies) an isotopic steady state is achieved. Muscle intracellular free enrichments will be used as the precursor. We will use phenylalanine as the tracer as it may be considered the best amino acid for measurement of FSR for several reasons (146). Phenylalanine has one of the smallest gradients between extra- and intracellular enrichments, thus minimizing possible errors in measurement of precursor enrichments. Moreover, phenylalanine has a relatively low endogenous turnover rate. Thus,

only a small amount of tracer is necessary. Leucine is often an alternative chosen to measure FSR, since whole body protein synthesis and breakdown may be measured simultaneously with leucine. However, phenylalanine is a better choice for our study, since the subjects will be ingesting leucine, thus making maintenance of an isotopic steady-state problematic.

### 1.2.3 Muscle protein synthesis, exercise and nutrition

Exercise of sufficient intensity can be a powerful stimulus to increase rates of MPS in the post-exercise period. However, following resistance exercise, despite increases in MPS, NBAL remains negative (14, 113, 114). In order to obtain a positive NBAL ingestion of an amino acid source is required (15, 116, 134). Therefore, a combination of exercise and provision of amino acids leads to the greatest increase in NBAL. Thus, in order to maximise muscle mass gain a combination of exercise and amino acid ingestion is required.

#### *1.2.3.1 Protein and amino acid ingestion*

Increased circulating amino acids are critical for increases in MPS. Both intravenous infusion (15) and ingestion (134) of crystalline amino acids created hyperaminoacidemia which resulted in positive NBAL and increased MPS following exercise. Further research demonstrated that NEAA were not required for increases in MPS either at rest (135) or following resistance exercise (21, 100, 134). Thus, the EAA are critical for increasing MPS. Furthermore, large increases in MPS were observed with only small amounts of EAA (21, 100, 130). However, the practicality of ingesting pure amino acids may be questioned due to taste and expense. Therefore, studies have been performed in an effort to investigate the effects of ingesting proteins on muscle protein metabolism. Ingestion of intact proteins also increases MPS. Ingestion of whey, soy, casein and egg protein as well as milk and beef have all been shown to increase muscle anabolism (101, 126, 128, 129, 131, 132, 144).

*1.2.3.2 Co-ingestion of carbohydrate and amino acids*

The ingestion of carbohydrate alone has been proposed to enhance MPS at rest (64). The responsible mechanism is likely through the enhanced delivery of nutrients to the muscle, secondary to the insulin induced vasodilation and capillary recruitment (32, 63). However, without provision of amino acids the stimulatory effect of carbohydrate is reduced (16). Previous studies have been designed to determine the effect of MPS following resistance exercise when carbohydrate is co-ingested with amino acids or protein. Ingestion of oral amino acids and carbohydrate following resistance exercise also has been shown to increase MPS during the post exercise period (100, 116). However, comparison of protein ingestion to the ingestion of protein plus high or low carbohydrate demonstrated no additional benefit on MPS of the carbohydrate (82). Furthermore, a bolus ingestion of carbohydrate and protein resulted in no further increases in MPS when compared to protein only (122). Glynn and colleagues (67) demonstrated no differences in MPS or MPB when a low or high carbohydrate dose was added to a high dose of EAA. Thus, in situations where there is ample protein provided carbohydrate does not play a synergistic or additive role on rates of MPS (67, 122).

*1.2.3.3 Effect of branched chain amino acids*

Following exercise, several studies have reported greater increases in MPS when higher plasma leucine concentrations are observed (101, 112, 129, 140). Different types of protein have different amino acid compositions. The ingestion of different types of protein results in different rates of MPS (112, 129, 144). For example whey protein ingestion following resistance exercise has been shown to increase MPS to a greater extent than casein or soy (112, 129). It has been suggested that it is the higher leucine content in whey protein that may be the cause of the higher MPS rates (112, 129). However, there appears to be a maximal rate of MPS even with increased leucine content. This notion was demonstrated by Moore and

colleagues (101) who showed that when egg protein dose was doubled from 20 to 40 g there were no further increases in MPS despite increases in leucine availability. Studies have investigated the effects of combining the ingestion of leucine with whey protein or EAA (38, 61, 68, 84-86, 133). Leucine ingestion did increase MPS rates when compared to a non amino acid containing control (38, 61, 84). However, the muscle anabolic response was not greater when leucine was added to whey protein or EAA if compared to protein/EAA intake at rest (68) or following exercise (85, 86, 133). Therefore, it appears there is no further effect of leucine ingestion in situations where there is already ample amino acids ingested. Rodent studies have suggested that ingestion of BCAA, and in particular leucine, results in increased rates in MPS (2, 3). In humans there are limited studies that have focussed on the effects of BCAA ingestion alone on muscle protein metabolim. When measured by phenylalanine disposal and branched chain keto acid release there was no increase in protein synthesis, however proteolysis was reduced with BCAA infusion (92). Furthermore, Nair and colleagues (104) demonstrated that an infusion of leucine decreased protein degradation more than a saline infusion. Thus, there appears to be a role for BCAA in reducing MPB. The effect of BCAA ingestion alone, compared to a placebo, on MPS has never been investigated. Thus, in chapter 4 of this thesis the effect of BCAA ingestion following resistance exercise will be investigated.

### 1.2.4 Anabolic signalling

Following exercise, the response of MPS to exercise and nutrition is due to increased translation initiation (80). Rodent models have provided the majority of data examining the effect of nutrition and exercise on anabolic signalling (2, 3, 5, 17, 19, 20, 50, 88). Only recently have studies in humans measured the phosphorylation status of signalling proteins

simultaneously with MPS in an attempt to mechanistically determine the cause of increased MPS with exercise and nutrition.

For many signalling proteins there is more than one site that can be phosphorylated. The phosphorylation status of S6K1 is commonly measured at two sites, Thr421/Ser424 and Thr389. It has been shown that muscular contractions have more influence on phosphorylation at Thr421/Ser424 (87, 145) and is not heavily influenced by circulating insulin or amino acid concentrations (83). Conversely phosphorylation of S6K1 at Thr389 is influenced by amino acid availability following exercise (38, 78, 83). Therefore, it is important to measure phosphorylation at the site that is appropriate for the assessment of the response of interest. For example, if a nutritional intervention is being investigated the phosphorylation status of S6K1 at Thr 389 should be measured. In chapter 4, we were interested in investigating the response to ingestion of BCAA following resistance exercise. Thus, we measured phosphorylation status of S6K1 at both Thr389 and Thr421/Ser424.

Furthermore it appears there are many different signalling proteins which interact with each other. An insulin infusion alone resulted in increased phosphorylation of Akt, however leucine infusion did not (70), yet both resulted in increased phosphorylation of the downstream signalling protein S6K1. Thus, the data from that study (70) suggest that insulin and leucine result in phosphorylation of S6K1 via different signalling pathways. Thus in chapter 4 we measured an assortment of signalling proteins associated with mTOR including mTOR, Akt, Pras40, 4E-BP1, S6K1 and eEF2.

*1.2.4.1 Anabolic signalling: the effect of nutrition*

There are several studies that have measured the effect of nutritional intake only on intracellular signalling proteins (61, 66, 69-71, 73). Amino acids have been shown to activate mTOR with only basal insulin levels (71, 73, 138). However, many studies provide additional

carbohydrates that would enhance the insulin-stimulated phosphorylation of mTOR by the upstream kinase Akt (61, 69). The presence of insulin results in enhanced phosphorylation of signalling molecules both upstream and downstream of mTOR (40, 73). However , in vitro studies (79, 148) have demonstrated that amino acids can activate mTOR independently of insulin, thus explaining how in the absence of hyperinsulinemia mTOR associated signalling proteins have enhanced phosphorylation when only amino acids are ingested (35, 70, 91).

There appears to be some inconsistencies in the findings of nutrition on signalling proteins and this may be related to the inherent difficulties with the measurements or differences in biopsy timing or the composition of the nutrient intake. Several authors have demonstrated an increased S6K1 phosphorylation with amino acid feeding/infusion (61, 69-71, 73), conversely others have shown no differences in phosphorylation status (66). Furthermore, Fujita et al. (61) demonstrated that a leucine-enriched EAA and carbohydrate beverage increased phosphorylation status of S6K1, demonstrating the requirement of elevated insulin levels. Conversely, Greenhaff et al (69) reported that an intravenous infusion of amino acids without elevated insulin levels increased phosphorylation status of S6K1 above basal levels. Other signalling proteins (mTOR, Akt, 4EBP1) have also been shown to both increase and decrease in phosphorylation status in response to nutrient intake only (61, 66, 69-71, 73), for review see (41). Pras40 is another signalling protein that is influenced by insulin levels and influences the phosphorylation of mTOR through Akt (137). Thus, the measurement of the phosphorylation status of pras40 in chapter 4 will allow us to determine if changes in mTOR are via insulin mediated pathway and Akt.

Of interest to the current thesis is the effect of ingesting only the BCAA on signalling proteins. It is proposed that leucine can regulate rodent MPS via enhancement of translation initiation (2-6, 18). Specifically leucine is involved in the phosphorylation of proteins which

control the 40S ribosomal subunit and mRNA binding. Furthermore, leucine has been shown to increase hyperphosphorylation of 4E-BP1 and increase phosphorylation of S6K1 (2, 5, 6), and will therefore both will be measured in chapter 4. Additionally, leucine exerts its' effect directly on eIF4E and therefore, the eIF4F complex (2).

The chosen methodological design implemented in chapter 4 did not allow us to measure the impact of nutrition alone on intracellular signalling proteins as we did not take biopsies from the rested leg. However, in the supplement we provided post exercise there were only the BCAA to allow for determination the signalling response without provision of additional insulin.

*1.2.4.2 Anabolic signalling: the effect of resistance exercise*

The mTOR signalling pathway has also been demonstrated to play a role in the regulation of MPS following resistance exercise. Drummond and colleagues (42) demonstrated that an infusion of a mTOR inhibitor resulted in a lack of increased post exercise MPS. During resistance exercise the increase in AMPK phosphorylation inhibits mTOR (37). However, in the recovery period from resistance exercise performed in a fasted state there are many studies that show an altered phosphorylation status of mTOR and downstream signalling proteins. (mTOR (39, 60), S6K1$^{Thr421/Ser424}$ (46, 66, 87) and ribosomal protein S6 (rpS6) (66)). Phosphorylation of S6K1 is markedly increased following resistance exercise (36, 39, 46, 60, 66, 87). Several studies (9, 39, 42) have demonstrated that eEF2 is dephosphorylated following resistance exercise. These results suggest that resistance exercise also influences the elongation phase of translation.

In chapter 4 although a placebo trial will be implemented, participants will have had a breakfast prior to the exercise and therefore we will be unable to determine the effect on signalling proteins of exercise alone.

*1.2.4.3 Anabolic signalling: the effect of exercise and nutrition*

The combination of exercise and nutrition can influence MPS by regulating intracellular signalling proteins that control translation initiation. In general, there is a synergistic action of exercise and nutrition on phosphorylation status of mTOR associated signalling proteins (21, 38, 62, 78, 86, 100, 116). Following resistance exercise protein and EAA ingestion have been demonstrated to alter phosphorylation status of S6K1 and 4E-BP1 (38, 62, 66, 78, 83). However, it should be noted that several studies have included carbohydrate in the feeding strategies (38, 66, 83). Thus, the altered phosphorylation status may be induced through insulin mediated pathways. Therefore, further studies are required, where only amino acids are provided, to fully elucidate the synergistic effect of resistance exercise and amino acid ingestion. Interestingly resistance exercise appears to maximally stimulate eEF2, with no additional effect observed when amino acids are ingested (38, 39, 66, 83). These results (38, 39, 66, 83) suggest that there is no synergistic effect of resistance exercise and nutrition on the elongation phase of translation. There are very few studies that have investigated the combination of resistance exercise and BCAA ingestion on various mTOR related signalling proteins (78). In this study (78) it was demonstrated that repeated ingestion of branched chain amino acids led to an increased phosphorylation of S6K1 at both Thr 389 and Ser424/Thr 421 sites. Additionally rpS6 was stimulated with BCAA ingestion. In chapter 4 we are going to measure Akt and mTOR associated signalling proteins following resistance exercise and BCAA ingestion.

**1.2.5 MPS and intracellular signalling**

To try and correlate differences in MPS and anabolic signalling, recent studies have combined the two measurements. Mixed muscle FSR was shown to increase by 41% alongside increases in phosphorylation of both mTOR and S6K1 2 h following exercise (39).

However, there is not always a correlation between MPS and signalling (69, 101, 122). Initially, increases in MPS following protein ingestion are associated with increases in phosphorylation of signalling proteins (10). However, these authors (10) demonstrated that when MPS returned to basal levels the phosphorylation status of signalling proteins remained elevated. The association of phosphorylation status and MPS when there is a combination of exercise and nutrition has not yet been fully investigated in humans. Thus, the relationship between signalling proteins and MPS still requires further investigation. mTOR signalling was enhanced and increased MPS rates were reported when a leucine, EAA and CHO drink was ingested and compared to a placebo (38). However, the measurement of MPS alongside signalling proteins, following the ingestion of BCAA following exercise has not been completed in humans. Thus, in chapter 4 we will simultaneously measure FSR and phosphorylation status of signalling proteins that are suggested to be influenced by both resistance exercise and BCAA ingestion.

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# Chapter 2: Branched-chain amino acid ingestion can ameliorate soreness from lengthening contractions

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*Med Sci Sports Exerc* 42: 962-970, 2010

## 2.1 Abstract

**Background:** Inadequate recovery from unaccustomed exercise can limit exercise performance and also the ability to perform habitual activities. Repeated lengthening contractions or unaccustomed exercise leads to loss of muscle strength, an increase in muscle soreness and putative blood markers of muscle damage. Recently, nutritional interventions including branched-chain amino acids (BCAA) have been suggested to alleviate these symptoms. However, supplementation with BCAA has led to equivocal findings. **Purpose:** The purpose of this study was to examine the role of branched chain amino acid (BCAA) supplementation during recovery from intense lengthening contraction based exercise. **Methods**: Twenty-four non-weight trained males were assigned to one of two groups; one group (SUP) ingested BCAA beverages (n=12); a second group (PLA) ingested artificially flavoured water (n=12). Diet was controlled throughout the testing period to match habitual intake. The exercise protocol consisted of 12x10 lengthening contractions of unilateral knee extension at 120% concentric one repetition maximum. On the day of the exercise supplements were consumed 30 min before exercise, 1.5 h following exercise, between lunch and dinner and before bed. On the following two days four supplements were consumed between meals. Muscle soreness, muscle function and putative blood markers of muscle damage were assessed pre and post (1, 8, 24, 48, 72 h) exercise. **Results:** Maximal muscle force production decreased after the exercise bout ($P<0.0001$) but was unaffected by BCAA ingestion (-51± 3% with SUP versus -48 ± 7% with PLA). A decrease in flexed muscle soreness was observed in SUP compared to PLA at 48 h (21 ± 3 mm vs. 32 ± 3 mm; $P=0.02$) and 72 h (17 ± 3 mm vs. 27 ± 4 mm; $P=0.038$). Flexed muscle soreness, expressed as area under the curve, was lower in SUP than PLA ($P=0.024$). **Conclusions:** BCAA supplementation may attenuate muscle soreness, but does not ameliorate lengthening

contraction-induced decrements in muscle function or increases in reputed blood markers of muscle damage, when consumed pre and for 3 days after a bout of multiple lengthening contractions.

**Keywords:** Nutrition, unaccustomed exercise, delayed onset muscle soreness, muscle function

## 2.2 Introduction

Unaccustomed lengthening contractions result in decreased maximal force production (10, 20), increased muscle soreness lasting several days (18) and increased concentrations of myofibre proteins (creatine kinase (CK) and myoglobin (Mb)) in the blood (6, 18). Thus, an impaired functional capacity of the muscle may prevent repeated exercise sessions which are required for adaptations to training i.e. an improved tolerance to the stress of resistance exercise or muscle hypertrophy. Nutritional strategies aimed at reducing the deleterious impact of lengthening contractions may be advantageous by allowing repeated exercise bouts of a superior quality to be performed.

Recent studies have focused on nutritional interventions during exercise recovery in an attempt to alleviate muscle soreness and impairments in muscle function (18, 22, 24). Post exercise supplementation of protein and amino acids has been suggested to reduce the negative impact of lengthening contractions (6, 18, 23). However the results of these studies are equivocal. Muscle soreness and blood concentrations of myofibre proteins were decreased with supplementation of mixed free amino acids ingested in a fed state 30 min pre exercise, post exercise and during the 4-day recovery period (18). Supplementation of mixed amino acids twice a day for 10 days resulted in an attenuated loss of isometric contraction force following lengthening contractions (24). Similarly, during the first week of a 4-week intensified training resistance protocol impairments in maximum strength of squat and bench press were attenuated with $0.4 g \cdot kg^{-1} \cdot day^{-1}$ of amino acid supplementation (11).

Conversely, mixed amino acid supplementation of shorter duration pre and post exercise in a fasted state had no impact on muscle soreness or blood myofibre proteins (18). Likewise, White *et al.* (31) demonstrated no differences in muscle function or soreness when comparing a protein/carbohydrate supplement to a placebo administered in a fasted state pre or post

lengthening contraction quadriceps exercise. Discrepancies between studies may be caused by the type or amount of amino acids in the supplements, whether the supplements are coingested with other nutrients, the duration of supplementary period, the degree of dietary control or the measurement tool used to assess the negative indices associated with intense lengthening contractions. Therefore, the effectiveness of amino acid or protein supplementation on attenuating muscle function loss, and elevation in muscle soreness and blood myofibre proteins appears somewhat equivocal at present.

Positive effects of amino acid supplementation have been claimed to be related to the branched-chain amino acids (BCAA) (18). Recent interest has focused on the effectiveness of BCAA for reducing the negative symptoms associated with intense exercise (6, 8). To our knowledge only one preliminary study has investigated BCAA supplementation and recovery from intense lengthening contractions (23). These authors reported a reduction in muscle soreness following squat exercise with supplementation of only 5g BCAA in females (23). However, BCAA were ingested only once. At present no data examining the effect of ingestion of multiple supplements containing BCAA following multiple lengthening contractions in males. Therefore despite some evidence for the efficacy of BCAA in amelioration of adverse indices associated with intense exercise, the effectiveness of BCAA is not clearly established.

Pilot data from our laboratory suggest high doses of whey protein ingestion may impact muscle soreness following resistant exercise. Mixed amino acids have also been shown to impact muscle soreness (18). BCAA have been suggested to be the active ingredient (18, 23). The aim of this study was to investigate the effectiveness of BCAA supplementation for the attenuation of the deleterious impact of an intense lengthening contractions in healthy males with no previous experience of regular resistance exercise. In accordance with Nosaka *et al*.

(18) showing an effect of amino acid supplementation, we provided supplements before, and in the four days following the exercise bout. In contrast to many previous studies, this study implemented strict dietary control, in order to ensure any differences that were observed could be attributed to the additional BCAA intake.

## 2.3 Method

### 2.3.1 Participants

Twenty-four non-weight trained males were recruited for this study, which was approved by the University of Birmingham, School of Sport and Exercise Sciences Ethics Subcommittee. Subjects were made aware of procedures including the risks and benefits, gave written informed consent (Appendix A) and completed a general health questionnaire (Appendix B). Participants were able to withdraw from the study at any point without provision of reason. All participants refrained from physical activity and alcohol consumption for two days prior to testing and throughout the testing period. Participants were excluded if they had previous history of resistance training or had completed high intensity lengthening contraction based exercise within the last 6-9 wk (5).

### 2.3.2 General Design

Subjects were assigned to either a supplementary (SUP) or placebo (PLA) group and performed one single-blind experimental trial. Groups were matched for weight and one repetition maximum (1RM). Subject characteristics are shown in Table 2.1. An exercise protocol consisting of lengthening contractions was implemented to allow comparison of putative blood markers of muscle damage, muscular dysfunction and muscle soreness

between SUP and PLA. Subjects consumed four supplements daily between meals. The composition of the supplement consumed by participants in SUP was 3.5 g leucine, 2.1 g isoleucine and 1.7g valine mixed in 300ml artificially sweetened and flavoured water. The control supplement contained 300 mL of artificially sweetened and flavoured water. The artificial sweetener was free of amino acids.

**Table 2.1: Participant characteristics**

| | Weight (kg) | Height (m) | 1 RM (kg) | Leg Volume (ml) |
|---|---|---|---|---|
| SUP | 72.8 ± 10.4 | 1.80 ± 0.1 | 61.2 ± 9.3 | 5512 ± 1353 |
| PLA | 74.9 ± 9.1 | 1.80 ± 0.1 | 62.3 ± 9.5 | 5725 ± 1094 |

Values are expressed as means ± SD
1RM = one repetition maximum
Leg volume = volume of upper leg which was exercised calculated via anthrompetry (32).

### 2.3.3 Pre testing

Subjects visited the laboratory for preliminary measurements on two occasions. On the first, leg volume of the non-dominant leg was calculated (32). Determination of 1 RM of their non-dominant leg followed using a Cybex leg extension machine (Cybex international, USA). Subjects lifted an initial load which was then increased in increments of 2.5 kg. The subjects were instructed to lift the weight, hold it out-stretched for 2 s and then slowly lower it. Subjects rested for 3 min between lifts to minimise the impact of fatigue. Subjects were given a second attempt after the rest period if they failed with any given lift. This session also served as familiarization for the exercise protocol that followed. The familiarisation involved two lifts at 50% of their concentric 1 RM in which the weight was lifted by the researchers for the subject to lower. Subjects were strapped into the chair to ensure that the weight was lifted using only their leg. The subjects' non-dominant leg was positioned at $90^{o}$ to their torso with their knee flexed to approximately $110^{o}$. The lift started with the exercising leg positioned at approximately $15^{o}$ of flexion. Two testers lifted the weight to its full extension; a 'contract'

command prepared the subject for the weight to be lowered. This command was followed by the command 'release' and the weight was released onto the subjects' leg, and was then lowered by the subject, resisting the load. The weight was then lowered through $95^{o}$ to bring the weight back to rest. Participants aimed to control the weight in the downward movement for 4 secs, if the movement was shorter in duration another repetition was completed. On the second visit to the laboratory, preliminary measurements were taken. These involved the measurements taken at each time-point, i.e. visual analogue scale (VAS), a blood draw and muscle function tests.

### 2.3.4 Experimental Protocol

On the day of the exercise protocol, subjects reported to the laboratory at ~06.00 h following an overnight fast (Figure 2.1). The subjects ingested their first supplement followed by a 30 min rest. The exercise protocol was then performed which lasted approximately 45 minutes. Subsequently, subjects rested for an hour before the first measurements occurred. Pain was assessed using the VAS, followed by a blood draw. Finally, participants' muscle function was assessed via maximal isometric strength (MIS) and low frequency fatigue (LFF) as described below. The order of these measurements was standardised for the 8, 24, 48, and 72 h post exercise to eliminate measurement order from confounding results. Following completion of the 1 h testing, participants consumed another supplement before breakfast. A third supplement was ingested between lunch and dinner and the fourth at bedtime. On the three days following the exercise bout, all measurements were taken in the morning in the fasted state with the exception of the 8 h time-point. To maximise measurement reliability, all

measurements were carried out by the same investigator for each subject.

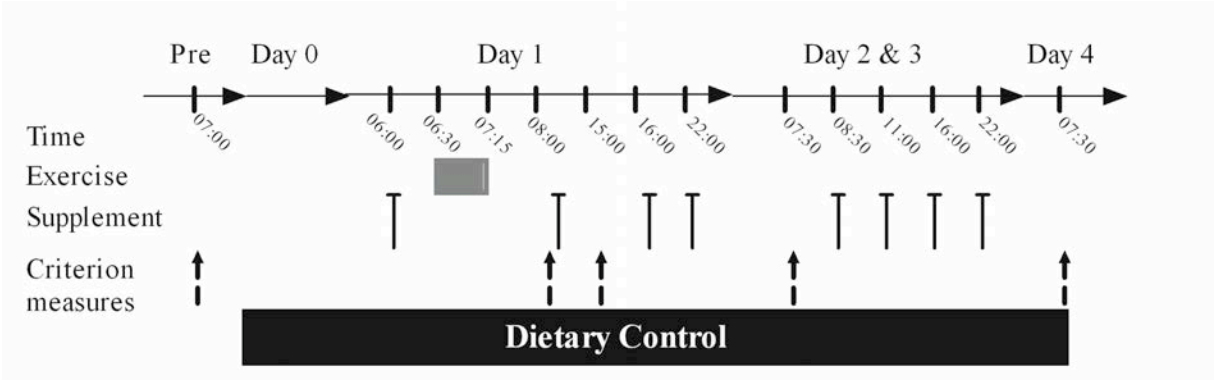



**Figure 2.1: Schematic protocol depicting the duration of the study.**
Criterion measures include; muscle soreness measures, blood draw, muscle function tests. Exercise = 12 sets of 10 repetitions of lengthening contractions of the quadriceps at 120% concentric 1RM. Supplements were consumed 4 times on the day of and two days following the exercise and were either BCAA or placebo.

### 2.3.5 Diet and activity

Prior to each trial, subjects were instructed to record a weighed three day diet record including at least one week day and one weekend day. An average energy intake from these three days was calculated and used to determine individuals' energy requirements. All food was prepared and supplied to the subjects to ensure that any differences observed could be attributed to the supplements and diets were comparable between subjects. The diet was composed of 55% carbohydrates, 1.5 g protein / kg body mass (BM) with the remainder of the energy from fat. Prepared food was administered the day before, the day of, and two days following the exercise protocol. Food composition for the study is shown in Table 2.2.

**Table 2.2: Diet composition of food provided to the subjects**

| | Energy | Protein | | Carbohydrate | | Fat | |
|---|---|---|---|---|---|---|---|
| | kJ | g/kgBM/d | % En | g/kgBM/d | % En | g/kgBM/d | % En |
| SUP | 12305 ± 1677 | 1.5 ± <1 | 15 ± 3 | 5.7 ± 1.1 | 55 ± <1 | 1.4 ± 0.4 | 30 ± 3 |
| PLA | 12026 ± 2074 | 1.5 ± <1 | 16 ± 3 | 5.3 ± 0.8 | 55 ± 4 | 1.3 ± 0.4 | 29 ± 6 |

Values are expressed as means ± SD.
% En: percentage of total energy intake.

### 2.3.6 Exercise protocol

Subjects were seated as described previously and completed 12 sets of 10 lengthening at 120% concentric 1RM, with 5 s rest between each repetition and 1 min rest at the end of each set. If the subject failed to control the weight for two consecutive repetitions, a rest period of 30 s was given. During each contraction the subject was verbally encouraged to aid maximum effort.

### 2.3.7 Muscle function

Maximal isometric strength (MIS) was assessed using electrical stimulation with a Tornvall chair (7). Participants were seated so the knee was at $90^{o}$ with a cord fastened round the ankle. This cord was attached to a strain gauge interfaced with a computer. Two arm straps held the subject in place in the chair. Damp electrodes (13 by 8 cm) were secured to the thigh over the proximal and distal ends of the quadriceps. Participants were electrically stimulated at their maximal electrical stimulation three times before being encouraged to push out with their leg against the cord as hard as possible. Three electrical stimulations were superimposed while contraction was occurring to ensure that the contraction was maximal. This procedure occurred three times at each time-point. The highest force obtained for each leg represented the subjects' MIS. Another measurement of muscle function is LFF, i.e. a loss of force at low frequency stimulation common in damaged muscle (7, 10). Force frequency measurements occurred at one third of maximal electrical stimulation current. Electrical stimulation of the quadriceps at 20 and 100Hz for 2 s was performed in random order. The relationship between 20 and 100 Hz was used as an assessment of LFF. This procedure with electrical stimulation has been shown to not affect the response to initial exercise bout (14).

### 2.3.8 Muscle soreness

Subjects rated general muscle soreness and soreness with the knee flexed and extended using a VAS. The VAS consisted of a 50mm line with anchor points of 'no soreness' on the left and 'extremely sore' on the right. Subjects marked a point along the line which represented the perceived soreness felt in the quadriceps muscle group. Pain was given a value by measuring the distance from the left anchor point. It has been shown that 90% of pain ratings can be repeated within 9mm using the VAS (2).

### 2.3.9 Blood Analysis

Plasma concentrations of CK and Mb were used to assess commonly measured putative markers of muscle damage. Serum was collected and analysed for interleukin-6 (IL-6) as an indirect marker of inflammation. Blood was drawn from the forearm vein of each subject at each testing point. Plasma samples were placed on ice, and serum samples were left at room temperature for 30 min to allow clotting. Samples were centrifuged at 3000 revolutions per min for 10 min at 4$^{o}$C. Plasma and serum were extracted and stored in the freezer at -20$^{o}$C until further analysis. Enzymatic analysis for plasma concentrations of CK (CK NAC CP, ABX diagnostics, UK) and Mb (myoglobin CP, ABX diagnostics, UK) were performed in duplicate at each time-point using a semi-automated analyser (COBAS MIRA S-plus, ABX, UK). Serum concentrations of IL-6 were analysed using commercially available sandwich enzyme-linked immunoabsorbent assay's (ELISA's) [High sensitivity Human IL6 assay, R&D systems, UK]. Plates were read in duplicate on a Labsystems Original Multiskan MS at selected wavelengths (490nm with correction at 630nm) Reported sensitivity of the ELISA is 0.039 pg/mL.

### 2.3.10 Data presentation and statistical analysis.

Data were analysed using statistical package for social sciences (SPSS) 15.0 for Windows (SPSS Inc., Chicago, IL). Differences across time for blood concentrations, force loss and VAS scores were analysed by a mixed design, two-way ANOVA with two between (SUP, PLA) and six or four (depending on variable analysed) within (time-points) subject variables. Significance was set at the 0.05 level of confidence. Where significance was found, least significant difference (LSD) post hoc tests were run to distinguish differences between supplementary groups. Missing data points were averaged from the other data sets for that time-point and group. This manipulation allowed a higher number of subjects to be included in the analysis to improve statistical power. VAS scores are shown as raw values and area under the curve (AUC) to give an indication of soreness over the 72 h experimental period. AUC baseline values were taken as a mean of preliminary values). Force frequency data were obtained by averaging values over a 1 s time period (0.8-1.8 s). Force frequency is shown as the percentage change from pre of the ratio between force value at 20Hz and 100Hz. Due to technical difficulties in this measure, data are shown for only n=10 in each group. MIS data were taken from the best value of three attempts and converted into Newtons from the volts value obtained. MIS values are shown as a percentage of the pre-exercise value to show the force that was lost, and using the pre value as a baseline. It was not possible to collect blood from one subject in PLA. Plasma CK, and Mb are depicted as percentage of each individual participants maximum. Serum IL-6 concentrations are shown as the mean concentrations.

Data were also analysed using inferential statistics using 90% confidence limits to produce magnitude-based values about the probabilities of the outcome (1). For overall flexed soreness, an effect was assumed to be a change of 143mm*72h, and for extended soreness this value was 137mm*72h. These values were derived from the smallest standardized

(Cohen) difference in mean (0.2) multiplied by the standard deviation of PLA. The likelihood of the outcome being of beneficial or detrimental nature was also determined using a published spreadsheet (9). When the value is determined to be <1% detrimental or beneficial it is almost certainly not; 1-5% very unlikely, 5-25% unlikely, 25-75% possible, 75%-95% likely, 95-99% very likely and >99% almost certain.

## 2.4 Results

### 2.4.1 Muscle Function

A decrease in MIS (Figure 2.2a) and in force frequency ratio (Figure 2.2b) was apparent in all subjects following exercise without difference between groups. The maximal percentage of force loss assessed using MIS was observed 48 h post exercise in both groups (SUP -51 ± 3 %, PLA -48 ± 7 %). The lowest force frequency ratio was observed at 1 h post the exercise bout(SUP -42 ± 6 %, PLA -47 ± 5 %).

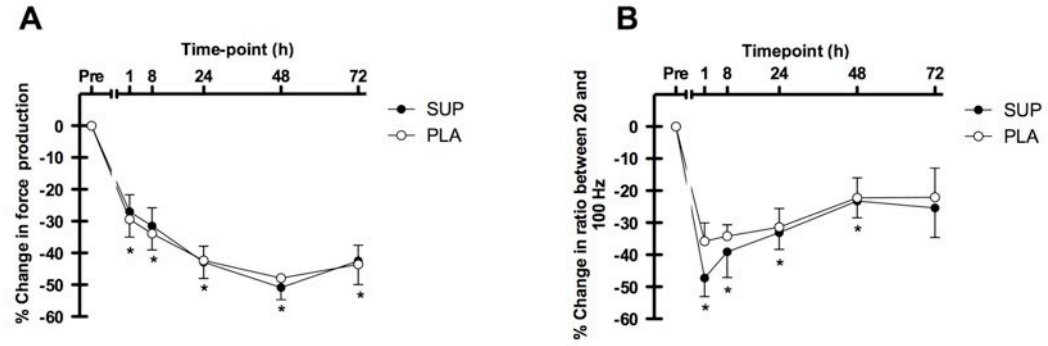
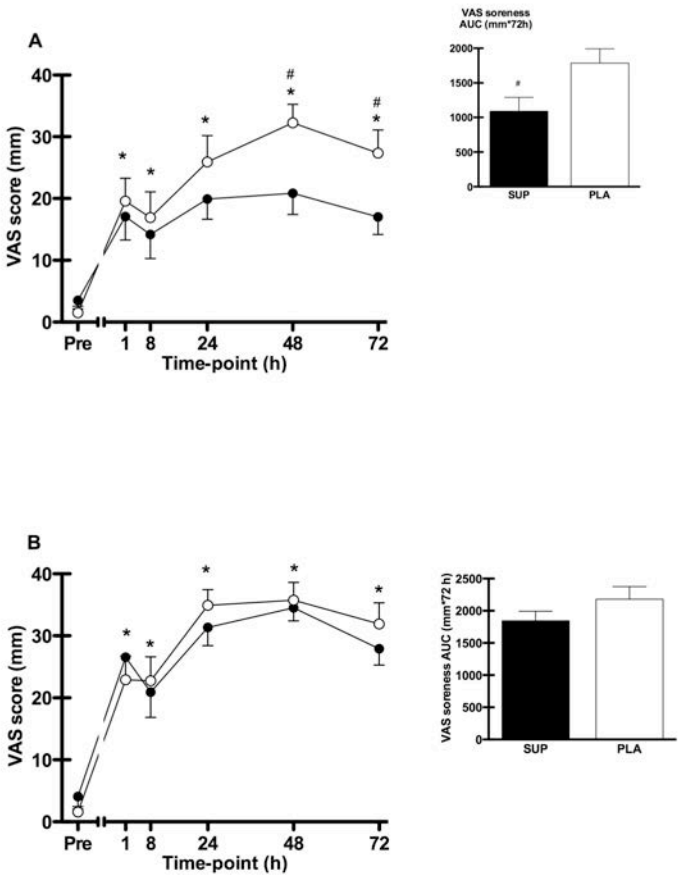



**Figure 2.2: Percentage change in leg muscle function pre and following 120 lengthening contractions. Depicted as a) electrically stimulated maximal isometric strength, b) 20:100Hz force frequency relationship.**
SUP = closed circles (Figure 2.2a, n=12, Figure 2.2b n=10) and PLA = open circles (Figure 2.2a, n=12, Figure 2.2b n=10). Data expressed as means ± SE. * signifies significant difference from baseline in both groups.

### 2.4.2 Muscle Soreness and Inflammatory Responses

The response of muscle soreness to the exercise bout is illustrated in Figure 2.3. The exercise protocol resulted in increased overall soreness in both groups. However no statistical differences were detected between SUP and PLA. Muscle soreness was also increased both with the knee flexed and extended. Soreness ratings tended to be lower at 8 h compared with 1 h. Soreness then continued to significantly increase to peak soreness observed at 48 h. A main effect of SUP was observed ($P<0.05$). SUP resulted in a decrease in quadriceps muscle soreness with the knee flexed compared to PLA at both 48 h (21 ± 3 mm vs. 32 ± 3 mm; $P=0.02$) and 72 h (17 ± 3 mm vs. 27 ± 4 mm; $P=0.038$) post exercise (Figure 3a). There were no significant differences between SUP and PLA with the knee in an extended position. Over the total 72 h experimental period when data are expressed as AUC, muscle soreness with the knee flexed was 64% less for SUP compared to PLA ($P=0.024$) (Figure 2.3a). There were no statistical differences between SUP and PLA AUC with the knee in an extended position.

Evaluation using inferential statistics showed SUP was 96.7% very likely beneficial, (700

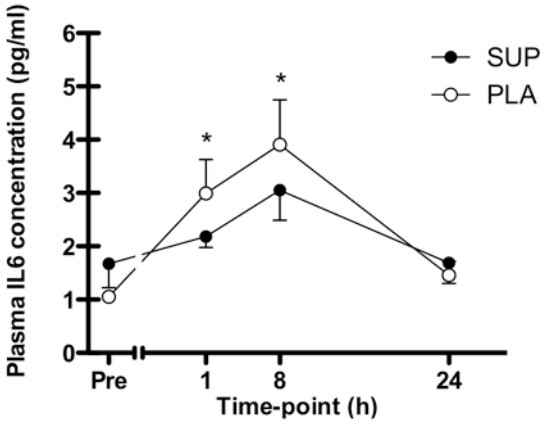


± 490 mm, 90 % CL) for amelioration of muscle soreness with the knee flexed. Furthermore, the positive effects of BCAA in the extended position was 78.5 % likely beneficial effect (300 ± 420 mm 90 % CL) (Figure 2.3b).

**Figure 2.3: Muscle soreness pre and following 120 lengthening contractions measured using a 50mm visual analogue scale (VAS). a) knee in a flexed position, insert shows area under the curve b) knee in an extended position, insert shows area under the curve.**
SUP black bar/closed circles (n=12) and PLA white bar/open circles (n=12). * signifies significant difference from baseline. # significant difference of SUP from PLA.

### 2.4.3 Blood parameters

Serum IL-6 data are depicted in Figure 2.4. IL-6 concentrations significantly increased following the exercise protocol in both groups (P=0.02). At 24 h post exercise IL-6




concentration had returned to normal. There were no differences in IL-6 concentrations observed between SUP and PLA.

**Figure 2.4: Serum interleukin-6 concentration measured pre-, 1, 8 and 24 h post 120 lengthening contractions.**
SUP closed circles (n=12) and PLA open circles (n=11). Data is means ± SE. * significant difference from baseline in both groups

Plasma CK (P<0.001) and Mb (P<0.001) concentrations increased post exercise and remained elevated throughout the testing period (Figure 2.5a and 2.5b respectively). Peak CK

concentrations were observed between 8 and 24 h post exercise. Peak Mb concentrations were observed between 1 and 8 h post exercise. There were no significant differences observed between groups for plasma concentration of CK or Mb. In both blood metabolite concentrations a large inter-subject variability was observed.

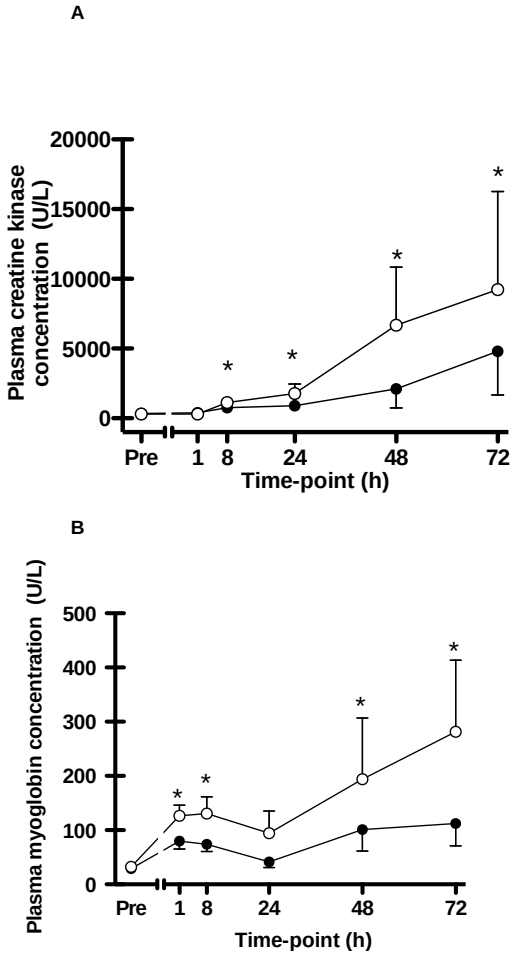



**Figure 2.5: Myofibre concentrations in the plasma pre and following 120 lengthening contractions, expressed as raw values a) Creatine kinase concentration, b) Myoglobin concentration.**
SUP closed circles (n=12) and PLA open circles (n=11). Data is means ± SE. * significant difference from baseline

## 2.5 Discussion

In the present study we investigated the effects of BCAA supplementation on recovery from multiple lengthening contractions under conditions of strict dietary control. Our findings

are the first to suggest a beneficial role for BCAA supplementation following intense lengthening contraction based exercise in males. We observed a reduction of overall soreness in the quadriceps muscle with the knee in the flexed position. Additionally, we calculated that there was a likely beneficial effect of BCAA supplementation with the knee in an extended position. No differences in plasma myofibre concentrations, indirect measurement of inflammation or muscle function were detected between BCAA or placebo supplementary conditions when supplements were provided pre and for three days following multiple lengthening contractions.

Amino acids intake may have an impact on muscle soreness. Supplementation with mixed amino acids (9 essential and 3 non essential) has previously been reported to decrease muscle soreness (18). In the present study, non trained subjects ingested only three amino acids (leucine, valine and isoleucine) at multiple time points post ex. Our results demonstrated that muscle soreness following the exercise bout was attenuated in the supplementation group, suggesting that BCAA are the active ingredient in protein for reducing muscle soreness. Interestingly, no effect of nutritional supplements containing BCAA in combination with other amino acids on muscle soreness have been observed in other studies (18, 31). Reasons for this discrepancy may be related to length of supplementary period and the total quantity of BCAA ingested. It would be of interest to determine if the beneficial effect on soreness we observed was replicated in a situation where only one of the BCAA were ingested.

The amount of BCAA ingested may be a particularly important factor for amelioration of muscle soreness. Conflicting findings between present and previous studies may be explained by the length of supplementary period and consequently total intake of amino acids. Our subjects consumed supplements pre exercise and throughout the three day recovery period resulting in reductions in muscle soreness. Conversely, previous studies (18, 31) which

supplemented on only one or two occasions observed no differences in muscle soreness between experimental and control conditions. Interestingly, data in a review paper (23) demonstrated reduced muscle soreness following squat exercise with only a single bolus (5g) of BCAA. However this effect was observed in females but not males. The authors (23) attributed the difference to the fact that the females consumed a larger amount of BCAA relative to their muscle mass compared to the males, rather than a sex effect per se. Despite not having female subjects our findings may lend some support to this assertion. In our male subjects the total amount of BCAA intake per kg BM was even greater than in the females in the aforementioned study (23) resulting in decreased soreness. Thus, it seems feasible to suggest that a larger BCAA intake than in the previous study (23) may be necessary for any reductions in muscle soreness.

Taken together the results of our study and those reported by Shimomura *et al*. (23) may suggest a threshold amount of BCAA relative to individuals muscle mass and to observe a decrease in muscle soreness this threshold must be reached. Therefore future studies should investigate BCAA intake relative to muscle mass

The exact mechanism by which BCAA supplementation decreases muscle soreness cannot be deduced by our study design. Intense lengthening contractions are known to lead to an inflammatory response and an increased concentration of IL-6 (3). The exact cause of muscle pain is unknown, however it has been suggested that inflammation of the perimysium or epimysium may be causing the pain (12). The inflammatory response results in a sensitization of the muscle noiceptors leading to the common feeling of soreness (20). In the present study we measured circulating IL-6 concentrations. In accordance with previous studies which implemented carbohydrate feeding, no differences in IL-6 concentration were observed at any time-point with BCAA supplementation when compared to the placebo condition (13). Our

results suggest that BCAA supplementation did not alter this Il-6 response and therefore the BCAA are unlikely to mediate soreness via an IL-6 inflammatory pathway. Alternatively, soreness was reduced in both groups at the 8 h post exercise when subjects were in an early postprandial state. It may therefore be suggested that the extra energy intake from the supplements of ~117 kcal/day in SUP may have led to the decrease in soreness observed, however no mechanism is clearly indicated.

Soreness may be modulated by feeding. Lower muscle soreness scores were recorded at 8 h compared to 1 h post exercise in 19 of the 24 subjects. Measurements collected 8 h following the exercise were typically performed at ~ 15:00 h when participants were in a postprandial state, having eaten lunch within the previous 3 hours. We therefore propose that feeding may be responsible for this temporary reduction in perceived feelings of soreness. The present study is the first to record criterion measurements within 3 h of a meal. Recently, a novel experimental design was implemented (18) whereby lengthening contractions were performed in a fed and supplemented state. Analogous to our findings, mixed amino acid supplementation provided pre- and for four days post-exercise attenuated muscle soreness compared to a placebo group (18). Taken together, these data suggest amino acid supplementation combined with food intake may attenuate muscle soreness, possibly due to a larger availability of all amino acids. The association between soreness and feeding is an interesting concept; future studies should investigate whether there is a synergistic relationship. Other nutrients including carbohydrate and fat particularly in association with protein or amino acids should be investigated with regards to the impact on muscle soreness following intense lengthening contractions. Alternatively the delivery pattern of the amino acids may be modulated when combined with other nutrients, thus resulting in the reduced soreness observed in this study following a meal.

Intense bouts of lengthening contractions induce decrements in muscle force production in untrained individuals (18, 24, 31). In the present study, BCAA supplementation failed to ameliorate impairments in MIS. However previously Sugita *et al*. (24) exercised the elbow extensor muscles and provided a mixture of 12 amino acids for 10 days post exercise consisting of lengthening contractions. These authors (24) reported attenuations in reductions of strength on days 2 and 3 post exercise. Some additional amino acids were ingested in the aforementioned study compared to the present study. It may be suggested that all essential amino acids, not only BCAA, may be necessary for successful amelioration of loss of muscle function. However, more recent studies (18, 31) that supplemented a mixture of amino acids or protein showed no effect on muscle function compared to placebo. Thus, future studies are required to fully elucidate how muscle function can be improved via nutritional interventions following intense lengthening contractions.

The shift in force frequency relationship we observed mirrors those associated with LFF that is common with damaged muscle. Consistent with previous studies (14-16, 21) these data in the present study demonstrated a greater reduction in force at low frequencies compared to high frequencies following intense lengthening contractions (10). A comparison between the force production at the two frequencies may indicate if there has been greater reductions in force at lower frequencies. Reduction in force at low frequencies indicates that there is an impairment of excitation-contraction coupling (30). The present study was the first to investigate the effect of amino acid supplementation on LFF. However, BCAA ingestion did not impact upon the observed effect. Thus in the present study we observed no effect of BCAA on either measure of muscle function. Therefore our data demonstrating BCAA ingestion decreased soreness but had no effect on decrements in muscle function supports

previous research (29) which suggests that the relationship between muscle function and soreness is unclear.

Muscle function following intense exercise may be, decreased due to increased muscle soreness. Twist and Eston (27) demonstrated that exercise-induced muscle damage increases perceived exertion and decreases performance during intense exercise. Whereas our study was not designed to investigate a relationship between muscle soreness and function, our result may offer some insights into this relationship. In the present study, soreness increased and muscle function decreased following the exercise bout as expected. However, whereas BCAA supplementation decreased muscle soreness, it has no impact on impaired muscle function. The most likely explanation for the apparent disconnect between the alleviation of muscle soreness with impaired muscle function is methodological. We used electrically-stimulated maximal voluntary contraction to test function of the muscle independent of motivation - or lack thereof - thus minimizing, if not eliminating, any reluctance to exert maximal force due to elevated muscle soreness. Alternatively, or additionally, the decrease in muscle soreness with BCAA supplementation may not have been sufficient to influence muscle function. More investigations specifically designed to investigate the relationship between soreness and function should be conducted.

Previous authors attributed the attenuation of deleterious indices of lengthening contractions with amino acid supplementation to a modulated protein turnover (23). It is clear that essential amino acids stimulate muscle protein synthesis following exercise (25, 26), and resistance exercise with lengthening component stimulates muscle protein synthesis (19). Muscle protein turnover rates are low compared with other tissues (28) thus increases in the rate of muscle protein remodelling is likely to be insufficient to remodel enough proteins to explain these results in the short recovery period implemented in the present and previous

studies. No study to date has measured protein synthesis and breakdown in an experimental model of lengthening contraction based exercise and nutrition supplementation. It is difficult to comprehend that increased protein synthesis would account for improvements observed during only 2-3 days of recovery. Furthermore, it is difficult to imagine how increased net muscle protein balance could alleviate soreness even if turnover rates were very high.

Intense lengthening contractions are known to elicit the release of CK and Mb into the blood. In the present study we observed increased plasma concentrations of both CK and Mb following exercise, however, BCAA ingestion did not alter the response. When comparing the magnitude of the CK response to endurance exercise in previous studies (6) with that observed in the present study, lengthening contractions if induces a higher CK response than the majority of endurance exercise. A reduction in CK activity with BCAA supplementation has previously been observed following lengthening contractions (6). CK and Mb levels are often erroneously considered as markers of muscle damage, in fact they are actually indicative of membrane disruption and 'leaking' of proteins from the muscle cell into the circulation. We measured myofibre concentrations to allow comparisons between other studies however, results showed a large variation in CK values between individuals. We demonstrate the peak response to be at 8 h post the exercise protocol which is earlier than previously demonstrated (29). However, within our results some subjects showed very high levels of CK compared to other individuals that showed very little increase in CK. Large inter-individual variability often observed in CK concentrations (4) may have accounted for the discrepancies in peak between this and previous studies (29). There were no correlations between the increase in CK and loss of muscle force, which is a more practical measure of the ability of the muscle to perform. These data add to existing data which suggest that myofibre protein responses are individualised and therefore may be considered weak predictors of muscle damage (29).

It is important to consider the practical implications of these results. Any attempt to extrapolate data generated in the present study to a trained population maybe non-sensical since untrained subjects were recruited. Athletes may respond differently due to the repeated bout effect (5, 14, 17) and the associated adaptations. However, these data may be applied to individuals starting an exercise regime or athletes returning to training following injury or off-season may benefit from supplementing their diet with BCAA's.

In summary, supplementing a controlled diet with 29.2g/day of BCAA directly before and during short term recovery resulted in a decrease in soreness with the knee flexed and extended. However BCAA supplementation failed to ameliorate impairments in muscle function typically associated with intense lengthening contractions. No alterations in levels of myofibre proteins were observed in the plasma with BCAA supplementation. In conclusion BCAA is an effective aid in reducing soreness following intense lengthening contractions in healthy, untrained males. However, further studies are required to determine the mechanisms by which BCAA ingestion may lead to reductions in muscle soreness following multiple lengthening contractions.

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# Chapter 3: Whey protein ingestion attenuates muscle soreness and reduces decrements in muscle function in the first few hours following unaccustomed intense lengthening contractions

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## 3.1 Abstract

**Purpose:** Repeated lengthening muscle contractions or unfamiliar exercise leads to loss of muscle strength, an increase in muscle soreness and elevated concentrations in the blood of myofibre proteins. Recently, nutritional interventions, including protein, have been suggested to alleviate these symptoms. The aim of the present study was to measure the impact of whey protein supplementation on the negative indices associated with multiple lengthening contractions under controlled conditions. **Method:** The present study was a parallel, matched, single-blind design. Twenty four non-weight trained males ingested either whey protein beverages (WP) or carbohydrate beverages (CHO) for 3 d following an intense bout of lengthening contractions. Diet was controlled throughout the testing period to match habitual energy intake. The exercise consisted of 12x10 repetitions of unilateral lengthening contractions of the knee extensor muscles at 120 % concentric one repetition maximum. Four supplements were consumed each day over a 3 d recovery period. Muscle soreness, muscle function and putative blood markers of muscle damage were assessed pre and post (1, 8, 24, 48, 72 h) exercise. **Results:** Maximal isometric strength decreased after the exercise bout ($P<0.001$). Additionally, there was a beneficial effect of ingesting WP on muscle function at all time-points post exercise. Protein ingestion resulted in reduced muscle soreness compared to carbohydrate ingestion at 8 h in general (WP: 10 ± 3 mm; CHO: 19 ± 3 mm) and in the flexed (WP: 12 ± 2 mm; CHO: 22 ± 4 mm) and extended (WP: 19 ± 3 mm; CHO: 32 ± 4 mm) positions. There were no differences in creatine kinase or interleukin-6 concentrations between conditions. **Conclusion:** These data suggest that protein supplementation may alleviate muscle soreness and reduce decrements in maximal force production.

**Keywords:** Nutrition, unaccustomed exercise, delayed onset muscle soreness, muscle function

## 3.2 Introduction

It is well established that unaccustomed lengthening contractions results in temporary decrements in maximal force production, low frequency fatigue, reduced range of motion, increased muscle soreness, increased inflammation and an increase in concentrations of myofibre proteins in the blood (6, 12, 18, 20). Recently, nutritional interventions, including protein and /or amino acids, aimed to reduce the negative indices associated with intense lengthening contractions have received considerable attention.

Whereas the effectiveness of ingesting a source of amino acids to attenuate the decrease in muscle force production following lengthening contractions has been investigated, the effect of these amino acids remains equivocal. Whey protein hydrolysate was shown to accelerate the recovery of muscle force production following 100 lengthening contractions (4). Impairments in maximum strength of squat and bench press were attenuated with 0.4 $g \cdot kg \cdot d^{-1}$ of amino acid supplementation in the first week of a 4 wk overreaching resistance protocol (14). Similarly, supplementation with a selection of 12 amino acids resulted in a decrease in the loss of muscle force following exercise involving lengthening contractions (23). However, in another study (18), when these 12 amino acids were ingested pre and post lengthening contractions and when supplementation was continued over 4 d following the exercise bout no improvements were observed in maximal voluntary contraction compared to a placebo. We previously demonstrated that there were no differences in strength loss when branched chain amino acid (BCAA) beverages were compared with a placebo four times a day over a 3 d recovery period (11). Differing results between studies may be attributed to the differences between the methods used to measure muscle function, the type of supplement and length of supplementation. In the present study, we replicated the exact exercise protocol that we previously implemented (11) to allow for comparison between our present and previous

results. Additionally, we used electrical stimulation when measuring maximal muscle force productions to ensure that the values obtained were not influenced by voluntary effort or soreness of the subjects.

In addition to differences in the exercise bout (intensity, muscles exercised), dietary control may help explain discrepancies between studies. Many studies did not control diet. Changes in energy and/or protein intake may have increased the variability in the response to the multiple lengthening contractions that may have obscured any response to the interventions. Therefore it is difficult to determine whether discrepancies between studies are caused by the supplementation or variations in habitual energy and protein intake. Thus, we provided all food for all subjects during the entire period of the study.

Despite equivocal findings in relation to amino acid ingestion and the effect on muscle force production following lengthening contractions, evidence suggests that muscle soreness can be attenuated with amino acid supplementation. We recently demonstrated that under conditions of strict dietary control, supplementation of BCAA resulted in decreased muscle soreness (11). Other authors also have observed decreased soreness with supplementation of BCAA (22) and mixed amino acids that included BCAA (18). Thus, it is possible that the BCAA are the active ingredient in amino acid mixtures shown to attenuate soreness. On the other hand, other studies contradict these findings (18, 29). Different findings between studies may be due to the length of supplementation. The studies that observed no differences in muscle soreness with amino acid supplementation only provided supplements on the day of the exercise (18, 30), whereas those that reported decreased soreness provided the amino acid source for at least 3 d following the exercise (11, 18). Therefore in the present study we had a 3 d supplementary period and matched the BCAA content to that of our previous study.

The aim of the current study was to determine if whey protein supplementation could attenuate the loss in maximal force production, rise in soreness and rise in inflammation when diet was controlled over a 3 d recovery period from intense exercise bout consisting of lengthening contractions. We hypothesised that similar to BCAA ingestion whey protein supplementation would ameliorate the increased muscle soreness observed following the exercise bout.

## 3.3 Method

### 3.3.1 Participants

Non–resistance trained males (n=24) were recruited for this study, which was approved by the University of Birmingham, School of Sport and Exercise Sciences Ethics Subcommittee. Subjects were made aware of procedures including the risks and benefits, gave written informed consent (Appendix A) and completed a general health questionnaire (Appendix B). Participants were able to withdraw from the study at any point without provision of reason. All participants refrained from physical activity and alcohol consumption for 2 d prior to testing and throughout the testing period. Participants were excluded if they had a previous history of resistance training or had completed high intensity lengthening contractions within the last 6 wk.

### 3.3.2 General Design

Subject characteristics are shown in Table 3.1. Subjects were assigned to either a whey protein (WP) or iso-energetic carbohydrate (CHO) group and performed one single-blind experimental trial. Groups were matched for weight and one repetition maximum (1RM). An

intense exercise protocol consisting of lengthening contractions was implemented to allow comparison of putative blood markers of muscle damage, muscular dysfunction and muscle soreness between WP and CHO. Subjects consumed four supplements daily between meals. The composition of the supplement consumed by participants is detailed in Table 3.2.

**Table 3.1: Composition of supplements**

| | Body mass (kg) | Height (m) | 1RM (kg) | Leg volume (mL) |
|---|---|---|---|---|
| WP | 71.3 ± 4.3 | 182 ± 2.5 | 59.7 ± 9.2 | 5617 ± 1354 |
| CHO | 72 ± 10.8 | 177 ± 4.3 | 62.5 ± 9.8 | 5795 ± 1289 |

Values are expressed as means ± SD
1RM = one repetition maximum
Leg volume = volume of upper leg which was exercised calculated via anthrompetry (31).

**Table 3.2: Composition of supplements**

| | Protein (g) | Carbohydrate (g) | Flavouring (g) | Water (mL) |
|---|---|---|---|---|
| WP | 25.8 | 0 | 3.5 | 300 |
| CHO | 0 | 25.8 | 3.5 | 400 |

### 3.3.3 Preliminary testing

Subjects visited the laboratory for preliminary measurements on two occasions. On the first, leg volume of the non-dominant leg was calculated (31). Determination of concentric 1RM of their non-dominant leg followed using a leg extension machine (Cybex international, USA). Subjects lifted an initial load that was then increased in increments of 2.5 kg. The subjects were instructed to lift the weight, hold it out-stretched for 2 s and then slowly lower it. Subjects rested for 3 min between lifts to minimise the impact of fatigue. Subjects were given a second attempt after the rest period if they failed with any given lift. This laboratory visit also served as familiarisation for the lengthening contractions exercise protocol that followed. The familiarisation involved two lifts at 50 % of their concentric 1RM in which the weight was lifted by the researchers for the subject to lower. Subjects were secured into the chair to ensure that the load was lifted using only their leg. The subjects' non-dominant upper leg was positioned at $90^{o}$ to their torso with their knee flexed to ~$110^{o}$. The lift started with

the exercising knee positioned at ~15$^{o}$ of flexion. Two testers lifted the weight to its full extension; a 'contract' command prepared the subject for the weight to be lowered. This command was followed by the command 'release' and the weight was released onto the subjects' leg, and was then lowered by the subject while resisting the load. The weight was then lowered through 95$^{o}$ to bring the load back to its resting position. Subjects aimed to control the weight in the downward movement for 4 s. On the second visit to the laboratory, preliminary measurements were taken. These involved the measurements taken at each time-point, i.e. visual analogue scale (VAS), a blood draw and a muscle function test.

### 3.3.4 Diet and activity

Prior to each trial, subjects were instructed to record a weighed 3 d diet record. An average energy intake from these 3 d was calculated and used to determine the energy requirements of each subject. All food was prepared and supplied to the subjects to ensure that i) any differences observed could be attributed to the supplements and ii) diets were comparable between subjects. The diet was composed of 55 % carbohydrates, 1.5 g protein/kg body mass (BM) with the remainder of the energy from fat, which was similar to their habitual diet. Prepared food was administered the day before, the day of, and 2 d following the exercise protocol.

### 3.3.5 Experimental Protocol

On the day of the exercise protocol, subjects reported to the laboratory at ~06:00 h following an overnight fast (Figure 3.1). The subjects ingested their first supplement followed by 30 min rest. The exercise protocol was then performed which lasted ~45 min. Subsequently, subjects rested for 60 min before the first measurements were carried out. Pain

was assessed using the VAS, followed by a blood draw from a forearm vein using a standardised venepuncture technique. Finally, muscle function was assessed via maximal isometric strength (MIS) as described below. The order of these measurements was standardised for the 8, 24, 48, and 72 h post exercise. Following completion of the 1 h testing period, participants consumed another supplement before breakfast. A third supplement was ingested between lunch and dinner and the fourth at bedtime. On the following 2 d supplements were consumed between meals. All measurements were taken in the morning in the fasted state with the exception of the 1 and 8 h time-point. To maximise measurement reliability, the same investigator carried out all measurements for each subject.

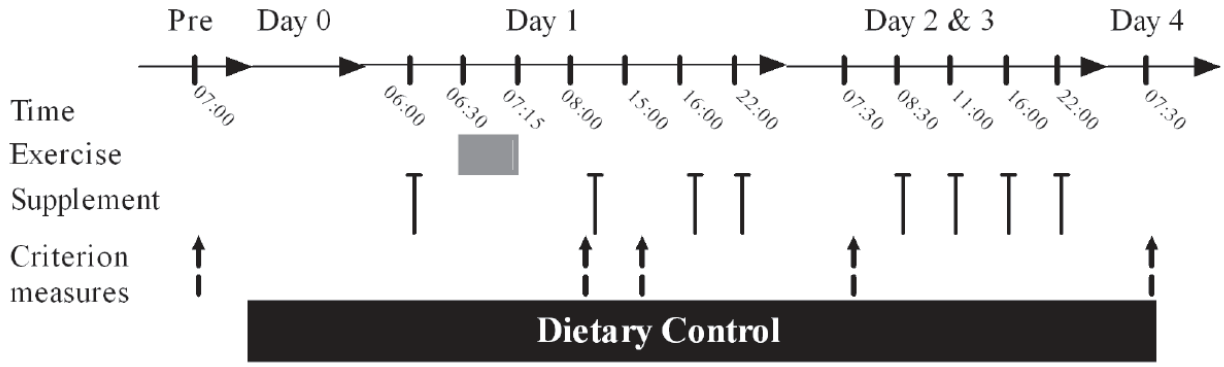


**Figure 3.1: Schematic protocol depicting the duration of the study.**
Criterion measures include; muscle soreness, blood draw, muscle function test. Exercise = 12 sets of 10 repetitions of lengthening contractions of the knee extensor muscles at 120% concentric 1RM. Supplements were consumed 4 times on the day of and two days following the exercise and contained either protein (WP) or carbohydrate (CHO).

### 3.3.6 Lengthening contractions protocol

Subjects were seated as previously described (11) and completed 12 sets of 10 lengthening contractions at 120 % concentric 1RM, with 5 s rest between each repetition and 1 min rest at the end of each set. If the subject failed to control the weight for two consecutive repetitions, a rest period of 30 s was enforced. During each lengthening contraction subjects were verbally encouraged to aid maximum effort.

### 3.3.7 Muscle function

MIS was assessed using electrical stimulation with a Tornvall chair (7). Participants were seated so the knee was at $90^{o}$ with a cord fastened round the ankle. This cord was attached to a strain gauge interfaced with a computer. Two arm straps held the subject in place in the chair. Damp electrodes (13 by 8 cm) were secured to the thigh over the proximal and distal ends of the quadriceps. On the first visit the subjects were familiarised to the electrical stimulation and current was increased until there was no further increases in force production. 1.3 times greater than this current was then used for the superimposed electrical twitch simulations. Participants were electrically stimulated at their maximal electrical stimulation three times before being encouraged to push out with their leg against the cord with as much force as possible. Three electrical twitch stimulations were superimposed during muscle contraction to ensure that the contraction was maximal. This procedure occurred three times at each time-point with a rest of 3 min between attempts. The highest force obtained for each leg represented the subjects' MIS.

### 3.3.8 Muscle soreness

Subjects rated muscle soreness generally and with the knee flexed and extended using a VAS. The VAS consisted of a 50 mm line with anchor points of 'no soreness' on the left and 'extremely sore' on the right. Subjects marked a point along the line that represented the perceived soreness experienced in the quadriceps muscle group. Pain was given a value by measuring the distance from the left anchor point. It has been shown that 90% of pain ratings can be repeated within 9mm using the VAS (2).

### 3.3.9 Blood Analysis

Plasma concentration of CK was measured as a putative marker of muscle damage. Serum was collected and analysed for interleukin-6 (IL-6) as an indirect marker of inflammation. Plasma samples were placed on ice, and serum samples were left at room temperature for 30 min to allow clotting. Samples were centrifuged at 3000 revolutions per min for 10 min at 4 $^{o}$C. Thereafter, plasma and serum were extracted and stored in the freezer at -20 $^{o}$C until further analysis. Enzymatic analysis for plasma concentrations of CK (CK NAC CP, ABX diagnostics, UK) was performed in duplicate at each time-point using a semi-automated analyser (COBAS MIRA S-plus, ABX, UK). Serum concentrations of IL-6 were analysed using a commercially available sandwich enzyme-linked immunoabsorbent assay (ELISA) (High sensitivity Human IL6 assay, R&D systems, UK). Plates were read in duplicate on a Labsystems Original Multiskan MS at selected wavelengths (490 nm with correction at 630 nm). Reported sensitivity of the IL-6 ELISA is 0.039 pg/mL.

### 3.3.10 Data presentation and statistical analysis.

MIS data were taken from the best value of three attempts and converted into Newtons from the volts value obtained. MIS values are displayed as a percentage of the pre-exercise value to show the force that was lost. The MIS value recorded at pre was used as the baseline.

VAS scores are shown over the course of the study period. Moreover, we calculated area under the curve (AUC) to give an indication of soreness over the total 72 h experimental period. Since any differences between groups seemed to be due primarily to changes in the first 24 h, we also chose to calculate AUC of soreness for only the first 24 h period. AUC baseline values were calculated from the mean of preliminary values. Plasma CK concentrations and serum IL-6 concentrations are shown as the mean of the raw concentrations.

Data were analysed using statistical package for social sciences (SPSS) 17.0 for Mac (SPSS Inc., Chicago, IL). Differences across time for blood concentrations, force loss and VAS scores were analysed by a mixed design, two-way ANOVA with two between (WP, CHO) and six or four (depending on variable analysed) within (time-points) subject variables. AUC values were compared using an unpaired students t-test. Significance was set at the 0.05 level of confidence. Where significance was found, least significant difference (LSD) post hoc tests were run to distinguish differences between supplementary groups. Missing data points were averaged from the other data sets for that time-point and group. This manipulation allowed a higher number of subjects to be included in the analysis to improve statistical power.

Data also were analysed using inferential statistics with 90 % confidence limits to produce magnitude-based values about the probabilities of the outcome (1). The likelihood of the outcome being of beneficial or detrimental nature also was determined using a published spreadsheet (9). The *P* value (Probability value), , degrees of freedom, percentage level for confidence, threshold values (meaning of an effect) and an effect statistic were entered. The value of an effect statistic was calculated as the difference between the mean of each group. For overall general soreness, an effect was assumed to be a change of 137 mm * 72 h and 50 mm * 24 h for flexed soreness, this value was 146 mm * 72 h, and 54 mm * 24 h and for extended soreness this value was 137 mm * 72 h and 50 mm * 24 h. These values were derived from the smallest standardized (Cohen) difference in mean (0.2) multiplied by the standard deviation of CHO (control condition). For MIS each time-point post exercise was compared back to the corresponding pre value and inferences were made on the comparison of these differences between groups. Inferential values were derived from the log of raw data and calculated using a published spreadsheet (8). When the value is determined to be <1%

detrimental or beneficial it is almost certainly not; 1-5 % very unlikely, 5-25 % unlikely, 25-75 % possible, 75%-95 % likely, 95-99 % very likely and >99 % almost certainly.

## 3.4 Results

### 3.4.1 Muscle Function

A decrease in MIS (Figure 3.2) was apparent in all subjects following exercise. The mean maximal percentage of force loss assessed using MIS was observed 24 h post exercise in WP (-59 ± 7 %) and 48 h post exercise in CHO (-57 ± 7 %). Using inferential statistics protein ingestion was likely beneficial at 1 (95 % beneficial) and 8 h (80% beneficial) post exercise.

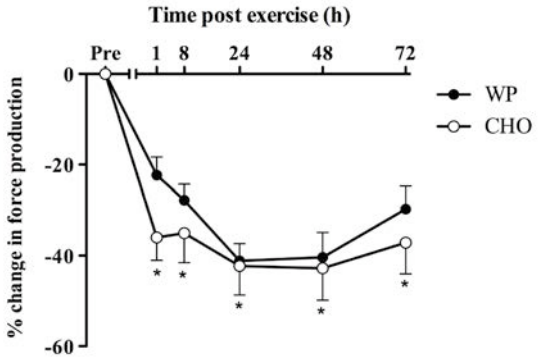


**Figure 3.2: Percentage change in leg muscle function pre and following 120 lengthening contractions.** Depicted as electrically stimulated maximal isometric strength. Data expressed as means ± SE. * significant difference from baseline in both groups. Supplements were consumed 12 times over the testing period and contained either protein (WP) or carbohydrate (CHO). Inferential statistics show a beneficial effect of WP compared to CHO at 1 and 8 h.

### 3.4.2 Muscle Soreness

The temporal pattern of muscle soreness to multiple lengthening contractions over the 72 h period was similar in all three positions. Soreness with the knee in a flexed position is shown in Figure 3.3A. The exercise protocol resulted in increased overall soreness in both groups. Soreness ratings were lower at 8 h compared with 1 h (p=0.03) in both groups. In all positions of the leg, peak soreness was observed at 1 h in CHO and at 48 h in WP. Additionally

soreness was significantly lower in an extended position at 1 h post exercise in WP compared to CHO.

Over the total 72 h experimental period when data are expressed as AUC, muscle soreness with the knee flexed was 30 % less for WP compared to CHO (Figure 3.3B). However, t-test indicated no statistical difference between groups. Inferential statistics showed WP was likely beneficial for amelioration of overall muscle soreness in all knee positions (Table 3.3).

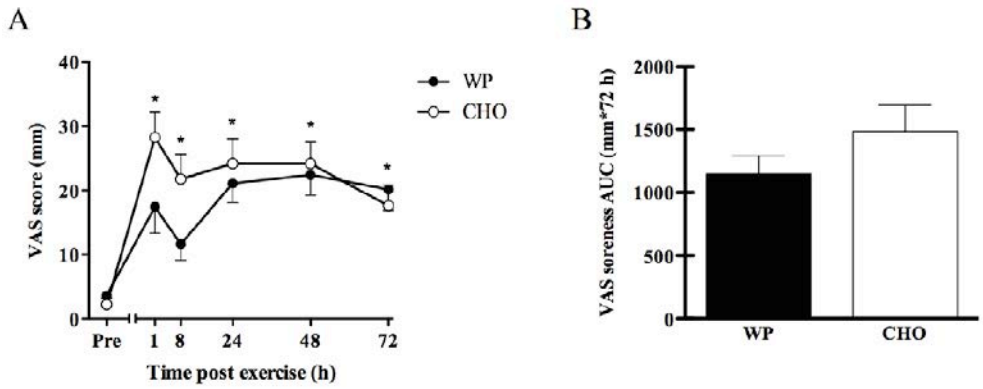


**Figure 3.3: Muscle soreness pre and following 120 lengthening contractions measured using a 50 mm visual analogue scale (VAS). a) knee in a flexed position over 72 h, b) area under the curve of flexed muscle soreness over the entire 72 h period.**
Data are expressed as means ± SE. * significant difference from baseline in both groups. Supplements were consumed 12 times over the testing period and contained either protein (WP) or carbohydrate (CHO). Inferential statistics show a beneficial effect of WP compared to CHO.

**Table 3.3: Muscle soreness inferentials for 72h period**

| Knee position | P value | Value ± 90% confidence limits | Outcome |
|---|---|---|---|
| General | 0.1369 | 290 ± 320 | 79% (likely) beneficial |
| Flexed | 0.1 | 340 ± 340 | 83% (likely) beneficial |
| Extended | 0.1848 | 330 ± 420 | 79% (likely) beneficial |

Over the first 24 h period only, total soreness in all three positions expressed as AUC was significantly lower in WP than CHO (Figure 3.4). Evaluation using inferential statistics showed WP was very likely beneficial for amelioration of overall muscle soreness in the first 24 h following the exercise bout with the knee in all positions (Table 3.4).

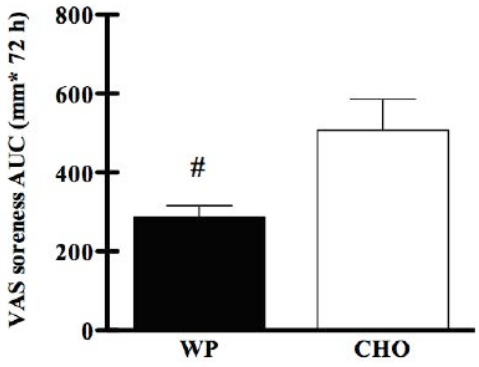


**Figure 3.4: Muscle soreness with knee flexed for the first 24 h as area under the curve (AUC) following 120 lengthening contractions measured using a 50 mm visual analogue scale (VAS).**
Data are expressed as means ± SE. # significant difference between groups. Supplements were consumed 12 times over the testing period and contained either protein (WP) or carbohydrate (CHO). Inferential statistics show a beneficial effect of WP compared to CHO.

**Table 3.4: Muscle soreness inferentials for the 24h period**

| Knee position | P value | Value ± 90% confidence limits | Outcome |
|---|---|---|---|
| General | 0.0153 | 190±130 | 97% (very likely) beneficial |
| Flexed | 0.0077 | 220 ±130 | 98% (very likely) beneficial |
| Extended | 0.004 | 250±130 | 99% (almost certainly) beneficial |

### 3.4.3 Blood parameters

Serum IL-6 data are depicted in Figure 3.5. IL-6 concentrations significantly increased following the exercise protocol in both groups. IL-6 concentration had returned to baseline by 24 h post exercise. No differences in IL-6 concentrations were observed between WP and CHO.

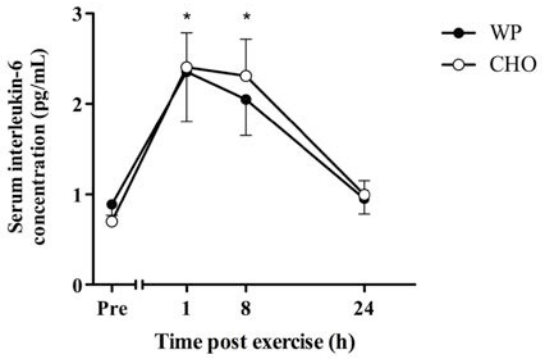


**Figure 3.5: Serum interleukin-6 concentration measured pre-, 1, 8 and 24 h post- exercise.**

Data are expressed as means ± SE. * significant difference from baseline in both groups. Supplements were consumed 12 times over the testing period and contained either protein (WP) or carbohydrate (CHO).

Plasma CK concentration increased post exercise and remained elevated throughout the recovery period (Figure 3.6). Peak CK concentrations were observed at 8 h post exercise in CHO and 72 h post exercise in WP. No significant differences were observed between groups for plasma concentration of CK. A large inter-subject variability was observed in CK responses, with peak values ranging 100-fold (184 -18150 U/L).

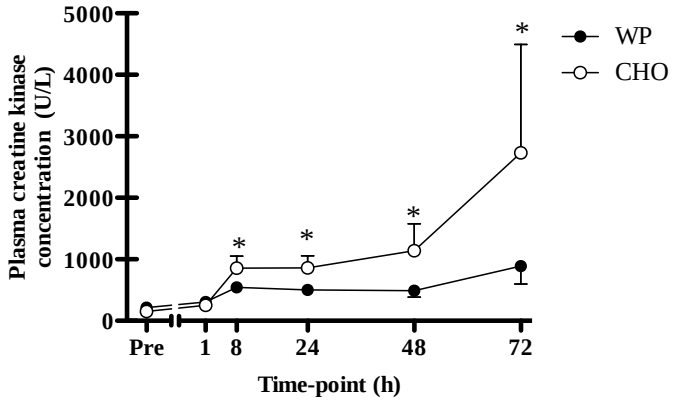


**Figure 3.6: Plasma creatine kinase concentrations in the plasma pre and following 120 lengthening contractions.**
Data are expressed as means ± SE. * significant difference from baseline in both groups. Supplements were consumed 12 times over the testing period and contained either protein (WP) or carbohydrate (CHO).

## 3.5 Discussion

The present study investigated whether the ingestion of whey protein, providing BCAA in amounts previously demonstrated (11) to reduce muscle soreness before and following intense lengthening contractions, improved indices of recovery when compared to an isoenergetic beverage. We observed a reduction in overall soreness in the quadriceps muscle over the first few hours following the exercise bout with whey protein supplementation. Furthermore, we determined that there was a likely beneficial effect of whey protein supplementation on muscle soreness over the entire 72 h period. Additionally, there

was a likely beneficial effect of whey protein on muscle function at 1 and 8 h post exercise. However, no differences in plasma concentration of a putative marker of muscle damage (CK), or an indirect measurement of inflammation (IL-6) were detected between whey protein and an isoenergetic placebo when supplements were provided pre and for 3 d following an intense bout of lengthening contractions. Thus, it seems whey protein ingestion following multiple intense lengthening contractions impacts muscle soreness and muscle function within the first few hours of recovery.

The impact of whey protein ingestion on muscle soreness seems to be due primarily to the amino acid content of the protein rather than other factors such as energy content. In the present study we aimed to compare the effects of a complete amino acid source with that of an isoenergetic non amino acid containing beverage. We recently demonstrated that supplementing a controlled diet with BCAA's only resulted in reduced overall soreness at 48 h and 72 h post exercise (11). Additionally, a preliminary study (22) reported reduced muscle soreness following squat exercise. Supplementation with mixed amino acids (9 essential and 3 non essential), including BCAA, has previously been reported to reduce muscle soreness (18). In the present study untrained subjects ingested all amino acids, including the same amount of BCAA as in our previous study (11) and we observed a reduction in muscle soreness. Therefore, taken together, these data suggest that the beneficial effect of the whey protein on muscle soreness may be related to the BCAA content. Moreover, the addition of other amino acids as in the present and a previous study (18) do not hinder this amelioration of muscle soreness. It would be interesting to compare the effects of a whey protein containing beverage with a beverage containing only the NEAA to see if the NEAA are required.

Negligible effects of supplementation on muscle soreness also have been previously reported. Interestingly, mixed amino acids (18) and protein plus carbohydrate (30)

supplements have been shown to have no influence on muscle soreness. The reason for the discrepancy between the present and previous studies is unknown. Careful comparison between study designs reveals a range of exercise intensities, limbs used, supplements provided, timing of supplementation and, for many, a lack of dietary control. Another factor that seems to be important is the length of supplementation period. When protein was provided pre or post exercise only no effect was observed on muscle soreness (30). We provided multiple supplements over a 3 d recovery period and observed improvements in soreness in the present and previous study The importance of an extended supplementary period is further supported by Nosaka et al (18), who demonstrated that with the same supplement content and exercise implemented (intensity and limb used), there was only a reduction in soreness when supplements were provided over 3 d of recovery. Thus, even within the same research group and when the same supplement is used the length of supplementation appears to influence the results. However, there are some studies that observed decreased soreness with only one dose of amino acid ingestion (21, 22). Therefore, a systematic investigation of the various aspects of study design and effect on muscle soreness should occur before solid recommendations can be made.

Whereas in the present study we observed a reduction in soreness with whey protein supplementation, the mechanism cannot be deduced using our data. It previously has been suggested that the feeling of pain is caused by inflammation of the perimysium or epimysium (15). A sensitization of the muscle nociceptors caused by the inflammatory response results in the common feeling of soreness (20). Intense lengthening contractions are known to lead to an inflammatory response which may be represented by an increased the circulating concentrations of the inflammatory cytokine IL-6 (3). However, as in previous studies (11, 16) in the present study we observed no differences between supplementation groups in the

response of IL-6 when whey protein or carbohydrate was ingested. Thus, our data do not support the suggestion that reduced muscle soreness with whey protein supplementation was modulated via an inflammatory response. However, the measurement of IL-6 is only looking at one inflammatory pathway, and does not solely represent muscle inflammation. Future studies are required to fully elucidate the mechanisms that result in the decreased soreness with amino acid supplementation, these studies may include other measures of inflammation including magnetic resonance imaging to measure oedema or swelling in the exercised limb.

It is possible that muscle soreness may contribute to the reduction of muscle function. It is known that an increase in perceived pain decreases performance (26). It also has been suggested that the cause of force loss with lengthening contractions, as in the present study, is partially driven by central mechanisms, but mainly caused by peripheral mechanisms (10). In the current study we employed electrical stimulation to obtain MIS. The use of electrical stimulation allowed us to measure muscle function independently of motivation, therefore likely diminishing resistance to maximally exert force due to pain. Hence, we attempted to reduce central mechanisms that might confound results. Thus, we compared force generated with whey protein supplementation and the control condition observing only peripheral mechanisms. Even when using electrical stimulation, we still observed decrements in muscle force in both conditions, suggesting that the force loss was caused by a peripheral mechanism. Furthermore, there was a reduced loss of muscle function and amelioration in soreness with whey protein ingestion compared with carbohydrate. Therefore, protein may impact peripheral mechanisms that cause decreased muscle force following intense lengthening contactions. However, from the data collected in the current study we are unable to deduce mechanisms to explain how ingestion of whey protein impacts peripheral mechanisms related to force production.

The timing of supplementation or the form of ingested protein may be important for attenuating force loss from lengthening exercise. In the present study we provided whey protein isolate and observed an amelioration of the decrement in muscle function over the first few hours following resistance exercise. Our results contradict those of Buckley *et al.* (4) who demonstrated that whey protein hydrolysate, but not whey protein isolate, resulted in an attenuation of the decrement in muscle function following lengthening contractions. We provided supplements pre and post exercise, however Buckley *et al.* (4) provided the first supplement after the post exercise measurements. Thus, it is possible that the provision of whey protein isolate post the exercise bout only, does not effectively attenuate decrements in muscle function. Thus, we suggest whey protein isolate may be effective for attenuating loss of maximal force production after intense lengthening contractions if provided both pre and post exercise. However, more studies should be completed in order to fully understand if there are any differences in muscle function recovery between ingesting whey protein hydrolysate and isolate following lengthening contractions.

From our data the cause of improved muscle function over the first few hours of recovery with ingestion of whey protein compared to carbohydrate is unclear. Previous authors attributed the attenuation of deleterious indices of multiple lengthening contractions with amino acid supplementation to a modulated protein metabolism (22). However, it is highly unlikely that any differences in muscle function in the first few hours can be attributed to increased protein synthesis. It is known that protein ingestion can stimulate muscle protein synthesis (13, 24, 25). However, the turnover rate of muscle protein synthesis is low (28), thus any increases in muscle protein synthesis is unlikely to remodel proteins sufficiently in the short time frame in which we observed changes in muscle function. Therefore, future studies

should look to examine the mechanisms by which decrements in muscle function are reduced with whey protein ingestion.

Plasma CK concentration increases following lengthening contractions. Myofibre proteins are often used as markers of muscle damage. However, elevated concentrations of myofibre proteins in the blood are more likely indicative of loss of membrane integrity due to disruption of structural elements within the muscle (29). This notion is further supported by evidence that the release of myofibre proteins into the blood can be prevented in rats if vitamin E is provided (27) due to the interaction with the membrane phospholipids (19). In our hands, whey protein had no impact on reducing CK concentrations following intense lengthening contractions. Previously, blood concentrations of myofibre proteins were decreased with supplementation of mixed free amino acids ingested in a fed state 30 min pre exercise, post exercise and during a 4 d recovery period (18). However, various other amino acid supplementation strategies have failed to reduce the increase in myofibre proteins observed following intense lengthening contractions (11, 18). Reasons for these discrepant findings are likely due to substantial variability observed between individual CK responses to eccentric exercise. We observed a wide variation in responses - up to 100-fold. Our observation of high inter-subject differences is consistent with existing literature (29) and further highlighted the inherent problems associated with using CK as a predictor of muscle damage. We used parallel group design, thus different subjects in each group may have contributed to the lack of observed differences between experimental groups.

The practical implications of the results from this study are not entirely clear. These results suggest that ingestion of whey protein, as a supplement, may be beneficial for reducing soreness and decrements in muscle function primarily during the first few hours following exercise when compared to ingestion of carbohydrate. However, if compared to previously

published data (11) ingestion of whey protein reduces the decrement in muscle function more than water in the later stages of recovery. We chose to measure the responses in untrained individuals to maximise our ability to detect any differences. The responses may very well be different in those individuals more accustomed to this type of exercise (5, 17). Thus, the impact of whey protein on soreness and muscle function may be limited to untrained individuals and may not apply to those who train regularly. It is possible that individuals beginning a training regime or athletes returning to training following an injury may show similar responses and therefore benefit from whey protein ingestion. However, as discussed previously (11), the physiological relevance of this finding may be questioned. Particularly for previously sedentary individuals, it is possible that reduced soreness immediately following exercise from ingesting whey protein may help to increase exercise adherence. Thus, our results may be used to suggest ingestion of whey protein as an aid to help increase exercise participation.

In summary, supplementing a controlled diet with 103 g/d of whey protein directly before and during short-term recovery from multiple lengthening contractions resulted in a decrease in muscle soreness and attenuated decrements in muscle function over the first few hours of recovery. In conclusion whey protein is an effective aid in reducing soreness and reducing muscle function decrements following intense lengthening contractions in healthy, untrained males.

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# Chapter 4: Branched-chain amino acid ingestion stimulates muscle protein synthesis following resistance exercise.

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Work funded by GlaxoSmithKline Nutritional Healthcare

## 4.1 Abstract

**Purpose:** Ingestion of essential amino acids are known to stimulate muscle protein synthesis. It is often speculated that BCAA ingestion is important for increases in muscle protein synthesis (MPS). However, no study has investigated the effect of ingesting only BCAA following resistance exercise on MPS. Therefore, the purpose of this study was to investigate the effect of ingesting BCAA on MPS, in association with leg resistance exercise. **Method:** Using a counterbalanced, crossover, double blind design, ten weight-trained males completed two trials consuming either a BCAA containing beverage or a placebo (PLA) post resistance exercise. Diet was controlled for two days prior to each infusion trial to match habitual caloric intake and dietary composition. The exercise consisted of 4 x 10 repetitions at 70 % one repetition maximum on a leg press machine and 75 % on a leg extension machine. Trials were performed following intake of a high protein (30 % energy) breakfast. Protein synthesis was measured over the 4 h recovery period by a primed, constant infusion of L-[*ring* $^{13}C_6$]phenylalanine. Muscle biopsies were obtained from the vastus lateralis immediately, 1 h and 4 h post resistance exercise and blood samples were collected throughout the trial. Western blotting was used to determine the phosphorylation status of P70 ribosomal protein S6 kinase (p70S6K) (Thr389 and Ser424/Thr421), ribosomal protein S6 (Ser235/236), 4E-binding protein (Thr37), protein kinase B (Thr308) and pras 40 (Thr246). **Results:** Plasma BCAA concentrations were significantly higher in BCAA than PLA following drink ingestion. Peak leucine (652 ± 43 nmol/L), isoleucine (355 ± 24 nmol/L) and valine (785 ± 47 nmol/L) were observed at 0.5 h post drink ingestion in BCAA and remained elevated for 3 h. FSR was 22 % higher in BCAA (0.11 ± 0.009%/h) than PLA (0.09 ± 0.006 %/h) over the 4 h recovery period. There were no differences between conditions in any of the signaling

proteins measured. **Conclusion:** Our results indicate that MPS can be further increased from resistance exercise by ingesting BCAA.

**Keywords:** Nutrition, intracellular signaling, protein, fractional synthetic rate

## 4.2 Introduction

Branched chain amino acids have long been associated with muscle anabolism. In vitro studies demonstrated that there was no increase in protein synthesis when the branched chain amino acids were omitted from amino acid perfusions (24). Studies in resting humans demonstrated a role of ingesting a specific branched chain amino acid, leucine, in muscle anabolism (29). In a rats leucine supplementation increased muscle protein synthesis following a running exercise above exercise alone (1, 2). However, the exercise resulted in decreased muscle protein synthesis, leaving the rats in a catabolic state. Therefore, leucine ingestion returned muscle protein synthesis levels to pre exercise levels, rather than increasing muscle protein synthesis above basal levels. The restoration of muscle protein synthesis rates was accompanied by increases in phosphorylation status of intracellular signalling proteins (1). In humans a decrease muscle protein synthesis has never been shown in the recovery period following exercise.. Additionally, the effect of leucine or branched chain amino acids on muscle protein synthesis in humans following exercise is unknown.

The exact mechanisms by which leucine or the branched chain amino acids exert their anabolic action remain unclear. However, increases in intracellular signalling lead to increased protein synthesis through elevated translation initiation and elongation in vitro and in animals. Leucine increases translation initiation and elongation signalling in resting humans (12). A recent study by Karlsson et al. (17) demonstrated that repeated ingestion of branched chain amino acids led to increase in phosphorylation of p70 protein kinase S6 (S6K1) at both Thr 389 and Ser424/Thr 421 sites following resistance exercise. Additionally, ribosomal protein S6 (rpS6) was stimulated with branched chain amino acid ingestion. Whereas no measure of synthesis was made by Karlsson *et al*. (17), it was assumed that this difference in phosphorylation of S6K1 and rpS6 would result in differences in muscle protein

synthesis (6, 15). However, it is important to note that assessment of anabolic signalling only provides a snapshot of potential translational control. It is for these reasons that direct measurements of muscle protein synthesis through stable isotopic tracer methodology are now widely employed in parallel with measures of signalling protein phosphorylation status.

It is well known that the ingestion of essential amino acids and protein following resistance exercise results in increases in muscle protein synthesis (8, 36, 42, 44). Whereas, the ingestion of leucine alone following resistance exercise has never been studied, the co-ingestion of leucine with both essential amino acids (EAA) (10) and whey protein (20-22, 41) has been investigated. However, the effect on muscle protein synthesis of ingesting additional leucine remains equivocal (10, 20-22, 41). The ingestion of leucine with protein or EAA resulted in greater post exercise muscle protein synthesis rates compared to exercise alone (10, 20). However, in these studies (10, 20) the effect of leucine is difficult to distinguish from that of the protein or EAA that was co-ingested, as there was no comparison to the ingestion of only EAA or protein following exercise. However, other studies that have investigated muscle protein synthesis rates following exercise after ingestion of leucine plus protein have made comparisons to protein ingestion alone and exercise (21, 22). This comparison allows the effect of leucine to be determined. These studies (21, 22) suggest that there was no additional effect on muscle protein synthesis with the ingestion of leucine plus protein compared to protein only. Similarly there is no further increase in muscle anabolism following exercise when leucine is added to whey protein compared to whey protein alone (41).

To date no studies have investigated the effect of branched chain amino acids ingestion alone following resistance exercise. There is reason to believe the ingestion of leucine alone results in a fall in the concentrations of other branched chain amino acids (41) likely due to increased branched chain alpha keto acid dehydrogenase activity (5). Therefore, to prevent a

fall in the concentrations of isoleucine and valine it would be logical to provide all branched chain amino acids within a supplement investigating the muscle protein synthesis. Hence, we provided all branched chain amino acids following a bout of resistance exercise. Branched chain amino acids have been shown to increase phosphorylation status of signalling proteins, however not alongside a measure of muscle protein synthesis. Thus, we measured signalling proteins simultaneously with muscle protein synthesis in trained human males. Resistance trained athletes typically train 2 -3 h after a meal. Hence, for practical reasons the study design was such that we provided a high protein breakfast so that the exercise would be completed following food intake. We hypothesised that the ingestion of branched chain amino acids following resistance exercise would not only increase the phosphorylation status of mammalian target of rapamycin (mTOR) related signalling proteins, but also increase muscle protein synthesis.

## 4.3 Method

### 4.3.1 Subjects

Eleven healthy males who completed regular resistance training of the lower body (≥ 2/ wk ≥ 6 mo) were recruited for the present study. Due to subject illness only 10 subjects completed both trials. Therefore data are shown only for 10 subjects. The mean (± SD) age was 20.1 (± 1.3) y, body mass 83.3 (± 11.7) kg, and percentage lean mass 86.1 (± 4.1) %. The design, purpose and potential risks associated with the study were explained to the subjects before obtaining written, informed consent (Appendix C). Exclusion criteria were metabolic disorders (as determined by health questionnaire (Appendix B)), food intolerances/allergies, allergies to local anaesthetic, current participation in other clinical trials, blood donations within three months of the initial screening visit and the prescription of medication or

nutritional/dietary supplements suggested to affect protein metabolism. All trials were conducted in the laboratories of the School of Sport and Exercise Sciences, University of Birmingham, following ethical approval by National Research Ethics Service ethics board (Warwickshire).

### 4.3.2 Study Design

Subjects reported to the laboratory on five separate occasions including two experimental trials separated by ≥ 3 wk. During the initial visit body composition was assessed using Dual-Energy X-ray Absorptiometry (DEXA) and one repetition maximum (1RM) was predicted for each leg individually. Approximately 1 wk later, subjects returned to the laboratory to confirm their 1RM on one leg. Two days later subjects performed their first blinded trial in which they consumed either a branched-chain amino acid containing beverage (BCAA) or a placebo (PLA) (Lucozade, GlaxoSmithKline, Brentford, UK) (Table 4.1). Subjects performed a unilateral bout of resistance exercise prior to consuming the test drink in each trial. Myofibrillar protein synthesis was determined by measurement of the incorporation of L-[ring-$^{13}C_6$] phenylalanine into muscle protein during a primed continuous infusion and urea production was measured through infusion of $^{15}N_2$ urea. The complete testing procedure was repeated on the contralateral leg ~3 wk after the first trial. Trial and exercised leg order were counter-balanced and randomised.

**Table 4.1: Contents of test drinks**

| | BCAA | PLA |
|---|---|---|
| Calories (Kcal) | 39 | 13 |
| Leucine (g) | 2.6 | 0 |
| Isoleucine (g) | 1.4 | 0 |
| Valine (g) | 1.6 | 0 |
| Carbohydrate (g) | 3.1 | 4.6 |
| Fat (g) | 0.01 | 0.02 |
| Sodium (g) | 0.2 | 0.2 |

Drinks were consumed after the unilateral bout of resistance exercise and were either a branched chain amino acid containing beverage (BCAA) or a placebo (PLA).

### 4.3.3 Preliminary testing

*4.3.3.1 Exercise sessions*

At least 6 d before and < 2 wk prior to the first infusion trial session, 1RM was predicted for both legs of each subject using a previously published method (45). Subjects warmed up each leg, before completing the maximum number of repetitions at 80 % of the subjects own prediction of 1RM. This load was then inserted into equation 1 to estimate 1RM (30).

$$1RM = \frac{load}{\left(1.0278 - 0.0278\right) \bullet reps} \quad (1)$$

Three days prior to the infusion trial days subjects returned to the laboratory to confirm their 1RM (23) on the leg that was to be exercised in the forthcoming trial. Briefly, the load was set to 90 % of the estimated 1RM from equation 1. Subjects completed the maximum number of repetitions possible. The load was then increased by 5-10% until only one repetition could be completed. A rest period of 3 min was given between each load.

*4.3.3.2 Diet and physical activity:*

Participants completed a weighed 3 d diet record prior to commencement of the testing period to provide an estimate of habitual macronutrient composition as analysed by a commercially available software program (Wisp V3.0, Tinuviel software). Food was provided for 2 d prior to the infusion trial and matched that of their individual habitual intake. Subjects were instructed to consume all food provided and asked to refrain from eating after 22:00 on the evening prior to the infusion trial. During this 2 d period subjects were asked to refrain from exercise and alcohol.

*4.3.3.3 Body Composition:*

Body composition was assessed using DEXA (Hologic Discovery W, Hologic Inc., Bedford, Massachusetts, USA).

### 4.3.4 Experimental Protocol

A schematic of the trial protocol is displayed in Figure 4.1. Subjects reported to the laboratory at ~ 06:15 following an overnight fast where standard measures of height and weight were taken. A cannula was inserted into a forearm vein of one arm, and a resting blood sample was obtained. Thereafter, subjects were fed breakfast (30 kJ/kg body mass (BM)) with 30% total energy provided by protein. After breakfast a primed, continuous infusion of $^{15}N_2$ Urea (Cambridge Isotope Laboratories Inc, Andover, Massachusetts, USA) was started and continued throughout the protocol. The priming dose relative to body mass was 88 μmol/kgBM and the infusion rate 0.227 μmol·min $^{-1}$·kgBM$^{-1}$. Subjects rested for ~ 75 min before a primed (2 μmol/kgBM) constant infusion (0.050 μmol·min$^{-1}$·kgBM$^{-1}$) of L-ring $^{13}C_6$ phenylalanine (Cambridge Isotope Laboratories Inc, Andover, Massachusetts, USA) was started.

105 min later, subjects performed a single bout of unilateral leg resistance exercise which lasted ~25 min. Subjects completed a warm up on the leg press machine (Cybex VR3, Cybex International, Medway, Massachusetts, USA), which consisted of 12 repetitions at 40 % 1RM, 10 repetitions at 50 % 1RM, eight repetitions at 60 % 1RM and two repetitions at 70 % 1RM. The warm up was followed by a 2 min rest period. The resistance exercise protocol consisted of four sets of 10 repetitions at 70 % and 75 % 1RM on the leg press and leg extension machine (Cybex VR3, Cybex International, Medway, Massachusetts, USA), respectively. Subjects rested for 2 min between each set and were free to consume water *ad libitum*. A floor standing fan was used to cool subjects and verbal encouragement was provided to all subjects throughout the protocol. If a subject could not complete a full set, the load was lowered by 4.5 kg. On completion of each set subjects were asked to rate their perceived exertion using a Borg scale (BORG). Immediately after exercise (t=0 min), an

arterialised blood sample and a muscle biopsy were collected followed by the ingestion of the test drink. Arterialised blood samples were also collected at t = -240, -145, -85, -25, 0, 15, 30, 45, 60, 75, 90, 120, 150, 180, 240 min. Two further muscle biopsies were collected at t = 60 and 240 min.

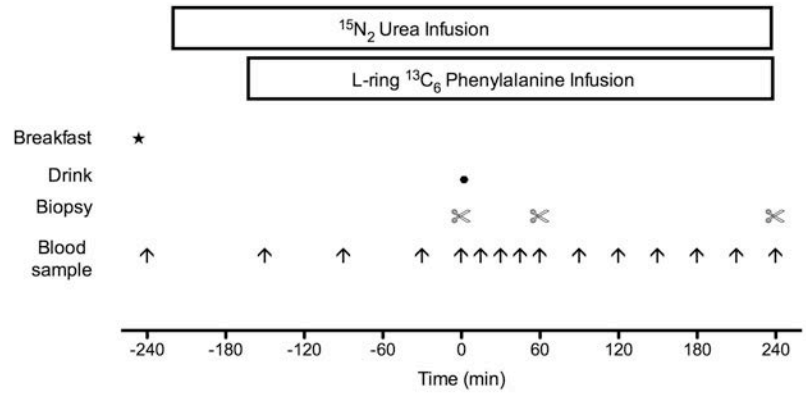


**Figure 4.1: Schematic diagram of the infusion protocol.**
Drink ingestion was of either a placebo or branched chain amino acid containing beverage.

### 4.3.5 Muscle biopsy collection and analysis

Muscle biopsies were obtained from the vastus lateralis of the exercised leg under local anaesthesia (1 % lidocaine) using 5 mm Bergstrom needles with suction. Different incisions (~1 cm apart) were used for each biopsy in an attempt to minimize the impact of local inflammation from the previous biopsy sample. Biopsy samples were immediately rinsed, blotted of excess blood, removed of visible fat and connective tissue and divided into aliquots, before being frozen in liquid nitrogen, and stored at -80°C until later analysis. Muscle samples were analysed for enrichment of L- [*ring*-$^{13}C_6$] phenylalanine in the intracellular pool and bound myofibrillar protein fractions, Furthermore, muscle was analysed to measure phosphorylation of mTOR related signalling proteins.

#### *4.3.5.1 Myofibrillar protein enrichment*

Muscle tissue was analysed for enrichment of L- [*ring*-$^{13}C_6$] phenylalanine in the myofibrillar protein fraction. Myofibrillar proteins were isolated from ~30 mg tissue as previously described (32). Briefly muscle was snipped in ice-cold homogenizing buffer (50

mM Tris-HCL, 1 mM EDTA, 1 mM EGTA, 10 mM β-glycerophospahte and 50 mM sodium fluoride). The homogenate was shaken for 10 min prior to being centrifuged at 1000 g for 10 min at 4 $^{o}$C. The pellet was then re-suspended in homogenisation buffer, before being and shaken and centrifuged as above. The myofibrillar fraction was separated from any collagen by dissolving the pellet in 0.3 M NaCl and heating for 30 min at 37 $^{o}$C. The proteins were then precipitated by combining the supernatant with 1M PCA before being centrifuged for 20 min at 3500 rpm at 4 $^{o}$C. The pellet was then washed with 70% ethanol. The remaining myofibrillar pellet was hydrolysed overnight at 110 ºC in 0.1 M HCl/Dowex 50W-X8 100-200 (Bio-Rad laboratories INC, USA) and the liberated amino acids purified on cation-exchange columns (Bio-Rad laboratories INC, USA). Amino acids were then converted to their N-acetyl-n-propyl ester derivative and the phenylalanine labelling determined by gas-chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS, Delta-plus XL, Thermofinnigan, Hemel Hempstead, UK). Ions 44/45 were monitored for unlabelled and labelled $CO_2$ respectively.

*4.3.5.2 Intracellular protein enrichment*

Intracellular amino acids were liberated from ~20 mg of muscle. The frozen tissue was powdered under liquid nitrogen using a mortar and pestle and 500 μL of 1 M perchloric acid (PCA) was added. The mixture was centrifuged at 10,000 g for 10 min. The supernatant was then neutralised with 2 M potassium hydroxide and 0.2 M PCA and combined with 20 μL of urease for removal of urea. The free amino acids from the intracellular pool were purified on cation-exchange columns as described above. Intracellular amino acids were converted to their MTBSTFA derivative and $^{13}C_6$ phenylalanine enrichment determined by monitoring at ions 234/240 using gas chromatography mass spectrometry (GCMS) (model 5973; Hewlett Packard, Palo Alto, CA).

*4.3.5.3 Western blots:*

20-30mg of muscle tissue was powdered on dry ice. Approximately 20mg of powdered muscle was homengised in lysis buffer (50mM Tris pH 7.5; 250mM Sucrose; 1mM EDTA; 1mM EGTA; 1% Triton X-100; 1mM $NaVO_4$; 50mM NaF; 0.50% PIC), using a hand-held homogenizer (PRO200, UK). Samples were shaken for 1h at 4$^{o}$C, before being centrifuged for 5min at 6000g. The supernatant was then used for determination of protein. A DC protein assay (Bio Rad, Hertfordshire, UK) was used for protein concentration. Equal amounts of protein were then boiled in Laemmli sample buffer (250mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 0.01% bromophenol blue; 5% β-mercaptoethanol) and separated on SDS polyacrylamide gels (10-15%) for ~1h at 58mA. Proteins were then transferred to a protran nitrocellulose membrane (Whatman, Dassel, Germany) at 100V for 1h. Membranes were blocked using milk solution and then incubated overnight at 4$^{o}$C with the appropriate primary antibody. The primary antibodies used were, rpS6$^{Ser235/236}$ (Cell signalling 2217), S6K1$^{Thr389}$ (Cell signalling 9234), S6K1 $^{Ser421/Thr424}$ (Cell signalling 9204), Akt$^{Thr308}$ (Cell signalling 4056), Akt$^{ser473}$ (Cell signaling 3787), 4E-BP1$^{Thr37}$ (Santa Cruz SC6025), eEF2$^{Thr56}$ (Cell signalling 2332), and PRAS40$^{Thr246}$ (Cell Signalling 2610). The following morning the membrane was rinsed in wash buffer (TBS with 0.1% Tween-20). The membrane was then incubated for 1h at room temperature with the appropriate secondary antibody, either horseradish (HRP)-linked anti-mouse IgG (New England Biolabs, 7072; 1:1,000) or anti-rabbit IgG (New England Biolabs, 7074; 1:1,000) diluted in wash buffer. The membrane was then cleared of the antibody using wash buffer. Antibody binding was detected using enhanced chemiluminescence (Millipore, Billerica, MA). Imaging and band quantification were carried out using a Chemi Genius Bioimaging Gel Doc System (Syngene, Cambridge, UK).

### 4.3.6 Blood collection and analysis

Blood was collected in LH- and EDTA-containing and serum separator tubes and centrifuged at 3500 rpm for 15 min at 4 °C. Blood was collected into the different tubes for different analysis, some of which my be affected by the anticoagulants within the tubes. Aliquots of plasma and serum were then frozen at -80 °C until later analysis. Plasma glucose and urea concentrations were analysed using an instrumentation laboratory automated blood metabolite analyser (Instrumentation Laboratory 650, Instrumentation Laboratory, Cheshire, UK). Serum insulin was measured using a commercially available ELISA (DRG Diagnostics, USA) following manufacturers instructions.

#### *4.3.6.1 Amino acid concentrations and enrichments*

$^{13}C_6$ enrichments of tyrosine and phenylalanine were determined by GCMS (model 5973; Hewlett Packard, Palo Alto, CA) by monitoring ions 234/240. Once thawed, plasma samples were mixed with diluted acetic acid and purified using a cation-exchange column (Bio-Rad laboratories INC, USA). The amino acids were then converted to their N-tert-butyldimethyl-silyl-N-methyltrifluoracetamide derivative. Simultaneously, concentrations of phenylalanine, leucine, threonine, isoleucine and valine were determined using an internal standard method (42, 44). Briefly, plasma samples were weighed to obtain ~ 300 µL, 30 µL of an internal standard containing U-$^{13}C_9$-$^{15}N$ phenylalanine (ions 336/346), U-$^{13}C_6$ leucine and isoleucine, (ions 302/308), U-$^{13}C_4$-$^{15}N$ threonine (ions 405/409) and U-$^{13}C_5$ valine (ions 288/293) was then added and weighed. From knowing the weight of both sample and internal standard we could obtain a tracer to tracee ratio. As we also knew the concentration of the amino acids in the internal standard we converted the tracer to tracee ratio into a concentration of each amino acid in the plasma.

*4.3.6.2 Urea enrichments*

$^{15}N_2$ enrichments of urea were determined from plasma collected in LH vacutainers. Once thawed, plasma samples centrifuged at 3500 rpm for 10 min at 4 $^{o}$C. 10 µL of plasma was then mixed with 120 µL ethanol. Samples were then left in the fridge for 30 min prior to centrifugation at 13000 rpm for 20 min at 4 $^{o}$C. The supernatant was then removed and dried under nitrogen. TBDMS and acetonitrile were then added to the dried sample prior to heating at 90 $^{o}$C for 90 min. Samples were then run on GCMS (model 5973; Hewlett Packard, Palo Alto, CA) and ions 231 and 233 were monitored.

### 4.3.7 Calculations

*4.3.7.1 Fractional synthetic rate*

The fractional synthetic rate (FSR) of myofibrillar proteins was calculated using the standard precursor-product method:

$$FSR\left(\% \cdot h^{-1}\right) = \left(\frac{\Delta E_m}{E_P \cdot t}\right) \cdot 100 \quad (2)$$

Where $\Delta E_m$ is the change in bound $^{13}C_6$ phenylalanine enrichment between two biopsy samples, $E_p$ is the intracellular precursor enrichment and *t* is the time between muscle biopsies.

*4.3.7.2 Urea production*

The contribution of labelled urea infusion to endogenous production of urea was calculated as previously (16) described (equation 3).

$$Production = \left(\left(\frac{E_{ui}}{E_{up}}\right) - 1\right) \bullet i \quad (3)$$

where i is the infusion rate of the urea tracer ($\mu mol \cdot h^{-1} \cdot kgBM^{-1}$) and $E_{up}$ and $E_{ui}$ are the enrichments of urea in the plasma and infusate respectively.

*4.3.7.3 Phenylalanine kinetics*

Phenylalanine was chosen as the tracer as it is not oxidised in the muscle or synthesized in the body. Therefore new appearance of phenylalanine in the blood can be attributed to protein breakdown. Whole body phenylalanine appearance was calculated according to equation 4 (43).

$$Ra = \frac{i}{E_p} \qquad (4)$$

where i is the infusion rate of the tracer ($\mu mol \cdot h^{-1} \cdot kgBM^{-1}$) and $E_p$ is the enrichment of phenylalanine.

Phenylalanine oxidation was calculated by using the phenylalanine balance model (40). Briefly, whole body phenylalanine oxidation ($Q_{pt}$) was determined from the calculation of the hydroxylation of L-[*ring*- $^{13}C_6$]phenylalanine to L-[*ring*- $^{13}C_6$]tyrosine using the equation 5 (40).

$$Qpt = \frac{P_t}{P_p} \bullet \frac{Q_p^2}{\left(\frac{E_p}{E_t} - 1\right) \bullet \left(i_p + Q_p\right)} \qquad (5)$$

where $P_t/P_p$ is estimated to be 0.73 (40). $Q_p$ is the phenylalanine flux ($\mu mol \cdot h^{-1} \cdot kgBM^{-1}$), i is the infusion rate of the tracer ($\mu mol \cdot h^{-1} \cdot kgBM^{-1}$) and $E_p$ and $E_t$ are the respective enrichments of phenylalanine and tyrosine.

*4.3.7.4 Area under the curve*

Area under the curve (AUC) has previously been used to detect differences in situations that have multiple time points (35). The benefit of using AUC is that the magnitude of response and changes over time are represented as a single number, irrespective of differing time intervals between measurements (11, 35). Hence, calculation of AUC allows comparison between interventions of a physiologically interesting time frame.

AUC for serum insulin concentrations and phenylalanine Ra and oxidation rates were calculated using Graphpad Prism V5.0 (Graphpad software incorporation, La Jolle,

California, USA). Baseline values for insulin AUC were set at the concentration of insulin at t=0. A value of zero was used when calculating AUC for phenylalanine Ra and oxidation.

### 4.3.8 Statistical analyses and data presentation

Plasma concentrations were analysed using a two-way repeated measures analysis of variance (ANOVA). Exercise variables, FSR and AUC of serum insulin concentrations and phenylalanine kinetics for the post exercise period were analysed using a paired samples one-tailed t-test. Western blot data are expressed as percentage change from t=0, and data were analysed using two way repeated measure ANOVAS. Significance for all analyses was set at $P < 0.05$. Where significance was observed a least significant difference correction was used in post hoc analysis. All statistical tests were completed using statistical package for social sciences version 17.0 (Chicago, Illinois, USA). All values unless otherwise stated are presented as means ± standard error of the mean (SEM) unless otherwise stated.

## 4.4 Results

### 4.4.1 Exercise variables:

There were no differences between conditions in the mass of lean tissue in the exercised leg ($P > 0.05$). There were no between-trial differences in RPE or total exercise volume ($P > 0.05$). An RPE of at least 19 was reached in all trials. Total exercise volume completed, including the warm up, was 11055±837 kg in BCAA and 11089±913 kg in PLA.

### 4.4.2 Blood analytes

Taken together urea concentrations significantly decreased in both drink conditions (main effect of time) towards the end of the recovery period. However no differences were observed between BCAA and PLA (Figure 4.2).

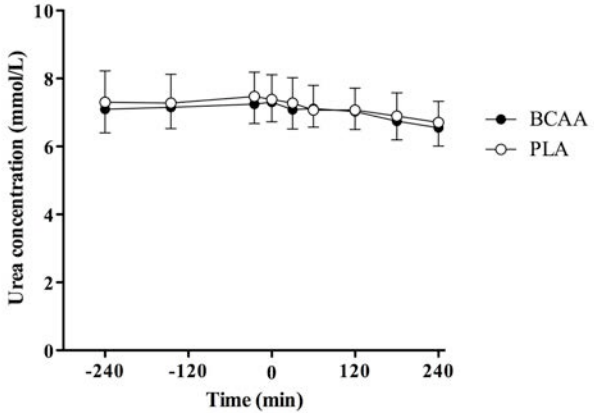


**Figure 4.2: Plasma urea concentrations pre and post either a branched chain amino acid (BCAA) or placebo (PLA) beverage consumed following resistance exercise.**

There were no differences between BCAA and PLA in the production of urea (Figure 4.3). Taken together, urea production significantly decreased (main effect of time) from immediately post exercise to the end of the 4h recovery period.

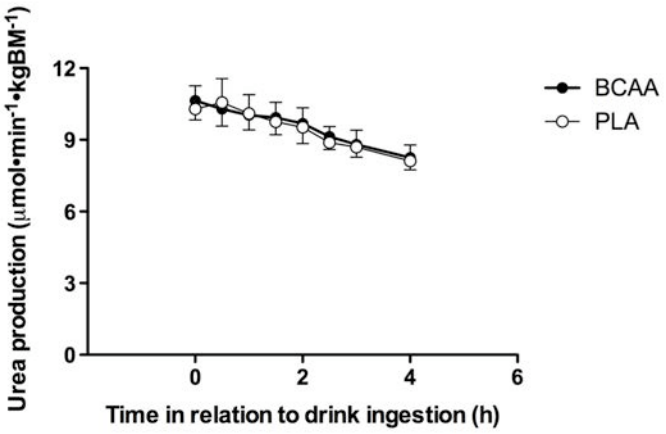


**Figure 4.3: Urea production following either a branched chain amino acid (BCAA) or placebo (PLA) beverage consumed following resistance exercise.**

Glucose concentrations did not change over time and were not different between groups (Figure 4.4). Serum insulin concentrations increased in both trials following breakfast ingestion. There were no differences in serum insulin concentrations following drink ingestion (Figure 4.5).

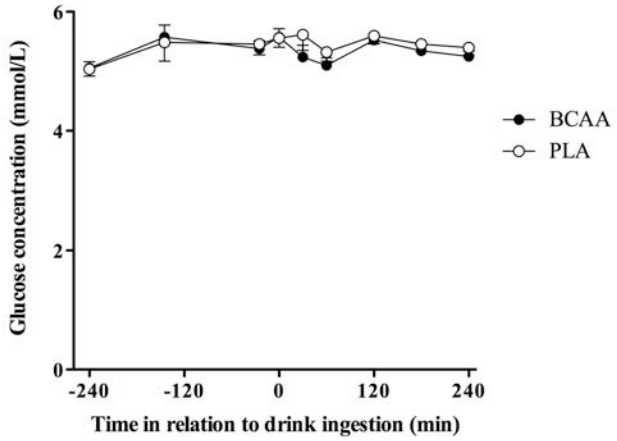


**Figure 4.4: Plasma glucose concentrations pre and post either a branched chain amino acid (BCAA) or placebo (PLA) beverage consumed following resistance exercise**

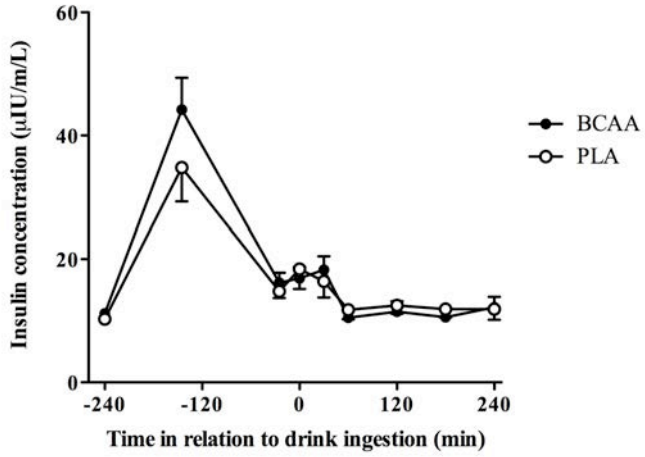


**Figure 4.5: Serum insulin concentrations pre and post either a branched chain amino acid (BCAA) or placebo (PLA) beverage consumed following resistance exercise**

*4.4.2.1 Amino acid concentrations*

Amino acid concentrations across time are shown in Figure 4.6. Branched chain amino acid concentrations were significantly higher following drink ingestion in BCAA than PLA. Peak branched chain amino acid concentrations were observed 30 min following drink ingestion. Plasma valine concentration increased 3-fold, isoleucine and leucine concentration increased 4-fold at 30 min following drink ingestion compared to basal. Threonine and phenylalanine concentrations were significantly increased post breakfast and decreased following the exercise. There were no differences in threonine concentrations between trials.

Phenylalanine concentrations were significantly higher towards the end of the recovery period in PLA compared to BCAA.

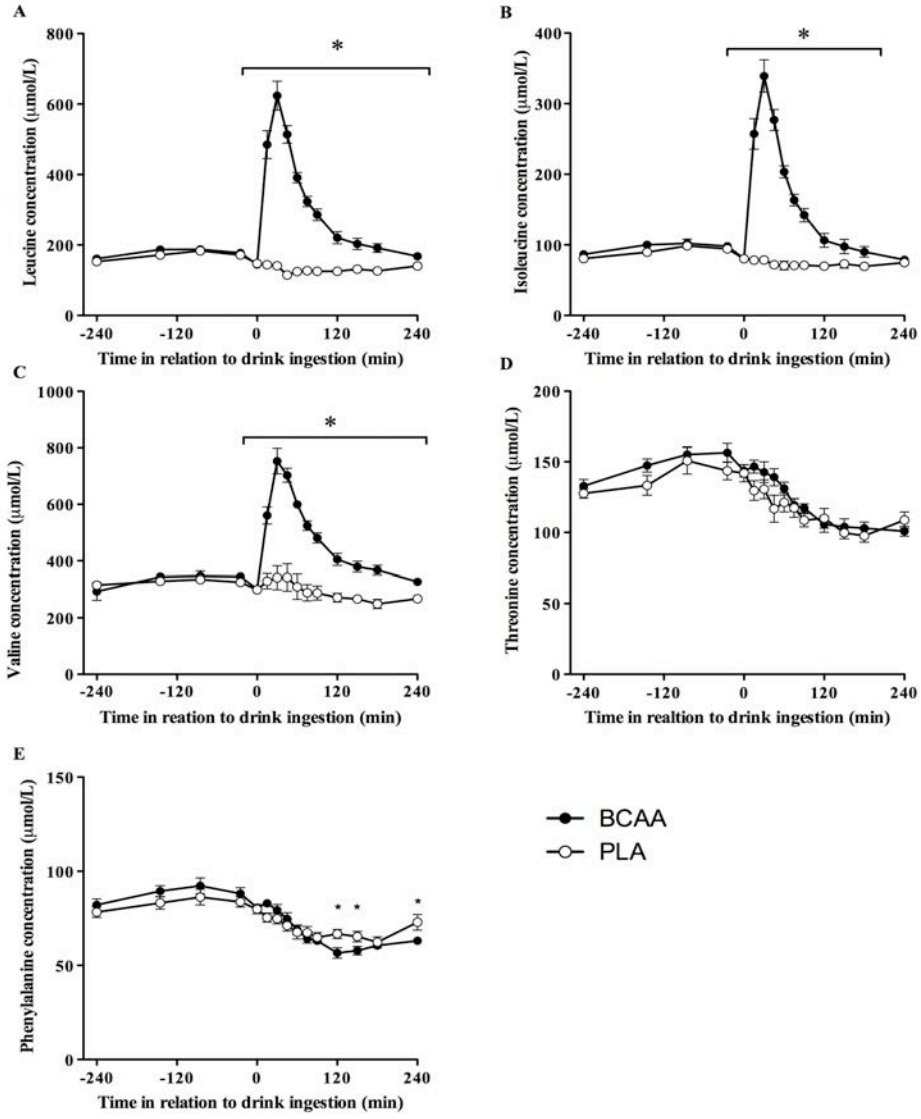


**Figure 4.6: Plasma concentrations of A) leucine, B) isoleucine, C) valine, D) threonine and E) phenylalanine both pre and following either a branched chain amino acid (BCAA) or placebo (PLA) beverage consumed following resistance exercise.**
* significant difference between trials

### *4.4.2.2 Plasma $^{13}C_6$ phenylalanine and tyrosine enrichments*

Plasma $^{13}C_6$ phenylalanine enrichment was stable across the time of tracer incorporation, immediately post-exercise to 4 h post-exercise for BCAA and PLA (Figure 4.7). Plasma $^{13}C_6$ tyrosine enrichment increased throughout the testing period. Intracellular $^{13}C_6$ phenylalanine

enrichment was stable across the time of tracer incorporation for BCAA and PLA (3.9 ± 0.2 and 3.8 ± 0.2% t/T, respectively). Trial order did not influence the tracer enrichment in plasma or in intracellular samples.

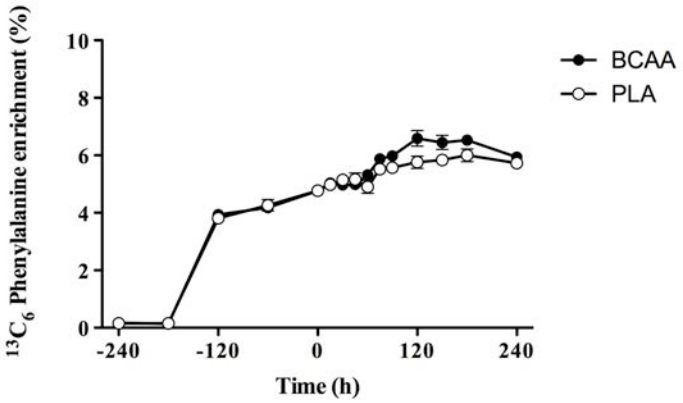


**Figure 4.7: Plasma enrichment of $^{13}C_6$ both pre and following either a branched chain amino acid (BCAA) or placebo (PLA) beverage consumed following resistance exercise.**

### 4.4.3 Phenylalanine kinetics

No difference in phenylalanine Ra was detected over the 4 h recovery period (Figure 4.8A). However, the overall phenylalanine Ra (AUC) was 6% ($P$ =0.0028) lower in BCAA compared to PLA (Figure 4.8B).

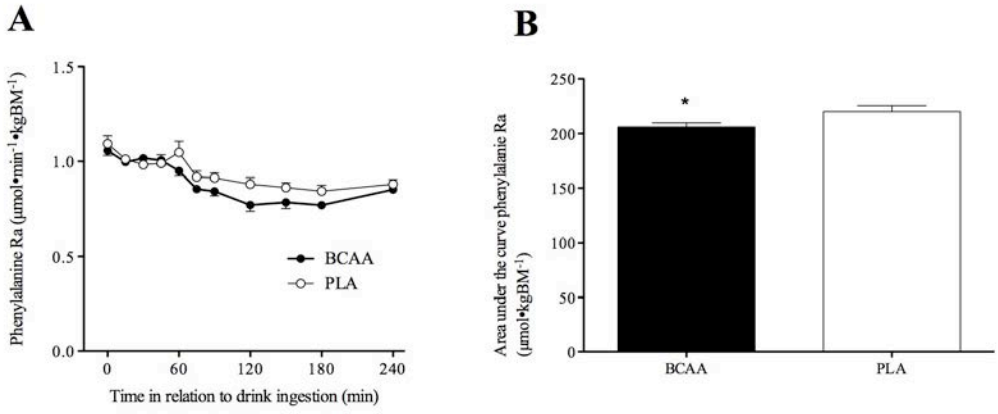


**Figure 4.8: Phenylalanine rate of appearance following either a branched chain amino acid (BCAA) or placebo (PLA) beverage consumed following resistance exercise, depicted A) over time and B) as area under the curve.**
* significant difference between trials

There was a tendency (*P* =0.055) for a time by trial interaction for phenylalanine oxidation (Figure 4.9A). Additionally, AUC of phenylalanine oxidation was 16% (*P* =0.0006) lower in BCAA compared to PLA (Figure 4.9B).

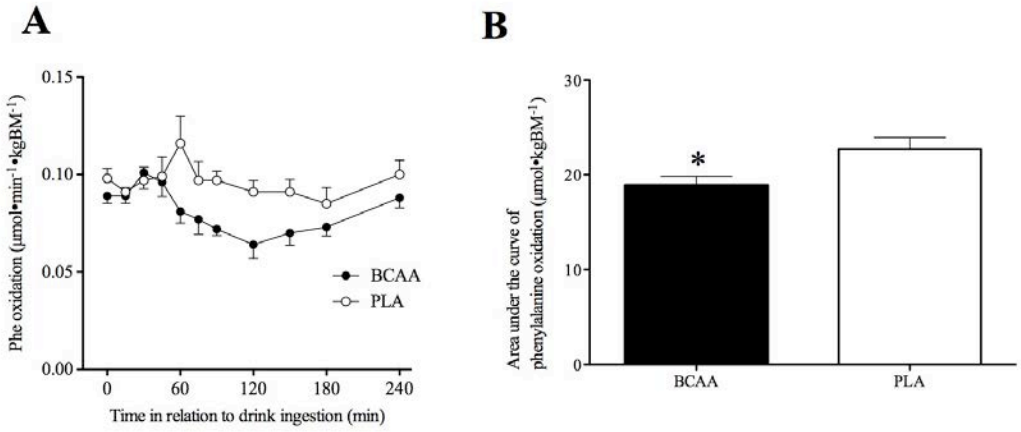


**Figure 4.9: Phenylalanine oxidation following either a branched chain amino acid (BCAA) or placebo (PLA) beverage consumed following resistance exercise, depicted A) over time and B) as area under the curve.**
* significant difference between trials

### 4.4.4 Anabolic signalling proteins

There were no statistical differences between BCAA and PLA in the phosphorylation status of S6K1$^{Ser421/Thr424}$, 4E-BP1$^{Thr37}$, AKT$^{Thr308}$ or rpS6$^{(Ser235/236)}$. When the trials were taken together there was a significant effect of time on the phosphorylation status of PRAS40$^{Thr246}$ (P=0.025) (Figure 4.10). Additionally, the phosphorylation of AKT$^{(Ser473)}$ was significantly higher compared to immediately post drink at 60 min than 240 min. There was a strong tendency (*P* =0.058) in the phosphorylation status of (Figure 4.11), at 60 min. The phosphorylation of S6K1$^{Thr389}$ in BCAA was 12 ± 6 % higher than PLA at 60 min post drink ingestion.

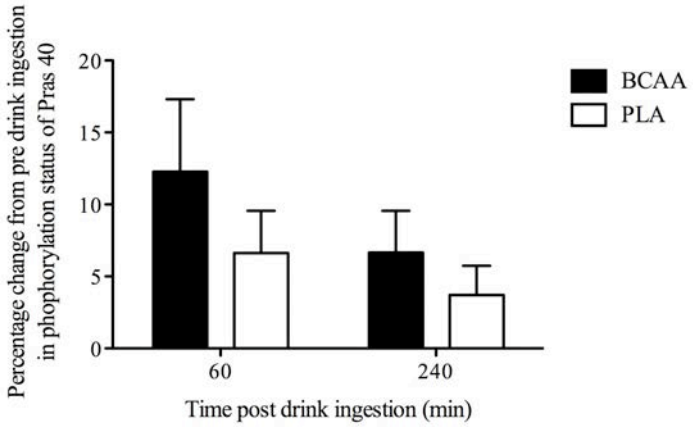


**Figure 4.10: Percentage change from immediately post exercise in phosphorylation status (means ± SE) of PRAS40$^{Thr246}$ 60 and 240 min following either a branched chain amino acid (BCAA) or placebo (PLA) beverage consumed following resistance exercise.**

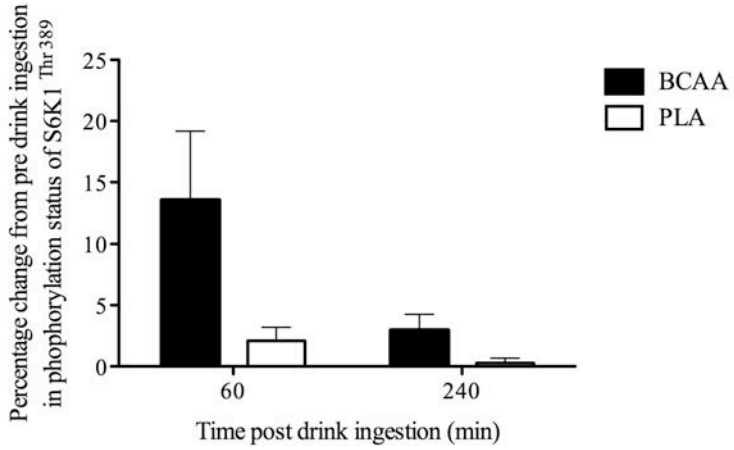


**Figure 4.11: Percentage change from immediately post exercise in phosphorylation status (means ± SE) of S6K1$^{Thr389}$ 60 and 240 min following either a branched chain amino acid (BCAA) or placebo (PLA) beverage consumed following resistance exercise.**

### 4.4.5 Myofibrillar protein synthetic response

Myofibrillar fractional synthetic rate (FSR) was ~22% greater ($P$ =0.012) over the 4h recovery period in BCAA compared to PLA (Figure 4.12).

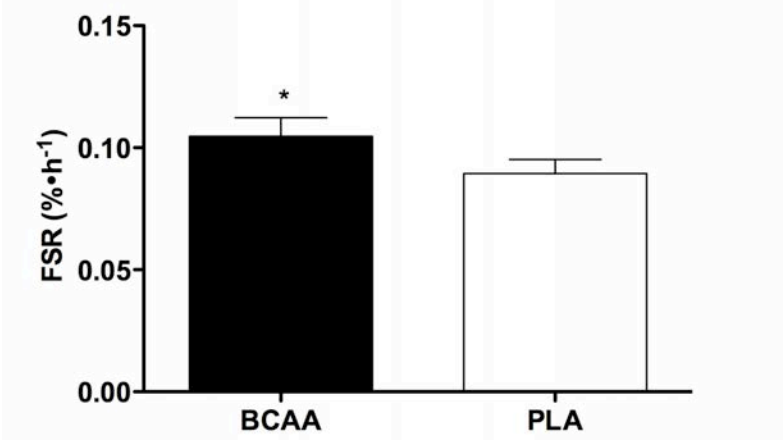


**Figure 4.12: Fractional synthetic rate following either a branched chain amino acid (BCAA) or placebo (PLA) beverage consumed immediately following resistance exercise**
* significantly higher in BCAA

## 4.5 DISCUSSION

The present study is the first to investigate the response of muscle myofibrillar protein synthesis to the ingestion of branched chain amino acids alone following resistance exercise with intake of a high protein breakfast. Our primary finding was that post-exercise myofibrillar muscle protein synthesis was greater with ingestion of a branched chain amino acid containing drink compared with a placebo. Additionally, the increased muscle protein synthesis was accompanied by a 12 % greater phosphorylation of $S6K1^{Thr389}$.

These data are the first to report that muscle protein synthesis can be increased following resistance exercise in trained human males with the ingestion of branched chain amino acids alone. Of all branched chain amino acids it is suggested that leucine is the most important as an anabolic agent. Studies that provided leucine plus protein (21, 22) observed no increase in muscle protein synthesis, when compared to protein alone. Furthermore, there was no greater anabolic response when leucine was added to whey protein (41). We provided only the branched chain amino acids and observed increases in synthesis above exercise alone. Therefore, taken together these data suggest that when muscle protein synthesis is already

stimulated with exercise and amino acids there is no further benefit of leucine. However, when only the branched chain amino acids are ingested muscle protein synthesis in increased above exercise alone.

Ingestion of essential amino acids is known to increase muscle protein synthesis. The ingestion of as little as 5 g protein following exercise has been demonstrated to increase muscle protein synthesis above exercise alone (31). Furthermore, these authors (31) reported that 20 g of egg protein, containing 10 g EAA (4.3 g branched chain amino acids), maximally stimulated muscle protein synthesis. Thus, ingestion of 20g of whey protein may be classed as optimal to stimulate muscle protein synthesis. Previously when leucine has been co-ingested with both EAA (10 and ~ 21.7 g) (10, 14) and protein (33.3 g) (22) there has been no further increase in muscle protein synthesis. However, in these studies (10, 14, 22) there was a greater than optimal amount of EAA or protein ingested. In situations where there are suboptimal (< 20 g protein) amounts of protein ingested muscle protein synthesis rates are lower (31). Therefore, muscle protein synthesis can be further increased. We demonstrated that branched chain amino acids ingestion alone increased muscle protein synthesis. In situations where there are suboptimal values of protein ingested it is unknown if additional branched chain amino acids would increase muscle protein synthesis. Thus, it would be interesting to investigate the effects of branched chain amino acids in combination with suboptimal levels of protein or essential amino acids.

It is well known that ingestion of amino acids post exercise increases phosphorylation status of several signalling proteins (10, 13, 19). Leucine specifically is known to activate mTOR signalling cascade (1, 3, 7). Recently Karlsson et al. (17) demonstrated increased phosphorylation of $S6K1^{Ser421/Thr424}$, $S6K1^{Thr389}$ (~3.5 fold increase) and the downstream rpS6 with branched chain amino acid ingestion and exercise compared to a placebo. In addition to

an augmentation of muscle protein synthesis we observed an ~ 5 fold increase above exercise in phosphorylation of S6K1$^{Ser389}$ with branched chain amino acid ingestion. However, the data failed to reach statistical significance. Our results may lack statistical significance due to a lack of power, for most of the signalling proteins observed power < 0.05. The lack of statistical difference and low power could be attributed to a large inter-individual difference or the large differences in responses to branched chain amino acid ingestion. Our data suggest there to be a difference in the individual response of phosphorylation of signalling proteins to branched chain amino acid ingestion. This difference in response is prevalent in S6K1$^{Thr389}$. The range in change of phosphorylation status between the two conditions over the first hour was ~2 – 50 arbitrary units. Therefore there are large changes between subjects in their response to branched chain amino acids. These data add to the growing body of evidence highlighting the differential response of signalling proteins in humans to anabolic stimuli, such as exercise and nutrition.

The amount and feeding pattern of branched chain amino acids may influence phosphorylation status of signalling proteins. We saw a 12 % increase in phosphorylation status of S6K1$^{Thr389}$ at 60 min post exercise. However, there were no differences in S6K1$^{Thr421/Ser424}$ and rpS6 phosphorylation at 60 min post exercise. In contrast, Karlsson et al. (17) reported increases in S6K1$^{Thr389}$, S6K1$^{Thr421/Ser424}$ and rpS6 phosphorylation at 60 min and 120 min post exercise. The timing and amount of nutrient ingestion differed between the present study and Karlsson et al. (17). We provided 5.6 g of branched chain amino acids following resistance exercise. In contrast, Karlsson et al. (17) provided branched chain amino acids totalling ~ 7.4 g before during and after resistance exercise. The contrasting feeding pattern may have accounted for the different results in phosphorylation status of signalling

proteins between the current and previous study (17). Perhaps, more amino acids are required in regular doses to increase phosphorylation status of S6K1$^{Thr421/Ser424}$ and rpS6.

Feeding prior to exercise may minimise the chance of observing the effect of nutritional interventions following exercise. In the present study, we provided a high protein breakfast, to make our findings more practical. This breakfast may have altered the phosphorylation status of the signalling proteins. It is known that in humans following ingestion of protein the phosphorylation status of some signalling proteins remain elevated for > 3 h (4). If the phosphorylation status of signalling proteins had not returned to basal levels when the branched chain amino acids were ingestion, this could explain why there was not a difference between groups in the signalling proteins that we monitored. Therefore, the ingestion of breakfast may have masked any differences in phosphorylation status of signalling proteins between branched chain amino acid ingestion and placebo.

Furthermore, the breakfast pre exercise may have reduced the effect of branched chain amino acids ingestion on muscle protein synthesis. Following resistance exercise, in a fasted state, protein ingestion increases muscle protein synthesis between ~35 and 90 % (20-22, 31, 32, 38, 39). In the present study ingestion of amino acids increased muscle protein synthesis by ~22 % over a placebo. Qualitatively at least, our findings suggest that branched chain amino acids do not increase muscle protein synthesis to the same extent as complete or essential amino acid sources. However, feeding and high protein breakfast pre resistance exercise increases muscle protein synthesis above breakfast alone (47). Thus, muscle protein synthesis rates in the placebo condition of the present study may have been stimulated through a combination of the high protein breakfast and exercise. Therefore, the effect of ingesting branched chain amino acids on muscle protein synthesis may have been diminished through the ingestion of breakfast. At present it is unclear as to whether protein ingestion

stimulates muscle protein synthesis more than branched chain amino acids. Therefore, further studies are required to make a direct comparison between the effect of branched chain amino acid ingestion and protein on muscle protein synthesis.

Leucine has been ingested in all previous studies that have reported increases in muscle protein synthesis with exercise and provision of amino acids. The importance of leucine also may be highlighted when the effects of whey and casein protein on muscle protein synthesis are compared. Whey protein has higher leucine content than casein. Ingestion of whey protein results in greater muscle protein synthesis than casein (34). It is suggested that the increase in blood leucine concentration needs to reach a threshold in order to reach a maximal rate of muscle protein synthesis. Thus, the leucine content of casein is insufficient for blood leucine concentrations to reach this threshold. A recent study (46) compared whey protein supplied as a bolus ingestion or in small pulses. Total amount of amino acids and leucine ingested were the same in each condition. However, when the amino acids were given as 10 individual doses, the increase in synthesis was less than when a bolus of whey was ingested. Blood leucine concentrations peaked at a lower level with separated ingestion than bolus ingestion (46). The lower increase in blood leucine concentrations may have meant that blood leucine concentrations did not reach the threshold. Therefore, muscle protein synthesis was not increased as much with repeated ingestion compared to bolus ingestion. As may be expected with our higher dose of leucine provided in this study, our data show a greater peak in leucine concentrations than those observed by West and colleagues (46). Hence, we suggest that perhaps the increase in plasma leucine concentrations we observed reached the threshold value. Therefore, the increase in plasma leucine concentrations we observed was important in our observation of increased muscle protein synthesis with branched chain amino acid ingestion.

Our data further support the suggested notion that the availability of leucine drives muscle protein synthesis. Many studies have suggested that leucine concentrations are important for muscle protein synthesis (9, 12, 18, 37, 39). In the present study when branched chain amino acids were ingested, there was a rapid rise in plasma leucine concentrations. Leucine concentrations were then elevated for 4 h of recovery. The rise in plasma leucine concentrations indicates an increased availability of leucine. Thus, the increase in muscle protein synthesis we observed with branched chain amino acid ingestion may have been partially caused by increased leucine availability.

Infusion of leucine decreases protein degradation to a greater extent than a saline infusion (33). Furthermore proteolysis is suppressed with a branched chain amino acid infusion (25).However, the impact of branched chain amino acid ingestion, on muscle protein breakdown following resistance exercise, remains unknown. Muscle anabolism depends on both muscle protein synthesis and muscle protein breakdown. Leucine ingestion has been suggested to reduce muscle protein breakdown in resting conditions (33). However, protein containing an artificially increased leucine content did not influence net balance despite increasing the insulin response (14). To date no study has measured muscle protein breakdown following resistance exercise with the ingestion of branched chain amino acids only. In the present study we did not measure protein breakdown on a muscle level. However, we demonstrated a reduced whole body phenylalanine Ra and oxidation when branched chain amino acids were ingested, suggesting that whole body protein breakdown may be reduced. These findings are in line with Koopman et al. (22) who demonstrated that the ingestion of carbohydrate, protein and leucine resulted in a lower amino acid oxidation rate. The reduction in phenylalanine oxidation and Ra may suggest that ingesting branched chain amino acids results in amino acids being synthesised into proteins and not broken down. Our data suggest

that branched chain amino acids may have a role in reducing protein breakdown on a whole body level. However, it is unclear as to whether whole body measures are representative of what is happening in the muscle. Therefore future studies also should aim to investigate muscle protein breakdown, to determine a greater understanding of muscle anabolism with branched chain amino acid ingestion.

Our urea production data show no differences between the two conditions, and therefore may suggest a lack of support for reduction in whole body protein breakdown with ingestion of branched chain amino acids. Ingestion of branched chain amino acids leads to an increase in ammonia (26-28). Whereas, not measured in the current study the lack of differences in urea production and urea concentrations between the two drink conditions suggest that ammonia concentration may not have increased with 5.6 g of branched chain amino acids. Our data may indicate that if proteins are being broken down the nitrogen is not being used for urea synthesis. Therefore, the nitrogen from amino acids may be used for synthesis of other nitrogen containing compounds. Thus, perhaps ingestion of branched chain amino acids perhaps maintains the nitrogen within body compounds. Hence, there was not an increase in urea production when branched chain amino acids were ingested. Alternatively the provision of the high protein breakfast may have caused a lack of difference between the two trial conditions. It is likely that some of the protein from the breakfast will be broken down, possibly creating urea, increasing urea production. This increase in urea production would have occurred in both trials. Thus, differences attributed to branched chain amino acid ingestion may be masked.

In conclusion our results demonstrate that ingesting branched chain amino acids following a breakfast and resistance exercise increase myofibrillar muscle protein synthesis.

## 4.6 References

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# CHAPTER 5: General Discussion

**Chapter 5: General Discussion**

## 5.1 Summary and overview

This thesis describes experiments that investigated the whole body and muscle response to ingestion of branched chain amino acids (BCAA) and protein following intense exercise. The aim of the first study (chapter 2) was to assess whether BCAA could reduce the negative symptoms that are associated with intense lengthening contractions. These negative indices included decreased muscle function, increased muscle soreness, inflammation and increased plasma concentration of myofibre proteins. The findings provide novel data for the current literature as it was demonstrated that BCAA was effective at reducing muscle soreness following intense lengthening contractions when compared to a placebo. However, there were no differences between BCAA and placebo ingestion regarding the decrement of maximalforce production, inflammation or myofibre protein concentration in the blood.

The second study (chapter 3) in this thesis was designed to obtain further information about nutritional interventions aimed at reducing negative indices associated with muscle damage. In chapter 3 the same experimental approach as chapter 2 was adopted. However, a whey protein source, containing the same amount of BCAA, as in chapter 2 was compared to an isoenergetic control. The findings demonstrated that whey protein ingestion resulted in a reduced decrement in maximal muscle force production. Furthermore, whey protein ingestion had a likely beneficial effect, compared to an isoenergetic control, of attenuating the decrease in force production; however, whey protein ingestion did not ameliorate rises in inflammation or myofibre protein concentrations in the blood.

The third study of this thesis (chapter 4) is the first study that has investigated muscle protein synthesis (MPS) with ingestion of only BCAA following resistance exercise. The main finding from this investigation was that ingestion of BCAA following resistance exercise increased rates of MPS.

## 5.2 Amino acid ingestion and eccentric exercise

### 5.2.1 Summary

Unaccustomed, exercise consisting of lengthening contractions leads to decreased maximal force production and range of motion and increased muscle soreness, plasma concentrations of myofibre proteins and inflammation. There has been much interest in the use of amino acid sources to alleviate these negative indices associated with lengthening contractions (10, 12-14, 26, 37, 42, 44, 51). However, the results of these studies offer little agreement over the efficacy of amino acid sources to ameliorate the negative indices associated with lengthening contractions. Two studies (Chapters 2 and 3) from this thesis contribute novel data to this sector of literature. The same exercise protocol, supplementary pattern, and method of assessing recovery from the exercise bout were employed and most importantly diet was strictly controlled in both studies. Both whey protein and BCAA ingestion reduced muscle soreness following intense lengthening contractions. However, only whey protein ingestion resulted in an attenuated decrement in maximal force production. The mechanisms underpinning the reduction in soreness and attenuation of force production decrements with nutritional interventions remain unclear.

The dose of amino acids may be important for reductions in negative indices associated with lengthening contractions. In chapter 2 we provided 7.3 g of BCAA in each dose as four doses per day for 3 d totalling 87.6 g. A more recent study by Kirby and colleagues (26) provided 250 mg/kg body mass, which equated to ~ 19 g BCAA within each dose. Kirby et al (26) provided supplements pre and post exercise and the in the morning of the following day so total leucine ingestion was ~57 g. However, taken together, differences in experimental design in addition to the amount of BCAA ingested do not allow a direct comparison between

the outcomes. Kirby et al. (26), provided more leucine but less amino acids than the experiment described in chapter 2. Leucine ingestion resulted in a better maintenance of force production compared to the control group (26). Therefore when taken together, these data support the notion that perhaps when consuming leucine or the BCAA, a bigger dose at the start of the day results in better maintenance of strength. Alternatively, smaller boluses throughout the day reduce muscle soreness.

*5.2.1.1 Muscle soreness and function*

Habitual movement may help to reduce muscle soreness. In both studies (chapter 2 and 3) indices of muscle soreness were assessed 8 h following the exercise bout. Interestingly soreness was lower in all four groups at 8 h compared with 1 h post exercise. Two factors make this time point unique in relation to the other time points we chose in the study design. First, the 8 h time point is measured with the volunteers in a post-prandial/fed state. Therefore, it is possible that feeding can influence subjective feelings of soreness. However, the 8 h measurement was completed when the subjects had been weight bearing and completing light exercise on the exercised muscle. In contrast, the morning measurements were taken in a situation after the exercised leg muscle had not been moved for approximately 8 h. Light exercise has been shown to be effective in reducing symptoms commonly associated with muscle damage (5). Therefore, performing activities of daily living may be effective at reducing muscle soreness. Thus, decrease in muscle soreness at 8 h may be attributed to daily exercise.

There is a difference in the temporal pattern of soreness and muscle function between ingesting BCAA (chapter 2) and whey protein (chapter 3) pre and post exercise. In chapter 2 when only BCAA were ingested, reductions in muscle soreness were reported at 48 h and 72 h post exercise compared with placebo. In contrast, ingestion of whey protein reduced muscle

soreness only within the first 24 h following the exercise protocol compared to carbohydrate ingestion. Furthermore, from 24 -72 h post exercise onwards soreness scores reported with carbohydrate and whey protein ingestion were similar. Additionally, BCAA ingestion did not impact muscle function (low frequency fatigue or maximal isometric strength), however whey protein ingestion resulted in reductions in the decrements of maximal force production compared to carbohydrate. Thus, it appears that whey protein ingestion only affects the initial 24 h period and fails to ameliorate feelings of soreness or decrements in maximal muscle force production thereafter.

On the other hand the isoenergetic control (i.e. the carbohydrate) may influence the observed findings, hence, making the temporal pattern of BCAA and whey protein effect appear different. The same experimental design was implemented in both experiments, thus, permitting a direct comparison of the data from all four groups. Comparison of the complete recovery period is shown in Figure 5.1. Whey protein and BCAA ingestion results in similar muscle soreness over the whole recovery period. Carbohydrate ingestion results in the largest initial increase in muscle soreness scores post exercise and then decreased to similar scores as both whey protein and BCAA ingestion. Ingestion of the flavoured water placebo resulted in higher soreness scores than BCAA, carbohydrate and placebo ingestion towards the end of the recovery period. Thus, our results suggest that perhaps early in the post exercise period carbohydrate has a negative impact on feelings of muscle soreness. However, in the later part of recovery whey protein, BCAA and carbohydrate are effective at reducing muscle soreness. Therefore, it would appear that whey protein actually has the same temporal pattern as BCAA when compared to the same control condition.

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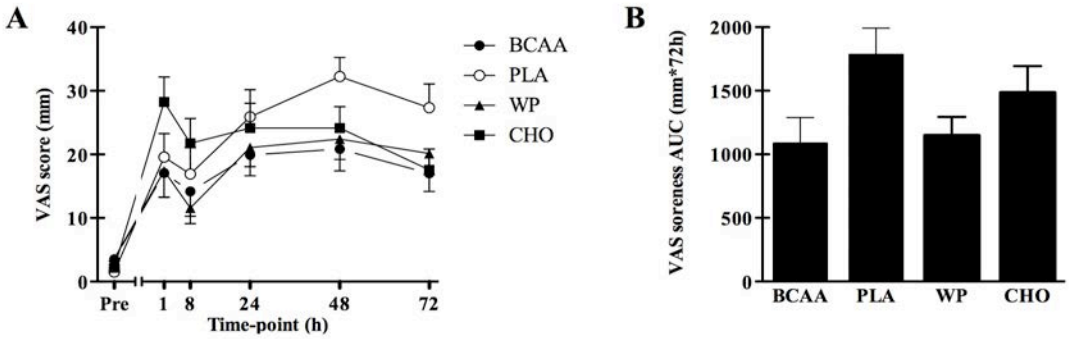


**Figure 5.1: Muscle soreness shown as A) raw values and B) area under the curve of flexed muscle soreness over the entire 72 h period.**
Data are expressed as means ± SE. Supplements were consumed 12 times over the testing period and contained branched chain amino acids (BCAA), placebo (PLA), whey protein (WP) or carbohydrate (CHO).

Resistance exercise, particularly lengthening contractions, results in the deformation of the proteins responsible for maintaining the structural integrity of the skeletal muscle architecture. The findings in this thesis suggest all amino acids are required for recovery of maximal muscle force production. Chapter 2 demonstrated that ingestion of BCAA had no effect on maximal muscle force production. However, the ingestion of whey protein when compared to a carbohydrate control, showed a likely beneficial effect on minimising the loss in maximal muscle force production, although the significance of this is questionable as this reduction in force production was apparent only over the first few hours.

The control condition may affect the interpretation of results. As with muscle soreness the results of maximal force production can be combined (Figure 5.2). There is a likely detrimental effect of carbohydrate compared to whey protein, BCAA and flavoured water 1 h post exercise. However, when comparing between supplements at the end of the recovery period there is a likely beneficial effect of both carbohydrate and whey protein compared with BCAA and the flavoured water placebo. In chapter 3 when carbohydrate and whey protein are compared there is no effect on maximal isometric strength at the end of the experimental period. However, when compared to a flavoured water placebo there is an effect at reducing

the decrements in maximal force production with whey protein ingestion. Therefore, these data further support the importance of drawing conclusions about nutritional interventions when differing control conditions are employed.

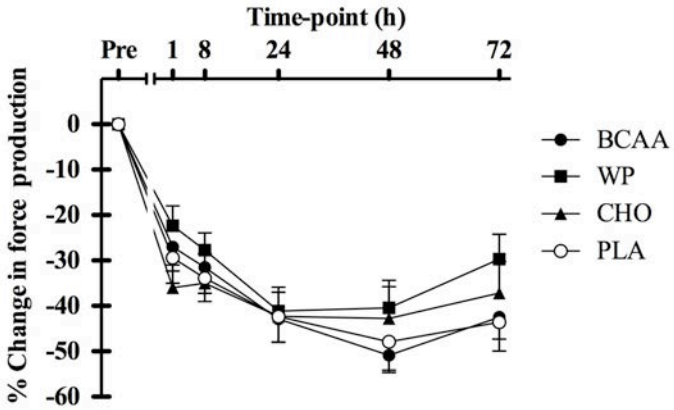


**Figure 5.2: Muscle function shown percentage force loss over the entire 72 h period.**
Data are expressed as means ± SE. Supplements were consumed 12 times over the testing period and contained branched chain amino acids (BCAA), placebo (PLA), whey protein (WP) or carbohydrate (CHO)

*5.2.1.2 Future studies*

If comparisons between nutritional conditions are being investigated, extrinsic factors, such as diet, should be strictly controlled to avoid confounding results. Therefore, future studies should aim to strictly control diet. Diets should be controlled so that the energy intake matches the individual's habitual intake, thus ensuring subjects remain in energy balance during the testing period.

There appears to be a difference in how male and females respond to nutritional interventions following lengthening contractions. There is only one study (42) that has investigated the impact of nutritional interventions following lengthening contractions in females. This study (42) investigated females since the authors' preliminary study (43) had demonstrated the effect of supplementation was more pronounced in females compared to

males. Thus, further work should investigate these potential sex differences in effectiveness of supplementation following intense resistance exercise.

The beneficial effects of exercise on human health have been widely explored. However, as we have demonstrated, an unaccustomed exercise bout is often accompanied by negative symptoms, including muscle soreness, decreased muscle function and possibly inflammation. Recommendations have suggested that aged individuals should adopt exercise programmes to allow them to live a healthy, independent and greatly improved quality of life (32). Unaccustomed exercise can be related to an unaccustomed length of the muscle, unaccustomed number of repetitions or unaccustomed forces (45). Aged individuals that start an exercise programme are likely to experience symptoms similar to those reported in chapters 2 and 3. Therefore, it may be useful if nutritional and dietary interventions are developed to reduce the negative indices associated with skeletal muscle damage for individuals to ingest after their first few exercising sessions. The nutritional effects on the negative indices associated with lengthening contractions is yet to be elucidated in aged individuals. It may therefore be interesting to see if supplementation of BCAA or whey protein reduces these negative symptoms especially that of muscle function and muscle soreness in an aged population. Thus, future studies should investigate the response of BCAA or whey protein following multiple lengthening contractions in aged individuals.

In chapters 2 and 3 we measured interleukin 6 (IL-6) as a marker of inflammation. However it may not have been the most suitable in response to the lengthening contractions as elevated levels are observed after many exercise modalities. The inflammatory response can be described as acute or prolonged, and relate to a muscle or whole body level. IL-6 and pre albumin have been shown to increase and return to baseline levels following a long distance triathlon (21). However, levels of C-reactive protein increased gradually after exercise

cessation and were at the peak values measured 16 h after exercise cessation (21). Thus, it may be interesting to measure the effect of whey protein and BCAA ingestion on other inflammatory markers which have a more delayed response following lengthening contractions. Additionally, the use of magnetic resonance imaging to monitor swelling and or oedema may also assist in understanding what is happening in the muscle when nutritional interventions are provided as a method to alleviate symptoms associated with lengthening contractions.

Muscle damage has been associated with whole body and myofibrillar protein breakdown (16, 17). Furthermore, animal studies indicate that damaging exercise increases muscle protein breakdown (MPB) and transiently decreases MPS resulting in loss of muscle proteins, particularly the force-generating myofibrillar proteins (20, 31). Myofibrillar protein synthesis was shown to be elevated at 8.5 h following damaging exercise in humans (34), but MPB was not examined. Furthermore, these measures were made shortly (up to 8.5h) following exercise and no study has examined later time points in humans as has been done in rats. Thus, no systematic examination of protein metabolism associated with muscle damage in humans has yet been made. Therefore, it would be of interest to quantify MPS and MPB in response to damaging exercise. It is important to know what happens to MPS and MPB as well as net protein synthesis to develop feeding strategies that optimise net protein synthesis and improve recovery from damaging exercise.

## 5.3 Branched chain amino acid ingestion and muscle protein synthesis

### 5.3.1 Summary

## Chapter 5: General Discussion

Resistance exercise combined with ingestion of BCAA increases myofibrillar MPS. Exercise of sufficient intensity (30) is known to stimulate mixed MPS in young healthy males. Ingestion of amino acids is required for a positive net balance, resulting in muscle mass gain (7, 40, 50). In a fasted state, the combined effect of nutrition and exercise to stimulate MPS is well established (7, 9, 15, 19, 28, 29, 35, 38, 40, 46-50). However, many athletes will consume a meal prior to their workout and an amino acid source following their exercise session. However, no study had investigated the combined effect of two nutritional interventions (meal before and amino acid source post) with resistance exercise. Therefore in chapter 4 we provided a high protein breakfast prior to the exercise bout. Following exercise BCAA or a placebo beverage was provided. We demonstrated that myofibrillar MPS could be increased in a postprandial state with the ingestion of BCAA.

The control condition may influence the outcome of the experiment. In chapter 4 the subjects were provided with a breakfast. The combination of exercise and breakfast has previously been shown to increase MPS more than feeding alone (53). Thus, the MPS rates in the placebo condition (feeding and exercise) potentially were elevated above those commonly reported with exercise alone. Therefore, the relatively small difference in MPS between placebo and BCAA in chapter 4 may be explained by the elevated MPS in the placebo condition. It may be difficult to compare the effect of interventions between studies that have a different control condition. For example Moore et al. (36) showed an ~93% increase in MPS comparing an exercised leg with an exercised leg and the ingestion of 25 g egg protein. The comparison in this study (36) was between an exercised and an exercised-fed situation. Consequently, until a study has made a direct comparison between the ingestion of protein and BCAA, it is unknown which nutritional intervention would result in higher MPS rates.

**Chapter 5: General Discussion**

There are many signalling proteins that control both transcription and translation. The phosphorylation status of some signalling proteins were measured in chapter 4 when either a placebo or 5.6 g of BCAA were ingested following resistance exercise. We observed a strong tendency for higher phosphorylation of S6K1$^{Thr389}$ at 60 min with BCAA ingestion following exercise. This is consistent with observations by Karlsson et al (22). However, these authors (22) also observed differences in phosphorylation status of S6K1$^{Thr421/Ser424}$ and rpS6 with BCAA ingestion and resistance exercise compared with exercise alone. These authors (22) provided BCAA before, during and after exercise totalling ~7.4 mg. Therefore a greater amount of BCAA were ingested in Karlsson et al. (22) than our study in chapter 4. This ingestion of greater amounts of BCAA may have been a reason for the discrepancies observed in signalling. Alternatively, the inconsistencies in the findings between the two studies could be attributed to various the feeding strategies employed, including bolus vs frequent feeding. On the other hand contradicting conclusions between the results in chapter 4 and Karlsson et al. (22) may be due to wide variations in the measurements of signalling proteins. It is important to note that current methods to assess phosphorylation status of signalling proteins are highly qualitative. Furthermore, phosphorylation of a protein only represents one form of activation for review see Kimball et al. (24).

Similarly to results of MPS, the phosphorylation status of signalling proteins may have been influenced by the breakfast we provided. It is known that in humans following ingestion of protein the phosphorylation status of some signalling proteins remain elevated for > 3 h (6). Furthermore, the synergistic effect of exercise and nutrition on S6K1$^{Thr389}$ has been shown to occur 5 h post protein ingestion (33). The 1 h biopsy in chapter 4 was 5 h post ingestion of the high protein breakfast. Therefore the phosphorylation status may have been elevated in both conditions at 1 h due to the combined effect of the breakfast and the exercise. Hence we

observed no difference in phosphorylation status of S6K1$^{Thr389}$ when the BCAA were ingested compared to the placebo condition. The elevation in phosphorylation status of signalling proteins from the breakfast may have minimised the chance of observing an effect of BCAA ingestion. Hence, our results showing no difference between the conditions in phosphorylation status of the measured signalling proteins.

### 5.3.2 Future studies

Of interest to many exercisers is the dose of protein that is required to maximally stimulate MPS. Two studies investigating the ingestion of egg (35) and whey (52) protein have demonstrated that 20 g of protein maximally stimulates MPS. This equates to ~ 4.3 - 4.6 g of BCAA, which is less than was used in chapter 4. In chapter 4 it was demonstrated that 5.6 g BCAA were all that was required to stimulate MPS. If only the BCAA are required for increases in MPS it would be of interest to investigate the dose response of BCAA alone.

There is no effect on MPS or muscle anabolism of ingesting leucine plus either protein (28, 29, 49) or essential amino acids (EAA) at rest or following exercise when compared to protein / EAA only (19, 28, 29, 49). When a difference in MPS has been observed with ingestion of leucine and an amino acid source ingestion the control group does not ingest protein / EAA, thus the effect of the leucine is difficult to determine from the protein (15, 18, 27). Previous studies have suggested that 20 g protein is optimal to maximally stimulate MPS following resistance exercise (35, 52). In the studies that have investigated leucine plus amino acid sources >20 g protein or amino acids were ingested. Therefore, it seems that when leucine has been co-ingested with protein and EAA there have been optimal amounts of protein / EAA ingested. Thus, MPS may not have been increased with the addition of leucine since the rate of MPS was already maximised due to the protein / EAA ingestion. No study has combined

doses of protein that may be classed as suboptimal (< 20 g protein) and additional leucine. Thus, based on the findings in chapter 4, that BCAA can stimulate MPS, it may be interesting to combine BCAA and suboptimal amounts of protein / EAA. The combination of protein and leucine may result in increases in MPS and phosphorylation status of signalling proteins.

Of all three BCAA, leucine has received the most attention as an anabolic stimulus in rats (1, 2). Recent studies have suggested that the availability of leucine is important (11, 18, 25, 41, 47). Furthermore, studies have shown that ingestion of leucine up regulates signalling proteins (3, 8, 15). However, in humans the effect on MPS of ingesting leucine alone has never been investigated. Thus, a study that would add further information to the current thoughts on the requirements for leucine would be to investigate the effect on MPS of providing only leucine. If successful, the dose that provides a maximal protein synthetic rate could be sought.

A recent study combined the ingestion of carbohydrate, protein and leucine (28). These authors (28) suggested that the extra leucine would increase phosphorylation of signalling proteins and therefore increase MPS. These authors (27, 28) found no further increase in MPS with the additional leucine than when compared to protein and carbohydrate alone, and there were no differences in the response between young and aged. Furthermore, a study completed by the same research group (27) observed no difference in the response of protein plus leucine on MPS following exercise. Conversely, Katsanos et al. (23) demonstrated greater MPS in elderly at rest when an EAA mixture was enriched to 41 % leucine. When providing BCAA post exercise we observed a greater increase in MPS than exercise alone (chapter 4). To date no study has investigated the effect of BCAA and exercise in the elderly. Therefore, it would be of interest to investigate if the effect of BCAA on MPS would be present in an aged population.

## Chapter 5: General Discussion

During prolonged exercise the muscle may become catabolic. MPS is reduced in rats following prolonged exercise (2). Leucine has been shown to restore MPS back to baseline levels following exercise that resulted in decreased MPS (2, 25). The restoration of MPS was associated with a restoration of intracellular signalling leading to enhanced translation initiation (2, 4, 25). These data suggest that leucine (BCAA) may have a greater impact on muscle during catabolic situations, such as prolonged, intense exercise. In chapter 4 we demonstrated that BCAA increased MPS during an anabolic situation. A future study could be designed to investigate the effect of BCAA or leucine on MPS following prolonged intense exercise.

As previously discussed the breakfast that was provided in chapter 4 may have had an influence on the phosphorylation status of the signalling proteins and MPS. However, with the experimental design implemented in chapter 4 it was not possible to determine if the breakfast influenced our findings. Therefore, a future study could be designed to compare the effect of BCAA ingestion and resistance exercise on MPS and phosphorylation status of intracellular signalling proteins with and without the provision of a breakfast.

The exact mechanisms that orchestrate molecular signalling activity are yet to be fully elucidated. Although there has been a great deal of work completed in rodent models, the exact time course for signalling protein in humans has only recently been investigated (6, 33). Atherton and colleagues (6) demonstrated a disconnect between changes in phosphorylation status of signalling proteins and changes in MPS. The peak in MPS was observed ~ 90 min after ingestion of a high dose (48 g) of whey protein (6). The phosphorylation status of signalling proteins matched the increase in synthesis, however as the rate of synthesis declined there was little change in the phosphorylation status of the cell. Therefore, the change in phosphorylation status of signalling proteins has a longer duration than the increase

in synthesis. Conversely, the phosphorylation of S6K1$^{Thr389}$ has been shown to follow a similar pattern to MPS after resistance exercise and ingestion of 25 g of whey protein. If the peak in phosphorylation of signalling proteins is at 90 min post drink ingestion we may have missed the peak in phosphorylation of signalling proteins. However, it should be noted that the peak at 90 min in phosphorylation status was observed in a rested situation (6). Exercise alone can increase mTOR related signalling proteins (39). As the present study included exercise the combined stimulus of exercise and BCAA ingestion may have altered the time course of phosphorylation of mTOR related signalling proteins. Further work should investigate the time course of signalling proteins phosphorylation alongside MPS following exercise and amino acid ingestion.

## 5.4 Summary and Conclusions

The data from this thesis point towards current thoughts that BCAA are important, but may not be all that is needed for complete recovery. We have shown in untrained males that BCAA reduces muscle soreness, but not muscle function, following multiple lengthening contractions. However both muscle function and muscle soreness are reduced with whey protein ingestion. BCAA ingestion in trained males increases MPS rates, however perhaps not to the same extent as essential amino acids (EAA) or protein.

- BCAA ingestion reduces muscle soreness in untrained subjects following a bout of intense lengthening contractions.
- Whey protein reduces muscle soreness and results in reduced decrements in muscle function in untrained subjects following a bout of lengthening contractions.
- BCAA ingestion increases MPS following resistance exercise.

# Chapter 5: General Discussion

**The data from this thesis suggest that BCAA have a role in recovery from damaging exercise and the growth of myofibrillar tissues.**

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# Appendices

**Appendix A**

## School of sport and exercise sciences

## Consent form

Investigation; Effect of ingestion of an amino acid source on muscle function and markers of muscle damage and soreness.

Investigators: Dr Kevin Tipton, Mr Oliver Witard, Miss Sarah Jackman,

Subject Number:

Name:

Address:

DOB:

I have read the attached information sheet and discussed the investigation with ……. ……who has explained the procedures to my satisfaction. I am willing to undergo the investigation but understand that I am free to withdraw at any time without having to give an explanation and that doing so will not affect my treatment or care I receive

Signed…….

Witnessed……..

Date…….

**Appendix B**

# The University of Birmingham
## School of Sport and Exercise Sciences
### General Health Questionnaire

**Name:** .....
**Address:** .....
.....
.....
**Phone:** .....

**Name of the responsible investigator for the study:**

.....

Please answer the following questions. If you have any doubts or difficulty with the questions, please ask the investigator for guidance. These questions are to determine whether the proposed exercise is appropriate for you. Your answers will be kept strictly confidential.

| | | | |
|---|---|---|---|
| 1. | You are..... | Male | Female |
| 2. | What is your exact date of birth?<br>Day..... Month.....Year..19.....<br>So your age is..... Years | | |
| 3. | When did you last see your doctor? In the:<br>Last week..... Last month..... Last six months.....<br>Year..... More than a year..... | | |
| 4. | Are you currently taking any medication? | YES | NO |
| 5. | Has your doctor ever advised you not to take vigorous exercise? | YES | NO |
| 6. | Has your doctor ever said you have "heart trouble"? | YES | NO |
| 7. | Has your doctor ever said you have high blood pressure? | YES | NO |
| 8. | Have you ever taken medication for blood pressure or your heart? | YES | NO |
| 9. | Do you feel pain in your chest when you undertake physical activity? | YES | NO |
| 10. | In the last month have you had pains in your chest when not doing any physical activity? | YES | NO |
| 11. | Has your doctor (or anyone else) said that you have a raised blood cholesterol? | YES | NO |

**Appendix B**

| | | | |
|---|---|---|---|
| 12. | Have you had a cold or feverish illness in the last month? | YES | NO |
| 13. | Do you ever lose balance because of dizziness, or do you ever lose consciousness? | YES | NO |
| 14. | a) Do you suffer from back pain<br>b) if so, does it ever prevent you from exercising? | YES<br>YES | NO<br>NO |
| 15. | Do you suffer from asthma? | YES | NO |
| 16. | Do you have any joint or bone problems which may be made worse by exercise? | YES | NO |
| 17. | Has your doctor ever said you have diabetes? | YES | NO |
| 18. | Have you ever had viral hepatitis? | YES | NO |
| 19. | If you are female, to your knowledge, are you pregnant? | YES | NO |
| 20. | Do you know of any reason, not mentioned above, why you should not exercise? | YES | NO |
| 21. | Are you accustomed to vigorous exercise (an hour or so a week)? | YES | NO |

I have completed the questionnaire to the best of my knowledge and any questions I had have been answered to my full satisfaction.

**Signed:** .....

**Appendix C**

**Study Number:**

**Patient Identification Number for this trial:**

**CONSENT FORM**

**Title of Project:** 'Effects of branched chain amino acid supplementation on post- exercise muscle protein metabolism'

**Name of Researcher:**

Please initial box

1. I confirm that I have read and understand the information sheet dated..... (version.....) for the above study. I have had the opportunity to consider the information, ask questions and have had these answered satisfactorily. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that relevant sections of my medical notes and data collected during the study, may be looked at by individuals from *GlaxsoSmithKline*, from regulatory authorities or from the NHS Trust, where it is relevant to my taking part in this research. I give permission for these individuals to have access to my records. ☐

4. I agree to my GP being informed of my participation in the study. ☐

5. I agree to take part in the above study. ☐

| Name of participant | Date | Signature |
|---|---|---|

| Name of Person taking consent | Date | Signature |
|---|---|---|