The impact of post-exercise protein-leucine ingestion on subsequent performance and the systemic, metabolic and skeletal muscle molecular responses associated with recovery and regeneration

A Thesis

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TITLE: The impact of post-exercise protein-leucine ingestion on subsequent performance and the systemic, metabolic and skeletal muscle molecular responses associated with recovery and regeneration

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ABSTRACT

The objective was to determine the effect of post-exercise protein-leucine coingestion with carbohydrate and fat on subsequent endurance performance and investigate whole-body and skeletal-muscle responses hypothesised to guide adaptive-regeneration. ***Methods***. *Study-1A* Twelve trained-men ingested protein/leucine/carbohydrate/fat (20/7.5/89/22 $g \cdot h^{-1}$) or carbohydrate/fat (control, 119/22 $g \cdot h^{-1}$) supplements after intense cycling over six days. Glucose and leucine turnover, metabolomics, nitrogen balance and performance were examined. *Study-1B* Immune-function responses to supplementation were investigated via neutrophil O_2^- production, differential immune-cell count, hormones and cytokines. *Study-2A* Twelve trained-men ingested low-dose protein/leucine/carbohydrate/fat (23.3/5/180/30 g), high-dose (70/15/180/30 g) or carbohydrate/fat control (274/30 g) beverages following 100-min of intense cycling. *Vastus lateralis* biopsies were taken during recovery (30-min/4-h) to determine the effect of dose on myofibrillar protein synthesis (FSR), and mTOR-pathway activity inferred by western blot. *Study-2B* The transcriptome was interrogated to determine acute-phase biology differentially affected by protein-leucine dose. ***Results.*** Protein-leucine increased day-1 recovery leucine oxidation and synthesis, plasma and urinary branch-chain amino acids (BCAAs), products of their metabolism, and neutrophil-priming plasma metabolites versus control. Protein-leucine lowered serum creatine kinase 21-25% (±90% confidence limits 14%) and day 2-5 nitrogen balance was positive for both conditions, yet the impact on sprint power was trivial. Protein-leucine reduced day-1 neutrophil O_2- production (15-17 ±20 $mmol \cdot O_2^- \cdot cell^{-1}$) but on day-6 increased post-exercise production (33 ±20 $mmol \cdot O_2^- \cdot cell^{-1}$) having lowered pre-exercise cortisol (21% ±15%). The increase in FSR with high-dose (0.103%·h^{-1} ± 0.027%·h^{-1}) versus low-dose (0.092%·h^{-1} ± 0.017%·h^{-1}) was likely equivalent. High-dose increased serum insulin (1.44-fold ×/÷90% confidence limits 1.18), 30-min phosphorylation of mTOR (2.21-fold ×/÷1.59) and p70S6K (3.51-fold ×/÷1.93), and

240-min phosphorylation of rpS6 (4.85-fold ×/÷1.37) and 4E-BP1-α (1.99-fold ×/÷1.63) versus low-dose. Bioinformatics revealed a biphasic dose-responsive inflammatory transcriptome centred on interleukin (IL)-1β at 30-min (high-dose) and IL6 at 240-min (high-dose, low-dose) consistent with regulation of early-phase myeloid-cell associated muscle regeneration. ***Conclusions.*** Protein-leucine effects on performance during intense training may be inconsequential when in positive nitrogen balance, despite saturating BCAA metabolism, protein synthesis, and attenuating cell-membrane damage. 24 g of protein and 5 g leucine near saturated post-exercise myofibrillar FSR and simulated an early inflammatory promyogenic transcriptome common to skeletal muscle regeneration that was accentuated with 3-fold higher protein-leucine dose.

ACKNOWLEDGEMENTS

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STATEMENT OF CONTRIBUTION

CHAPTER 3: A protein-leucine supplement increases BCAA and nitrogen turnover but not performance.

Study conception was by Dr David Rowlands and Andre Nelson, and study design by Dr David Rowlands, Dr Trent Stellingwerff, Prof. Mark Tarnopolsky and Prof. Stuart Phillips. Ethics proposal was written by Dr David Rowlands. Subjects were recruited and the study co-ordinated by Andre Nelson. Lead-in and experimental-block controlled diets were designed and co-ordinated by Andre Nelson. Supplements were produced by Lionel Bovetto and Alain Fracheboud at the Nestle Research Center, Lausanne, Switzerland. The data was collected primarily by Andre Nelson with help from Dr David Rowlands and assistance by Jim Clarke, Lara Jackson, Marjolein Ros and Jasmine Thomson. Blood and expired breath-gas collection was by Andre Nelson and Dr David Rowlands. Urine and sweat samples were collected and prepared by Andre Nelson. Blood creatine kinase and glucose and urinary and sweat urea and creatinine were analysed by Andy Hollings with help from Andre Nelson using standard kits. Blood amino acids and stable isotopes for whole-body glucose and protein turnover were analysed at McMaster University, Toronto, Canada by Tracey Rerecich. Blood and urine samples for metabolomics were analysed at the Nestle Research Center, Lausanne, Switzerland by Serge Rezzi, Stephen J. Bruce, Isabelle Breton, Anita Thorimbert and Philippe A. Guy. Statistical analyses were performed by Dr David Rowlands and Andre Nelson. The manuscript was written and prepared by Andre Nelson with guidance from Dr David Rowlands and feedback from Prof. Stuart Phillips, Dr Trent Stellingwerff, Jim Clarke, Dr Suzanne Broadbent and Dr Daniel Moore.

CHAPTER 4: Post-exercise protein-leucine feeding affects neutrophil function via immunomodulatory plasma metabolites and attenuated cortisol during a 6-day block of intense cycling.

Immune and inflammatory study design was by Dr David Rowlands, Dr Suzanne Broadbent and Andre Nelson. Study conception, ethics approval, data collection, and statistical analyses were as detailed for Chapter 3. The neutrophil oxidative burst assay and differential immune-cell counts were conducted by Lara Jackson with assistance from Andre Nelson. Blood cortisol, testosterone and sex-hormone binding globulin were determined by Andy Hollings, and neutrophil elastase concentration by Andy Hollings and Andre Nelson, using standard kits. Immunoglobulin-A was determined by LabPlus, Auckland City Hospital, New Zealand. Interleukin-6 and 10 were analysed via Bioplex by Fliss Jackson at Massey University, Palmerston North, New Zealand. The manuscript was written by Andre Nelson with guidance from Dr David Rowlands and Dr Suzanne Broadbent and feedback from Dr Trent Stellingwerff and Jim Clarke.

CHAPTER 5: Acute phase fractional muscle protein synthetic and signalling responses to the ingestion of low and high saturating doses of a protein-leucine-carbohydrate supplement following high-intensity endurance exercise.

Study design was by Dr David Rowlands, Andre Nelson, Dr Trent Stellingwerff, Dr Dan Moore and Prof. Stuart Phillips. Ethics approval was written by Dr David Rowlands with assistance by Andre Nelson. Subjects were recruited and the study co-ordinated by Andre Nelson. Lead-in and experimental-period controlled diets were designed and co-ordinated by Andre Nelson. Supplements were produced by Garry Radford at Massey University, Palmerston North, New Zealand. Muscle biopsies were taken by Dr Murray Leikis, Dr Kevin

Bell and Dr Sarah Beable. The data were collected primarily by Andre Nelson with help from Dr David Rowlands and assistance by Jim Clarke, Dr James Faulkner, Daniel Wadsworth and Wendy O'Brien. Blood samples were collected by Andre Nelson and Dr David Rowlands. Blood glucose and lactate were determined using an automated analyser by Wendy O'Brien with assistance by Andre Nelson. Blood amino acid concentrations and blood and muscle stable isotopes for myofibrillar and mitochondrial protein fractional synthesis rates were analysed at McMaster University, Toronto, Canada by Tracey Rerecich and Dr Nick Burd. Muscle homogenates for were made by Andy Hollings and Andre Nelson. Western blots were performed by Andy Hollings with assistance from Andre Nelson at Massey University, Wellington, New Zealand; Chandra Kirana at Wakefield Hospital, Wellington, New Zealand; and Dr Leigh Breen at McMaster University, Toronto, Canada. Statistical analyses were performed by Andre Nelson with guidance by Dr David Rowlands. The manuscript was written and prepared by Dr David Rowlands and Andre Nelson with feedback from Dr Trent Stellingwerff, Dr Daniel Moore and Professor Stuart Phillips.

CHAPTER 6: The action of protein-leucine feeding and dose on the acute-phase skeletal muscle transcriptome after endurance exercise.

Study conception and data collection were as detailed for Chapter 5. Microarray methods by Frederic Raymond and microarray data analyses were by Robert Mansourian. Bioinformatics was by Dr David Rowlands (Ingenuity Pathway Analysis) and Andre Nelson (Database for Annotation, Visualization and Integrated Discovery). The manuscript was written and prepared by Dr David Rowlands and Andre Nelson.

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RESEARCH ETHICS

Ethics approval was obtained from the Central Regional Ethics Committee for the studies conducted. The potential risks, and management of the risk involved, are detailed below:

All participants were screened via a health history questionnaire for pre-existing conditions to ensure they were physically healthy and able to take part in the studies. Participants were limited to athletes in regular training, and individuals who were neither disabled nor elderly and at increased risk of discomfort during the exercise and performance portions of the research. Fatigue during the exercise and performance trials was to be expected, however, this was anticipated to be of a similar level to that experienced by participants during their own endurance training and competition. Maximal efforts were requested of the participants during both the maximal aerobic power (VO_2max) testing and performance trials and the associated discomfort is normal for the level of athlete recruited, and in fact adaptive and beneficial to health. There may have been some discomfort and a minor risk of infection with venipuncture and cannulation procedures and muscle biopsies. This discomfort was minimised in each instance by having subjects lying prone on a hospital bed, with blood and biopsy procedures performed by trained phlebotomists and medics with experience in the procedures. It was considered that the amount of blood and muscle tissue samples taken posed no risk of adverse health effects. Risk of infection were minimised by following sterile procedure guidelines.

Social and psychological risks were minimised by ensuring privacy and confidentiality of participants throughout data collection and data storage periods. Initially we obtained informed consent and communicated to participants their right to discontinue or withdraw

from the studies at any time. We ensured that there were adequate change and shower facilities and we minimised the number of observers in the laboratory at any one time while the participants were being examined and/or tests conducted. Following data collection, any identifying information has been stored securely in a locked filing cabinet in a locked office with access to only those principally involved in the studies. We aimed to reduce the economic risk to participants by reimbursing them for their travel and time where necessary.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SUPPLEMENTARY DATA

Supplementary Data 2.1. Koopman 2009 ES and Inferences.xlsx.

Supplementary Data 2.2. Moore 2009 ES and Inferences.xlsx.

Supplementary Data 2.3. Moore 2009 ES and Inferences.pptx.

Supplementary Data 2.4. Wilkinson 2008 ES and Inferences.xlsx

Supplementary Data 2.5. Wilkinson 2008 ES and Inferences.pptx

Supplementary Data 2.6. (Folder) Muscle Protein Synthesis ES and Inferences

Supplementary Data 3.1. (Folder) Participant Nutrition and Exercise Loading

Supplementary Data 3.2. (Folder) Infusion Pump Calibration

Supplementary Data 3.3. Flow Meter Calibration.xlsx.

Supplementary Data 3.4. Isotope Infusions.xlsx.

Supplementary Data 3.5. Leucine Turnover and Breath Data Calculations.xlsx.

Supplementary Data 3.6. Glucose Turnover Calculations.xlsx.

Supplementary Data 3.7. Nitrogen Balance Calculations.xlsx.

Supplementary Data 3.8. Metabolomics Methods.docx.

Supplementary Data 3.9. Metabolomics Outcomes.xlsx.

Supplementary Data 3.10. Koopman 2005 ES and Inferences.xlsx.

Supplementary Data 5.1. Final Ingredients for Manufacture of Supplement at Massey.xlsx

Supplementary Data 5.2. Correlations and Regressions of PlasmaAA mTOR-pathway phosphoproteins and myofibrillar FSR.xlsx.

Supplementary Data 5.3. Infusion Pump Validity and Flow Rates.xls.

Supplementary Data 6.1. p0.001ANOVAsAllContrastsFoldESCIs.xlsx.

Supplementary Data 6.2. Muscle Growth & Development and Immune Related Functions.xlsx.

Supplementary Data 6.3. IPA Functions.xlsx.

Supplementary Data 7.1. Study 1 Descriptive Data.xlsx

Supplementary Data 7.2. Study 2 Descriptive Data.xlsx

LIST OF GENE ABBREVIATIONS AND ACRONYMS

ABCA1, ATP-binding cassette sub-family A member 1

ABCE1, ATP-binding cassette sub-family E (OABP) member 1

ACADVL, acyl-CoA dehydrogenase very long chain

ACHE, acetylcholinesterase

ACP1, acid phosphatase 1 soluble

ACTB, actin beta

ACTC1, actin cardiac muscle 1

ADAMTS9, ADAM metallopeptidase with thrombospondin type 1 motif 9

ALOX5AP, arachidonate 5-lipoxygenase-activating protein

ANGPTL2, angiopoietin-like 2

ANKRD1, ankyrin repeat domain 1

ANTXR1, arachidonate 5-lipoxygenase-activating protein

ANXA1, annexin 1

ANXA2, anthrax toxin receptor 1

APP, amyloid beta (A4) precursor protein

AQP4, aquaporin 4

ATP1B1, ATPase Na+/K+ transporting beta 1 polypeptide

ATP2A1, ATPase fast twitch 1

ATP2A2, ATPase slow twitch 2

BARX2, BARX homeobox 2

BCL6, B-cell lymphoma 6

BDNF, brain-derived neurotrophic factor

BGN, biglycan

BHLHE40, basic helix-loop-helix family, member e40

C21orf33, chromosome 21 open reading frame 33

CCND1, cyclin D1

CD2, cluster of differentiation 2

CD14, cluster of differentiation 14

CD36, cluster of differentiation 36

CD44, cluster of differentiation 44

CD86, cluster of differentiation 86

CD93, cluster of differentiation 93

CD97, cluster of differentiation 97

CD163, cluster of differentiation 163

CDKN1A, cyclin-dependent kinase inhibitor 1A

CEBPA, CCAAT/enhancer binding protein (C/EBP) alpha

CFH, complement factor H

CHRNA1, cholinergic receptor nicotinic alpha 1

CHRND, cholinergic receptor nicotinic delta

CHRNG, cholinergic receptor nicotinic gamma

CIDEC, cell death-inducing DFFA-like effector c

CLIC4, chloride intracellular channel 4

COL1A2, collagen type I alpha 2

COL3A1, collagen type III alpha 1

COL4A1, collagen type IV alpha 1

COL5A1, collagen type V alpha 1

COL5A2, collagen type V alpha 2

COL6A1, collagen type VI alpha 1

COL6A2, collagen type VI alpha 2

COL6A3, collagen type VI alpha 3

COLQ, collagen-like tail subunit of asymmetric acetylcholinesterase

CREB1, cAMP responsive element binding protein 1

CSF1R, colony stimulating factor 1 receptor

CSRP3, cysteine and glycine-rich protein 3

CTGF, connective tissue growth factor

CTSG, cathepsin G

CXCL2, chemokine (C-X-C motif) ligand 2

CYR61, cysteine-rich angiogenic inducer 61

DAB2, disabled homolog 2

DACH1, dachshund homolog 1

DCN, decorin

DDB1, damage-specific DNA binding protein 1

DDIT3, DNA-damage-inducible transcript 3

DDIT4, DNA-damage-inducible transcript 4

DIO2, deiodinase iodothyronine type II

DMD, dystrophin

DNMT3L, DNA (cytosine-5-)-methyltransferase 3-like

DUSP1, dual specificity phosphatase 1

DUSP10, dual specificity phosphatise 10

DYRK1A, dual-specificity tyrosine-(Y)-phosphorylation regulated kinase 1A

EFEMP1, EGF containing fibulin-like extracellular matrix protein 1

EGR1, early growth response 1

EIF4G2, eukaryotic translation initiation factor 4 gamma 2

F2RL1, coagulation factor II receptor-like 1

FBXO32, F-box protein 32

FMOD, fibromodulin

FOS, FBJ murine osteosarcoma viral oncogene homolog

FSTL1, follistatin-like 1

GADD45A, growth arrest and DNA-damage-inducible alpha

GADD45B, growth arrest and DNA-damage-inducible beta

GADD45G, growth arrest and DNA-damage-inducible gamma

GEM, GTP binding protein overexpressed in skeletal muscle

GJA1, gap junction protein alpha 1

H19, imprinted maternally expressed transcript

H6PD, hexose-6-phosphate dehydrogenase

HBP1, high mobility group box transcription factor 1

HK2, hexokinase 2

HLA-DQA1, major histocompatibility complex, class II, DQ alpha 1

HMGA1, high mobility group AT-hook 1

ID2, inhibitor of DNA binding 2

IFIT3, interferon-induced protein with tetratricopeptide repeats 3

IGF1, insulin-like growth factor 1

IGFBP1, insulin-like growth factor binding protein 1

IGFBP3, insulin-like growth factor binding protein 3

IL10RB, interleukin 10 receptor beta

IL1B, interleukin 1-beta

IL6, interleukin-6

ING5, inhibitor of growth family member 5

IRAK1, interleukin-1 receptor-associated kinase 1

IRF1, interferon regulatory factor 1

ITGB1, integrin beta 1

JUN, jun proto-oncogene

KLF10, Kruppel-like factor 10

KLF2, Kruppel-like factor 2

KLF4, Kruppel-like factor 4

LCP1, lymphocyte cytosolic protein 1

LDLR, low density lipoprotein receptor

LUM, lumican

LYVE1, lymphatic vessel endothelial hyaluronan receptor 1

MAP2K2, mitogen-activated protein kinase kinase 2

MAP4, microtubule-associated protein 4

MB, myoglobin

MGP, matrix Gla protein

MLYCD, malonyl-CoA decarboxylase

MRAS, muscle RAS oncogene homolog

MRVI1, murine retrovirus integration site 1 homolog

MT2A, metallothionein 2A

MTPN, myotrophin; SMAD1, mothers against decapentaplegic homolog 1

MYBPH, myosin binding protein H

MYC, v-myc myelocytomatosis viral oncogene homolog

MYCN, v-myc myelocytomatosis viral related oncogene, neuroblastoma derived

MYH1, myosin heavy chain 1

MYH11, myosin heavy chain 11

MYL4, myosin light chain 4

MYOD1, myogenic differentiation

MYOG, myogenin

NAMPT, nicotinamide phosphoribosyltransferase

NDRG2, N-myc downstream regulator 2

NFIC, nuclear factor 1 C-type

NFKB1, nuclear factor of kappa light polypeptide gene enhancer in B-cells 1

NFKBIA, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha

NR4A3, nuclear receptor subfamily 4 group A member 3

OGT, O-linked N-acetylglucosamine transferase

PDE4D, phosphodiesterase 4D

PELI3, pellino 3

PHGDH, phosphoglycerate dehydrogenase

PHLDA1, pleckstrin homology-like domain family A member 1

PI1, glutathione S-transferase pi 1

PIM1, pim-1 oncogene

PKM2, pyruvate kinase muscle 2

PLA2G7, phospholipase A2 group VII

PLEC, plectin

PLEKHO1, pleckstrin homology domain containing, family O member 1

PLIN2, perilipin 2

PLTP, phospholipid transfer protein

PMP22, peripheral myelin protein 22

POSTN, periostin

PPARD, peroxisome proliferator-activated receptor delta

PPARG, peroxisome proliferator-activated receptor gamma

PPP1R15A, protein phosphatase 1 regulatory (inhibitor) subunit 15A

PRDX2, peroxiredoxin

PRLR, prolactin receptor; cytoplasm

PTGER4, prostaglandin-endoperoxide synthase 2

PTGS2, prostaglandin-endoperoxide synthase 2

PTP4A3, protein tyrosine phosphatase type IVA member 3

RASA1, RAS p21 protein activator

RELA, v-rel reticuloendotheliosis viral oncogene homolog A

RGS2, regulator of G-protein signaling 2

RORA, RAR-related orphan receptor A

RPLP1, ribosomal protein large P1

RRM2B, ribonucleotide reductase M2 B

RRS1, ribosome biogenesis regulator homolog

RTN4, reticulon 4

S100A6, S100 calcium binding protein A6

S100A8, S100 calcium binding protein A8

S100A9, S100 calcium binding protein A9

S100A10, S100 calcium binding protein A10

SCD, stearoyl-CoA desaturase

SCN4A, sodium channel voltage-gated type IV alpha subunit

SETD3, SET domain containing 3

SGCA, sarcoglycan alpha

SGK1, serum/glucocorticoid regulated kinase 1

SLC37A4, solute carrier family 37 (glucose-6-phosphate transporter), member 4

SMAD3, mothers against decapentaplegic homolog 3

SMAD4, mothers against decapentaplegic homolog 4

SMAD7, mothers against decapentaplegic homolog 7

SOCS3, suppressor of cytokine signaling 3

SP4, Sp4 transcription factor

SPARC, secreted protein acidic cysteine-rich

SPI1, spleen focus forming virus (SFFV) proviral integration oncogene

SPRR2A, small proline-rich protein 2A;

SREBF1, sterol regulatory element binding transcription factor 1

STAT3, signal transducer and activator of transcription 3

TAGLN, transgelin

TAP1, transporter associated with antigen processing 1

TFRC, transferrin receptor

TGFBI, transforming growth factor, beta-induced

TGFBR2, transforming growth factor beta receptor 2

THBD, thrombomodulin

THBS2, thrombospondin 2

THBS4, thrombospondin 4

THRB, thyroid hormone receptor, beta

TIMP2, tissue inhibitor of metalloproteinase 2

TNC, tenascin C

TNFAIP6, tumor necrosis factor alpha-induced protein 6

TNFRSF12A, tumor necrosis factor receptor superfamily member 12A

TNRC6A, trinucleotide repeat containing 6A

TOM1, target of myb1

TP63, tumor protein p63

TPM3, tropomyosin 3

TRDN, triadin

TRIM29, tripartite motif containing 29

TXN2, thioredoxin 2

TXNIP, thioredoxin interacting protein

TYROBP, TYRO protein tyrosine kinase binding protein

UBC, ubiquitin C

UCP2, uncoupling protein 2

UCP3, uncoupling protein 3

USF2, upstream transcription factor 2 c-fos interacting

VCAM1, vascular cell adhesion molecule 1

VCAN, versican

VEGFA, vascular endothelial growth factor alpha

VIM, vimentin; ACTG2, actin gamma 2

WASF2, WAS protein family, member 2

WISP2, WNT1 inducible signaling pathway protein 2

ZBTB16, zinc finger and BTB domain containing 16

ZFAND6, zinc finger AN1-type domain 6

ZFHX3, zinc finger homeobox 3

CHAPTER 1 INTRODUCTION

Crucial to athletes engaging in repeated training or competition bouts of fatiguing endurance-type exercise is a period of post-exercise recovery in order to prevent a substantial decline in subsequent performance. Furthermore, optimising recovery can promote enhanced adaptation to the training stimulus. Insufficient recovery from exercise can result in a performance decrement (Halson et al. 2002) or impaired training adaptation (Smith 2000) and is of particular importance to endurance athletes, whom frequently incorporate into their training periods of high exercise-intensity and volume, including training more than once per day (Petibois, Cazorla, Poortmans and Déléris 2002). Recovery is necessary to promote optimal rehydration, replenish fuel stores, restore muscle cell homeostasis and repair damaged myofibres, and to initiate molecular and metabolic processes of adaptation (Burke 1997, Ivy 2004, Mahoney et al. 2005, Hood, Irrcher, Ljubicic and Joseph 2006). Total recovery duration may be the most important factor impacting on recovery of performance and adaptation, but endurance athletes must balance their time in order to optimise the training-stimulus and recovery. For these reasons, specific nutritional recovery strategies are undertaken by endurance athletes to provide adequate macronutrients (with an emphasis on carbohydrate and protein) and energy, and to rehydrate in order to facilitate optimal recovery and potentially augment training adaptation (Hawley, Tipton and Millard-Stafford 2006, Beelen, Burke, Gibala and van Loon 2010, Maughan and Shirreffs 2012).

DIETARY CARBOHYDRATE UNDERPINS ENDURANCE ATHLETE NUTRITION

Muscle and liver glycogen are the key fuel stores determining endurance performance (Bergström, Hermansen, Hultman and B. 1967, Coggan and Coyle 1987); low muscle

glycogen is detrimental to the performance of moderate and high intensity exercise (Hargreaves and Richter 1988) and repletion of glycogen may be the most important factor impacting the time needed to recover exercise performance (Ivy 2001, Beelen, Burke, Gibala and van Loon 2010). Dietary carbohydrate ingestion is well-established as the key factor impacting on the muscle glycogen resynthesis rate (Ivy et al. 1988, Burke, Collier and Hargreaves 1993, Burke et al. 1996, Tsintzas et al. 2003, Stevenson, Williams and Biscoe 2005). For example, ingesting carbohydrate-rich meals following glycogen-depleting exercise enhances skeletal muscle glycogen repletion (Burke, Collier and Hargreaves 1993, Stevenson, Williams and Biscoe 2005) and consuming a high-carbohydrate diet during a period of intensified training can benefit endurance performance (Achten et al. 2004, Burke, Kiens and Ivy 2004). Provision of nutrients immediately after exercise takes advantage of several important aspects of post-exercise physiology that may enhance the effectiveness of nutrients on cellular processes suspected of regulating muscle recovery. Blood flow to the exercised skeletal muscle is increased, enhancing delivery of nutrients (Biolo, Tipton, Klein and Wolfe 1997). Exercised muscle also exhibits an acute but transient increase in substrate uptake (Goodyear et al. 1990, Biolo et al. 1995) via upregulation of nutrient (glucose, amino acid) transporters (Goodyear et al. 1990, Etgen Jr, Farrar and Ivy 1993, Drummond et al. 2011) and enhanced insulin sensitivity (Maarbjerg, Sylow and Richter 2011) which further increases transport and uptake (Richter, Garetto, Goodman and Ruderman 1982, Biolo et al. 1995, Biolo, Williams, Fleming and Wolfe 1999, Timmerman et al. 2012). Unsurprisingly, the highest rates of muscle glycogen storage occur in the first hour after exercise (Ivy et al. 1988). These factors appear to contribute to enhanced rates of synthesis of glycogen (Ivy 1998), and also muscle proteins (Biolo, Declan Fleming and Wolfe 1995, Levenhagen et al. 2001), in the immediate few hours after exercise. Full glycogen restoration following depleting exercise may require ~20 h (~100 mmol·kg muscle^{-1} at an average rate of ~5

mmol·kg^{-1}·h^{-1}) and it follows that carbohydrate availability in the immediate post-exercise recovery period and the subsequent daily diet is the primary nutritional concern of the endurance athlete (Burke, Kiens and Ivy 2004).

PROTEIN-CARBOHYDRATE COINGESTION AFTER ENDURANCE EXERCISE HAS INCONSISTENT EFFECTS ON RECOVERY OF PERFORMANCE: INSUFFICIENT GLYCOGEN RESTORATION?

Perhaps owing to the primacy of carbohydrate to endurance performance, there have been relatively fewer investigations into the impact of ingesting protein and amino acids in the 'crucial window' of few hours constituting the immediate post-exercise recovery period. Early work recognized an impact of protein-carbohydrate coingestion upon glycogen restoration (Zawadzki, Yaspelkis and Ivy 1992). Additional investigation has established that co-ingesting protein with carbohydrate appears to benefit the rate of glycogen resynthesis, but only if the rate of carbohydrate provision is sub-optimal (< 1.2g·kg^{-1}·h^{-1}) (Jentjens et al. 2001, Berardi, Noreen and Lemon 2006). Co-ingesting protein with carbohydrate increases insulin secretion and activity (van Loon et al. 2000, Betts et al. 2005) which might enhance the rate of glycogen resynthesis (van Loon, Saris, Kruijshoop and Wagenmakers 2000) in part by promoting greater muscle glucose uptake and activity of glycogen synthase, the enzyme that controls the rate limiting step of glycogen synthesis (Ivy 1991). However, evidence for an impact of post-endurance exercise protein-carbohydrate coingestion on the recovery of endurance performance during a subsequent bout of exercise is inconsistent.

A number of recent studies investigating the role of protein-carbohydrate, protein-leucine-carbohydrate, or flavoured milk coingestion on the recovery of endurance performance over acute (several hours to nearly a day) and short-term (~40 h to six days) timeframes (Niles et al. 2001, Williams, Raven, Fogt and Ivy 2003, Betts et al. 2005, Millard-Stafford et al. 2005, Berardi, Noreen and Lemon 2006, Karp et al. 2006, Betts, Williams, Duffy and Gunner 2007, Luden, Saunders and Todd 2007, Rowlands et al. 2007, Berardi, Noreen and Lemon 2008, Rowlands et al. 2008, Watson, Love, Maughan and Shirreffs 2008, Pritchett et al. 2009, Thomas, Morris and Stevenson 2009, Kerasioti et al. 2010, Ferguson-Stegall et al. 2011, Thomson, Ali and Rowlands 2011, Goh et al. 2012, Lunn et al. 2012). For the majority of these investigations, the extent of glycogen depletion and restoration between bouts is most likely the primary mechanism influencing subsequent performance; variability in elements of the diverse study designs utilised could impact upon the relative concentration of muscle and liver glycogen prior to testing of performance and might, therefore, account for the inconsistent performance outcomes. For example, factors that might have impacted on glycogen resynthesis and thus performance outcome variation between studies include differences in the energy content of the protein-carbohydrate interventions versus carbohydrate-only or placebo conditions (Williams, Raven, Fogt and Ivy 2003, Millard-Stafford et al. 2005, Betts, Williams, Duffy and Gunner 2007), rates of post-exercise carbohydrate provision that range from inferior (e.g. (Betts, Williams, Duffy and Gunner 2007)) to optimal (≥ 1.2 $g \cdot kg^{-1} \cdot h^{-1}$) (e.g. (Rowlands et al. 2007)), and the extent of time allowed between the initial exercise bout and the subsequent performance test with a range spanning from 2 h (Niles et al. 2001, Millard-Stafford et al. 2005) out to18-19 h (Rowlands et al. 2007, Pritchett et al. 2009) for acute recovery-of-performance investigations. Additional factors, such as the various exercise protocols and performance tests utilised, micronutrients in milk beverages (e.g. (Ferguson-Stegall et al. 2011)), or the addition of electrolytes to

protein-carbohydrate (Williams, Raven, Fogt and Ivy 2003) might also have contributed to between-study variability in performance outcomes.

SKELETAL MUSCLE RECOVERY AND ADAPTATION TO ENDURANCE EXERCISE: WHAT IS THE ROLE FOR PROTEIN?

In addition to fuel substrate resynthesis, optimal skeletal muscle recovery from endurance-type exercise also involves restoration of muscle homeostasis, repair of damaged tissue proteins and cellular apparatus, and the accumulation of enzymes and other proteins, organelles (e.g. mitochondria) and extracellular constituents with an adaptive benefit, culminating in successful muscle adaptive remodelling in response to the exercise stimulus (Timmons et al. 2005, Fluck and Hoppeler 2006, Hood, Irrcher, Ljubicic and Joseph 2006). Adaptive processes are guided by the specific molecular programmes of the post-exercise transcriptome (Puntschart et al. 1995, Pilegaard, Ordway, Saltin and Neufer 2000, Fluck and Hoppeler 2003, Mahoney et al. 2005, Timmons 2011). Acute endurance exercise stimulates the expression of genes involved in the oxidative stress response, electrolyte shuttling, metabolism, transcription, proteolysis, and cell growth and death (Mahoney et al. 2005) and the transcriptional regulation of metabolic genes may be driven by the metabolic changes associated with exercise and during subsequent recovery (Pilegaard et al. 2005) and transduced via key regulatory signalling protein networks (Atherton et al. 2005, Nader and Esser 2011). By impacting on these processes of recovery and adaptation, ingesting insulinotropic protein and amino acids post-exercise may have additional benefit to skeletal muscle recovery on top of accelerating glycogen repletion when carbohydrate availability is low.

Insulin potently regulates post-exercise muscle physiology

Insulin activates intracellular signalling pathways such as that of the mammalian target of rapamycin (mTOR) (Morrison, Hara, Ding and Ivy 2008) thereby regulating an increase in the resting-fasted rate of muscle protein synthesis (Timmerman et al. 2010). Although insulin does not contribute to the increase in muscle protein synthesis seen with the provision of essential amino acids (EAAs), it substantially impacts the rate of muscle protein breakdown with or without amino acid provision (Greenhaff et al. 2008) thereby improving net protein balance (the sum of the rates of synthesis and breakdown) and, therefore, muscle anabolism. Under conditions of increased amino acid availability, the anabolic impact of insulin might be primarily the result of its potent endothelial vasodilatory effect that increases blood flow (and therefore nutrient flow) to skeletal muscle (Timmerman et al. 2010). Insulin also has widespread effects on gene transcription (Rome et al. 2003). In rested skeletal muscle insulin regulates approximately 800 genes (Rome et al. 2003, Wu et al. 2007) and with diverse temporal effects on patterns of gene expression (Di Camillo et al. 2012). Thus, insulinotropic protein and amino acids coingestion with carbohydrate could impact on glycogen-dependent and non-glycogen dependent mechanisms of repair and recovery following endurance exercise by enhancing the secretion of insulin. While insulin is probably an important regulator of post-exercise recovery mechanisms, not all studies show a substantial impact of protein-carbohydrate coingestion on the blood concentration of insulin, relative to control conditions (e.g. (Ferguson-Stegall et al. 2011, Goh et al. 2012)). More importantly, the most potent effects of dietary protein and amino acid ingestion upon the post-exercise muscle environment are probably more direct and related to enhanced amino acid availability in the blood and at the muscle.

Dietary protein-derived amino acids and the post-exercise muscle environment

A primary reason to consume protein in the post-exercise period is to provide exogenous amino acids to replace the small quantities oxidised as a fuel during exercise (Wagenmakers 1998, Tarnopolsky 2004, Gibala 2007). Of even greater importance is that dietary protein provides the substrate for and stimulates an increase in the rate of muscle protein synthesis following endurance exercise (Howarth, Moreau, Phillips and Gibala 2009, Breen et al. 2011, Lunn et al. 2012) which could be a key mechanism impacting on the rate of renewal of damaged muscle proteins and synthesis of new adaptive proteins (Tipton and Wolfe 2004, Phillips, Moore and Tang 2007, Phillips and van Loon 2011). In terms of the timing of nutrient intake following exercise, Levenhagen et al. (Levenhagen et al. 2001) showed that the immediate ingestion of a small quantity of protein, carbohydrate and fat (10/8/3 g, respectively) enhanced uptake of blood glucose and amino acids and leg and whole-body protein synthesis rates (thereby promoting greater accretion of muscle and body protein) compared to ingesting the same supplement 3 h into recovery, emphasising the heightened responsiveness of muscle tissue protein turnover to feeding in the immediate hours post-exercise.

Amino acid (leucine) ingestion and intracellular signalling

Amino acids, particularly the branch-chain amino acid (BCAA) leucine, activate the intracellular signalling pathways that increase the initiation and elongation steps of messenger ribonucleic acids (mRNA) translation (Anthony et al. 2000, Frøsig et al. 2007, Suryawan et al. 2008, Dickinson et al. 2011), thereby enhancing the rate of cellular protein synthesis (Kimball and Jefferson 2004). In particular, the mTOR signalling pathway has been heavily investigated, and it has been established that leucine is the key amino acid for

enhancing mTOR activity (Anthony et al. 2000, Crozier et al. 2005, Frøsig et al. 2007, Suryawan et al. 2008, Atherton et al. 2010, Dickinson et al. 2011). It follows that enriching dietary protein (which is needed to provide all essential and non-essential amino acids as substrates) with leucine might further increase mTOR-pathway signalling and protein synthesis (Murgas Torrazza et al. 2010, Casperson, Sheffield-Moore, Hewlings and Paddon-Jones 2012) but reduced overall nitrogen (protein) load. This could be important to muscle recovery as it has been shown that a high intake of dietary protein can increase protein breakdown and amino acid oxidation without increasing protein synthesis at rest (Gaine et al. 2007) and negatively impact the rate of muscle protein synthesis following endurance exercise (Bolster et al. 2005). Furthermore, endurance athletes should maintain a low body-fat mass to maximise their power-to-mass ratio and, therefore, total caloric intake is important; potentiating the signalling potency of a protein with leucine can reduce the total energy content of feeding.

Post-exercise protein feeding regulates muscle adaptive gene expression

Intracellular signalling networks, including mTOR, also augment the transcription of a wide array of transcription factors, genes and gene networks (Ducluzeau et al. 2001, Thong et al. 2003, Boonsong et al. 2007, Duvel et al. 2010). As about 40% of variability in the cellular abundance of proteins appears to be explained by mRNA levels, at least in mouse fibroblasts (Schwanhäusser et al. 2011), repeated activation of exercise-induced gene programs is likely to contribute substantially to the overall direction and magnitude of adaptation in response to the training stimulus (Pilegaard, Ordway, Saltin and Neufer 2000, Perry et al. 2010); superimposition of amino acid regulated gene activity over exercise-regulated gene expression could summatively affect aspects of the adaptive response. Therefore, it might

also be advantageous to consume protein post-exercise in order to regulate gene networks important to the recovery and adaptive responses. In support of this assertion, it has recently been shown that protein feeding during recovery from an acute bout of endurance exercise upregulated the expression of gene ontology allied to the wound-healing response in skeletal muscle; at 3 h following exercise, these ontology included extracellular matrix (ECM), stress response, myocellular growth and development, and immunity and defense (Rowlands et al. 2011). The upregulated immunity and defense ontology is of special interest, as here is an accumulating body of evidence that the magnitude, direction and duration of the inflammatory response is important for rapid and effective muscle healing following exercise-induced trauma (Shen et al. 2005, Tidball 2005, Arnold et al. 2007, Segawa et al. 2008, Villalta et al. 2009, Serrano and Muñoz-Cánoves 2010, Tidball and Villalta 2010, Mann et al. 2011). Furthermore, dietary nutrients play important roles in regulating aspects of immune cell function and muscle and systemic inflammation following exercise (Gleeson 2006, Moreira et al. 2007, Walsh et al. 2011) and mTOR signalling is emerging as a key intracellular effector of nutrient-induced immune and inflammatory regulation (Weichhart et al. 2008, Thomson, Turnquist and Raimondi 2009). Thus, exercise-damaged muscle tissue requires repair and remodelling guided by the post-exercise transcriptome; protein and amino acids ingested post-exercise provides substrate for protein synthesis and regulates protein turnover, gene expression and potentially immune function and inflammation, regulated at least in part via the nutrient-responsive intracellular signalling pathway mTOR and might, therefore, improve post-exercise recovery and adaptation.

Protein effects on non-muscle adaptations to endurance exercise: cardiovascular changes

It is well established that chronic endurance training leads to not only skeletal muscle but also cardiovascular and systemic metabolic adaptations, for instance increased cardiac stroke volume and output (Ekblom et al. 1968) and blood, plasma and erythrocyte volumes (Sawka et al. 2000). Long-term protein-carbohydrate supplementation following exercise has also been shown to improve the rate of endurance training adaptation relative to isocaloric carbohydrate feeding (Okazaki et al. 2009, Ferguson-Stegall et al. 2011, Robinson et al. 2011). While most of these changes require longer timeframes (e.g. weeks to months) than are employed with acute and short-term recovery of performance studies (hours to several days), some adaptations are more rapidly attained; plasma volume is regulated by changes in the extracellular fluid and circulating protein volumes, and plasma volume expansion can occur within 24 h of exercise (Sawka et al. 2000). Therefore, there is some suggestion that the impact of dietary protein and amino acids during post-exercise recovery on mechanisms of aerobic exercise adaptation in organ systems other than the exercising skeletal muscle could also be important to the performance of subsequent exercise.

Protein effects on non-muscle adaptations to endurance exercise: immune function

Endurance athletes will undertake periods of high training intensities and/or volumes, known as overreaching, in order to apply sufficient physiological stimulus to induce further training adaptations that, with adequate recovery, improve subsequent performance (Halson et al. 2002). Cross-sectional and longitudinal studies have shown that during periods of high-intensity exercise and/or large training volumes there is an increased risk of some illnesses, including an increased incidence of upper respiratory tract infection (URTIs) (Gleeson 2007,

Walsh et al. 2011). A partial explanation for the increased risk of URTIs may be the observed reduction in cell-mediated immune function associated with the post-exercise immunosuppression, and mediated by exercise-induced alterations in cytokines such as interleukins IL-1, IL-6 and IL-10, and endocrine hormones (e.g. (Neiman 1997, Gleeson and Bishop 2005, Gleeson 2007, Walsh et al. 2011)). A key endocrine hormone affected by exercise is cortisol, which can exert an immunosuppressive effect with a lag of several hours, and has widespread impact on immune cell migration and function (Pedersen and Hoffman-Goetz 2000).

Lymphocytes are key immune cells of the acquired immune system. Intense exercise impairs lymphocyte function by reducing their activation, proliferation, secretory function, and target-cell killing (Walsh et al. 2011). Although lymphocyte function is important, so too is their migration into peripheral tissues, such as the lungs and skin, in order to increase the likelihood of detecting infected cells. Typically the circulating blood lymphocyte concentration increases during exercise 2- to 3-fold, followed by a post-exercise lymphopenia where concentrations fall to ~50% of the normal circulating concentration within 1-2 h (Gleeson and Bishop 2005) as they move into the migrate to peripheral tissues. Witard et al. (Witard et al. 2012) showed that during a period of high-intensity training (shown earlier to induce a performance decrement characteristic of athletic overreaching (Witard et al. 2010)), the redistribution of $CD8^+$ T lymphocytes (which primarily target viral antigens (van Lier, ten Berge and Gamadia 2003) out of the central circulation to the periphery was impaired. These data suggest that during athletic overreaching, the capability of the cell-mediated immune response to detect virus-infected cells might be impaired, and could be a contributing factor to the increased risk of URTI (Witard et al. 2012).

Neutrophils are an important component of the innate arm of the immune system, but also play a role in the muscle-tissue regeneration response to injury and exercise (Tidball and Villalta 2010). Neutrophils are among the first cells recruited to exercised skeletal muscle, where they regulate tissue repair by phagocytosis of exercise-damaged cells and cellular debris, modulate the function of other immune cells nearby, and facilitate initiation of myogenic programmes in muscle-tissue cells (Tidball and Villalta 2010). It has been shown that the neutrophil oxidative burst response (the primary debris-clearing and cell-killing mechanism of the neutrophil) declines following intense endurance exercise, and prolonged endurance training can further suppress microbicidal activity (Hack, Strobel, Weiss and Weicker 1994) which could impact on innate immunity and also muscle repair following hard or prolonged endurance exercise training.

Consuming adequate dietary carbohydrate, with a high rate of ingestion in the periexercise period, is a well-established method nutritional practice to offset exercise-induced changes in cytokines, and immune cell concentrations and function (Walsh et al. 2011). Endurance athletes are already recommended to consume a high carbohydrate diet (usually >7 $g \cdot kg^{-1} \cdot d^{-1}$) during heavy training and competition to maintain glycogen stores (Burke, Hawley, Wong and Jeukendrup 2011). Consuming a high carbohydrate diet (>70% of daily energy) during a period of intense training has also been shown to reduce plasma concentrations of cytokines IL-6 and IL-10 cytokine concentrations by 2-fold and the cytokine IL-1 receptor activator by 9-fold relative to placebo, relative to a low carbohydrate diet (<10% of daily energy) (Bishop et al. 2001). Furthermore, in untrained males who cycled for 60 min (70% of VO_2max), consumption of a high carbohydrate diet (75% of daily energy) for 3 days prior to exercise

resulted in a lower rise in cortisol during exercise and a reduced neutrophilia post-exercise, compared to a low carbohydrate diet (7% of energy) (Gleeson et al. 1998), High carbohydrate availability could, therefore, attenuate the exercise-induced immunosuppression by altering the concentration of immunoregulatory hormones and cytokines. Additionally, the ingestion of large quantities of carbohydrate before, during, and following endurance exercise can attenuate exercise-induced impairments in immune cell function through a reduction in the release of immunosuppressive stress hormones (e.g. cortisol; (Scharhag et al. 2006)) and contraction-induced cytokine transcription (e.g. IL-6; (Keller et al. 2001, Febbraio et al. 2003)). For example, in six well-trained cyclists who completed 2.5 h of cycling at 85% of their ventilatory threshold, it was found that carbohydrate ingestion during exercise (6% solution, total carbohydrate of 3.2 $g \cdot kg^{-1}$ body mass) reduced impairment of T-lymphocyte function by decreasing cell death within mitogen-stimulated cell cultures of extracted blood lymphocytes, relative to placebo (Green, Croaker and Rowbottom 2003). Consuming carbohydrate (1.2 $g \cdot kg^{-1}$ body mass) after 2 hr of running at 75% VO_2max prevented a post-exercise decrease in bacterially stimulated neutrophil degranulation relative to a placebo condition (Costa, Walters, Bilzon and Walsh 2011). Nevertheless, endurance athletes during training and competition already consume large amounts of carbohydrate and, therefore, should already be experiencing any preventative effects on the exercise-induced immunosuppression.

Endurance athletes are also recommended to ingest protein post-exercise to stimulate muscle repair via muscle protein synthesis (Tipton et al. 2004). Altering the dietary protein intake has been suggested as another nutritional countermeasure to the immunosuppressive effects of intense exercise (Calder and Kew 2002, Gleeson, Nieman and Pedersen 2004). Amino acids

(particularly glutamine) are important metabolic and fuel substrates for immune cells such as lymphocytes and neutrophils (Curi et al. 1999, Castell et al. 2004, Curi et al. 2005, Costa et al. 2009, Matsumoto et al. 2009, Costa, Walters, Bilzon and Walsh 2011). Multiple *in vitro* studies demonstrate that neutrophil and lymphocyte functional processes including proliferation, cytokine secretion, and cytotoxicity are in part, dependent on amino acid availability (e.g. (Waithe, Dauphinais, Hathaway and Hirschhorn 1975, Curi et al. 1999, Calder and Jackson 2000). Amino acid-induced intracellular signalling has been shown to be important regulator of the function of neutrophils and lymphocyte (Säemann et al. 2009, Powell and Delgoffe 2010, Dodd and Tee 2012). Leucine is the key amino acid activator of mTOR pathway signalling (Anthony et al. 2000, Crozier et al. 2005, Atherton et al. 2010) and as such could be an important amino acid regulating immune function. Glutamine is an important fuel substrate for immune cells (Curi et al. 1997, Curi et al. 1999, Moinard et al. 2002, Pithon-Curi et al. 2002, Castell et al. 2004, Curi et al. 2005) but the extracellular concentration of glutamine also regulates leucine intracellular availability via the glutamine/leucine bidirectional cotransporters present in cell plasma membranes (Nicklin et al. 2009). Leucine and glutamine are perhaps the best candidate amino acids to supplement in order to reduce or prevent an exercise-induced immunosuppression and, indeed, there are numerous studies that have investigated the immunological impact of supplementing the daily diet with small doses of the branch-chain amino acids valine, isoelueice and leucine (e.g. (Bassit et al. 2000, Bassit et al. 2002, Negro, Giardina, Marzani and Marzatico 2008, Matsumoto et al. 2009)) and glutamine (e.g. (Castell and Newsholme 1997, Castell et al. 1997, Rohde, MacLean and Pedersen 1998, Walsh et al. 2000, Lagranha et al. 2005, Lagranha, Hirabara, Curi and Pithon-Curi 2007)). There may also be bioactive peptides derived from digestion of whole whey protein that could benefit immune function; Rusu et al. (Rusu et al. 2009, Rusu et al. 2010) found that whey-protein peptide digests exert beneficial

effects on immune cell autophagy and survival, and neutrophil function. Several recent studies in humans have investigated the effect of increased dietary protein post-exercise or in the daily diet on function of the innate immune system (neutrophils) (Costa et al. 2009, Costa, Walters, Bilzon and Walsh 2011) and acquired immune function (lymphocytes) (Witard et al. 2013)Costa et al. (Costa, Walters, Bilzon and Walsh 2011) found that post-exercise dietary carbohydrate, rather than protein, prevented a decline in neutrophil function during recovery. Having earlier shown that exercise-induced lymphocyte redistribution was impaired by intense exercise characteristic of overreaching (Witard et al. 2012), Witard et al. (Witard et al. 2013) investigated whether a high protein diet was able to ameliorate the impairment. The authors (Witard et al. 2013) found that consuming a high protein diet (3 $g \cdot kg^{-1}$ body $mass \cdot day^{-1}$) during a week-long period of high-intensity endurance cycling returned leukocyte kinetics (CD8+ T-lymphocyte mobilization and extravasation from the bloodstream) to levels that were observed during normal-intensity training, relative to an energy and carbohydrate-matched control diet (1.5 g $protein \cdot kg^{-1}$ body $mass \cdot day^{-1}$). Importantly, there were fewer self-reported symptoms of URTIs with the high protein diet, suggesting that the protein-mediated enhancement of immune surveillance (inferred by greater T-lymphocyte effector cell mobilisation and extravasation) might contribute to improved immune function and reduced sickness (Witard et al. 2013).

Fat is a consideration in post-exercise feeding

It may also be prudent to include some fat in post-exercise nutrition. During moderate-intensity aerobic exercise, the oxidation of fats contributes approximately half of the overall energy requirements, with plasma free fatty acids accounting for about half of total fat oxidation (van Loon et al. 2001) and intramuscular triglycerides (IMTGs) constituting the

other major portion of fat substrate (van Loon et al. 2001, Schrauwen-Hinderling, Hesselink, Schrauwen and Kooi 2006). Endurance exercise depletes IMTG stores (Décombaz et al. 2000, Décombaz et al. 2001, van Loon et al. 2001, Larson-Meyer, Newcomer and Hunter 2002) and consumption of additional fat in the diet may be beneficial to enhance the rate of IMTG repletion (Décombaz et al. 2000, Larson-Meyer, Newcomer and Hunter 2002) and supercompensation of IMTG storage (Décombaz et al. 2001) as a result of enhanced fat availability (Schrauwen-Hinderling, Hesselink, Schrauwen and Kooi 2006). The dietary fatty acid profile impacts on cell membrane fatty acid composition, thereby influencing the function of membrane-linked cellular processes such as the Na^+/K^+ pump (Hulbert, Turner, Storlien and Else 2005). Additionally, dietary lipid also regulates the expression of many genes (Clarke 2000, Grimaldi 2001), such as (but not limited to) those involved in fat metabolism (Afman and Müller 2012), and can modulate protein expression by influencing mRNA processing and decay, and post-translational modifications (Xu, Nakamura, Cho and Clarke 1999, Clarke 2004). Fatty acids, in particular the polyunsaturates, can also impact on inflammation (Patterson et al. 2012) and augment the basal muscle protein synthesis response to infused amino acids and insulin (Smith et al. 2011, Smith et al. 2011). Only a handful of investigators looking at nutritional recovery of endurance performance have also incorporated fats into post-exercise interventions (Karp et al. 2006, Rowlands et al. 2007, Rowlands et al. 2008, Watson, Love, Maughan and Shirreffs 2008, Pritchett et al. 2009, Thomas, Morris and Stevenson 2009, Thomson, Ali and Rowlands 2011) and it is by and large incidentally included as the lipid-component of low-fat milks.

CONSUMING PROTEIN OR PROTEIN-LEUCINE POST-ENDURANCE EXERCISE SUBSTANTIALLY ENHANCES SUBSEQUENT PERFORMANCE

In order to investigate the effect of protein or protein with added leucine on putative non-glycogen mechanisms of recovery of endurance performance, experimental study designs need to allow for sufficient time between exercise bouts and provide adequate carbohydrate provision in the supplemental recovery period and background diet. As previously mentioned, most investigations have looked at subsequent performance *less than* 20 h following initial exercise, despite full glycogen resynthesis possibly requiring that much time or more. Two recent investigations have utilized short-term (several day) study designs to capture possible slow or delayed nutrient-mediated mechanisms of recovery (Rowlands et al. 2008, Thomson, Ali and Rowlands 2011). Rowlands et al. (Rowlands et al. 2008) found using a well-controlled crossover design and standardized high-carbohydrate background diet that ingesting a very large dose of protein (0.7 $g \cdot kg^{-1} \cdot h^{-1}$) with sufficient carbohydrate to saturate glycogen resynthesis (1.4 $g \cdot kg^{-1} \cdot h^{-1}$) and some fat (0.26 $g \cdot kg^{-1} \cdot h^{-1}$) during a 4-h recovery from 2.5 h of intense interval cycling provided no clear benefit to performance of a repeated sprint test the next day (15 h later), relative to an isocaloric low-protein (0.1 $g \cdot kg^{-1} \cdot h^{-1}$), high-carbohydrate (2.1 $g \cdot kg^{-1} \cdot h^{-1}$) and identical fat control condition, confirming earlier work (Rowlands et al. 2007). However, by day 4 (~60 h following initial exercise) there was a substantial 4.1% (95% confidence limit (CL) ±4.1%) improvement in repeated sprint mean power with the high-protein condition. Notably, this performance effect with high-protein recovery feeding was associated with positive nitrogen balance during day 1 recovery (compared to negative nitrogen balance with the control) indicative of enhanced whole-body tissue protein retention.

In a subsequent study, Thomson et al. (Thomson, Ali and Rowlands 2011) confirmed a delayed benefit of post-exercise protein feeding on the recovery of high-intensity endurance cycling performance (2.5 ±99%CL 2.6%). The investigators used a unique crossover design that isolated the specific post-exercise effect of high-protein recovery feeding by providing the alternate supplement at the opposite end of the day, enabling dietary protein intake to be clamped at 1.6 $g \cdot kg^{-1} \cdot d^{-1}$ with a controlled background diet and thereby removing total daily protein intake as a variable (Thomson, Ali and Rowlands 2011). By adding leucine (0.1 $g \cdot kg^{-1} \cdot h^{-1}$) to the protein, carbohydrate and fat (0.4/1.2/0.2 $g \cdot kg^{-1} \cdot h^{-1}$, respectively) recovery feeding, intracellular signalling potency was maintained with a reduced nitrogen load; a criticism of the earlier study (Rowlands et al. 2008) being that the protein intake was unreasonably high (Thomson, Ali and Rowlands 2011). Interestingly, the plasma concentration of the muscle membrane damage marker creatine kinase was 19% lower and perceived overall tiredness during repeated sprint performance tests reduced 13% with protein-leucine feeding, relative to the control, despite negative nitrogen balance with both conditions (Thomson, Ali and Rowlands 2011) suggesting a benefit to recovery even when whole-body protein metabolism (as gauged indirectly by the nitrogen balance method) may not be optimal. However, it should be noted that it is possible that the true effect of protein or protein-leucine feeding on the recovery within several days of endurance performance is trivial, given that the confidence interval for the mean effect from both studies (Rowlands et al. 2008, Thomson, Ali and Rowlands 2011) overlaps zero.

In summary, there are several plausible mechanisms that might explain preliminary evidence of a delayed effect of post-endurance exercise ingestion of protein-carbohydrate (with or without free-leucine and/or fat) on the recovery of subsequent endurance performance, but

limited direct mechanistic evidence. Elevated post-exercise rates of whole-body and skeletal muscle protein synthesis and turnover could expedite repair of cellular constituents and accrual of adaptive proteins. Some elements of the post-exercise skeletal muscle transcriptome guiding adaptation are nutrient responsive. By three hours post-exercise, an effect of recovery protein feeding is apparent on wound-healing related gene ontology including the ECM, muscle growth and development, and immunity and defense; with regards to the latter, an accumulating body of evidence suggests that the immune response is critical to muscle repair and recovery from exercise-induced damage. However, there are gaps in our understanding of how protein nutrition regulates these processes in vivo and in well-trained humans. The impact of post-exercise protein-carbohydrate-fat and protein-leucine-carbohydrate-fat coingestion on subsequent high-intensity endurance performance (Rowlands et al. 2008, Thomson, Ali and Rowlands 2011) requires confirmation, but also represents an opportunity to concurrently investigate the impact of feeding on whole-body immune and inflammatory responses and metabolism during intense training in a well-controlled setting. Oral intake of amino acids, especially leucine, increases the global rate of muscle protein synthesis, but the impact of post-endurance exercise protein-carbohydrate on muscle protein fractions (myofibrillar and mitochondrial) in well-trained humans has only recently been investigated (Breen et al. 2011). Nutrient and hormone-responsive intracellular signalling governs protein translation initiation and elongation but may also guide the expression of genes and biologically-related gene functions important to muscle cell recovery and adaptation. Importantly, the effect of the amino acid dose on skeletal muscle protein synthesis, intracellular signalling and gene expression is unknown following endurance exercise. Recent advances in high-throughput 'omics technologies are now being applied by exercise scientists (Walsh et al. 2011) and will undoubtedly offer new opportunities and

insights into the mechanisms regulating post-exercise human muscle repair and adaptation that are influenced by nutrition.

PURPOSE OF THE THESIS

The two clinical trials described in this thesis (herein detailed in four separate chapters) were designed to investigate the effects of post-endurance exercise leucine-protein, carbohydrate and fat recovery supplementation on subsequent performance, systemic immune function and inflammation, and putative mechanisms of muscle cell recovery and adaptation to endurance exercise, relative to isocaloric post-exercise carbohydrate and fat.

The aims of the first study were to investigate a possible benefit of post-exercise protein-leucine, carbohydrate and fat feeding during a 6-day block of intense cycling to:

1. subsequent high-intensity endurance performance and whole-body protein and amino acid metabolism, and
2. markers of systemic inflammation and immune cell concentration and function.

Notable elements of the study design included: the use of modern techniques including whole-body stable isotope methodology to study leucine and glucose turnover, and contemporary mass-spectrometry based metabolomics to assess large-scale changes in metabolites in response to supplementation; providing saturating quantities of essential amino acids, particularly leucine (for protein synthesis), and carbohydrates (for glycogen resynthesis) in the recovery supplements, and the inclusion of a moderate quantity of fat to simulate normal dietary practice; stringent control of daily energy and macronutrient intake

and energy expenditure, based around a high-carbohydrate background diet containing a moderate intake of high-quality (animal-derived) proteins and some fat, typical of normal athletic practice; and an intense 6-day cycle training protocol designed to simulate an extremely hard period of training, overtraining or multiday competition and providing a strong training stimulus, coupled with periods of rest and active recovery. It was hypothesised that protein-leucine, carbohydrate and fat supplementation would result in an enhancement of repeated-sprint cycling performance, improve whole-body leucine, protein and nitrogen balance, increase branch-chain amino acid turnover and attenuate muscle damage, relative to the isocaloric carbohydrate-fat control supplement. Furthermore, we hypothesised that the protein-leucine supplement would attenuate markers of systemic inflammation and a predicted decline in immune cell function.

The aims of the second study were to determine the effect of protein-leucine dose on:

1. skeletal muscle myofibrillar and mitochondrial protein synthesis,
2. translation initiation signalling via mTOR-pathway activation, and
3. on the post-exercise transcriptome.

We hypothesised that the quantity of protein-leucine in the low-dose was sufficient to saturate the mitochondrial and myofibrillar fractional protein synthesis rate (FSR) and would, therefore, result in no substantial difference in FSR compared to the high-dose condition. We also hypothesised that key acute-phase repair and regeneration biology of the skeletal muscle transcriptome that was previously found to respond to high-protein recovery feeding would also be regulated to protein-leucine feeding. Furthermore, we sought to determine if this transcriptome biology, predicted to involve muscle growth and development, immunity and

defense, ECM and the cellular stress response, would respond in a dose-dependent manner to protein-leucine feeding.

CHAPTER 2 LITERATURE REVIEW

Methodologies, Considerations and Outcomes from Investigations into the Effect of Protein Ingestion on Skeletal Muscle-Protein Synthesis Following Endurance Exercise

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PURPOSE

The purpose of this review was to discuss the measurement and magnitude of the impact of dietary protein ingested in the immediate hours following endurance exercise on skeletal muscle protein synthesis. The first part of the review will briefly introduce the rationale for providing protein and amino acids in post-exercise recovery feeding following intense endurance exercise; muscle protein synthesis is a primary candidate mechanism regulating muscle repair and recovery, and the rate of synthesis is modified at rest and after endurance-type exercise by protein feeding and amino acids. Secondly, methodological considerations for the measurement of the muscle protein synthetic rate are briefly reviewed, including common current stable-isotope infusion approaches, recent innovations and future directions. Next, the digestion characteristics of dietary proteins are discussed as they are a key determinant of subsequent tissue amino acid use. Rapidly digested proteins may be the most efficacious proteins at stimulating muscle protein synthesis in the immediate hours after endurance exercise because of their capacity to rapidly increase plasma amino acid concentrations. Fourth, the findings of investigations utilizing these methodologies to assess the impact of protein-carbohydrate and protein-leucine-carbohydrate coingestion following endurance exercise on post-exercise recovery muscle fractional synthetic rate (FSR) will be outlined and compared using magnitude-based inferential statistics (effect sizes and confidence intervals). Finally, plausible mechanisms to explain recent findings of a delayed benefit to short-term recovery of performance, including the possible impact of increased muscle protein synthesis, will be briefly discussed. This review should be of interest to researchers with a good understanding of molecular mechanisms of change in muscle (e.g. cell and molecular biologists) but with a less comprehensive background in nutrition and exercise physiology, or to emerging researchers who wish to familiarize themselves with current and future methodologies, practical considerations, and unresolved questions.

BACKGROUND

High-intensity endurance exercise involves prolonged periods of cyclical muscle contraction and concomitant high energy turnover to provide adequate adenosine triphosphate (ATP) (Trump, Heigenhauser, Putman and Spriet 1996, Gastin 2001, Watt, Heigenhauser, Dyck and Spriet 2002, De Feo et al. 2003). In man, liver and muscle glycogen are the primary fuel sources for intense exercise (Bergström, Hermansen, Hultman and B. 1967, Coggan and Coyle 1987). Prolonged intense exercise depletes liver and muscle glycogen to sustain a high rate of energy production (Hargreaves and Richter 1988) and is associated with muscle cell metabolic changes that can induce damage to cellular constituents (Faulkner, Brooks and Opiteck 1993, Tee, Bosch and Lambert 2007, Powers, Talbert and Adhihetty 2011) and apoptosis triggered by increased oxidative stress (Brancaccio, Lippi and Maffulli 2010). The exercise associated increases in oxygen demand, catecholamines, prostanoids, and enhanced muscle mitochondrial respiratory chain activity lead to increases in reactive oxygen and nitrogen species (RONS) and high-intensity intermittent endurance exercise might also increase muscle tissue ischemia and reperfusion which promotes oxidase enzyme activity (e.g. xanthine oxidase, nicotinamide adenine dinucleotide phosphate (NADPH)-oxidase); exercise-induced trauma resulting in the breakdown of iron-containing proteins, and post-exercise mechanical microdamage associated with inflammation (e.g. phagocyte respiratory burst via NADPH-oxidase), proteolysis, and calcium homeostatic imbalance can further augment RONS (Clarkson and Hubal 2002, Fisher-Wellman and Bloomer 2009, Brancaccio, Lippi and Maffulli 2010, Sahlin et al. 2010, Powers, Talbert and Adhihetty 2011). It should be noted, however, that reactive oxygen species (ROS) also act as signalling molecules in muscle regulating the cellular response to the exercise stress (Hughes, Murphy and Ledgerwood 2005) and muscle inflammation via phagocyte infiltration (Aoi et al. 2004) and

are therefore important regulators of the muscle recovery and adaptation responses (Powers, Talbert and Adhihetty 2011).

Relative to work-matched concentric exercise, eccentric resistance exercise results in a higher proportion of muscle fibres exhibiting damage, and the damage is of a greater magnitude, while a higher training status attenuates the severity of disruption associated with an acute bout (Gibala et al. 1995, Gibala et al. 2000). Prolonged and intense exercise, and that with a high eccentric component (e.g. running) induces a greater degree of muscle myofibril damage than endurance exercise with predominantly concentric requirements (e.g. cycling or rowing) (Clarkson and Hubal 2002, Abbiss and Laursen 2005, Faria, Parker and Faria 2005, Millet, Vleck and Bentley 2009, Blake, Champoux and Wakeling 2012). For example, downhill runners and alpine ultramarathoners exhibit higher plasma concentrations of the muscle membrane-damage marker creatine kinase (CK), and myosin heavy-chain protein fragments derived from muscle contractile apparatus, compared to uphill runners and alpine long-distance cyclists, respectively (Koller et al. 1998). Concentric contraction of primarily *vastus lateralis* and *vastus medialis* develops most of the force during cycling exercise (Ryan and Gregor 1992, Bini et al. 2008) and because competitive cyclists typically push high gears for long durations (Lucia et al. 2004) mechanical damage to muscle is possible even with cycling. In professional cyclists, rises in the plasma activity of the indirect myofibril-damage markers aspartate aminotransferase, alanine aminotransferase, lactate dehydrogenase and alkaline phosphatase during the Vuelta Ciclista a Valencia (Tour of Valencia; 800 km) and Vuelta Ciclista a Espana (Tour of Spain; 2700 km) are likely the result of mechanical muscle-damage induced leakage of cell contents into the interstitium (Mena, Maynar and Campillo 1996). Similarly, in male amateur triathletes competing in an 800 km cycling relay (totalling

~200 km each in bouts of 20-25 min until exhaustion), creatine kinase increased ~300%, lactate dehydrogenase ~40% and aspartate and alanine aminotransferases by 140% and 250%, respectively (Bessa et al. 2008). Exercise-induced muscle damage is one mechanism that could contribute to a decline in force production and power output and increased fatigue during a subsequent bout of exercise (Abbiss and Laursen 2005) and leading to a decrement in performance.

Skeletal muscle recovery from the mechanical and metabolic effects of endurance exercise therefore requires cellular restoration of metabolic homeostasis and repair of damage, followed by adaptation, to ensure that future bouts are better tolerated (Mahoney et al. 2005, Hood, Irrcher, Ljubicic and Joseph 2006, Seene, Kaasik and Umnova 2009, Jose, Melser, Benard and Rossignol 2012). Repair of muscle damage requires the recycling of myofibril and mitochondrial and other sarcoplasmic cellular constituents damaged by mechanical and metabolic mechanisms, and the synthesis and accumulation of new and adaptive proteins (Seene, Alev, Kaasik and Pehme 2007, Egan et al. 2011, Seene, Kaasik and Alev 2011). Because this turnover of cellular protein is a crucial aspect of muscle recovery and adaptation, the rate of muscle protein synthesis within these cellular fractions following exercise is likely to be an important determinant of the rate of recovery, and may be predictive of future adaptation.

Amino acids are required to maintain the protein homeostasis of tissues, and they are readily available as a result of the dynamic free amino acid pool (Schutz 2011). This pool, however, is small and tightly regulated, so at times when the amino acid requirement increases, protein breakdown must also increase and to a greater extent (owing to less than perfect efficiency)

to shed amino acids for the synthesis of new adaptive proteins to replace damaged or obsolete ones (Schutz 2011). An acute bout of endurance exercise increases the activity of key intracellular signalling proteins, centered around the mammalian target of rapamycin (mTOR) protein, that are involved in translation initiation steps of protein synthesis (Mascher et al. 2007); correspondingly, acute endurance exercise increases the post-exercise mixed-muscle protein synthesis rate (Mascher, Ekblom, Rooyackers and Blomstrand 2011) requiring substrate amino acids for *de novo* synthesis of proteins. Furthermore, chronic training increases resting skeletal muscle protein turnover by increasing mixed-muscle protein synthesis (Short et al. 2004) but also breakdown, establishing a more negative net protein balance at rest (Pikosky et al. 2006) which could increase the requirement for dietary protein. There is some evidence for an attenuation of endurance-exercise induced muscle membrane damage, as assessed by creatine kinase, with post-exercise protein-carbohydrate ingestion (Luden, Saunders and Todd 2007, Rowlands et al. 2007, Rowlands et al. 2008, Pritchett et al. 2009) although not all studies agree (Millard-Stafford et al. 2005, Goh et al. 2012). Robinson et al. (Robinson et al. 2011) found that, in older individuals, chronic protein supplementation following endurance exercise does not appear to substantially impact long-term muscle protein synthesis rates measured with deuterated-water, relative to isocaloric carbohydrate. Regardless, the protein-feeding led to a greater improvement in maximal oxygen consumption (VO_2max: 12.2% ±SD 6.2% versus carbohydrate 3.3 ± 8.7%) after six-weeks training (Robinson et al. 2011) while post-exercise chocolate milk consumption following 4.5 weeks of aerobic training improved body composition (lean-mass:fat-mass) and resulted in moderate effect-sized increases in absolute (~0.15 $L \cdot min^{-1}$) and relative (~3 $mL \cdot kg^{-1} \cdot min^{-1}$) VO_2max versus isocaloric carbohydrate supplementation (Ferguson-Stegall et al. 2011). Additionally, protein-feeding following an acute bout of endurance exercise can increase plasma volume, which might be explained by an increased blood albumin concentration

relative to placebo (Okazaki et al. 2009); like myofibrillar protein synthesis, albumin synthesis responds in a dose-dependent manner to protein feeding after resistance exercise (Moore et al. 2009). Furthermore, greater plasma volume and improved cardiovascular adaptation and thermoregulatory function were also found with short-term (5-day) and long-term (8-wk) endurance training and protein supplementation (Okazaki et al. 2009, Goto et al. 2010). Therefore, by providing substrate amino acids sufficient for cellular and tissue metabolic-needs, including acutely stimulating and supporting post-exercise protein synthesis, additional dietary protein ingested during recovery could enable more rapid restoration of tissue amino acid and protein homeostasis and improve repair and adaptation, and should therefore be an important consideration to the endurance athlete.

METHODOLOGICAL CONSIDERATIONS IN THE MEASUREMENT OF THE RATE OF HUMAN SKELETAL MUSCLE PROTEIN SYNTHESIS

Maintenance of skeletal muscle mass and the turnover of muscle proteins during post-exercise repair and adaptation are dependent on the balance between the rates of protein synthesis and breakdown, i.e. balance = synthesis - breakdown. Protein turnover is dynamic and involves the double-flux of amino acids, in that following their release from proteins via protein breakdown they can be reused for protein synthesis. Application of stable isotope tracer methods to measure protein turnover has enabled the quantification of the impact of various acute or chronic exercise and nutritional interventions upon the rates of protein synthesis and breakdown in multiple body tissues. Measurement of the global (tissue) rate of protein synthesis may be of more importance than that of breakdown, as it is the largest contributor to variation in protein turnover (Kumar, Atherton, Smith and Rennie 2009). Technical improvements, predominantly new methods of tissue or protein-extract isolation,

have enabled study of the protein synthesis rates of various body tissues and protein fractions (e.g. (Guillet, Boirie and Walrand 2004), and even individual muscle fibres (Dickinson et al. 2010, Koopman et al. 2011). Whole-body protein turnover approaches have been extensively utilized, but might be less informative than tissue-specific tracer amino acid incorporation methods, as the relative contribution and interaction of metabolism between the tissue and protein pools composing the whole organism is poorly understood (Wagenmakers 1999). Nevertheless, whole-body methods provide some advantage in assessing gastrointestinal absorption kinetics and regional (between-tissue) changes in metabolic utilization in tissues that are difficult to access (Fouillet et al. 2009). Furthermore, advances in the development of multi-compartment interregional kinetic models to predict changes in nitrogen and amino acid metabolism in unsampled body pools, based on sampling from readily accessible pools into which a stable isotope-labelled protein or amino acid has been introduced (for instance, sampling of ileal effluent, blood and urine following digestion of ingested ^{15}N-labelled milk and soy proteins (Gaudichon et al. 2002, Fouillet et al. 2009)), has renewed the usefulness of whole-body type methodology.

In order to understand properly the mechanisms contributing to the metabolic or adaptive response of a tissue to a stimulus: for example, of skeletal muscle response to exercise, nutrition, or both; the target tissue should be directly investigated, and preferably specific protein-fractions or individual proteins within that tissue of interest should be analysed (Wagenmakers 1999, Guillet, Boirie and Walrand 2004). Notable recent advances in methods that are either now being applied by exercise scientists or that will be in the near future include the aforementioned method to assess fibre-type specific changes in FSR (Dickinson et al. 2010), application of heavy water (deuterium oxide, $^{2}H_2O$) as a universal biosynthetic

label to simultaneously assess the rate of synthesis of muscle proteins and DNA (Gasier, Fluckey and Previs 2010, Robinson et al. 2011), methods to assess for individual proteins the rates of synthesis (Jaleel et al. 2008) and breakdown (Holm and Kjaer 2010), and an analysis of remodelling of the human skeletal muscle mitochondrial proteome in response to 14-days of endurance training (Egan et al. 2011). Furthermore, a cutting edge *in vivo* method called SUnSET (SUrface SEnsing of Translation) has recently been used to measure and visualise differences in protein synthesis rates in mouse type 1, 2A, 2X and 2B fibres in response to food deprivation and synergist ablation-induced mechanical overload (Goodman, Kotecki, Jacobs and Hornberger 2012). The SUnSET method involves measuring the rate of incorporation of small amounts of the aminoacyl tRNA analog puromycin into newly synthesized proteins via a flourescence-activated cell-sorting based assay; the rate of puromycin incorporation *in vivo* directly reflects the rate of mRNA translation *in vitro*. If such technology can be successfully applied to humans (for instance, in combination with other new methods such as muscle microbiopsies (Pietrangelo et al. 2011)), it may allow more sensitive, rapid, cost-effective, accessible and ethical measurement of changes in tissue protein synthesis rates.

The arteriovenous balance method to indirectly measure muscle protein synthesis

An approach used until relatively recently (e.g. (Levenhagen et al. 2001, Levenhagen et al. 2002)) was to calculate net skeletal muscle protein balance by the dilution of a radiolabelled (weakly radioactive) or stable-isotope (non-radioactive) labelled tracer amino acid across a limb. Phenylalanine is particularly useful as it is an essential amino acid, and therefore not synthesized de novo by muscle, and it is also not oxidized in the muscle. Therefore, the rate of appearance (R_a) of unlabelled phenylalanine in a sampled tissue amino acid pool reflects

the rate of muscle protein breakdown, while the rate of disappearance (R_d) of labelled phenylalanine from the same pool estimates the rate of muscle protein synthesis (Gelfand and Barrett 1987).

This method is relatively invasive, in that indwelling catheters need to be placed in the artery supplying the limb or muscle group of interest, and the deep vein draining that limb/muscle (for instance, the brachial artery and the ipsilateral deep forearm vein for the forearm) for regular sampling of arterial and venous blood for measurement of tracer amino acid concentration and enrichment and blood flow rate, while via the contralateral arm the participant receives a primed continuous intravenous infusion of tracer (Gelfand and Barrett 1987). Amino acid concentrations would typically be determined by automated ion-exchange chromatography (Gelfand and Barrett 1987) or high-performance liquid chromatography (Levenhagen et al. 2001). The enrichment of radiolabelled proteins is relatively easily and cheaply measured using a scintillation counter following amino acid extraction from samples; non-radioactive stable-isotope labelled proteins require more time and expense because in order to measure the sample enrichment mass spectrometry is required, demanding not only access to a mass spectrometer but also relatively more sample preparation and technical expertise. The net muscle amino acid balance is calculated using the equation:

$$\text{Net muscle amino acid balance} = ([A]-[V]) \times BF$$

where A and V are the arterial and venous blood amino acid concentrations, respectively, and BF is the muscle blood flow, measured using dilution of an indocyanine green dye periodically infused arterially in the limb where isotopic measurement is taking place (Gelfand and Barrett 1987). R_d is calculated from tissue disposal of plasma tracer via the equation:

$$R_d = E \times [A] \times BF$$

Where E is the arteriovenous difference in tracer enrichment, and subsequently the muscle production of unlabelled (new) amino acid derived from protein breakdown can be calculated by reintegrating the measured net muscle amino acid balance (Gelfand and Barrett 1987).

$$R_a = R_d - \text{Net muscle amino acid balance}$$

An assumption is usually made about the skeletal muscle amino acid content based on previously measured values to avoid a biopsy; for instance, the muscle phenylalanine content is typically 3.8% (Levenhagen et al. 2001). Modern approaches are now to directly sample the muscle tissue to determine the rate of incorporation of a tracer amino acid into newly synthesized muscle proteins.

Direct measurement of muscle protein synthesis using stable isotope infusions

The most common approach currently used to quantify rates of muscle protein synthesis involves administering a continuous intravenous infusion of a stable-isotope labelled tracer amino acid, usually phenylalanine or leucine, in conjunction with multiple muscle-tissue biopsies. From the muscle samples, the amount of tracer that has incorporated into the muscle proteins is measured by mass spectrometry, and the average rate of synthesis is calculated by the change in enrichment (tissue-protein labeling) over the time between biopsies (Rennie, Smith and Watt 1994).

As with the arteriovenous balance primed isotope-infusion method, immediately prior to beginning the biopsy continuous infusion method, a large single dose of the tracer amino acid

is typically provided to prime the body amino acid pools, thereby rapidly increasing the proportion of labelled to non-labelled amino acid. For measurement of the rate of protein synthesis to be valid and reliable, tracer amino acid incorporation into new proteins needs to proceed linearly, and this is only the case if the precursor pool is steady (has stable enrichment) during the time between biopsies. A prime of tracer is typically provided at least 1 h prior to the first biopsy to reduce the time required to attain steady-state enrichment. Recent studies have validated protein labeling using the primed continuous infusion (Volpi, Chinkes and Rasmussen 2008, Smith et al. 2010). Alternatively, a flooding dose of tracer can be used to rapidly equilibrate the precursor pool and shorten the duration of an infusion protocol (Figure 2.1). The advantages of this approach are the ease of tracer application (as a bolus dose) and the ability to make repeated measures within a short period of time (~2 h); however, measured rates of muscle protein synthesis are 2-fold greater than for the continuous infusion method, which could be the result of a disturbance to protein metabolism owing to the relatively larger infused amino acid dose with the flooding method (Rennie, Smith and Watt 1994). The continuous infusion method also allows simultaneous determination of whole-body, limb or muscle turnover (Rennie, Smith and Watt 1994).

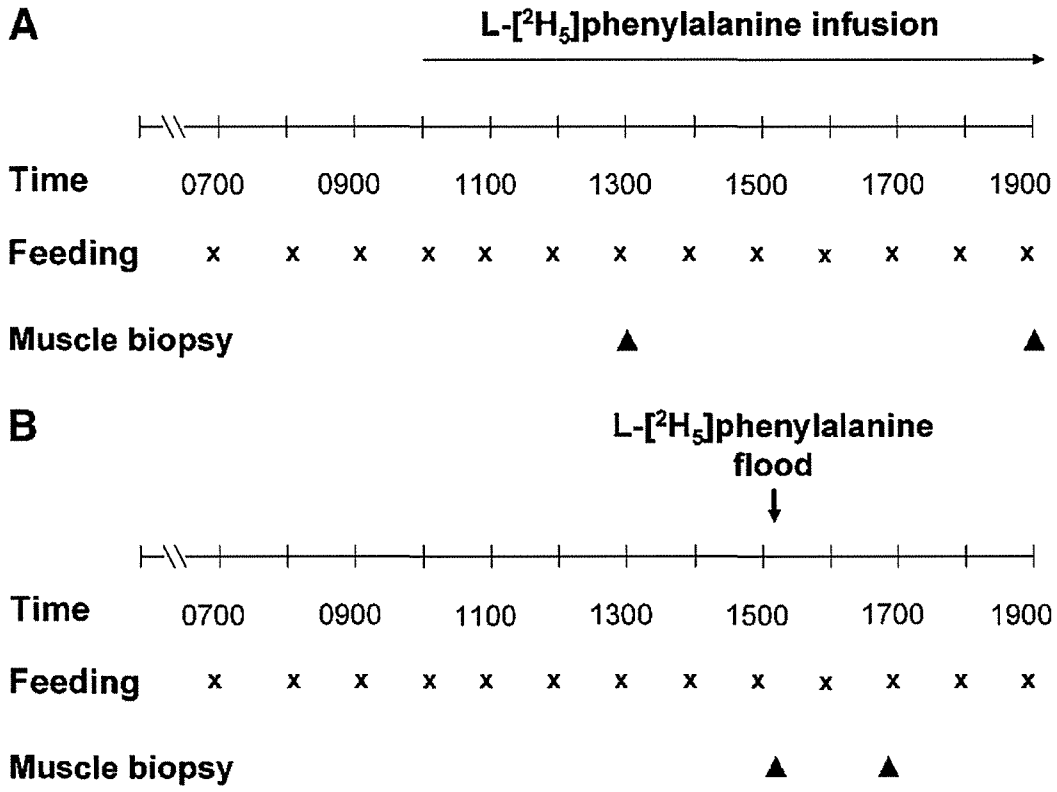

Figure 2.1. Comparison of the relative time advantage provided by the flooding-dose stable-isotope method (B), versus the primed continuous-infusion method (A) to assess the impact of feeding on resting muscle protein turnover. Using the flooding-dose method, a large dose of (43 mg·kg^{-1} body mass) of labelled tracer is infused, in this case [$^{2}H_5$]-phenylalanine. Repeated measurements (plasma samples to measure the isotopic enrichment of free phenylalanine, muscle biopsies) were then taken over 90 min to assess achievement of enrichment equilibrium and determine the acute change in muscle tissue protein turnover. In contrast, this constant infusion protocol required 9 h, with 6 h between biopsies. In this example, the infusion period prior to the first biopsy is long (1000 h to 1300 h); 60-90 min to achieve steady-state is typical (Smith et al. 2010); and the time between biopsies will typically vary from 2-6 h depending on the purpose of the investigation. This figure is reprinted with permission from Caso et al. (Caso et al. 2006).

A requirement of stable-isotope infusion approaches is that, to determine the fraction of muscle proteins that have been synthesized per hour, the increase in protein enrichment has to be divided by the enrichment of the precursor pool which provides the tracer-enriched amino acid substrate incorporated into newly synthesized proteins. Several choices are available as to which precursor pool is chosen. The pool providing amino acids directly to the ribosomal machinery that manufactures new proteins is the aminoacyl-tRNA pool. The constant infusion and flooding-dose methods both yield comparable protein synthesis rates when this pool is used, but it is inaccessible in human research studies due to the large muscle biopsies (~0.5 g) required to isolate sufficient substrate for analysis (Caso et al. 2006). Therefore, a more accessible precursor amino acid pool must be used, which are the free amino-acid or corresponding keto-acid pools in the plasma, or tissue fluid (i.e. the muscle free amino acid pool). Of these, the muscle free amino acid pool most closely reflects the enrichment of the muscle aminoacyl-tRNA (Ljungqvist, Persson, Ford and Nair 1997, Caso et al. 2002). Using one of these surrogates, calculation of the muscle protein FSR is then via the equation:

$$FSR\ (\%\cdot h^{-1}) = \Delta E_p / E_m \times 1 / t \times 100$$

where ΔE_p is the change in protein-bound tracer amino acid enrichment between biopsies, E_m is the average precursor pool enrichment across the two biopsies, and t is the time between biopsies. The plasma amino acid pool appears to be a good surrogate for the aminoacyl-tRNA pool enrichment for [ring-$^2H_5$]-phenylalanine (Caso et al. 2002) although the plasma ketoisocaproate (leucine keto-acid) enrichment in the plasma pool might underestimate the muscle protein synthesis rate as compared to the intracellular or aminoacyl-tRNA pools when using a [1-^{13}C]-leucine tracer (Ljungqvist, Persson, Ford and Nair 1997). The intracellular muscle free amino acid pool is still considered the best surrogate as, regardless of tracer, it

most closely reflects the aminoacyl-tRNA enrichment (Ljungqvist, Persson, Ford and Nair 1997).

The single biopsy approach

If a muscle biopsy is already being taken to measure the muscle protein enrichment, for instance to determine the baseline enrichment, the additional requirements of a larger muscle tissue sample and the procedures to isolate and measure the free amino acid enrichment and concentration are not overly onerous. In the situation where a participants has never previously been infused with the tracer being utilized, their baseline muscle enrichment can be estimated from the plasma protein precipitated from a preinfusion blood sample, enabling a single biopsy approach to be used (Miller et al. 2005, Tang et al. 2009, West et al. 2009, Burd et al. 2010, Burd et al. 2010). A single biopsy method could be preferable in some situations for practical, logistical and ethical reasons (Burd et al. 2011). For instance, a requirement of endurance exercise performance studies is that the test used to measure performance is sensitive, such as the validated high-intensity repeated-sprint performance test used by the Rowlands laboratory (Rowlands et al. 2007, Rowlands et al. 2008, Rowlands and Wadsworth 2010, Thomson, Ali and Rowlands 2011) or time-to-exhaustion or simulated time-trial tests. If an investigator wishes to determine performance and protein metabolism, the invasiveness of repeated muscle biopsies around exercise could substantially impact on the validity and reliability of the performance measure, and thus the outcome. A single biopsy approach might, therefore, have advantages over serial biopsies. While there is some concern that the single biopsy approach may provide unreliable measurements of muscle protein synthesis when an assumption of a zero background is made for the initial enrichment with deuterated tracer infusions (ring-[$^{2}H_5$]-phenylalanine and [5,5,5]-$^{2}H_3$-leucine) (Smith et al.

2010), other workers using carbon-labelled tracers have taken an initial, easily accessed body-protein (e.g. plasma or skin) sample in order to measure the baseline enrichment (Miller et al. 2005, Tang et al. 2009, West et al. 2009, Burd et al. 2010, Burd et al. 2010) indicating that it is a useful method, and this approach has recently been validated (Burd et al. 2011). Of course, if a laboratory has limited access to participants or particular cohort requirements (for instance, due to a small population of sufficiently trained individuals within a feasible distance to participate) often the same participants contribute to multiple investigations. Unless the laboratory continuously switches tracers (to ensure each participant is naive) this could limit some of the practicality of the single biopsy approach to smaller laboratories.

Additional practical considerations regarding stable isotope studies

A number of recent publications have investigated various aspects of stable isotope tracer methodology to determine the normal variance in FSR from within and between muscles, to validate techniques, and to discern if reliable comparisons of protein synthetic rates can be made between studies utilizing different tracers (Smith, Villareal and Mittendorfer 2007, Volpi, Chinkes and Rasmussen 2008, Harber et al. 2009, Smith et al. 2010, Burd et al. 2011, Harber et al. 2011, Smith, Patterson and Mittendorfer 2011). The timing of the initial biopsy under resting postabsorptive conditions (Smith et al. 2010) and sequential biopsies from the same spot (Volpi, Chinkes and Rasmussen 2008) appear to have little impact on the measurement of protein synthesis rates. The choice of the precursor amino acid pool and inter-individual variability appear to be the two main determinants of variation in FSR values obtained from resting, postabsorptive, untrained muscle taken from nonobese adults $\leq$50 y (Smith, Patterson and Mittendorfer 2011). Normal muscle FSR variance in the population appears to be on average 30%, and factors contributing to this would probably include dietary

variation and levels of habitual activity (Smith, Patterson and Mittendorfer 2011). For instance, myofibrillar and sarcoplasmic protein synthesis may remain elevated for 48-72 h after a single bout of endurance-like exercise (Miller et al. 2005). Therefore, it is important to control for diet, habitual activity and planned exercise in any study design investigating protein turnover in human muscle.

There is some controversy surrounding the effect of tracer selection on measured FSRs; Harber et al. (Harber et al. 2011) reported that tracer use of either a [ring-$^{2}H_{5}$]-phenylalanine or [ring-$^{2}H_{3}$]-leucine did not substantially impact post-absorptive resting or post-exercise FSR outcomes. Smith et al. (Smith, Villareal and Mittendorfer 2007) found that resting post-absorptive FSR rates differed between [5,5,5-$^{2}H_{3}$]-leucine and either [ring-$^{13}C_{6}$]-phenylalanine or [ring-$^{2}H_{5}$]-phenylalanine (with no substantial difference between phenylalanine isotopomers), although the magnitude of the FSR change following mixed-meal feeding did not substantially differ between tracers. Taken together, there is some evidence that comparisons between absolute FSRs measured with different tracers should be avoided, but that comparing the magnitude of the change may be appropriate, especially following feeding-induced changes.

For research investigating the effect of dietary protein or amino acids on muscle protein metabolism, unless the ingested protein is isotopically labelled a possible dilution of the precursor pool and thus disturbance to steady-state could occur because of the rapid influx of a large quantity of unlabelled dietary protein-derived amino acids. To circumvent this, a series of investigations from the laboratory of Luc van Loon have utilized intrinsically-labelled milk proteins, derived from dairy cows fed tracer-enriched feed, (Koopman et al.

2009, Koopman et al. 2009, Pennings et al. 2011, Pennings et al. 2012, Res et al. 2012). Otherwise, it has been common practice to add free amino acid tracer to the protein meal or beverage. The amount of added tracer is matched to the tracee content consistent with the predicted plasma free and intracellular tissue-pool enrichment (Burd et al. 2011). For example, for a [ring-$^2H_5$]-phenylalanine tracer, a protein drink would be enriched to ~8-9% of the total phenylalanine in the drink protein by dissolving into it free [ring-$^2H_5$]-phenylalanine tracer powder (e.g. (Howarth, Moreau, Phillips and Gibala 2009, Tang et al. 2009)). This approach was recently been brought into question, based on the argument that were the free amino acid tracer more rapidly absorbed than the protein-derived amino acid tracee, there would also be a disturbance to isotopic steady-state, presumably a rapid increase followed by a prolonged dilution (Reitelseder et al. 2011). Subsequently, this hypothesis was tested and the addition of free tracer to protein-containing beverages was not shown to disturb the isotopic steady-state of either the plasma nor intracellular free amino acid pools (Burd et al. 2011). However, the authors agreed with earlier suggestions (van Loon et al. 2009) that digestion and absorption kinetics are best assessed with intrinsically-labelled proteins (Burd et al. 2011).

With regard to instrumentation, isotope ratio mass spectrometry (IRMS) or gas chromatography (GC)-IRMS are more sensitive and can more reliably measure small enrichments versus gas chromatography mass spectrometry (GCMS), and are therefore preferred to measure the muscle protein enrichment (Mittendorfer et al. 2005, Smith, Patterson and Mittendorfer 2011). Smith et al. (Smith, Patterson and Mittendorfer 2011) also suggest that good laboratory practices can make GCMS a reliable platform; for instance, day-to-day variation in the range of values obtained can be in the order of 20-30%, so rapid-

throughput of all samples from a given investigation, with appropriate use of standards to minimize variability, is an important consideration.

While the most reliable FSR measurements appear to be from mixed-muscle, these values cannot be extrapolated to the myofibrillar and mitochondrial protein fractions that may be of interest to exercise and nutrition researchers, because they have different rates of turnover, although the myofibrillar FSR could be assumed to be below that of the mixed-muscle FSR (Smith, Patterson and Mittendorfer 2011). The overall FSR of the myofibrillar and sarcoplasmic protein fractions varies between muscles at rest and following amino acid infusion, although the degree of change from the resting rate induced by amino acids is proportional (Mittendorfer et al. 2005); however, with regards to the most commonly used endurance exercise modalities (running and cycling) the tissue sample from exercised muscle is commonly and readily obtained from *vastus lateralis*, and therefore these differences can be ignored.

A final consideration is perhaps more important than any of the methodological or technical considerations so far mentioned. Within the fields of exercise science and muscle metabolism, there are no established criteria for deciding what a meaningful or substantial change in the rate of muscle protein synthesis actually is. There has been the suggestion that for differences in resting muscle protein fraction FSRs (i.e. between muscles) a change in the order of that elicited by exercise or feeding (a 2-fold to 3-fold increase) might be important (Mittendorfer et al. 2005), but this is of no relevance to assessing the importance of the magnitude of a change between feeding interventions, and post-exercise. Otherwise, a pharmacokinetic threshold utilised in drug bio-equivalence trials is an option (Haidar et al.

2008). Applying this approach to a muscle protein synthesis outcome, the magnitude of the change in FSR is considered relative to a 25% reference standard; a larger change (>25%) is substantial, a smaller change trivial (equivalent). That is, to be considered bioequivalent the 90% confidence limits for the mean FSR change for the test condition (e.g. protein-carbohydrate-fat feeding) should be contained within the acceptance interval of less than a 25% change from the reference condition (e.g. isocaloric carbohydrate-fat only):

$$125.00\% \leq [90\%\ CI] \geq 80.00\%$$

Finally, European Union (EU) guidelines (http://www.emea.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/01/WC500070039.pdf) recommend that for narrow therapeutic index drugs a 90%-111.11%CV acceptance interval is used; the confidence interval should be no less that 5% greater or less than the 90-111% bioequivalent threshold to be clear, and if the difference and 90% confidence interval lies between a value 90-111% of the coefficient of variation (CV) for the outcome it is deemed equivalent. While rigorous, it is debatable if the latter is appropriate as dietary protein may not be considered a narrow therapeutic index drug. Therefore, until such time as research and expert debate can agree on a magnitude of change in the global FSR that might be meaningful (and if indeed there is such) the pharmacokinetic 25% standard could have some utility as a standard.

THE MUSCLE PROTEIN SYNTHESIS RATE AS A KEY MECHANISM BY WHICH PROTEIN FEEDING REGULATES POST-EXERCISE REPAIR AND ADAPTATION

Acute endurance exercise increases mixed-muscle protein synthesis in both untrained and trained muscle. In untrained, healthy adults, mixed-muscle protein synthesis following 4 h of

walking at a low intensity (40% VO_2max) was ~25% greater than at rest (Carraro et al. 1990). In a series of studies, Harber et al. (Harber et al. 2009, Harber et al. 2010, Harber et al. 2011) showed that in trained men undertaking cycling (60 min) or running (45 min) exercise, *vastus lateralis* mixed-muscle FSR was increased relative to rest by 1.4-fold to 1.6-fold. Pikosky et al. (Pikosky et al. 2006) found that in untrained men and women, a four week endurance training regimen increased resting mixed-muscle FSR by ~16%, while Short el al. (Short et al. 2004) found that a longer (16 week) endurance training program increased the resting rate of mixed-muscle protein synthesis by ~22% in previously untrained young and old men and women. Therefore, acute endurance exercise and training status impact on the resting and post-exercise rates of mixed-muscle protein synthesis.

In untrained, resting muscle, the average rate of mixed-muscle FSR is greater than that of the myofibrillar or myosin heavy chain FSR (Smith, Patterson and Mittendorfer 2011), indicating a substantial contribution of other protein fractions (e.g. mitochondrial) to the global muscle rate. Mitochondrial proteins in human *vastus lateralis* account for 22% of the total identified proteins (Højlund et al. 2007) but have a greater turnover rate than myofibrillar proteins (van Wessel, de Haan, van der Laarse and Jaspers 2010) and might, therefore, account considerably for changes in mixed-muscle FSR with aerobic exercise. In support, Wilkinson et al. (Wilkinson et al. 2008) found that acute endurance exercise increased skeletal muscle mitochondrial protein synthesis in untrained individuals in the fed state by 154%, relative to at rest, but only by 104% in the same individuals following ten weeks of cycle training. The authors (Wilkinson et al. 2008) indicated that, in the trained muscle, there was no statistically significant effect of cycling exercise on myofibrillar FSR (33% greater than at rest, $p=0.12$). However, reanalysis using a threshold for a substantial effect of a 25% difference between

treatment groups (also the *a priori* threshold of Wilkinson et al. (Wilkinson et al. 2008)) revealed a likely moderate increase in post-exercise myofibrillar FSR (ES 0.70 ±90%CL 0.75; see Supplementary Data 2.4 Wilkinson 2008 ES and Inferences.xlsx and Supplementary Data 2.5 Wilkinson 2008 ES and Inferences.pptx). Furthermore, following 1 h of 1-legged kicking at 67% of maximum workload (which could be considered endurance-like exercise) sarcoplasmic and myofibrillar FSR increased and peaked at 24 h post-exercise, and although the FSRs then declined, the myofibrillar fraction was still elevated above the resting rate at 72 h post-exercise (Miller et al. 2005). Therefore, in both the fasted and fed state and relative to at rest, the mitochondrial fraction contributes substantially to the magnitude of change in post-endurance exercise mixed-muscle FSR, but an increase in myofibrillar FSR also substantially supports the increased mixed-muscle FSR. Additionally, it is advisable to measure the synthesis rate within specific protein fractions so as not to extrapolate the global cellular rate to a particular fraction or protein.

As is the case with exercise, nutrition is a powerful modifier of the cellular environment (Hawley, Burke, Phillips and Spriet 2011) including protein metabolism. Athletes are also interested in maximizing the training adaptation. Post-endurance exercise protein-carbohydrate nutrition could reduce the recovery time needed between bouts, allowing more bouts of exercise within a given training phase. Although post-exercise whole-body and muscle FSR are increased by protein feeding (Levenhagen et al. 2002, Howarth, Moreau, Phillips and Gibala 2009), it is not well understood how an enhanced recovery rate of protein synthesis is responsible for some observations of improved endurance adaptation (Okazaki et al. 2009, Goto et al. 2010, Ferguson-Stegall et al. 2011) given that long-term rates of synthesis with chronic supplemental protein consumption might not be substantially different

to the resting rate, albeit in elderly participants (Robinson et al. 2011). Nevertheless, specific properties of the protein appear to impact on metabolic responses at the level of the whole-body and muscle-tissue, which could benefit training-induced adaptations by improving peripheral utilization of dietary-protein derived amino acids.

MUSCLE-TISSUE UTILIZATION OF AMINO ACIDS IS DEPENDENT ON THE CHARACTERISTICS OF THE PROTEINS THEY ARE DERIVED FROM

Effects of protein types on protein synthesis in resting humans

The characteristics of the ingested dietary protein used in mixed-meal feedings are important determinants of muscle amino acid availability and muscle protein synthesis. Whole milk and soy protein (and more recently rapeseed), and milk (whey and caseins) and soy protein-fraction concentrates, isolates and hydrolysates are commonly used as protein sources in both research and as sports supplements. The absorption of amino acids from the gut depends on the type of protein ingested, with rapidly digested proteins being coined 'fast' proteins (e.g. whey proteins) versus 'slow' proteins (e.g. caseins) with a relatively delayed absorption profile (Boirie et al. 1997). Caseins are the major protein component of milk (~80% of total milk-protein content) and precipitate to clots in the acidic media of the gut, whereas the whey protein fraction of milk remains soluble (Billeaud, Guillet and Sandler 1990, Mahe et al. 1996) which presumably enhances access of hydrolytic enzymes to amino acid cleavage sites within the proteins, enabling more rapid digestion and absorption relative to slow proteins. For example, whey protein isolates have rapid digestion and absorption culminating in a larger but transient peak blood amino acid concentration (Lacroix et al. 2006). Soy is primarily a soluble protein fraction (Bos et al. 2003) and is, therefore, a fast protein. Indeed, in healthy young adults at rest, postprandial blood glucose and insulin concentrations are

similar between soy and whole-milk protein in a mixed meal but the amino acid profile was reflective of the dietary protein source amino acid profile, and the rise in serum amino acid concentration was more rapid with soy, peaking 2.5 h post-feeding versus 3.9 h for the milk, reflecting more rapid digestion and absorption (Bos et al. 2003). Peripheral whole-body protein synthesis is lower at rest after soy protein intake relative to whole-milk protein ingestion, which is predicted by compartmental modeling to be the result of a greater proportion of soy-derived amino acids being deaminated or used for liver protein synthesis (Fouillet et al. 2002, Bos et al. 2003). In fact, the majority of dietary nitrogen (31.5%) derived from soy-protein appears to be retained in the splanchnic tissues at six hours following ingestion of a mixed meal, compared to that incorporated into splanchnic plasma proteins (7.5%) and peripheral proteins (21%) (Fouillet et al. 2003). Overall, in young adults at rest the consumption of slow-digestion proteins leads to a greater peripheral net protein gain than rapidly digested whey or soy proteins or free amino acid mixtures (Dangin et al. 2001, Dangin, Boirie, Guillet and Beaufrère 2002, Fouillet et al. 2002, Bos et al. 2003, Dangin et al. 2003, Fouillet et al. 2003, Morens et al. 2003, Lacroix et al. 2006, Deglaire et al. 2009, Fouillet et al. 2009).

This situation appears to be reversed in elderly men at rest, where there is evidence that whey protein is superior to casein in stimulating whole-body and muscle net protein gain. Relative to younger adults, the overall rate of whole-body protein turnover is reduced in the elderly when fasted (Short et al. 2004), following protein ingestion (Boirie, Gachon and Beaufrère 1997, Dangin et al. 2003), and post-resistance exercise (Koopman et al. 2006). In two separate studies, Dangin et al. (Dangin, Boirie, Guillet and Beaufrère 2002, Dangin et al. 2003) found greater whole-body net protein gain with whey-proteins than caseins, which may

not be explained by others observations of impaired digestion and greater splanchnic extraction reducing peripheral availability of amino acids such as leucine (Boirie, Gachon and Beaufrère 1997). Recently, Koopman et al. (Koopman et al. 2009) found no substantial difference in splanchnic amino acid extraction between ten young (23 ± 1y; 72 ± 2% extraction) and ten elderly (64 ± 1 y; 73 ± 1% extraction) subjects who consumed a single 35 g bolus of intrinsically [1-^{13}C]-phenylalanine-labelled micellar casein. While the trend was for a greater mixed-muscle protein synthesis rate with younger participants (0.063 ± 0.006%·h^{-1} versus and 0.054 ± 0.004%·h^{-1} in the older group), the difference was reported as not significant ($p = 0.27$) (Koopman et al. 2009) although reanalysis of their data using an inferential magnitude-based approach suggests that a small increase in FSR was possible with the elderly group versus the young (see Supplementary Data 2.1 Koopman 2009 ES and Inferences.xlsx). Subsequently, Pennings et al. (Pennings et al. 2011) showed that in elderly men, the resting mixed-muscle FSR response to the ingestion of a 20 g bolus of protein derived from whey, casein hydrolysate, or casein, was greatest with the whey and least with the casein (0.15 ± 0.08%·h^{-1}, 0.10 ± 0.04%·h^{-1} and 0.08 ± 0.04%·h^{-1}, respectively) and attributable to the measured digestion kinetics of each protein and the higher leucine content of the whey. Furthermore, of all the amino acids, the plasma concentration of leucine showed the strongest positive correlation to FSR ($r=0.66$) (Pennings et al. 2011). Altogether, while fast protein-derived amino acids might contribute primarily to whole-body protein turnover through enhanced splanchnic metabolism, their ability to induce a rapid and large rise in plasma amino acid concentrations, especially leucine, stimulates muscle protein synthesis to a greater extent at rest than slow proteins; this is probably because the extracellular amino acid concentration is a key regulator of muscle protein synthesis (Bohe, Low, Wolfe and Rennie 2003).

Effects of protein-types on protein synthesis following exercise

It follows that, regardless of possible age-related variability in resting protein turnover responses to protein ingestion, the relatively greater increase in blood amino acids associated with fast-protein feeding might be beneficial immediately post-exercise, when blood flow to the skeletal muscle and nutrient uptake and sensitivity is enhanced. Soy protein isolate shows reduced effectiveness in stimulating muscle protein synthesis post-exercise relative to skim milk (34% greater FSR) (Wilkinson et al. 2007) and milk-derived whey hydrolysate (31% greater FSR; 18% greater than at rest) (Tang et al. 2009) but appear to be superior to micellar casein in stimulating FSR at rest and post-resistance exercise (64% and 69% greater, respectively) (Tang et al. 2009). Combining the findings of two recent studies (Tang et al. 2009, Reitelseder et al. 2011), plasma concentrations of essential amino acids (EAAs) and insulin during post-resistance exercise recovery appear to be increased the most with whey protein ingestion, the least with casein, and moderately with soy protein, which has relevance to muscle protein metabolism because of the potent stimulatory effects on muscle protein synthesis and inhibition of protein breakdown associated with EAAs and insulin, respectively. Relative to whole skim milk (~20% whey and 80% casein), soy does not appear to substantially alter the blood insulin concentration during a 180-min recovery but does slightly increase the plasma amino acid concentration shortly (30 min) after resistance exercise (Wilkinson et al. 2007). In healthy males and females randomly assigned to ingest 20 g of casein (n=7) or whey (n=9) 1 h following a bout of resistance exercise, whey protein was associated with increased blood leucine, phenylalanine and insulin concentrations, and greater intramuscular leucine at 120-min into recovery, but with differential effects on the kinetics of leucine (greater leg net balance with whey) and phenylalanine (no significant effect but trending reduced leg net balance with whey) for protein synthesis (Tipton et al. 2004). The authors suggest this differential outcome may be attributable to greater oxidation

of whey-derived leucine, leading to an overestimation of leg net balance: phenylalanine is not oxidized in muscle and therefore is a 'true' indicator of leg net balance; and the possibility of an insufficient sample size (n=7) leading to a type II error (intersubject CVs for phenylalanine and leucine muscle concentrations were 27% and 28% respectively) (Tipton et al. 2004). The relative effectiveness of milk-derived whey proteins versus soy-derived proteins in stimulating increases in blood amino acids and insulin and enhancing muscle protein synthesis is synonymous with fast versus slow protein peripheral utilization at rest, in that it is most likely related to protein digestibility and amino acid absorption kinetics, culminating in superior amino acid availability (e.g. increased plasma and intracellular leucine) at the muscle. Leucine stimulates muscle protein synthesis by enhancing translation initiation via an mTOR-dependent mechanism (Anthony, Anthony and Layman 1999, Anthony et al. 2000, Crozier et al. 2005) and appears to be the most potent mTOR-regulating amino acid (Atherton et al. 2010).

In support, Cribb et al. (Cribb, Williams, Carey and Hayes 2006) supplemented the normal diet of bodybuilders with 1.5 $g \cdot kg^{-1} \cdot$body mass of either hydrolyzed whey isolate or casein over the course of a 10 week supervised resistance training regimen, and found greater gains in lean mass and strength, and a reduction in body fat with the whey condition. Hartman et al. (Hartman et al. 2007) found that chronic feeding of either fat-free soy (n=19) or fat-free milk (n=18) following resistance exercise (immediately and 1 h post-exercise; 5 $d \cdot week^{-1}$ over 12 weeks) in untrained young men resulted in greater increases in fat-free, bone-free and type II fibre mass with milk ingestion. Whether improved adaptation to endurance exercise might be impacted by feeding fast versus slow proteins is unclear and uninvestigated. Based on the resting and post-resistance exercise data, amino acid utilization by the muscle is greatest with

whey hydrolysates, isolates and concentrates, then soy, then casein. A combination of whey and casein might be optimal following exercise, as whey provides the initial stimulus to increase protein synthesis with high digestibility and greater peak insulin and amino acid concentrations, especially leucine, whereas casein provides a prolonged supply of substrate amino acids (Reitelseder et al. 2011). Future research could focus on manipulating the ratio of whey to casein in milk to determine if there is an optimal combination.

THE IMPACT OF CARBOHYDRATE ON PROTEIN METABOLISM

Carbohydrate availability has been shown to influence the rates of whole-body and skeletal muscle protein synthesis, degradation and net balance during endurance exercise. Intravenous infusions have shown that urea nitrogen excretion is inversely proportional to carbohydrate intake (Long, Wilmore, Mason Jr. and Pruitt Jr. 1977). Lemon and Mullin (Lemon and Mullin 1980) found that when exercise reduced endogenous carbohydrate availability, blood nitrogen levels increased suggestive of increased amino acid oxidation and protein degradation. Subsequent arterial-venous balance studies of the effect of glycogen availability on amino acid turnover indicated a net release of amino acids from muscle during exercise, indicating either an increase in protein degradation or a decrease in protein synthesis, or both (Blomstrand and Saltin 1999, Van Hall, Saltin and Wagenmakers 1999). In trained males cycling for 75 min at 80% of VO_2max trials and ingesting 125 g of (13(C)-glucose) carbohydrate or 10 g of tracer carbohydrate, protein oxidation estimated from urine and sweat urea was reduced with the larger carbohydrate feeding indicating a protein sparing effect of carbohydrate feeding during endurance exercise (van Hamont et al. 2005). Howarth et al. (Howarth et al. 2010) had six men cycle at 75% VO_2max until exhaustion in order to reduce body carbohydrate stores, and then fed them a high-carbohydrate (71% of total energy) or

low-carbohydrate (11% of total energy) diet for two days prior to a bout of two-legged kicking exercise at 45% of kicking VO_2peak. Leg net protein balance was lower with the low-carbohydrate diet compared with at rest and with the high-carbohydrate diet, primarily because of increased protein degradation (Howarth et al. 2010). Whole-body net protein balance was reduced in the low-carbohydrate group largely because of reduced whole-body protein synthesis, and whole-body leucine oxidation increased above the resting rate in the low-carbohydrate diet only, and greater than the leucine oxidation observed with the high-carbohydrate diet (Howarth et al. 2010). Thus, a convincing body of evidence exists highlighting the protein-sparing effect of increased dietary carbohydrate intake.

The most plausible mechanism regulating an impact of dietary carbohydrate on protein metabolism is the enhanced insulin secretion associated with a high carbohydrate diet. Increased dietary carbohydrate is associated with higher insulin concentrations, and although insulin has little effect on muscle protein synthesis when amino acid availability is limited (Castellino et al. 1987, Biolo, Williams, Fleming and Wolfe 1999, Bell et al. 2005, Greenhaff et al. 2008) it can impact muscle protein turnover by substantially reducing protein breakdown (Greenhaff et al. 2008).

Interestingly, dietary carbohydrates can also affect dietary protein uptake and utilization, in part by impairing the rate (but not efficiency) of amino acid absorption (Gaudichon et al. 1999) which mitigates differences in kinetics and metabolic utilization of slow versus fast proteins (Dangin, Boirie, Guillet and Beaufrère 2002). For example, sucrose slows absorption of purified milk protein relative to milk-fat and the milk protein alone, which reduces the transfer of ^{15}N-milk protein derived nitrogen to urea and thereby increases postprandial

protein utilization (Gaudichon et al. 1999). Whether the alterations in postprandial utilization would be substantial enough to impact on muscle tissue repair and recovery processes during intense training and effect performance remains to be tested.

INCREASING DIETARY PROTEIN INTAKE ENHANCES DEAMINATION AND REDUCES PERIPHERAL AMINO ACID UTILIZATION

A high dietary protein intake enhances splanchnic nitrogen extraction and deamination, reducing peripheral nitrogen availability, and accentuates differences between fast and slow proteins (Morens et al. 2003, Juillet et al. 2008, Fouillet et al. 2009). Eight healthy adults were adapted for 7 days to normal protein diet (1 gram of protein per kilogram of body mass per day; $g \cdot kg^{-1} \cdot d^{-1}$) and then to a high-protein diet (2 $g \cdot kg^{-1} \cdot d^{-1}$), and ingested a ^{15}N-labelled wheat protein meal. Nitrogen was measured in the blood and urine for 8 h, and kinetics in these and in unsampled pools was assessed using a multicompartment mathematical modelling (Juillet et al. 2008). Following adaptation to the high-protein diet, whole-body retention of the meal nitrogen declined at 8 h by 10% relative to following the low-protein diet; modelling suggested that splanchnic urea synthesis from dietary nitrogen was increased, reducing peripheral availability by 20-30% with the high-protein diet (Juillet et al. 2008). A subsequent study comparing soy and milk protein confirmed that when consuming a high-protein diet, splanchnic use of dietary nitrogen came at the expense of peripheral availability, but more importantly that this effect was more pronounced for the rapidly absorbed soy-protein compared to the whole-milk protein (Fouillet et al. 2009). Thus, while a diet high in total protein would provide more daily nitrogen and amino acids overall, there is a reduced efficiency of peripheral amino acid availability due to greater splanchnic deamination and/or oxidation, and this is accentuated for rapidly absorbed proteins. This may be of relevance to

the endurance athlete. Firstly, the additional caloric intake from greater than normal dietary protein consumption might be excessive, as maintenance of a high power: weight ratio is important to most endurance athletes, and it could contribute to a gain in body mass. Secondly, a high dietary protein intake could reduce the benefit of fast-digestion proteins provided in the immediate hours post-exercise and, putatively, impair mechanisms of recovery and adaptation. A high dietary protein intake (3.6 $g \cdot kg^{-1} \cdot d^{-1}$) has been shown to modify post-endurance exercise protein turnover in trained males relative to low and moderate protein diets (0.8 and 1.8 $g \cdot kg^{-1} \cdot d^{-1}$, respectively) by increasing protein oxidation at rest (Gaine et al. 2006) and following exercise (Gaine et al. 2007) and reducing the rate of mixed-muscle protein synthesis after exercise in a fasted state (Bolster et al. 2005).

WHAT IS THE EFFECT OF PROTEIN DOSE ON PROTEIN SYNTHESIS?

In older adults and in resting skeletal muscle, ingestion of a 35 g dose of whey protein increases mixed-muscle protein synthesis to a greater extent than either a 10 g or 20 g dose (Pennings et al. 2012). However, ingestion of a 40 g dose of egg white protein following resistance exercise did not substantially increase mixed-muscle FSR relative to a 20 g dose (see Supplementary Data 2.2 Moore 2009 ES and Inferences.xlsx and Supplementary Data 2.3 Moore 2009 ES and Inferences.pptx) indicating saturation of protein synthesis with the 20 g dose (Moore et al. 2009). Thus, resistance exercise either potentiates the effect of protein nutrition, or there is an additive effect of nutrition and exercise upon FSR that occurs up to the maximum rate of protein synthesis (which is probably limited by the ribosomal machinery themselves). The theoretical maximum obtainable rate of muscle protein synthesis following endurance exercise and protein feeding is most likely the same as post-resistance exercise, as the mechanisms limiting the absolute rate are no different; however, a dose-

response investigation comparing absolute FSR rates following each form of exercise has not yet been undertaken.

THE IMPACT OF PROTEIN INGESTION ON RATES OF MIXED-MUSCLE, MYOFIBRILLAR AND MITOCHONDRIAL PROTEIN SYNTHESIS DURING RECOVERY FROM ENDURANCE EXERCISE

The literature suggests that in order to compare the effectiveness of post-exercise protein and amino acid supplementation on nitrogen and amino acid metabolism outcome measures such as nitrogen balance, whole-body or muscle protein synthesis, or with new methods such as large-scale metabolomics analyses: 1) cohorts should be as homogenous as possible within a study, including for age, as absorption of fast-proteins appears to provide a relatively greater peripheral tissue benefit amongst older individuals than slow-proteins, and with the reverse true for young participants; and 2) the relative intake and composition of macronutrients (protein, but also carbohydrates and fats) in controlled diets should be held constant between parallel or concurrent investigations looking at similar parameters within the same laboratory group, to enable better comparison between outcome measures (including for exercise performance). While feasible within a single research group, this is unlikely to be standardised between different laboratories.

It is well-established that the ingestion of protein following resistance exercise enhances the global synthesis rate of mixed-muscle protein and muscle protein-fractions (e.g. see recent reviews by (Burd, Tang, Moore and Phillips 2009, Kumar, Atherton, Smith and Rennie 2009)). While several recent reviews have covered aspects of muscle protein turnover in

response to endurance training (Seene, Kaasik and Alev 2011) and exercise coupled to recovery protein feeding (e.g. (Burd, Tang, Moore and Phillips 2009, Kumar, Atherton, Smith and Rennie 2009, Atherton and Smith 2012)) a direct comparison of the magnitude of the post-exercise muscle protein and protein-fraction response to protein feeding has not been conducted. It is important to measure the rate in trained muscle as endurance exercise training increases the rates of mitochondrial and myofibrillar protein synthesis in the fed state (Wilkinson et al. 2008), and to consider cohort age as FSR declines by ~3.5% per decade (Short et al. 2004). In order to interpret data between studies utilizing differing tracers or methodologies, it has been suggested that qualitative comparisons should be made (Gasier, Fluckey and Previs 2010). The use of a magnitude-based inferential statistical approach to interpret the muscle protein synthetic responses between investigations may be superior, as it enables quantitative and qualitative comparisons inferred from the magnitude and certainty of the change (i.e. the uncertainty) in a parameter. Therefore, the small number of applicable studies (Levenhagen et al. 2002, Howarth, Moreau, Phillips and Gibala 2009, Harber et al. 2010, Breen et al. 2011, Lunn et al. 2012) were compared and the impact of protein feeding after endurance exercise presented as standardized effect sizes, with uncertainty as confidence intervals (Table 2.1).

Table 2.1. *Comparison of mixed-muscle, myofibrillar, and mitochondrial protein fractional synthesis rates (FSRs) obtained from recent acute recovery studies using endurance-type exercise and comparing post-exercise protein-carbohydrate ingestion to carbohydrate control or placebo conditions*

Reference Cohort Mean age ± SD Mean VO_2max/peak ± SD	Exercise mode Duration Intensity ± SD (%VO_2max/peak, if published)	Tracer protocol Recovery duration FSR biopsy times Muscle tissue fraction Notes	Background diet standardization/control Feeding regimen: type and number of serves Feeding regimen: timing of serves PRO/CHO/FAT g·serve^{-1} (kilojoules) PRO/CHO/FAT g·kg^{-1}	Mean FSR ± SD%·h^{-1} or leg protein synthesis (μg·min^{-1}·100 CC^{-1}) (Levenhagen et al. 2002)	Comparison ES ±90%CL[a] Inferences[b]
(Levenhagen et al. 2002) Ten healthy men and women (n=5 each) Pooled 31 ± 7 yr 39.1 ± 9.5 mL·kg^{-1}·min^{-1} Men 30 ± 7 yr 44.9 ± 6.5 mL·kg^{-1}·min^{-1} Women 33 ± 8 yr 33.2 ± 8.9 mL·kg^{-1}·min^{-1}	Cycle 60 min 60 ± 3%	Arteriovenous balance method [$^2H_5$]-phenylalanine GCMS of plasma samples 3 h recovery No biopsies	3-day controlled diet, eucaloric to estimated energy expenditure Single bolus Beverage immediately post-exercise Casein, sucrose and milk-fat beverage (PRO) 10/8/3 (407) g·kg^{-1} as above Sucrose milk-fat control (CON) 0/8/3 (247) g·kg^{-1} as above, except protein 0 g·kg^{-1} Placebo 0/0/0 (0)	PRO: 201 ± 146 CON: 52.0 ± 8.2 Placebo: 31.0 ± 20.9	PRO vs CON Pooled 1.44 ±1.17 Very likely large increase PRO vs Placebo Pooled 1.63 ±1.32 Very likely large increase
(Howarth, Moreau, Phillips and Gibala 2009) Six healthy men 22 ± 2 yr 48.9 ± 8.2 mL·kg^{-1}·min^{-1}		Primed continuous infusion [$^2H_5$]-phenylalanine Tissue-fluid pool surrogate GCMS for all samples 4 h recovery 0 and 4 h post-exercise Mixed-muscle	Diet standardized for 48 h prior to testing Repeat feeding Beverages every 15 min for 3 h Hydrolyzed whey concentrate and carbohydrate (PRO) 8/25/0 (553) 1.2/3.6/0 g·kg^{-1} Low-carbohydrate control (L-CHO) 0/25/0 (425) 0/3.6/0 g·kg^{-1} Isocaloric high-carbohydrate control (H-CHO) 0/33/0 (561) 0/4.8/0 g·kg^{-1}	PRO: 0.088 ± 0.015 L-CHO: 0.066 ± 0.018 H-CHO: 0.060 ± 0.018	PRO vs L-CHO 1.31 ±0.65 Likely large increase PRO vs H-CHO 1.60 ±0.80 Very likely large increase
(Harber et al. 2010) Eight trained men 25 ± 3 yr 52 ± 6 mL·kg^{-1}·min^{-1}	Cycle 60 min 72% ± 3%	Primed continuous infusion [$^2H_5$]-phenylalanine Tissue-fluid pool surrogate GCMS for all samples 6 h recovery 2 and 6 h post-exercise Mixed-muscle Tracer initiated 1 h post-feeding	Milk protein isolate, carbohydrate and fat beverage (PRO) Two beverages 0 and 1 h post-exercise Milk-protein isolate, carbohydrate and fat (PRO) 27/62/2 (1560) 0.37/0.83/0.03 g·kg^{-1} Placebo 0/0/0 (0)	PRO: 0.129 ± 0.040 Placebo: 0.112 ± 0.028	PRO vs Placebo 0.49 ±0.40 Likely small increase

Table 2.1 continued. *Comparison of mixed-muscle, myofibrillar, and mitochondrial protein fractional synthesis rates (FSRs) obtained from recent acute recovery studies using endurance-type exercise and comparing post-exercise protein-carbohydrate ingestion to carbohydrate control or placebo conditions*

Reference Cohort Mean age ± SD Mean VO_2max/peak ± SD	Exercise mode Duration Intensity ± SD (%VO_2max/peak, if published)	Tracer protocol Recovery duration FSR biopsy times Muscle tissue fraction Notes	Background diet standardization/control Feeding regimen: type and number of serves Feeding regimen: timing of serves PRO/CHO/FAT g·serve^{-1} (kilojoules) PRO/CHO/FAT g·kg^{-1}	Mean FSR ± SD%·h^{-1} or leg protein synthesis (μg·min^{-1}·100 CC^{-1})	Comparison ES ±90%CL[a] Inferences[b]
(Breen et al. 2011) Ten well-trained male cyclists 29 ± 6 yr 66.5 ± 5.1 mL·kg^{-1}·min^{-1}	Cycle 90 min 77 ± 3%	Primed continuous infusion [$^{13}C_6$]-phenylalanine Tissue-fluid pool surrogate GCMS for surrogate pool GC-C-IRMS for muscle 4 h recovery 0 and 4 h post-exercise Mitochondrial, myofibrillar	Diet standardized for 48 h prior to testing (PRO 1.5 ± 0.2 g·kg^{-1}·d^{-1}) Two beverages 0 and 30 min post-exercise Whey isolate and carbohydrate (PRO) 10/25/0 (585) 0.26/0.66/0 g·kg^{-1} Carbohydrate-only control (CON) 0/25/0 (425) 0/0.65/0 g·kg^{-1}	Mitochondrial PRO: 0.082 ± 0.032 CON: 0.087 ± 0.051 Myofibrillar PRO: 0.057 ± 0.024 CON: 0.057 ± 0.023	Mitochondrial PRO vs CON -0.10 ±0.07 Almost certain trivial effect Myofibrillar PRO vs CON 1.32 ±0.90 Likely large increase
(Lunn et al. 2012) Six trained male runners 23.7 ± 3.9 yr 53.1 ± 3.9 mL·kg^{-1}·min^{-1}	Run 45 min 65%	Primed continuous infusion [ring-$^2H_5$]-phenylalanine Tissue-fluid pool surrogate Mass spectrometry platform for FSR determination not published 3 h recovery 0 and 3 h post-exercise Mixed-muscle	7-day controlled diet, eucaloric to estimated energy expenditure (PRO 1.5 g·kg^{-1}·d^{-1}) Single bolus beverage 0 min post-exercise Fat-free chocolate milk (PRO) 16/58/0 (1243) 0.21/0.76/0 g·kg^{-1} High-carbohydrate control (CON) 0/74/0 (1243) 0/0.97/0 g·kg^{-1}	MILK: 0.110 ± 0.024 CON: 0.077 ± 0.024	MILK vs CON 0.99 ±0.78 Very likely moderate increase

All data are presented as mean ± SD; for data published as mean ± standard error of the mean (SEM), e.g. subject characteristics and fractional synthesis rate (FSR) (Levenhagen et al. 2001, Levenhagen et al. 2002, Howarth, Moreau, Phillips and Gibala 2009, Harber et al. 2010, Lunn et al. 2012); or FSR (Breen et al. 2011), the SD was determined (SD = SEM x √n).

Absolute VO_2max (L·min^{-1}) data (Howarth, Moreau, Phillips and Gibala 2009) were converted to relative VO_2max (mL·kg^{-1}·min^{-1}) based on the published participant mean body mass.

For studies presenting intervention nutrition as grams per kg body-mass per hour (Howarth, Moreau, Phillips and Gibala 2009, Breen et al. 2011), mean grams of macronutrients per serve were calculated from published participant mean body-mass and the feeding schedule. Kilojoules were calculated using mean per gram energy equivalents (protein, 16kJ; carbohydrate, 17kJ; fat, 37kJ).

For studies presenting intervention nutrition as grams of macronutrients per serve (Levenhagen et al. 2002, Lunn et al. 2012), mean grams of macronutrients per kg body-mass provided over the course of the recovery period, defined as the time between biopsies, was calculated from the total nutrition provided in grams divided by the published participant mean body-mass.

For effect size (ES) and inferential outcome calculations, see Supplementary Data 2.6 (Folder) Post-Endurance Protein-Carbohydrate FSR Comparisons.

For Levenhagen et al. (Levenhagen et al. 2002) only pooled participant data was available for protein synthesis; p-values were published as P <0.05 only.

[a]Add and subtract this number by the mean effect to obtain the upper and lower 90% confidence limits.

[b]Inferences are based on the smallest important effect, defined as the pharmacokinetic threshold criteria of a 25% difference. ES thresholds: <0.2 trivial, <0.6 small, <1.2 moderate, <2.0 large, <4.0 very large, >4.0 extremely large. Thresholds for assigning qualitative terms to chances of substantial effects: <0.5%, almost certainly not; <5.0%, very unlikely; <25%, unlikely; <75%, possible; >75%, likely; >95%, very likely; >99.5%, almost certain.

Only five studies have quantified an impact of post-endurance exercise protein-carbohydrate coingestion on rates of human muscle protein synthesis, relative to carbohydrate-matched or isocaloric high-carbohydrate control conditions (to enable estimation of the protein-specific effect), or non-caloric placebo (Table 2.1) (Levenhagen et al. 2002, Howarth, Moreau, Phillips and Gibala 2009, Harber et al. 2010, Breen et al. 2011, Lunn et al. 2012). Others have looked at the impact of dietary protein intake on post-exercise, post-absorptive muscle FSR (Bolster et al. 2005) or have used supplementation regimens that provided protein during (Pasiakos et al. 2011) or during and after exercise (Wilkinson et al. 2008) but these were excluded from the present summary, as a specific post-exercise effect of recovery protein feeding could not be elucidated. Furthermore, some authors have determined the impact of post-endurance exercise protein feeding on the intracellular signalling pathways regulating translation initation and elongation steps of cellular protein synthesis via mTOR-pathway activity (Ivy et al. 2008, Morrison, Hara, Ding and Ivy 2008, Kammer et al. 2009, Ferguson-Stegall et al. 2011, Rowlands et al. 2011), and while a qualitative assertion would be that muscle FSR is most likely increased based on the signalling response, in these studies there was no direct measurement of muscle FSR.

Based on those studies that have directly measured the muscle FSR response to post-endurance exercise protein-carbohydrate ingestion, the magnitude of the effect relative to isocaloric or carbohydrate-matched control conditions ranges from small (ES 0.49) to large (ES 1.60) effect size (see .xlsx and .pptx files in Supplementary Data 2.6 (Folder) Muscle Protein Synthesis ES and Inferences). A general finding was that some of the studies were relatively underpowered with only six (Howarth, Moreau, Phillips and Gibala 2009, Lunn et al. 2012) or eight (Harber et al. 2010) male participants; Levenhagen et al. (Levenhagen et al.

2002) and Breen et al. (Breen et al. 2011) had ten. Harber et al. (Harber et al. 2010) concluded that, despite a numerical increase in post-exercise mixed-muscle FSR versus at rest, the effect was not statistically significant with n=8, and suggested that their biopsy protocol and/or transient feeding or effect of feeding might be responsible. However, in the current reanalysis we found that a small increase in mixed-muscle FSR was likely (Table 2.1; threshold for a meaningful effect was 0.2 times the standard deviation in the reference condition). Rather than an immediate post-exercise and end-recovery biopsies, the authors (Harber et al. 2010) used a 2-h and 6-h recovery biopsy approach coupled to a [ring-$^{2}H_5$]-phenylalanine tracer started 1 h post-feeding, which might have missed a substantial portion of the increase in muscle FSR in the first 2 h of recovery. If the study of Harber et al. (Harber et al. 2010) is withdrawn, then the range of the response becomes large effect sized, from 1.31 ±90%CL 0.65 to 1.60 ±0.80, with the lower and upper 90% confidence intervals extending to an effect size range of moderate to very large.

Breen et al. (Breen et al. 2011) quantified the responses of the myofibrillar and mitochondrial protein fractions to post-exercise protein-carbohydrate, whereas the other investigations looked at leg or mixed-muscle. Some evidence suggests that the mitochondrial fraction contributes most to the change in mixed-muscle FSR after endurance exercise (Wilkinson et al. 2008) although the impact of the myofibrillar fraction cannot not be discounted (Supplementary Data 2.4 and 2.5). Interestingly, protein ingestion only increased myofibrillar FSR substantially, suggesting that either the mitochondrial fraction is less responsive in the time-frame of recovery assayed by Breen et al. (Breen et al. 2011) or that the synthesis rate of mitochondrial proteins is already maximal. Speculatively, the myofibrillar fraction might access protein-derived amino acids first and, therefore, 'starve' the mitochondria, perhaps

until the muscle (myofibrillar) portion is 'full' (as has been implicated by others (the 'muscle full effect' (Atherton et al. 2010)). However, further work is required to confirm the effect of endurance exercise and feeding on the myofibrillar and mitochondrial muscle protein fractions.

All investigators utilized phenylalanine tracers ([ring-$^{2}H_5$]-phenylalanine or [$^{13}C_6$]-phenylalanine) and GCMS for all samples, except Breen et al. (Breen et al. 2011) who used GC-C-IRMS for muscle protein enrichment, and Lunn et al. (Lunn et al. 2012) who did not report their platform. Therefore, variability due to methodology is probably small, but use of the more sensitive IRMS-based platforms is recommended. Levenhagen et al. (Levenhagen et al. 2001, Levenhagen et al. 2002) used the arteriovenous balance method rather than direct measurement of the muscle tissue incorporation of infused phenylalanine tracer. Furthermore, their cohort was mixed-sex (5 men and 5 women), relatively varied in age (range 20-41 yrs), and based on the published mean VO_2max data for each sex the cohort would be best classified as aerobically untrained. In fact, there was considerable variation in the relative fitness of participants between all of the studies, with only three studies using trained or well-trained men (Harber et al. 2010, Breen et al. 2011, Lunn et al. 2012). While comparison can be made between stable-isotope infusion studies that directly sample tracer amino acid incorporation into the muscle, as the change in muscle FSR in response to exercise and protein ingestion at rest appears to be proportional between different tracers, caution should be urged when comparing between direct (muscle) and indirect (arteriovenous) measurement methods. As previously mentioned, trained muscle appears to respond differently to untrained muscle, with an increased rate of protein turnover (synthesis and breakdown) at rest (Pikosky et al. 2006), and a reduced mitochondrial FSR following endurance exercise (Wilkinson et al.

2008). Unfortunately, the authors (Wilkinson et al. 2008) did not present separate muscle protein synthesis data for men versus women to enable comparison of rates between the sexes. It has recently been shown that in women, protein ingestion after resistance exercise results in a likely small increase in myofibrillar FSR (ES 0.69 ±0.81) over hours 1-3 of recovery, but a likely moderate decrease over hours 3-5 (ES -0.81 ±0.65), relative to men (West et al. 2012)(see Supplementary Data 2.7 West 2012 ES and Inferences.xlsx, and Supplementary Data 2.8 West 2012 ES and Inferences.pptx). Notably, the investigation of Levenhagen et al. (Levenhagen et al. 2002) derived the largest observed effect size for leg muscle protein synthesis (1.63 ±1.32) which was for the protein-carbohydrate versus non-caloric placebo comparison, despite providing a total of just 10 g of a slow-digestion casein protein in the intervention.

With regards to the nutrition provided in the protein-carbohydrate interventions, several protein-types were used, carbohydrate provision was suboptimal (below the recommended rate of intake to saturate glycogen resynthesis) in four of the five studies (Levenhagen et al. 2002, Harber et al. 2010, Breen et al. 2011, Lunn et al. 2012), and feeding protocols varied dramatically in terms of the total nutrition provided. Protein-types provided in the interventions were a fast-digestion hydrolyzed whey concentrate (Howarth, Moreau, Phillips and Gibala 2009) and a whey isolate (Breen et al. 2011), slow-digestion casein (Levenhagen et al. 2001, Levenhagen et al. 2002), and milk proteins intact or as an isolate (Harber et al. 2010, Lunn et al. 2012) which likely exhibit moderated digestion kinetics. While it has been suggested that ~25 g of high-quality protein might be optimal to saturate mixed-muscle protein synthesis following resistance exercise (Moore et al. 2009) there has yet been no comparable investigation following endurance exercise. Regardless, there is some speculation

(Rodriguez 2009) that the protein dose given by Howarth et al. (Howarth, Moreau, Phillips and Gibala 2009) was excessive. Only one investigation provided sufficient carbohydrate in the intervention (1.2 $g \cdot kg^{-1} \cdot h^{-1}$) and control conditions (1.2 and 1.6 $g \cdot kg^{-1} \cdot h^{-1}$) to ensure saturation of glycogen resynthesis, supported by a measured glycogen resynthesis rate that was not substantially different between trials (Howarth, Moreau, Phillips and Gibala 2009). A further consideration is the inclusion of fat (incidentally, as the lipid component of the low-fat milk used as the protein source) in one investigation (Harber et al. 2010). Quantifying the impact of mixed-meals that contain protein, carbohydrate and fat is important given that the majority of normal daily nutrition for athletes is provided in this way, and that the non-protein macronutrients could impact on the fed-recovery rate of muscle FSR by affecting absorption and uptake of protein and the derived amino acids, or via direct mechanisms. Supplementation of the diet with long-chain n-3 polyunsaturated fatty acids increased mTOR-pathway activity and further stimulated a hyperaminoacidemia-hyperinsulinemia-induced increase in resting rate of muscle protein synthesis in middle-aged (25-45 yrs) (Smith et al. 2011) and older adults (Smith et al. 2011). However, in this instance (Harber et al. 2010) the quantity of fat provided was so small (two serves of 2 g) it was unlikely to substantially impact FSR versus the amino acid-induced effects. Only Howarth et al. (Howarth, Moreau, Phillips and Gibala 2009) had both an isocaloric and a carbohydrate-matched control; both Breen et al. (Breen et al. 2011) and Lunn et al. (Lunn et al. 2012) used an isocaloric higher-carbohydrate control condition. Comparative conditions otherwise consisted of a non-caloric placebo (Harber et al. 2010) or were non-isocaloric (Levenhagen et al. 2002).

Mention should be made of the to the statistical approach used by all the mentioned studies, that is, use of a null-hypothesis test to declare if (in this case) the FSR difference between a protein-fed and non-protein condition is significant or not significant on the basis of the test-derived p-value. This approach has well-known drawbacks and is frequently misinterpreted (see (Batterham and Hopkins 2006) and there are numerous examples in the literature of trivial differences in the measured muscle FSR between experimental conditions misinterpreted as 'no effect', and several examples of substantial clear effects being reported as 'no significant difference', including in the present brief review (Wilkinson et al. 2008, Harber et al. 2010) (see Supplementary Data 2.4; and Supplementary Data 2.6 (Folder), Harber ES and Inferences.xlsx and .pptx). A more practical method that has been utilized in the current work is to express uncertainty of the true effect via the confidence limits, which define the likely range that the true value lies within (Batterham and Hopkins 2006). By then defining a threshold value of the measured effect that can be considered substantial (a meaningful or functional difference in a positive or negative direction) an inference can be made about the magnitude of the measured value, and a likelihood that the true population effect will share the magnitude expressed (e.g. likely large effect or difference) (Batterham and Hopkins 2006). As already mentioned, an unresolved issue with regard to expressing the utility of a change in FSR with an intervention, is that there is no currently accepted value for what a meaningful or functional change in muscle protein synthesis is. Therefore, we have used the pharmacokinetic threshold (a 25% difference) standard to drug trials.

Summary of the key information and future directions

Post-endurance exercise muscle protein synthesis is a key mechanism impacting the rate of renewal of exercise-damaged proteins and adaptive plasticity. Several methods are currently

employed to assess muscle protein synthesis directly, utilizing stable-isotope infusions and muscle biopsies coupled to mass spectrometry analysis. The digestion characteristics of proteins are an important feature impacting on their primary tissue site of metabolism; for peripheral muscle tissue after exercise, amino acids from rapidly digested and absorbed 'fast' proteins, such as whey hydrolysates, isolates and concentrates, provide the largest initial increase in muscle FSR, but might be best supported by later ingestion of 'slow' proteins (casein) to provide additional substrate amino acid. Based on an evaluation of five investigations of the muscle protein synthetic response to protein feeding after endurance exercise, it can be concluded that the overall quality of investigations to date is low. The study design parameters and methodological quality of future studies should be improved based on the presented recommendations, and those of others (Smith, Patterson and Mittendorfer 2011). Study design parameters to address include; larger sample sizes to ensure studies are well-powered to detect differences; the use of an inferential statistical approach to enable better interpretation and understanding of data, avoid misleading conclusions about effects, and also enable easier comparison in meta-analyses; using a cohort homogenous for age and training status, with well-trained muscle preferred or contrasted to untrained or moderately trained muscle; nutritional interventions that are realistic of athletic practice (if well-trained muscle is used), that is, containing carbohydrate and some fat; and a preference should be given to IRMS or GC-C-IRMS platforms for the analysis of intracellular free amino acid and muscle protein enrichment. An overriding issue is consensus on what the smallest important change in muscle protein synthesis to yield worthwhile changes in muscle and performance phenotype. Determination will require additional experiments well-designed to address that question.

CHAPTER 3 STUDY 1A

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A Protein-Leucine Supplement Increases BCAA and Nitrogen Turnover but not Performance

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ABSTRACT

Purpose. To determine the effect of post-exercise protein-leucine coingestion with carbohydrate-lipid on subsequent high-intensity endurance performance, and to investigate candidate mechanisms using stable isotope methods and metabolomics. **Methods.** In a double-blind randomized crossover, 12 male cyclists ingested a leucine/protein/carbohydrate/fat supplement (LEUPRO, respectively 7.5/20/89/22 $g \cdot h^{-1}$) or isocaloric carbohydrate/fat control (119/22 $g \cdot h^{-1}$) 1-3 h post-exercise during a 6-day training block (intense intervals, recovery, repeated-sprint performance rides). Daily protein intake was clamped at 1.9 (LEUPRO) and 1.5 $g \cdot kg^{-1} \cdot d^{-1}$ (control). Stable isotope infusions (1-^{13}C-leucine and 6,6-$^{2}H_2$-glucose), mass-spectrometry based metabolomics, and nitrogen balance methodology were used to determine effects of LEUPRO on whole-body branch-chain amino acid (BCAA) and glucose metabolism, and protein turnover. **Results.** Following exercise, LEUPRO increased BCAAs in plasma (2.6-fold; 90%CL x/÷1.1) and urine (2.8-fold; x/÷1.2), and products of BCAA metabolism plasma acylcarnitine C5 (3.0-fold; x/÷0.9) and urinary leucine (3.6-fold; x/÷1.3) and β-aminoisobutyrate (3.4-fold; x/÷1.4), indicating ingesting ~10 $g \cdot leucine \cdot h^{-1}$ during recovery exceeds the capacity to metabolise BCAA. Furthermore, LEUPRO increased leucine oxidation (5.6-fold; x/÷1.1), non-oxidative disposal (4.8-fold; x/÷1.1), and left leucine balance positive, relative to control. With the exception of day-1 (LEUPRO 17 ± 20 mg $N \cdot kg^{-1}$; control -90 ± 44 mg $N \cdot kg^{-1}$), subsequent (day 2-5) nitrogen balance was positive for both conditions (LEUPRO 130 ± 110 mg $N \cdot kg^{-1}$; control 111 ± 86 mg $N \cdot kg^{-1}$). Compared to control feeding, LEUPRO lowered the serum creatine kinase concentration by 21-25% (90%CL ±14%), but the impact on sprint power was trivial (day 4: 0.4% ±1.0%; day 6: -0.3% ±1.0%). **Conclusion.** Post-exercise protein-leucine supplementation saturates BCAA metabolism and attenuates tissue damage, but effects on

subsequent intense endurance performance may be inconsequential under conditions of positive daily nitrogen balance.

INTRODUCTION

Endurance athletes frequently undertake high-intensity exercise on consecutive days, such as during training camps or multi-day competition. Such exercise depletes glycogen stores (Kirwan et al. 1988) and can disrupt skeletal-muscle structural integrity (Koller et al. 1998), but is also an adaptive stimulus (Hawley, Gibala and Bermon 2007, Rowlands et al. 2011). Nutrition plays an important role in the restoration of muscle glycogen, and emerging evidence indicates a role for dietary protein and amino acids in attenuating skeletal muscle damage and increasing muscle protein turnover to promote adaptive remodelling (Hawley, Gibala and Bermon 2007, Howarth, Moreau, Phillips and Gibala 2009). For these reasons, there is considerable interest in the role of post-exercise protein, amino acid and carbohydrate ingestion in the mechanisms associated with recovery and training adaptation.

Evidence for an ergogenic effect of post-exercise protein-rich food on subsequent endurance performance was reported recently (Rowlands et al. 2008, Thomson, Ali and Rowlands 2011) (Rowlands et al. 2007, Rowlands et al. 2008, Thomson, Ali and Rowlands 2011). Only limited mechanistic investigation was undertaken, but the effect was associated with positive nitrogen balance and attenuation of plasma creatine kinase (CK) concentrations. Other evidence points to a post-exercise protein-nutrition mediated effect via adaptive remodelling of structural and contractile elements in the exercised skeletal muscle (Rowlands et al. 2011) supported by an enhanced fractional protein synthesis rate (FSR) (Howarth, Moreau, Phillips and Gibala 2009).

Recently our group revealed that feeding well-trained cyclists milk proteins (4 x 15 g·30 min^{-1} over 1.5 h) with additional free leucine (4 x 3.75 g·30 min^{-1}), carbohydrate and lipid following intense endurance cycling daily for three days, resulted in a small improvement in repeated-sprint mean power 2 days later, relative to an isocaloric low-protein-lipid control (Thomson, Ali and Rowlands 2011). Leucine is of special interest because it stimulates muscle protein synthesis (Anthony, Anthony and Layman 1999, Crozier et al. 2005) and inhibits protein degradation (Mordier et al. 2000), although some evidence suggests that leucine ingested at rest without recent exercise at doses near the maximum tissue oxidative capacity (estimated at ~40 g·d^{-1}; (Elango et al. 2010)) could be excessive and less useful to muscle protein metabolism when consumed out of proportion to valine and isoleucine (Harris, Joshi, Jeoung and Obayashi 2005). With evidence for a functional effect, the objective of the present study was to not only provide additional end-point phenotype information on the impact of a post-exercise protein-leucine rich supplement on subsequent performance, but to also investigate putative metabolic mechanisms that might explain the performance effect. Possible alterations to carbohydrate metabolism warranted further investigation, as did the effect of protein-leucine supplementation on BCAA metabolism.

To investigate these questions, we used whole-body stable isotope methodology to study leucine and glucose turnover, and contemporary mass-spectrometry based metabolomics to assess large-scale changes in metabolites in response to post-exercise protein-leucine ingestion. We also sought to confirm the benefit of the leucine-rich supplement on subsequent high-intensity endurance cycling performance several times throughout a 6-day block of controlled training and diet. Our hypothesis was that the protein-leucine recovery

supplement would increase BCAA metabolism and non-oxidative disposal and enhance subsequent high-intensity endurance cycling performance.

METHODS

Participants. Twelve well-trained male cyclists (mean ± SD: age, 35 ± 10 y; height, 182 ± 5 cm; body mass, 76.9 ± 6.5 kg) with a maximal oxygen uptake (VO_2max) of 64.8 ± 6.8 $mL \cdot kg^{-1} \cdot min^{-1}$ and peak power output (Wmax) of 355 ± 36 W completed the study. Cyclists had a 9 ± 4 y training history with a recent weekly training volume of 10 ± 1 h. Participants were screened for contraindications and excluded on the basis of failing to pass health screening, having recently given blood, or intake of caffeine, alcohol, medications/drugs or smoking during the experimentally controlled period. All participants were informed of the purpose of the study and associated risks, and provided written informed consent. The study was approved by the Central Regional Ethics Committee of New Zealand.

General Design. The study was a randomized, double-blinded, crossover to determine the effect of a leucine, protein-carbohydrate-fat (LEUPRO) post-exercise feeding intervention compared with an isocaloric carbohydrate-fat control on repeated-sprint cycling performance and mechanistic variables during a 6-day block of controlled high-intensity training and diet (Fig. 3.1). Prior to the first experimental block, participants recorded their habitual training and diet for 7 and 3 days, respectively; training was tapered in the 3 days prior to the experimental block, including a rest day prior to starting. To standardize lead-in fatigue and minimize diet variation this training and dietary regimen was repeated preceding block 2.

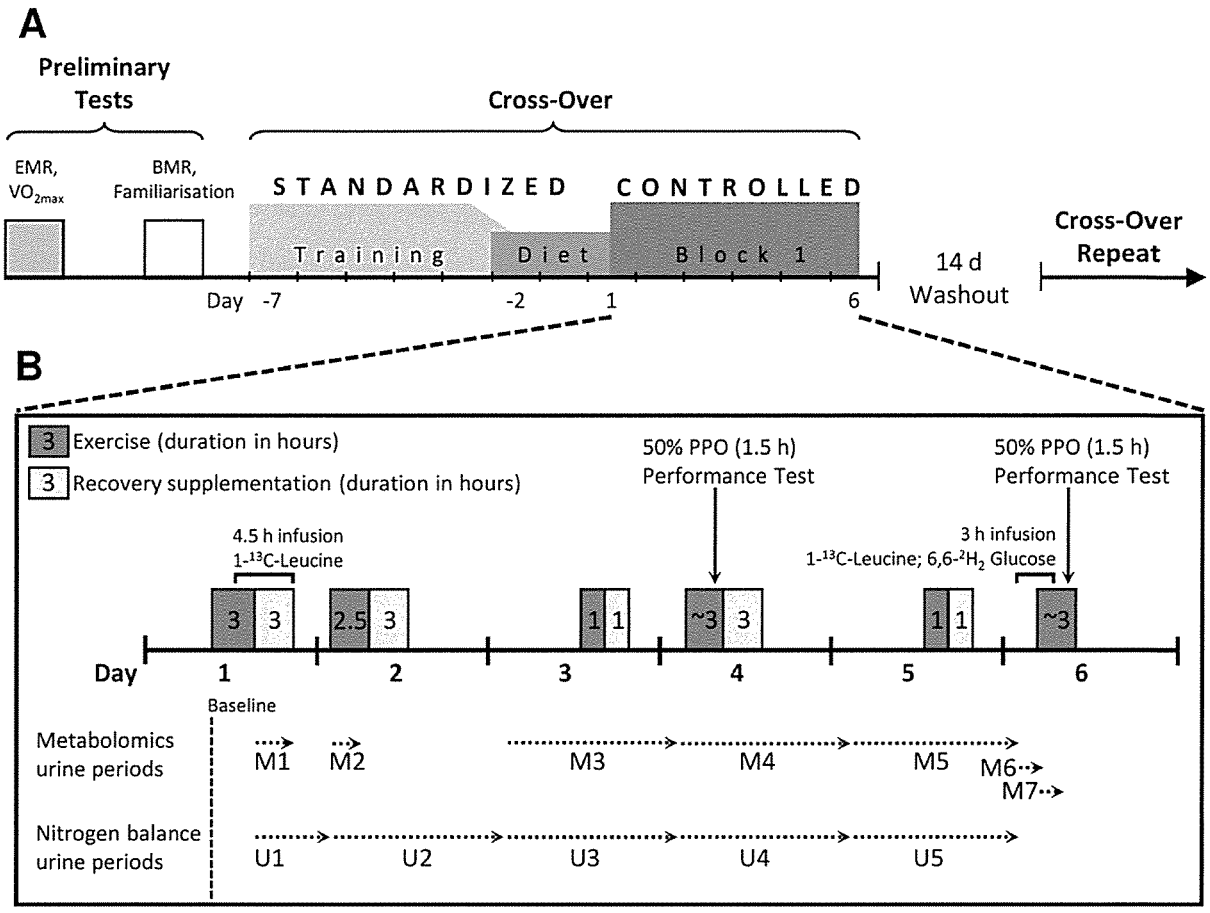

Figure 3.1. Study 1 experimental design. Shown are preliminary visits and standardized training and diet leading in to a 6-day experimental block (**A**), with one experimental block further outlined, including high-intensity cycling, recovery supplementation, and urine collection periods (**B**). M1-M7, metabolomics urine collection periods 1-7. U1-U5, urine collection periods 1-5.

Preliminary Testing. Two weeks prior to the first experimental block cyclists underwent exercise metabolic rate (EMR), VO_2max and Wmax tests on an electromagnetically-braked cycle ergometer (Velotron, Version 1.9 Software, Racer Mate, Seattle, USA). The exercise protocol consisted of 10 min at 100 W, and 3 x 6-min stages at 150 W, 188 W and 225 W (for EMR) after which workload was increased by 25 W every 2.5 min until volitional

exhaustion or failure to maintain a cadence of at least 60 rpm. Expired breath was collected into Douglas bags (Vacumed 1195-200, GBC BioMed, Auckland, New Zealand) over the last half of each increment, then continuously near exhaustion. Subsequent Wmax was calculated (Rowlands et al. 2007), and values used to establish workloads employed during the main experimental protocol. VO_2max was determined with minute volume measured by pneumotach (Hans Rudolp, Inc., Kansas City, MO) and oxygen and carbon dioxide analyzers (Sensormedics, Vmax Spectra Series, Sensor Medics Corp., CA, USA). During the three submaximal stages EMR ($kJ \cdot min^{-1}$) was calculated from oxygen consumption (VO_2) and carbon dioxide production (VCO_2) rates (Jeukendrup and Wallis 2005). Flow meter validity is detailed in Supplementary Data 3.3 Flow Meter Calibration.xlsx.

One week following, cyclists reported to the laboratory (06:00 h) in fasted condition for calculation of basal metabolic rate (BMR) via indirect calorimetry (Jeukendrup and Wallis 2005). Subjects were supine for 20 min followed by 30 min of expired breath collection. Subsequently, a small breakfast was provided before participants engaged in a full familiarization trial of the repeated-sprint performance test as described previously (Rowlands et al. 2007). During all laboratory visits, environmental conditions were 19.3 ± 1.4°C and 42 ± 7% relative humidity.

Experimental protocols

Exercise procedures. The riding schedule was a multi-day model adapted from that used previously (Rowlands et al. 2008) to simulate the physical stress involved in strenuous high-intensity training or competition. Workloads were pre-programmed at fixed percentages of

individual Wmax. Each block comprised four intermittent high-intensity rides. Day 1 (3 h cycling) consisted of a warm up (15 min at 30%, 10 min at 40%, 6 min at 50% Wmax) followed by loading intervals: 3 blocks of 10 x 2 min intervals at 90%/80%/70% Wmax (2 min at 50% Wmax between intervals, 6 min at 50% Wmax between blocks); and a cool down of 11 min at 30% Wmax. Day 2 (2.5 h cycling) comprised a warm up (10 min at 30%, 10 min at 40%, 5 min at 50% Wmax) followed by 2 blocks of longer loading intervals (4 x 5 min at 70% Wmax interspersed with 3 x 5 min at 50% Wmax; 3 x 4 min at 70% interspersed with 3 x 4 min at 50% Wmax) and separated by 3 x 1 min at 90% and 3 x 1 min at 80% Wmax (interspersed by 2 min at 50% Wmax). Days 4 and 6 comprised 90 min at 50% of Wmax followed by the repeated-sprint performance test as described by Rowlands *et al.* (Rowlands et al. 2007, Rowlands et al. 2008). Rides on days 3 and 5 comprised 60 min at 30% of Wmax. All rides were conducted at the same time of day for a given participant: between 14:00 to 18:00 h for days 1, 3, and 5 and 05:00 to 09:00 h for days 2, 4, and 6.

Energy expenditure. Total daily energy expenditure (ΣEE) for each day of the experimental block was estimated from RMR and EMR and daily activity energy expenditure: ΣEE (kJ) = PA + EMR + TEF, where PA is the sum of daily physical activities, and TEF is the thermogenic effect of food assumed as 10% of expended daily energy plus exercise energy expenditure (i.e. TEF = 0.1 x [PA + EMR]). To estimate PA, participants prospectively diary-logged all activities during the experimental block, which were converted to metabolic equivalents (METs; (Ainsworth et al. 2000). Uncompleted or extra activities were recorded and repeated during the subsequent experimental block. Energy expenditure during exercise was estimated from the regression of the three sub-maximal EMR versus workload samples obtained during VO_2max testing.

Control of diet. To reduce the endogenous ^{13}C breath background enrichment participants were instructed to avoid foods with carbohydrate sources naturally enriched in ^{13}C (maize, sugar cane, or sugar beet) starting 7 days prior to the experimental block. All food was provided during the 6-day experimental blocks. Inclusive of recovery supplementation, dietary protein intake was clamped at 1.5 $g \cdot kg^{-1} \cdot d^{-1}$ for control condition, mid-range within estimated requirements for endurance training men (Tarnopolsky 1999), and 1.9 $g \cdot kg^{-1} \cdot d^{-1}$ for the LEUPRO condition, with the protein difference provided in the post-exercise intervention. The daily protein intake for LEUPRO approximates the value estimated to achieve nitrogen balance from our previous 4-day high-intensity cycling protocol (Rowlands et al. 2008). The diet provided ≥8 g carbohydrate$\cdot kg^{-1} \cdot d^{-1}$ on heavy training days (days 1, 2, 4) for both conditions to ensure sufficient glycogen resynthesis (Burke, Kiens and Ivy 2004). Daily energy intake was designed to balance estimated daily energy expenditure. A balancing supplement comprising milk protein, maltodextrin and cream powder was provided with evening meals to ensure total daily requirements were met (see Table 3.1 for mean daily macronutrient and energy intake). Prior to morning rides (days 2, 4, 6), cyclists were provided a small carbohydrate-rich breakfast of toast, butter and honey to simulate normal practice (50/10/9 g carbohydrate/protein/fat). 30 min after ingesting the final serving of intervention on days 1, 2 and 4, participants received a small pasta meal (36/7.5/2.0 g carbohydrate/protein/fat). Cyclists ingested a 7.5% carbohydrate sports drink comprising 2:1 maltodextrin to glucose (tapioca and wheat origin, respectively), 1.17 $g \cdot L^{-1}$ NaCl, and lime juice, provided every 30 min during exercise (every 15 min during performance tests) at a rate of 790 ± 82 $mL \cdot water \cdot h^{-1}$.

Table 3.1. Daily and overall mean macronutrient and energy intake for protein-leucine and control supplemented diets.

	Day 1[b]	Day 2[c]	Day 3[c]	Day 4[c]	Day 5[c]	Overall Daily Mean[d]
Macronutrient[a] ($g \cdot kg^{-1} \cdot d^{-1}$)			*LEUPRO*			
Protein	1.2 ± 0.2	1.9 ± 0.1	1.9 ± 0.1	1.9 ± 0.2	1.9 ± 0.1	1.9 ± 0.1
Carbohydrate	4.0 ± 0.5	10.7 ± 1.1	7.2 ± 0.9	10.7 ± 0.9	7.4 ± 0.9	9.0 ± 1.9
Fat	0.9 ± 0.1	1.8 ± 0.2	1.7 ± 0.2	2.0 ± 0.2	1.4 ± 0.3	1.7 ± 0.3
Macronutrient[a] ($g \cdot kg^{-1} \cdot d^{-1}$)			*Control*			
Protein	1.0 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.5 ± 0.1
Carbohydrate	5.2 ± 0.7	11.0 ± 1.2	7.5 ± 1.0	11.3 ± 1.2	7.7 ± 0.9	9.4 ± 2.1
Fat	0.9 ± 0.1	1.8 ± 0.2	1.7 ± 0.2	2.0 ± 0.2	1.4 ± 0.3	1.7 ± 0.3
Energy[a] (MJ)						
Intake	9.0 ± 0.9	20.8 ± 1.7	16.0 ± 1.1	22.8 ± 1.5	16.0 ± 1.7	17.9 ± 1.0
Expenditure	3.8 ± 0.3	20.9 ± 1.7	15.5 ± 1.5	22.6 ± 1.8	15.1 ± 1.8	17.8 ± 1.2
Balance	5.3 ± 1.1	-0.1 ± 0.5	0.5 ± 0.6	-0.2 ± 0.5	0.5 ± 0.7	0.2 ± 0.6

[a]Data are means ± SD. Values for energy apply to both dietary conditions.
[b]From 1800 h to 0600 h following morning. Mean value represents intake for 12 h.
[c]0600 h to 0600 h following morning.
[d]Mean of days 2-5.
Abbreviations. LEUPRO, protein-leucine-carbohydrate-lipid supplement; control, carbohydrate-lipid supplement. MJ, megajoules.

Nutritional intervention. Following post-exercise urine collection and blood sampling, cyclists ingested the intervention beverages which comprised two identically-flavored milk-like emulsions prepared from dried ingredients. The supplements were prescribed for a model 360W rider, then scaled to each participant on the basis of Wmax (grams of leucine or macronutrient multiplied by Wmax/360W) so that riders with greater energy expenditures received more nutrition, with a per serve final intake of: LEUPRO, leucine 3.8 ± 0.4 g, protein, 10.0 ± 1.0 g, carbohydrate 44.0 ± 4.6 g, fat, 11.0 ± 1.1 g; control, carbohydrate 60.0 ± 6.2 g, fat 11.0 ± 1.1 g into 250 ± 72 mL water. One serving was provided every 30 min for a total 6 servings after exercise on days 1, 2, and 4, and 2 servings on days 3 and 5. Additionally, water was provided equating to weight lost during exercise. L-leucine was from Dolder AG (Basel, Switzerland). Whole-protein was a micellar whey-protein isolate (PROLACTA-90, Lactalis Industrie, Bourgebarré, France) containing 11.95 g leucine:100 g amino acid. Carbohydrate was 2:1 maltodextrin (Glucidex IT21W, Sugro AG, Basel, Switzerland) to fructose (Fructofin C, Danisco, Kotka, Finland) and fat was a palm oil

powder (Palmstearin 54, JuChem Food Ingredients Gmbh, Eppelborn, Germany). Both supplements also contained per serve 490 ± 51 mg of sucrose ester emulsifier (Ryoto S-1670, Mitsubishi Chemical Europe Gmbh, Düsseldorf, Germany), 300 ± 31 mg of aroma (CHOC505176, Firmenich SA, Meyrin, Switzerland) and 630 ± 65 mg of NaCl. To minimize disturbance to the plasma pool steady-state enrichment of 1-^{13}C-leucine during day 1 infusion, the LEUPRO supplement contained ~8% 1-^{13}C-leucine added during drink preparation. Supplementary Data 3.1 (Participant Nutrition and Exercise Load) contains details of participants' prescribed diets and supplemental nutrition, preliminary testing results and experimental block exercise loading.

LEUPRO supplementation was designed to saturate post-exercise muscle protein synthesis, suggested at the time of design conception to be at a leucine intake of ~0.135 g·kg^{-1} (~10 g·h^{-1}) (Crozier et al. 2005) and 6-20 g of EAA (Tipton et al. 1999, Miller et al. 2003). LEUPRO supplementation provided carbohydrate at 1.2 g·kg^{-1}·h^{-1} to saturate glycogen synthesis (Burke, Kiens and Ivy 2004) and moderate lipid was utilized for successful blinding between treatments and to aid in restoration of intramuscular lipid stores.

Whole body leucine and glucose turnover. Whole-body leucine turnover was measured using a primed constant 1-[^{13}C]-leucine infusion during the 3 h post-exercise recovery period on day 1 and at rest (pre-exercise) and during steady-state exercise on day 6. On day 1 the participants provided a urine sample and body mass measurement. A 20-gauge catheter was positioned in an antecubital vein of each arm; to the first catheter an extension line leading to an infusion pump (74900 Series, Cole-Parmer Instrument Company, Vernon Hills, IL; pump calibration provided in Supplementary Data 3.2 Infusion Pump Calibration) was attached,

while the second catheter was kept patent with 0.9% isotonic saline and closed with a 2-way valve. Following priming doses of 0.295 $mg \cdot kg^{-1}$ $NaH^{13}CO_3$ and 1 $mg \cdot kg^{-1}$ 1-[^{13}C]-leucine, the cyclists underwent a 4.5-h constant infusion of 1 $mg \cdot kg^{-1} \cdot h^{-1}$ 1-[^{13}C]-leucine starting 90-min into exercise. Blood samples for α-[^{13}C]-ketoisocaproate acid (α-KIC) enrichment and amino acid concentrations were taken pre-exercise, prior to infusion priming, following exercise immediately prior to supplement ingestion (defined as 0 min, the start of recovery), and at 30, 60, 90, 120 and 180 min into recovery.

On day 6, fasted participants provided body mass and urine and catheters were fitted as described. At rest, participants received priming doses of $NaH^{13}CO_3$, 1-[^{13}C]-leucine and [6,6-$^2H_2$]-glucose (3.1 $\mu g \cdot kg^{-1}$) before beginning constant infusions of 1-[^{13}C]-leucine and [6,6-$^2H_2$]-glucose (initially at 40 $\mu g \cdot kg^{-1} \cdot min^{-1}$) for 180 min. At the onset of exercise the [6,6-$^2H_2$]-glucose infusion rate was increased stepwise at 0, 5 and 10 min to 60, 80, and 100 $\mu g \cdot kg^{-1} \cdot min^{-1}$ (Devries et al. 2006) where it continued for the duration of the 90-min steady-state exercise. Blood was taken pre-infusion, pre-exercise and at 60, 75, and 90 min into exercise. Blood for α-KIC and [6,6-$^2H_2$]-glucose concentrations and enrichments were collected into EDTA and LH-treated evacuated tubes (Beckton-Dickson, Auckland, New Zealand), respectively, and samples were collected and stored at -80°C until analysis. All infusates were 99 atom% and sourced from Cambridge Isotopes (Andover, MA). Infusion pump working flow rates and infusate calculations are detailed in Supplementary Data 3.4 Isotope Infusions.xlsx.

Breath collection. Expired breath samples for indirect calorimetry were directed through a 5-litre mixing chamber attached to a Douglas bag for 10-12 min gas collections at rest and 3-5

min during exercise. From a port on the mixing chamber, 3 x 10 mL samples were collected into exetainers (Labco, High Wycombe, UK) for breath ^{13}C enrichment. On day 1, exetainer samples were collected during exercise prior to 1-[^{13}C]-leucine infusion (background), and at recovery times 0, 30, 60, 90, 120 and 180 min. On days 4 and 6, exetainer samples were collected at rest and at 60, 75 and 90 min into steady-state exercise.

Additional blood collection. Blood samples for additional parameters were collected on days 1 and 6 at sampling times as described for leucine/glucose infusions, as well as post-exercise on day 6; pre and post-exercise samples were taken on days 2 and 4 by venipuncture. Whole blood was collected into chilled EDTA vacutainers and centrifuged (15 min at 1750 g, 4°C) for plasma CK and glucose; blood for insulin sat for 30 min in a serum vacutainer and was then centrifuged (30 min at 2000 g). Perchloric acid extraction of plasma for amino acid analysis was performed as described by Moore *et al.* (Moore et al. 2005). For the analysis of plasma metabolites, 3 mL of whole blood sat for 3 min away from light in an ice-cold vacutainer and, then spun (2500 g, 4°C for 10 min). For all parameters, a sample of supernatant was extracted to Eppendorf tubes and immediately frozen at -80°C until analysis.

Urine and sweat collection. Throughout the 6-day block 24-h urine was collected for quantification of nitrogen excretion and urinary metabolite analysis (Fig. 1). From these data, the protein oxidation component to VO_2 and VCO_2 was calculated and corrected (Jeukendrup and Wallis 2005). Sweat was collected during exercise on day 2 and over the 90-min steady-state ride on day 6 using 7.5 cm^2 gauze pad set on parafilm within an adhesive patch and applied to two chest regions (superior to the nipple and ~5 cm lateral to the sternum) and two abdominal regions (mid-point between costal border and iliac crest, ~5 cm lateral to the

sternum). Following exercise, patches were removed and samples stored and frozen (-80°C) until analysis. Participants were asked to toilet, towel dry and take nude bodyweight before and after collection periods for weight change sweat loss calculations.

Analyses

Isotopic enrichments, leucine kinetics, and glucose R_a, R_d and MCR. Plasma enrichment of the t-BDMS derivative of α-KIC was measured by gas chromatography–mass spectrometry (GC-MS; Hewlett-Packard 6890; MSD model 5973 Network; Agilent Technologies, Santa Clara, CA). Calculations of leucine turnover were based on the reciprocal pool model where α-KIC enrichments were used as a proxy for plasma leucine enrichment, using equations described previously (Matthews et al. 1980). A bicarbonate retention factor of 0.81 at rest and 1.00 during exercise was used (Hamadeh, Devries and Tarnopolsky 2005). Calculations included a correction of 33% for first pass splanchnic extraction of 1-[^{13}C]-leucine (Boirie et al. 1997) ingested with LEUPRO supplementation. However, because of the quantity of leucine ingested during fed recovery on day 1, the rate of uptake of free 1-[^{13}C]-leucine oral tracer was unknown. Given the quantities of leucine, protein and carbohydrate delivered, the rate of breakdown was most likely to decrease during recovery (Boirie et al. 1997, Howarth, Moreau, Phillips and Gibala 2009); conservatively, we have assumed a constant rate of breakdown throughout recovery with the LEUPRO supplement that is equal to the resting sample prior to supplement ingestion (where $B = Q - i$), and this clamped breakdown rate was used for subsequent calculations (see Supplementary Data 3.5 Leucine Turnover and Breath Data Calculations.xlsx). Standard equations (i.e. without a clamped breakdown rate) apply for the control on day 1 (i.e. $I = 0$) and for day 6 (fasted) infusions for both conditions (Matthews et al. 1980).

Isotopic enrichment of glucose was determined using GC-MS of the trimethylsilyl derivative, and Steele equations modified for stable isotopes to calculate glucose R_a, R_d and metabolic clearance rate (MCR) using a volume of distribution of glucose of 100 $mL \cdot kg^{-1}$, as described by Phillips *et al.* (Phillips et al. 1996). Selected ion mass-to-charge ratio (*m/z*) was recorded at 205 and 207 atomic mass units. Glucose R_a and R_d were calculated at rest and at 60, 75, and 90 min during exercise and averaged throughout (see Supplementary Data 3.6 Glucose Turnover Calculations.xlsx).

Plasma amino acid, glucose and insulin concentrations. Plasma amino acid concentrations were measured from the perchloric acid extract by HPLC (Moore et al. 2005). Glucose was assayed by spectrophotometric measurement by automated analyzer (Roche/Hitachi 917, Roche, New Zealand) of enzyme-catalyzed NADPH formation from glucose, using an *in vitro* test kit (Roche/Hitachi Glucose Kit, Roche, New Zealand). Insulin was assayed via a standard immunoassay kit (Insulin ELISA IS130D 96 Tests, Calbiotech Inc., Spring Valley, CA).

Muscle membrane damage. Serum creatine kinase activity was measured spectrophotometrically using a kit (Roche/Hitachi Total Creatine Kinase kit, Roche, New Zealand).

Urea and creatinine. Net nitrogen balance was calculated over five ~24 h collections (day 1 being only ~12 h) to total 108 h (Fig. 1). Nitrogen intake was calculated from the protein

content of each dietary food item, divided by an appropriate amino acid-nitrogen, based on Australian Food Composition Tables (NUTTAB 2006). Measured nitrogen outputs were urinary urea (Thermo Electron Urea Assay, Thermo Fisher Scientific Inc., MA) and creatinine (Creatinine kit, Roche, New Zealand), with additional estimated nitrogen losses from sweat at rest and during exercise on days 2 and 6, and from faeces and other miscellaneous losses throughout the collection period based on the urine with sweat loss ratios reported previously (Tarnopolsky, MacDougall and Atkinson 1988). Full details of nitrogen intake estimates and nitrogen loss measurement is detailed in Supplementary Data 3.7 Nitrogen Balance Calculations.xlsx.

Metabolomic analysis of plasma and urine. Plasma samples were analysed using AbsoluteIDQ™ kits (Biocrates Life Sciences AG, Innsbruck, Austria) and liquid chromatography tandem mass spectrometry (LC-MS/MS). 20 µl samples were injected into the system (UltiMate 3000, Dionex AG, Olten, Switzerland) directed to the 3200 Q TRAP tandem mass spectrometer instrument equipped with a TurboIonSpray® ionization source (AB Sciex, Foster City, CA, USA). Met*IQ* software (Biocrates Life Sciences AG, Austria) was used to calculate metabolite concentrations and quality assessment. Urine samples were analysed using EZ:Faast ™ amino acid kits (Phenomenex Inc., Torrance, CA, USA) and GC-MS (Agilent 6890 GC coupled to Hewlett Packard 5975 MS; Agilent Technologies Schweiz AG, Basal, Switzerland). Samples were transferred into an insert within sample vials for analysis by gas chromatography (GC)-MS using GC conditions set to kit guidelines. An extended methodology including ions measured in GC-MS selective ion monitoring mode is described in Supplementary Data 3.8 Metabolomics Methods.docx. All samples were prepared and run in duplicate; %CV tolerance was set at 15%.

Sample size calculation. Sample size was generated based on sufficient power to declare the effect of treatment on performance was likely greater than the defined smallest worthwhile effect, when utilising inference by magnitude-based precision of estimation as described by Hopkins *et al.* (Hopkins, Marshall, Batterham and Hanin 2009). The smallest worthwhile effect of treatment on performance is between 0.3-0.7 times estimate for the coefficient of variation (CV) (Hopkins, Hawley and Burke 1999). The relationship between performance in our repeat-sprint test (CV=3.1%; (Rowlands et al. 2007) and competition performance has not been established, but the CV is within the range of other estimates for repeated-sprint tests and cycling competition (Paton and Hopkins 2006). Powered to detect 0.5 × CV yielded n=24; however, the magnitude of our anticipated effect was 4.1% (Rowlands et al. 2008) reducing n to 10. Two-thirds through data collection we added two further subjects to correct for a randomisation error in two cyclists to retain effective power within the crossover.

Statistical analysis. The effect of treatment on outcomes was estimated with mixed modelling (Proc Mixed, SAS Version 9.1, SAS Institute, Cary, NC). Most outcome variables were 100*log-transformed before modelling to reduce non-uniformity of error and to express outcomes as percentages (Hopkins, Marshall, Batterham and Hanin 2009), with the exception of data sets with negative values (nitrogen balance). Most outcomes and comparisons were generated from fixed effects models based on the interaction between the respective levels of treatment, test day, and order of treatment. For the analysis of sprint mean power, sprint number was a numeric effect (as in linear regression). Appropriate random effect models for each parameter included all or some of: between-athlete variation, additional treatment-associated variation, and additional variation associated with moving between test days.

Variability between blocks at baseline in blood measures was identified *a priori* as a potential confounder; value at baseline was hence included as a covariate within the appropriate model. All covariates were first normalised to and expressed as a proportion of the within-subject standard deviation for the covariate. In keeping with trends in inferential statistics, we utilised the magnitude-based approach to inferences as recently described (Rowlands et al. 2008, Hopkins, Marshall, Batterham and Hanin 2009). For performance, we used 0.93% (0.3 x 3.1%) as the threshold for small to align inferentially with our recent work (Rowlands et al. 2008, Thomson, Ali and Rowlands 2011); while for mechanistic outcome we used the standardised difference (effect size) (Rowlands et al. 2008, Hopkins, Marshall, Batterham and Hanin 2009).

RESULTS

Performance

The effect of LEUPRO on sprint mean power was trivial on day 4 (0.4% ± 1.0%; p=0.51) and day 6 (-0.3% ± 1.0%; p = 0.63) (Fig. 3.2); effects on fatigue (slope) on both day 4 (0.9% ± 5.4%; p=0.68) and day 6 (0.6% ± 3.4%; p=0.77) were inconclusive. The likelihoods that the observed mean effect of LEUPRO was substantially detrimental/trivial/beneficial were 1.4%/79.6%/19% on day 4 and 2.1%/83.4%/14.5% on day 6.

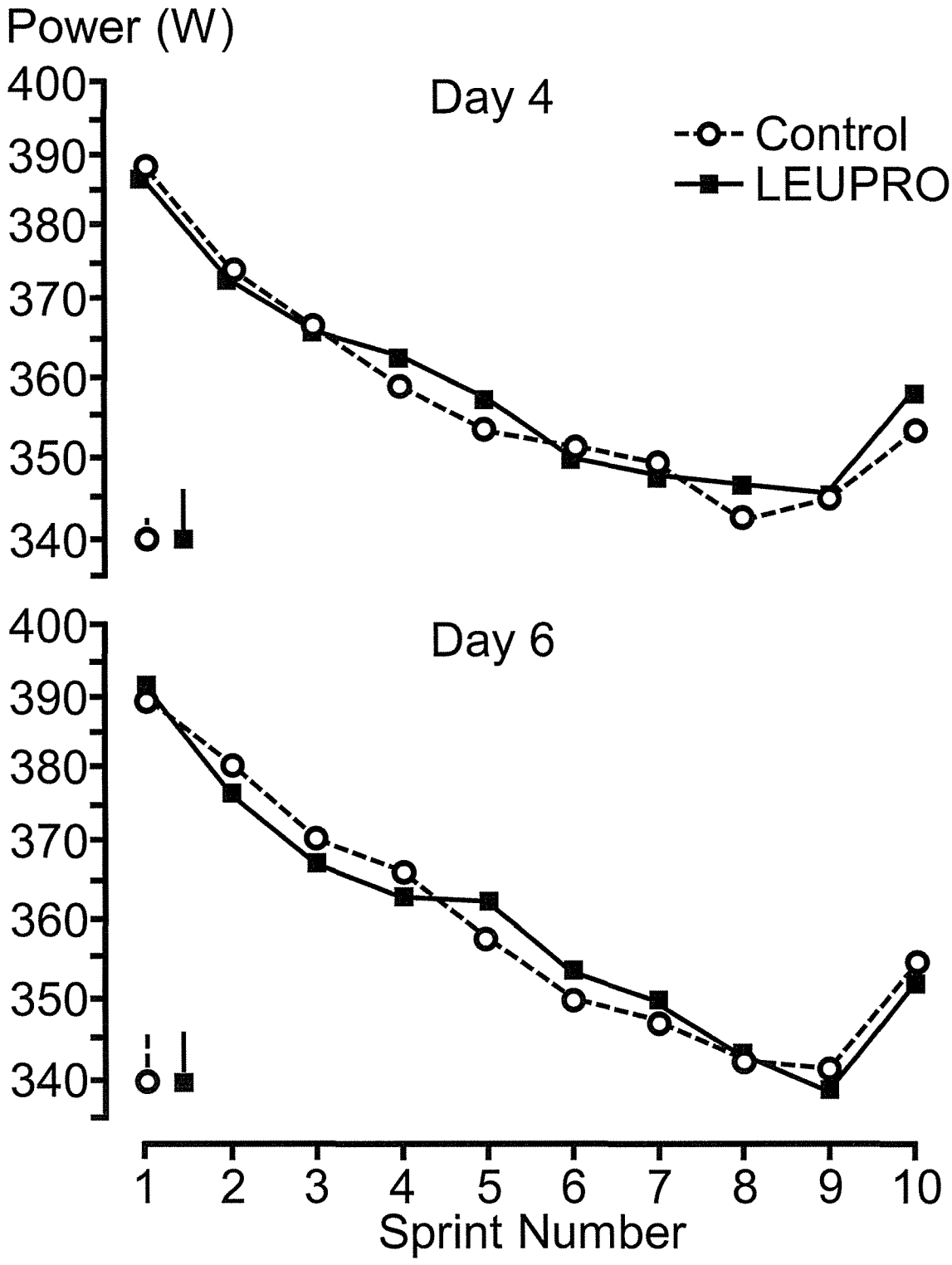

Figure 3.2. Sprint mean power during the performance tests on day 4 and day 6. Data are back log-transformed least-squares mean power outputs plotted on a log scale with composite

between-subject standard deviations (upper error bar represented on symbols at 340 W, sprint number 1) obtained from the analysis.

Whole-body leucine and glucose kinetics

During recovery on day 1 there were very-large increases in whole-body leucine flux, oxidation, and non-oxidative disposal with LEUPRO supplementation, relative to the control; while whole-body net leucine balance was positive with LEUPRO but negative for control (Table 3.2, Fig. 3.3). At rest on day 6, the effect of LEUPRO supplementation on leucine flux, oxidation, non-oxidative disposal, breakdown, and balance was trivial (Table 3.2). Day-6 exercise increased the rate of whole-body leucine oxidation 1.6-fold (90%CL x/÷1.1) and 1.9-fold (x/÷1.1) with LEUPRO and control, respectively, relative to rest. Whole-body leucine non-oxidative disposal was greater during day-6 exercise with LEUPRO (Table 3.2, Fig. 3.3). Breath ^{13}C and plasma α-KIC enrichments are shown in Fig. 3.4A, B.

At rest on day 6, there was a possible small increase in glucose R_a with LEUPRO of 3.1% (±3.6%; p=0.14) relative to control, but the increase in R_d of 1.7% (±6.2%; p=0.62) was trivial (R_a: LEUPRO 14.2 ± 1.0, control 13.8 ± 1.1 $\mu mol \cdot kg^{-1} \cdot min^{-1}$; R_d LEUPRO 14.2 ± 1.5, control 13.9 ± 1.1 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) (Fig 3.4C). During exercise on day 6, the effect of LEUPRO on glucose R_a was likely trivial (0.8% ±3.0%; p=0.63); outcomes were otherwise inconclusive (R_a: LEUPRO 16.9 ± 1.0, control 16.8 ± 1.1 $\mu mol \cdot kg^{-1} \cdot min^{-1}$; R_d LEUPRO 17.7 ± 9.0, control 17.0 ± 9.0 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) (Fig 3.4C).

Table 3.2. *Effect of protein-leucine relative to control supplementation on whole-body leucine kinetics during recovery from exercise on day 1, and during rest and exercise on day 6.*

Period	Parameter	Mean effect (fold)[a]; ×/÷90% CL[b]	Effect Size[c]; ±90% CL[b]	P-value	Inference[d]
Day 1 Recovery (60-180 min post-exercise)					
	Q	5.20; ×/÷1.10	14.6; ±5.1	6E-14	almost certain
	OX	5.62; ×/÷1.11	4.28; ±1.33	9E-16	almost certain
	B	0.76; ×/÷1.09	-0.77; ±0.44	26E-16	almost certain
	NOLD	4.79; ×/÷1.13	15.39; ±5.43	3E-12	almost certain
	BALANCE[a]	504; ±87	17.8; ±5.8	3E-9	almost certain
Day 6 Rest (Pre-exercise)					
	Q	1.02; ×/÷1.14	0.07; ±0.38	0.74	unclear
	OX	1.08; ×/÷1.12	0.19; ±0.27	0.20	possible
	B	1.02; ×/÷1.14	0.07; ±0.37	0.75	unclear
	NOLD	1.01; ×/÷1.16	0.04; ±0.43	0.88	unclear
	BALANCE[a]	-2.0; ±7.5	0.18; ±0.67	0.66	likely
Day 6 Exercise					
	Q	1.05; ×/÷1.11	0.28; ±0.57	0.37	unclear
	OX	0.90; ×/÷1.16	-0.38; ±0.58	0.25	possible
	B	1.06; ×/÷1.12	0.28; ±0.57	0.39	unclear
	NOLD	1.14; ×/÷1.13	0.50; ±0.48	67E-3	likely
	BALANCE[a]	2.7; ±5.6	-.18; ±0.38	0.42	unclear

[a]Data for Q, OX, B and NOLD are the fold-difference for the LEUPRO condition minus control. BALANCE is the absolute difference ($\mu mol \cdot kg^{-1} \cdot h^{-1}$).
[b]Multiply and divide this number by the mean effect to obtain the upper and lower confidence limits. For BALANCE, add and subtract.
[c]Effect size thresholds: <0.2 trivial; substantial outcomes; <0.6 small, <1.2 moderate, <2.0 large, <4.0 very large, >4.0 extremely large.
[d]Thresholds for assigning a qualitative inference to the likelihood of a substantial outcome: <1.0%, almost certainly not; <5.0%, very unlikely; <25%, unlikely; <75%, possible; >75%, likely; >95%, very likely; >99%, almost certain; an effect is unclear if its CI includes both substantial increases and decreases.
Abbreviations: *Q*, leucine flux; *OX*, leucine oxidation; *B*, leucine breakdown; *NOLD*, nonoxidative leucine disposal; *BALANCE*, whole-body net leucine balance.

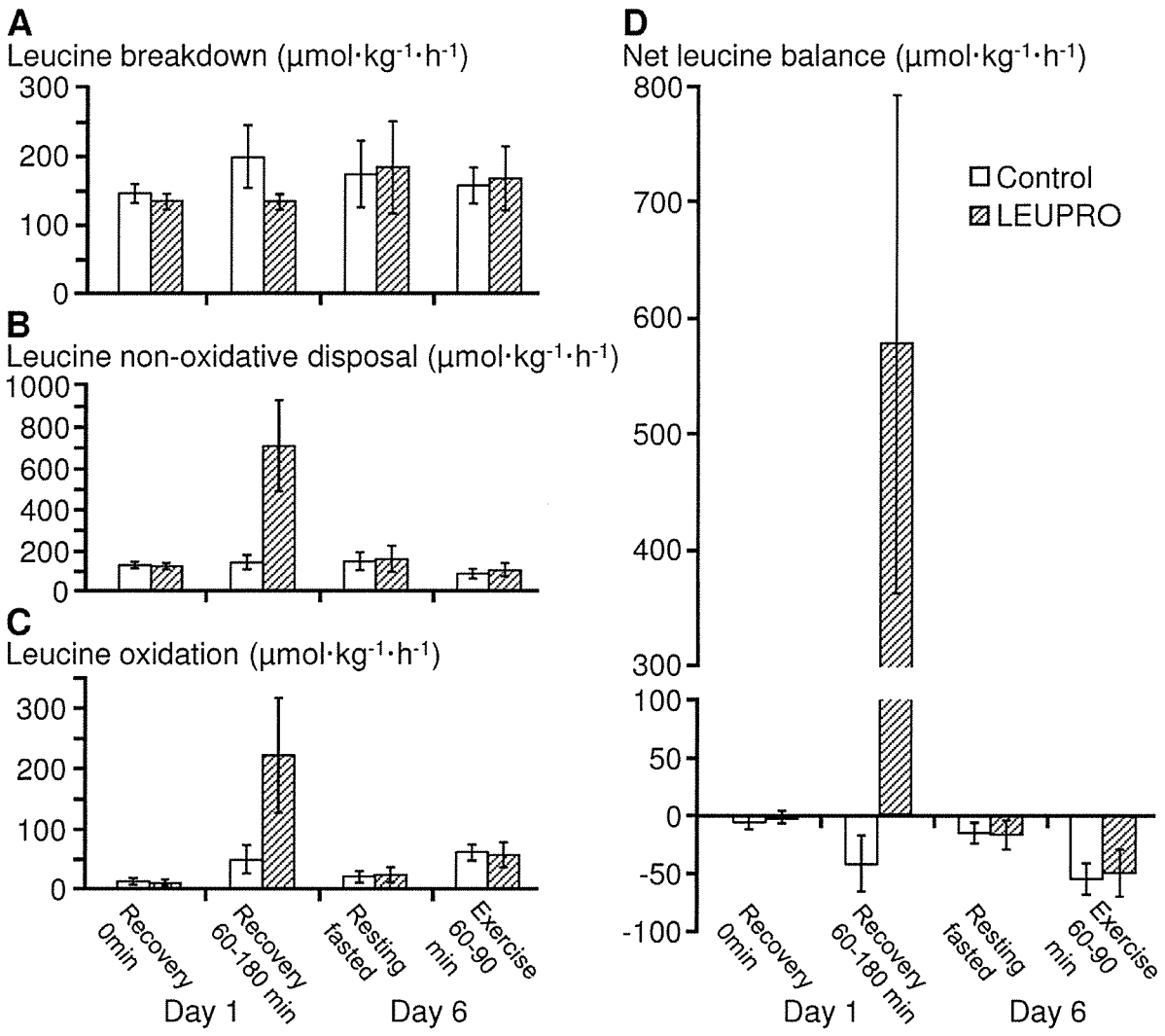

Figure 3.3. Whole-body leucine kinetics on day 1 and day 6. Whole-body leucine breakdown (**A**), non-oxidative disposal (**B**), oxidation (**C**), and balance (**D**) immediately post-exercise (recovery 0 min) and during recovery (60-180 min) on day 1, and at rest and during steady-state exercise on day 6. Data are means ± SD.

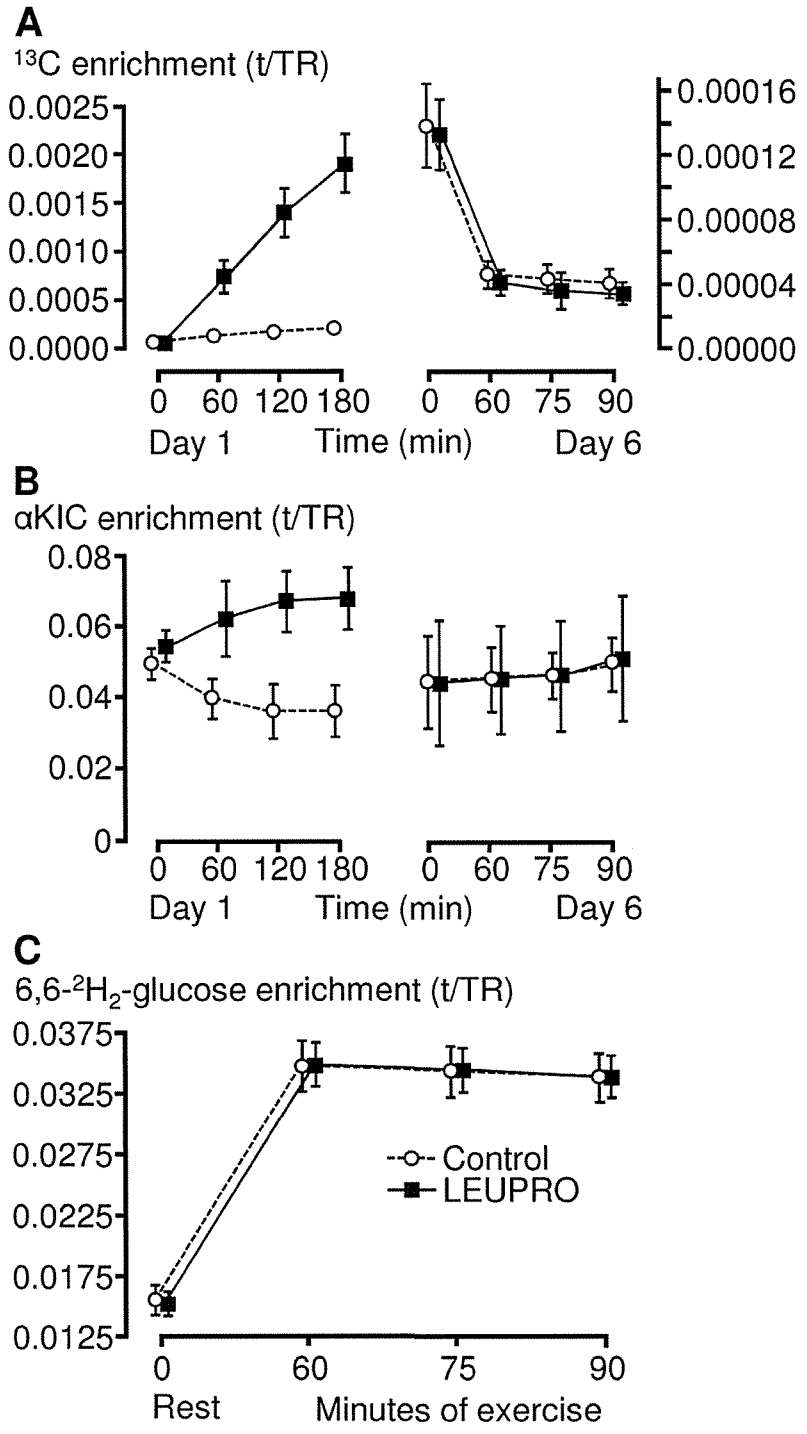

Figure 3.4. Isotopic enrichments on day 1 and day 6. Expired breath enrichment of $^{13}CO_2$ (**A**) and plasma enrichment of α-[^{13}C]-ketoisocaproate acid (α-KIC) (**B**) on day 1 immediately post-exercise (time 0 min) and during recovery (60-180 min) and on day 6 at rest (time 0 min) and at rest and during steady-state exercise on day 6; and enrichment of plasma [6-6-$^{2}H_2$] glucose (**C**) on day 6. Data are means ± SD.

Respiratory exchange ratio

There were no clear effects of treatment on the respiratory exchange ratio (range of means 0.86-0.90) during recovery from exercise on day 1, and at rest or during exercise on day 4 and day 6 (Table 3.3).

Table 3.3. *Respiratory exchange ratio during recovery from exercise on day 1 and during rest and exercise on days 4 and 6.*

	Mean RER		Outcome		
Period	LEUPRO	Control	Mean Effect; ±90%CL (%)	Effect Size; ±90% CL	Inference
			Day 1		
Recovery	0.88	0.88	-0.01; ±0.02	-0.20; ±0.58	Unclear
			Day 4		
Rest	0.90	0.90	0.00; ±0.04	0.03; ±0.79	Unclear
Exercise	0.87	0.86	0.02; ±0.05	0.73; ±1.44	Unclear
			Day 6		
Rest	0.84	0.86	-0.02; ±0.05	-0.31; ±0.86	Unclear
Exercise	0.87	0.86	0.01; ±0.05	0.34; ±1.43	Unclear

[a]RER, respiratory exchange ratio.

Nitrogen balance

Nitrogen balance was positive on all of the five 24-h collection periods with the LEUPRO supplement; with control, nitrogen balance was negative from the completion of exercise on day 1 through to the morning of day 2 (Fig. 3.5A). The net difference in nitrogen balance between LEUPRO and control on day 1 was large (ES 2.4; 90%CL ±0.60; p=8E-5) (Fig 3.5B); differences in 24-h nitrogen balance between LEUPRO and the control were otherwise inconclusive on days 2 and 3, and almost certainly trivial on days 4 and 5 (Fig. 3.5B). Over the entire 6-day block (days 1-5), there was large net nitrogen gain with LEUPRO (ES 0.95

±0.48; p=4E-03) but no clear gain with control (ES 0.15 ±0.52; p=0.61), with a very-large positive treatment differential (ES 2.8 ±1.6; p=2E-12); however, when day 1 nitrogen balance was excluded (i.e. over days 2-5) there was a large net nitrogen gain for both LEUPRO (ES 1.03 ±0.56; p=7E-03) and control (ES 0.90 ±0.52; p=0.01) and the treatment differential was unclear (ES 0.15 ±0.70; p=0.73) (Fig. 3.5B).

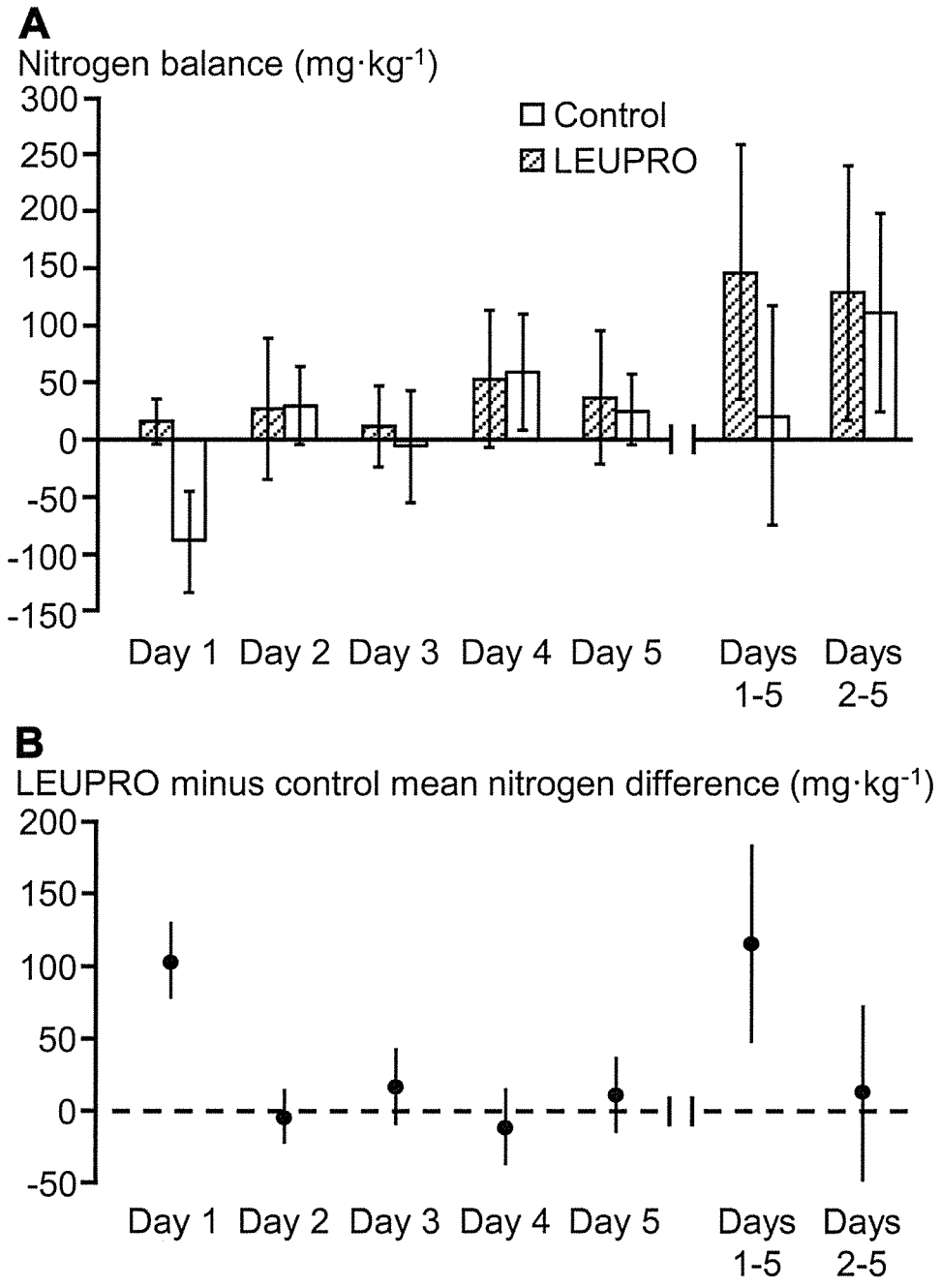

Figure 3.5. Effect of protein-leucine and control supplementation on nitrogen balance. Shown are mean ± SD daily nitrogen balance for collection periods on days 1 (~12 h) and days 2, 3, 4 and 5 (all 24 h), and total nitrogen balance over days 1-5 and days 2-5 (**A**), and the difference between treatments by collection period with the 90% confidence interval (**B**).

Plasma concentrations of glucose and insulin

Overall mean plasma glucose and insulin concentrations during the 3-h recovery period following exercise on day 1 in the control were 6.9 ± 1.6 mmol·L^{-1} and 89 ± 118 μIU·mL^{-1}; the LEUPRO supplement led to a 12% reduction (±7%; ES -0.80 ±0.51; p=7E-3) and a possible increase of 17% (±34%; 19 ±34; p=0.23), respectively.

Metabolomics

The effect of supplementation on the concentrations of urinary and plasma metabolites during 3-h of recovery on day 1 is shown in Fig. 3.6; nomenclature and the full listing of mean effect sizes and other statistics for the treatment differential can be found in Supplementary Data 3.9 Metabolomics Outcomes.xlsx. On day 1 in the control, the total plasma amino acid concentration declined with time from the start of recovery (Fig. 3.6A). However, LEUPRO supplementation led to an extremely large increase in plasma leucine (3.5-fold; 90%CL x/÷1.1) and very large increase in essential (2.2-fold x/÷1.1), and total amino acid (1.7-fold x/÷1.1) concentrations, relative to the control (Fig. 3.6A, B). LEUPRO supplementation also resulted in small, moderate, and large increases in the plasma concentration of acylcarnitines related to BCAA metabolism, C4 (1.2-fold x/÷1.2), C3 (1.5-fold x/÷1.1), and C5 (3.0-fold x/÷1.1), respectively. During exercise on day 6, LEUPRO caused small increases in plasma acylcarnitines C3 (1.1-fold x/÷1.1) and C5-M-DC (1.2-fold x/÷1.3) and a moderate increase in C2 (1.3-fold x/÷1.3), but a small decrease in C16 (0.92-fold x/÷1.2), relative to the control. The effect of LEUPRO supplementation on day 6 plasma amino acid concentrations and other plasma acylcarnitines, glycerophospholipids and sphingolipids were mostly trivial or inconclusive (Supplementary Data 3.9).

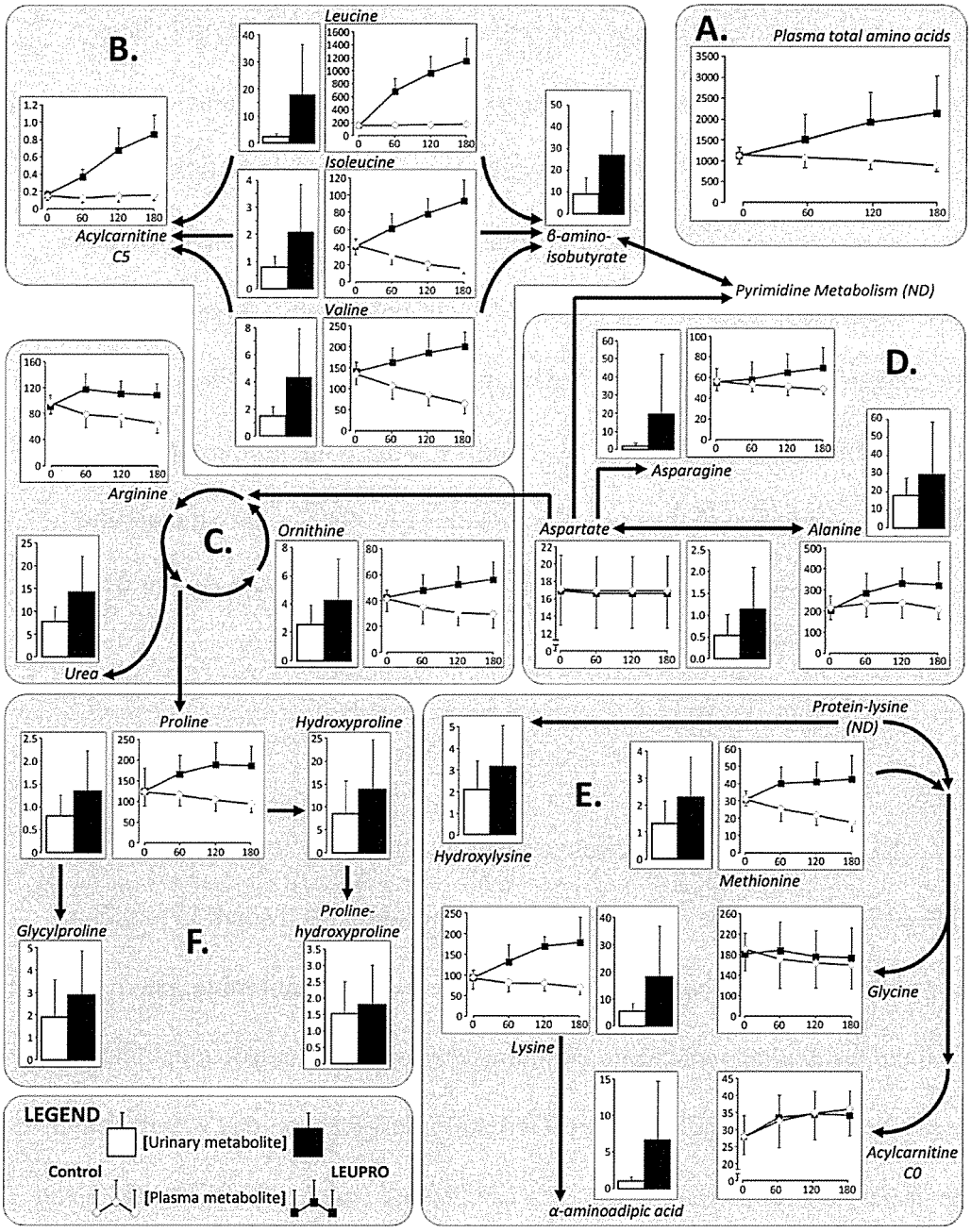

Figure 3.6. Plasma and urinary metabolite concentrations during recovery from exercise on day 1. Plasma essential and total amino acid concentration (**A**), and plasma and urinary concentrations of substrates and metabolites relating to the branch-chain amino acids (**B**), the urea cycle (**C**), the metabolism of alanine and aspartate (**D**), the degradation of lysine (**E**) or the metabolism of arginine and proline (**F**) during recovery on day 1. Data are means ± SD. Concentrations of plasma metabolites are in $\mu mol \cdot L^{-1}$ and urinary metabolites in $nmol \cdot L^{-1}$. ND, not determined.

LEUPRO ingestion during recovery on day 1 also resulted in small to extremely large increases in the urinary concentrations of products of the metabolism of branch-chain amino acids, alanine and aspartate, arginine and proline, lysine, and urea cycle metabolites (Fig. 3.6B-F), as well as cysteine and methionine, phenylalanine and tyrosine, and tryptophan, relative to control ingestion (Supplementary Data 3.9). During exercise on day 2 there were likely moderate reductions in proline (-18% ±18%), methionine (-19% ±19%), isoleucine (-20% ±18%) and α-aminobutyric acid (-17% ±17%) and a large reduction in asparagine (-19% ±19%) with LEUPRO, compared to the control; outcomes for other urinary metabolites during day 2 exercise, and on day 3, day 4 and day 5 were mostly inconclusive or trivial (Supplementary Data 3.9).

At rest on day 6, LEUPRO produced moderate reductions in urinary leucine (-26% ±19%), isoleucine (-23% ±17%) and valine (-36% ±22%) concentrations, and small to moderate reductions in products of the metabolism of proline, cysteine and methionine, phenylalanine and tyrosine, and the tryptophan metabolite kynurenate, relative to control supplementation (Supplementary Data 3.9). This effect was reversed during exercise on day 6, with small increases in the urinary excretion of leucine (27% ±30%) and isoleucine (26% ±26%) and small to moderate increases in methionine, phenylalanine and tyrosine, proline, tryptophan, and products of their metabolism (Supplementary Data 3.9).

Creatine kinase

Prior to and following exercise, mean ± SD plasma creatine-kinase concentration ($U \cdot L^{-1}$) in the control condition was respectively: day 4, 170 ± 88, 210 ± 94; day 6, 145 ± 63, 195 ± 72.

Relative to the control values, LEUPRO led to small-moderate reductions prior to (24-25% ±14%; ES -0.66 to -0.70 ±0.33; p=4E-3 to 7E-3) and following (21-22% ±14%; -0.59 to -0.61 ±0.33; p=0.01 to 0.02) exercise on days 4 and 6.

DISCUSSION

Post-exercise supplementation with LEUPRO increased the rate of whole-body non-oxidative leucine disposal and resulted in positive leucine and nitrogen balance during the immediate hours following intense endurance exercise. The supplement also attenuated the increase in creatine kinase during the 6-day cycling block, suggesting lower tissue disruption. These outcomes, however, were associated with trivial impact on subsequent high-intensity cycling performance, which might have been influenced by positive mean daily nitrogen balance. Furthermore, LEUPRO ingestion during recovery increased the concentration of plasma and urinary metabolic intermediates of BCAA degradation, indicative of protein and leucine intake that exceeded the whole-body capacity to metabolise BCAA.

Association between protein-leucine feeding, nitrogen balance, and performance

We reported previously that high protein-carbohydrate or protein-leucine and carbohydrate post-exercise feeding can improve subsequent repeated-sprint performance (Rowlands et al. 2008, Thomson, Ali and Rowlands 2011). Therefore, the likely trivial effect of LEUPRO on performance was somewhat unexpected. Our recent work indicated that the observed enhancements to repeated-sprint mean power were small (2.5%; 99%CL: ±2.6% (Thomson, Ali and Rowlands 2011)) to moderate (4.1%; 95%CL: ±4.1% (Rowlands et al. 2008)), with uncertainty allowing for very large to trivial performance outcomes. Given the overlap in

confidence intervals across all three investigations in this series of work, it is possible that sampling variation could account for mean differences in subsequent performance between studies. On the other hand, several design and outcome related factors could also explain the unremarkable performance effect, and yield new insight into the physiological responses resulting from post-exercise ingestion of protein-leucine-carbohydrate that could impact recovery processes and subsequent performance.

Firstly, post-exercise high protein feeding during a period of intense endurance training might not substantially benefit subsequent performance (relative to nil or low protein isocaloric control) if nitrogen balance is positive. Rowlands et al. (Rowlands et al. 2008) reported that low-protein high-carbohydrate feeding in the 4-h following exercise totalling 0.92 g protein·kg^{-1}·d^{-1} (including controlled diet at other times) was insufficient to meet nitrogen requirements when compared to high protein-carbohydrate feeding that provided 2.97 g protein·kg^{-1}·d^{-1}. To our knowledge, this was the first study to suggest that nitrogen balance status might be linked to performance. However, from the design (in which daily protein intake was not balanced) it was not possible to establish if improved performance was the result of positive daily nitrogen balance in the intervention versus negative in control, or to the post-exercise protein feeding. In the following study that provided further insight into the possible nitrogen balance-performance association, Thomson et al. (Thomson, Ali and Rowlands 2011) clamped dietary protein intake at 1.6 g·kg^{-1}·d^{-1} during the experimental block but observed in both protein-leucine and control post-exercise feeding conditions a mild average nitrogen deficiency, equivalent to 0.12 and 0.14 g protein·kg^{-1}·d^{-1}, respectively. This latter study showed that during a 5-day block of intense training, protein-leucine supplemental feeding over 1.5 h immediately post-exercise can benefit subsequent

performance when nitrogen balance is mildly negative. To our surprise, in the current study overall nitrogen balance was positive in both conditions over days 2-5 of the 6-day protocol (Fig. 3.5). Positive nitrogen balance may be assumed to represent net protein gain, which would likely reflect changes in metabolic processes, such as, an increased postprandial protein synthesis rate or reduced protein breakdown (Forslund et al. 1999). Therefore, sufficient tissue anabolism may have occurred in the control condition to have negated the relative positive protein synthetic and anti-catabolic effects of protein-leucine feeding. Taken together, our collective data suggest that a net relative negative nitrogen balance might be required for post-exercise high protein-carbohydrate or protein-leucine and carbohydrate feeding to benefit subsequent performance. Nevertheless, post-exercise protein-leucine ingestion still lowered plasma creatine-kinase concentrations, which suggests attenuation of membrane disruption may be insensitive to nitrogen balance.

Secondly, if protein intake during the experimental period differed considerably from the habitual intake, a period of adaptation would be required that could have impacted on nitrogen balance (Quevedo et al. 1994) and may have contributed to differences in nitrogen balance between the current study and that of Thomson et al. (Thomson, Ali and Rowlands 2011). Although the protein composition of the lead-in diet was not determined in the current study, substantially positive nitrogen balance in the control condition over days 2-5 could indicate that protein intake was greater with the experimental diet than with the habitual diet. Pre-study diet diaries collected by Thomson et al. (Thomson, Ali and Rowlands 2011) estimate mean habitual protein intake of 2.0 $g \cdot kg^{-1} \cdot d^{-1}$ by their cohort, which might explain their findings of negative nitrogen balance. Given that our cohort was extremely similar (and, therefore, likely consume similar diets), an alternative explanation to account for our findings

of positive nitrogen balance could be that the experimental diet in the current study provided a greater quantity of non-vegetable based proteins, and a more even distribution of protein across the daily diet. Nitrogen retention from animal-derived proteins appears to be superior (Fouillet et al. 2009) and consuming the majority of dietary protein at the opposite end of the day to exercise (Thomson et al. (Thomson, Ali and Rowlands 2011)) would limit the impact of dietary protein on post-exercise muscle protein synthesis versus dietary protein consumed evenly across the day, as in the current study. Further research intervening on chronic nitrogen balance with control of other covariates is warranted to explore the hypothesis that nitrogen balance has an important role in determining the efficacy of post-exercise high protein-carbohydrate feeding on skeletal muscle protein metabolism, recovery processes, and endurance performance.

Effects of protein-leucine feeding on leucine turnover during recovery

It has been suggested that the primary outcome of protein ingestion for endurance athletes should be to replace exercise associated oxidative protein losses and support amino acid requiring metabolic processes, especially an increased rate of post-exercise protein synthesis (Phillips, Moore and Tang 2007). Net muscle protein balance and fractional muscle protein synthesis following exercise is associated with extracellular essential amino acid concentration (Borsheim, Tipton, Wolf and Wolfe 2002, Moore et al. 2009). Without protein ingestion (control supplementation) during recovery, the plasma essential, leucine, and total amino acid concentration decreased with time, while whole-body leucine oxidation was increased, indicating that amino acid availability for metabolic processes could have become limiting. However, LEUPRO ingestion increased recovery plasma amino acid concentrations and established net positive whole-body leucine balance (Fig. 3.3). Furthermore, the

increased plasma amino acid availability would most likely have been sufficient to offset the elevated rate of whole-body protein oxidative losses and support the increased tissue protein synthesis rate.

Based on the large increases in post-exercise whole-body leucine kinetics with LEUPRO supplementation, and the findings of Howarth et al. (Howarth, Moreau, Phillips and Gibala 2009) and others (Tipton et al. 1999, Miller et al. 2003, Koopman et al. 2005), it is likely that LEUPRO ingestion increased the post-exercise muscle-protein synthesis rate in the immediate few hours of recovery, relative to the control. However, we acknowledge that whole-body protein kinetics might not necessarily reflect changes in skeletal muscle protein turnover (Koopman et al. 2005, Howarth, Moreau, Phillips and Gibala 2009) and that expansion of the intracellular α-KIC pool could explain some of the changes in leucine kinetics. Furthermore, we note that there is some discrepancy in whole-body leucine turnover findings among similar post endurance exercise protein-carbohydrate feeding studies in trained men utilising the 1-[^{13}C]-leucine infusion method. Levenhagen et al. (2002) reported only a 15% increase in non-oxidative leucine disposal with protein-carbohydrate feeding (8/5/3 g protein/carbohydrate/fat) following 1 h of cycling at 60% of VO_2max, relative to protein-free and placebo conditions. The different magnitude of the effect relative to the current study (~380% increase in non-oxidative leucine disposal) is most likely because Levenhagen et al. used only 8 g of protein, which Moore et al. (2009) has shown would not saturate mixed-muscle FSR post resistance exercise. Comparably, the recent investigation by Howarth et al. (Howarth, Moreau, Phillips and Gibala 2009) is more like the present in terms of exercise undertaken and post-exercise nutrition. The authors reported that the addition of protein to carbohydrate led to increased post-exercise leucine oxidation (4-fold versus 5.6-

fold in the current study) and positive whole-body net leucine balance, but most importantly that mixed-muscle FSR was increased by 50% (Howarth, Moreau, Phillips and Gibala 2009). However, non-oxidative leucine disposal was not significantly different (Howarth, Moreau, Phillips and Gibala 2009). It might be that the added leucine in the LEUPRO condition could explain our present observation of increased non-oxidative leucine disposal. Leucine potently stimulates tissue protein synthesis in the rodent model (Crozier et al. 2005), and when added to protein and carbohydrate ingested following resistance exercise in men led to a small increase in already positive whole-body net protein balance and moderate-sized increase in the rate of mixed-muscle FSR (Koopman et al. 2005) (see Supplementary Data 3.10 Koopman 2005 ES and Inferences.xlsx). Nevertheless, as we have no comparison condition, we cannot differentiate the effect of leucine (or protein) alone. Overall, it is reasonable to suggest that LEUPRO ingestion increased the muscle-protein synthesis rate in the hours following exercise, relative to the control (Koopman et al. 2005, Koopman et al. 2009). However, this did not translate to a functional (performance) improvement during the 6-day block of intense cycling suggesting that an increased muscle protein synthesis rate in the immediate hours post-exercise is, by itself, not an important mechanism governing protein nutrition-related improvement in short-term (45-60 h) subsequent performance.

Amino acid metabolomics

Not surprisingly, LEUPRO supplementation resulted in large increases in the plasma and urinary concentrations of amino acids and products of their metabolism during recovery from exercise on day 1. Elevated plasma BCAA concentrations and leucine turnover with LEUPRO supplementation would almost certainly explain the increases in plasma acylcarnitines C3, C3:1, C4, C5 and urinary β-aminoisobutanoate on day 1. Conversely, the

small increase in plasma acylcarnitine C3 during exercise on day 6 might instead be the result of a decline in the concentration of plasma palmitoylcarnitine, an inhibitor of the biosynthesis of acylcarnitine C3 (Bohmer 1968), or reduced metabolism to the C3 derivatives C3-OH and C3:1. Associated with the LEUPRO condition were reduced urinary losses of BCAAs, proline, methionine and β-aminoisobutyrate (a product of both valine and pyrimidine metabolism) during exercise on day 2, suggesting reduced turnover or retention of these amino acids and metabolites. The more widespread alterations to the concentrations of plasma and urinary amino acids and their metabolites at rest and during exercise on day 6 (Supplementary Digital Content 2) could be the result of adaptive processes in response to higher intakes of leucine and protein associated with LEUPRO ingestion.

Recently, a mean upper metabolic limit to oxidize leucine in men (ages 18-35) at rest was estimated at 556 $mg \cdot kg^{-1} \cdot d^{-1}$ (Elango et al. 2010). In comparison, LEUPRO supplementation during recovery provided leucine (free and protein-bound) at a rate of ~130 $mg \cdot kg^{-1} \cdot h^{-1}$ for 3 h. Increased concentrations of plasma acylcarnitines C3 and C5 indicates that leucine ingestion of ~130 $mg \cdot kg^{-1} \cdot h^{-1}$ exceed the capacity of some enzymes involved in the metabolism of leucine following an acute bout of endurance exercise. Accumulation of amino acids and their secondary metabolites in the plasma and urine may indicate an upper tolerable limit of intake, above which might increase toxicity risk (Pencharz, Elango and Ball 2008). However, we hypothesize that the increased whole-body leucine oxidation rate following endurance exercise might act to increase the upper tolerable limit and, therefore, post-exercise supplementation with leucine (and protein) may be better tolerated than in resting individuals; adaptive alterations of amino acid metabolism to chronic high intakes could also alter leucine tolerance. Given recent interest in the role of post-exercise protein ingestion to

elevate and saturate post-exercise whole-body and muscle-protein synthesis (Howarth, Moreau, Phillips and Gibala 2009, Moore et al. 2009) further work is needed to investigate metabolic responses to protein and leucine ingestion following exercise, or with chronic feeding. This work should include sensitive and high-throughput mass spectrometry-based metabolomics to elucidate subtle but possibly large-scale perturbations to metabolism, which could represent important mechanisms of recovery or adaptation to exercise and protein feeding.

Conclusion

Ingesting a protein-leucine supplement following high-intensity cycling resulted in a positive whole-body net leucine balance, reduced plasma creatine kinase, and led to the accumulation of plasma and urinary amino acids and their metabolites, during recovery from exercise. However, the supplement provided no clear benefit to subsequent performance, which contrasts to previous findings by our group. We hypothesize that the likely trivial performance differential between the protein-leucine and control conditions was primarily the result of positive nitrogen balance over the experimental period ameliorating the benefit of post-exercise protein-leucine feeding reported recently (Thomson, Ali and Rowlands 2011).

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CHAPTER 4 STUDY 1B

MANUSCRIPT DRAFT

Effect of post-exercise leucine-enriched protein feeding on neutrophil function, immunomodulatory plasma metabolites and cortisol during a 6-day block of intense cycling

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ABSTRACT

Purpose. We determined the effects of post-exercise leucine-enriched protein supplementation (including carbohydrate-lipid) on neutrophil function and immunological regulators during a period of intense cycling. **Methods.** In a double-blind randomized crossover, 12 male cyclists ingested leucine/protein/carbohydrate/fat (LEUPRO: 7.5/20/89/22 $g \cdot h^{-1}$, respectively) or isocaloric carbohydrate/fat control (CON: 119/22 $g \cdot h^{-1}$) beverages for 1-3 h post-exercise during 6-days of high-intensity training. Blood was taken pre- and post-exercise on days 1, 2, 4 and 6 for phorbol myristate acetate (PMA)-stimulated neutrophil superoxide (O_2^-) production, immune cell-counts, amino acid and lipid metabolism (metabolomics), hormones, and cytokines (immunoglobulin A, interleukin-6, interleukin-10). **Results.** During recovery on day-1 LEUPRO ingestion increased mean concentrations of plasma amino acids (glycine, arginine, glutamine, leucine) and fatty-acid acylcarnitines (C14, C14:1-OH) with neutrophil priming capacity, and reduced neutrophil O_2- production (15-17 $mmol \cdot O_2^- \cdot cell^{-1}$ ±90% confidence limits 20 $mmol \cdot O_2^- \cdot cell^{-1}$). On day-2, LEUPRO increased pre-exercise plasma volume (6.6% ±3.8%) but haematological effects were otherwise trivial or unclear. LEUPRO supplementation did not substantially alter neutrophil elastase, insulin, testosterone, or cytokine concentrations. By day 6, however, LEUPRO reduced pre-exercise cortisol 21% (±15%) and acylcarnitine C16 during exercise, but increased post-exercise neutrophil O_2^- production (33 ±20 $mmol \cdot O_2^- \cdot cell^{-1}$), relative to control. **Conclusion.** Altered plasma amino acid and acylcarnitine concentrations with protein-leucine feeding might partly explain the acute post-exercise reduction in neutrophil function and increased exercise-stimulated neutrophil oxidative burst on day-6, which could impact neutrophil-dependent processes during recovery from intense training.

INTRODUCTION

Prolonged and intense endurance exercise attenuates some functions of innate immune cells, possibly increasing the risk of symptoms of infection (Walsh et al. 2011). For example, in neutrophils the oxidative burst response declines following intense endurance exercise, and prolonged endurance training can further suppress microbicidal activity (Hack, Strobel, Weiss and Weicker 1994). Neutrophils are also among the first cells recruited to exercised skeletal muscle, where they regulate tissue repair by phagocytosis of exercise-damaged cells and cellular debris, and modulate the function of initial macrophage infiltrators (Tidball and Villalta 2010). Elite athletes often undergo periods of intense training and/or tour-style competitions, which has been shown to suppress immune function (Pyne et al. 1995).

Ingestion of carbohydrate during and following endurance exercise can attenuate the exercise-induced impairment of immune cell function, partly through a reduction in the release of immunosuppressive stress hormones (e.g. cortisol; (Scharhag et al. 2006)) and contraction-induced cytokine transcription (e.g. IL-6; (Keller et al. 2001, Febbraio et al. 2003)). Endurance athletes are also recommended to ingest protein post-exercise to stimulate muscle repair via muscle protein synthesis (Tipton et al. 2004). Furthermore, branch-chain amino acids (BCAA) and protein supplementation might also alter the post-exercise immune response (Costa et al. 2009, Matsumoto et al. 2009, Costa, Walters, Bilzon and Walsh 2011). The BCAA leucine potently activates translational regulators downstream of the mammalian target of rapamycin (mTOR) (Anthony et al. 2000) a central regulator of diverse cellular responses to environmental stimuli. However, beyond activation of muscle protein synthesis, mTOR modulates T cell differentiation and function in response to environmental cues and cellular metabolic demands (Powell and Delgoffe 2010) and could be a mechanism by which BCAAs exert immunomodulatory effects in inflammatory cells. Other amino acids may also

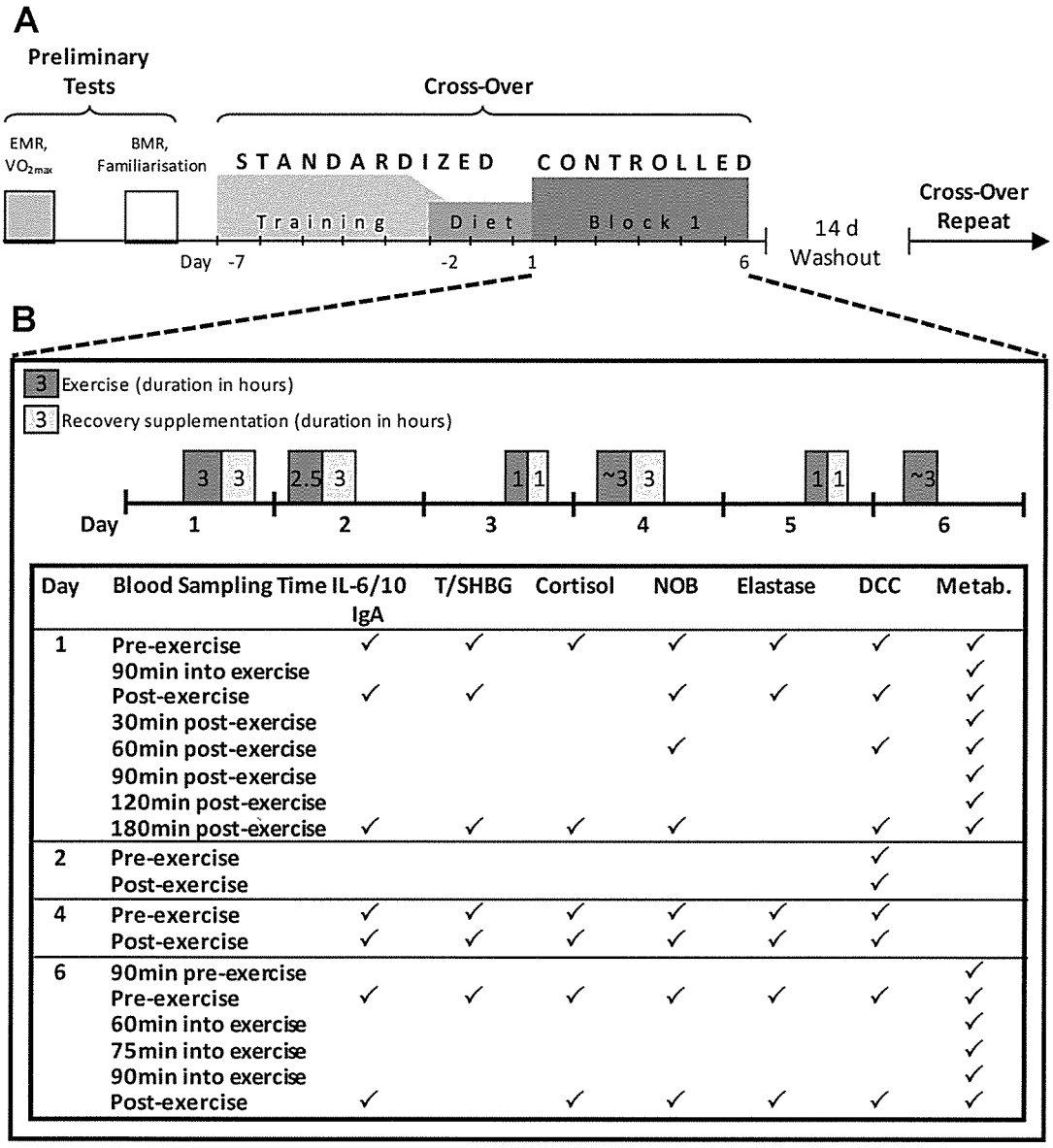
play a role. For example, cellular glutamine flux determines the rate of essential amino acid (EAA) entry into the cell, thereby regulating mTOR activity (Nicklin et al. 2009). Additionally, glutamine can be metabolised into arginine, which modulates neutrophil respiratory burst (Moinard et al. 2002) and bioactive whey-protein peptide digests appear to exert positive effects on immune cell autophagy and survival, and neutrophil function (Rusu et al. 2009, Rusu et al. 2010). Thus, adding protein/amino acids to carbohydrate supplementation might also support immune function following hard endurance exercise.

Accordingly, we determined the effects of post-exercise leucine-enriched whey-protein ingestion relative to an isocaloric carbohydrate-fat control on neutrophil function, immune-cell concentrations and selected hormonal and cytokine parameters during 6-days of extremely intense cycling training leading to an anticipated immunosuppression. An area of current interest in exercise nutrition and metabolism research is the use of sensitive, high-throughput 'omics approaches (Walsh et al. 2011). Consequently, we utilized a metabolomics approach to study changes in metabolites with immunomodulatory functions in response to post-exercise protein-leucine ingestion.

METHODS

The current study is the second unique dataset presented from the intervention trial detailed elsewhere (Nelson et al. 2012) with important aspects of exercise and nutritional control briefly summarised here. Twelve well-trained male cyclists or triathletes (mean ± SD: age, 35 ± 10 y; height, 182 ± 5 cm; body mass, 76.9 ± 6.5 kg; VO_2max, 64.8 ± 6.8 $mL \cdot kg^{-1} \cdot min^{-1}$; training history, 9 ± 4 y; recent weekly training volume 10 ± 1 h) completed a randomized, double-blinded, crossover to determine the effect of a leucine, protein-carbohydrate-fat (LEUPRO) post-exercise feeding intervention compared with an isocaloric carbohydrate-fat

control (CON) on neutrophil cell and other immune functions and plasma volume responses during a 6-day block of controlled high-intensity training and diet (Fig. 4.1). All participants were informed of the purpose of the study and associated risks, and provided written informed consent. The study was approved by the Central Regional Ethics Committee of New Zealand.



Day	Blood Sampling Time	IL-6/10 IgA	T/SHBG	Cortisol	NOB	Elastase	DCC	Metab.
1	Pre-exercise	✓	✓	✓	✓	✓	✓	✓
	90min into exercise							✓
	Post-exercise	✓	✓		✓	✓	✓	✓
	30min post-exercise							✓
	60min post-exercise				✓		✓	✓
	90min post-exercise							✓
	120min post-exercise							✓
	180min post-exercise	✓	✓	✓	✓		✓	✓
2	Pre-exercise						✓	
	Post-exercise						✓	
4	Pre-exercise	✓	✓	✓	✓	✓	✓	
	Post-exercise	✓	✓	✓	✓	✓	✓	
6	90min pre-exercise							✓
	Pre-exercise	✓	✓	✓	✓	✓	✓	✓
	60min into exercise							✓
	75min into exercise							✓
	90min into exercise							✓
	Post-exercise	✓		✓	✓	✓	✓	✓

Figure 4.1. Experimental design. Shown are preliminary visits and standardized training and diet leading in to a 6-day experimental block (A), with one experimental block, including

high-intensity cycling, recovery supplementation and blood sampling periods, further outlined (B). Abbreviations for sampled blood parameters are; IL-6/10, interleukin-6 and interleukin-10; T/SHBG, testosterone/sex-hormone binding globulin; NOB, neutrophil oxidative burst; DCC, differential cell count; Metab., metabolomics.

Preliminary testing. Cyclists underwent exercise metabolic rate (EMR), VO_2max and Wmax tests on an electromagnetically-braked cycle ergometer two weeks prior to the first experimental block; one week later, cyclists reported to the laboratory (06:00 h) in fasted condition for calculation of basal metabolic rate (BMR) via indirect calorimetry (Nelson et al. 2012). During all laboratory visits, environmental conditions were 19.3 ± 1.4°C and 42 ± 7% relative humidity.

Experimental protocols

Exercise procedures. The riding schedule was a multi-day model designed to simulate the physical stress of high-intensity training or competition. Each block comprised four intermittent high-intensity rides, with workloads pre-programmed at fixed percentages of individual Wmax. Day 1 (3 h cycling) consisted of a warm up (15/10/6 min at 30%/40%/50% Wmax, respectively) and loading intervals (3 blocks of 10 x 2 min intervals at 90%/80%/70% with 2 min between intervals and 6 min between blocks at 50%) and a cool down (11 min at 30%). Day 2 (2.5 h cycling) comprised a warm up (10/10/5 min at 30%/40%/50%, respectively), two blocks of longer loading intervals (4 x 5 min at 70% interspersed with 3 x 5 min at 50% Wmax; and 3 x 4 min at 70% interspersed with 3 x 4 min at 50%) separated by 6 x 1 min intervals (3 at 90% and 3 at 80%, with 2 min at 50% between each). Days 4 and 6

(~3 h) comprised 90 min at 50% Wmax followed by the repeated-sprint performance test as described by Rowlands et al. (Rowlands et al. 2007, Rowlands et al. 2008). Light rides on days 3 and 5 comprised 60 min at 30% Wmax. All rides were conducted at the same time of day for a given participant: between 14:00 to 18:00 h for days 1, 3, and 5 and 05:00 to 09:00 h for days 2, 4, and 6.

Energy expenditure and dietary control. Total daily energy expenditure was estimated from RMR, EMR (estimated from the regression of the sub-maximal EMRs versus workload samples obtained during preliminary testing) and daily activity energy expenditure; participants prospectively diary-logged all activities during the experimental block, which were then converted to metabolic equivalents (Nelson et al. 2012). All food was provided during the 6-day experimental blocks. The diet provided ≥8 g carbohydrate·kg^{-1}·d^{-1} on days 1, 2, 4 for both conditions with daily energy intake designed to balance estimated energy expenditure and dietary protein intake was clamped (control, 1.5 g·kg^{-1}·d^{-}1; LEUPRO, 1.9 g·kg^{-1}·d^{-1}) with the protein difference provided in the post-exercise intervention; cyclists ingested a 7.5% carbohydrate sports drink with 1.17 g·L^{-1} NaCl and lime juice, provided every 30 min during exercise (every 15 min during performance tests) at a rate of 790 ± 82 mL·water·h^{-1} (Nelson et al. 2012).

Blood collection. Blood was taken via 20-gauge catheter positioned in the antecubital vein on days 1 and 6 or venipuncture on days 2 and 4. For interleukin-6 (IL-6) and interleukin-10 (IL-10), blood was collected into chilled EDTA vacutainers (Beckton-Dickson, Auckland, New Zealand) and centrifuged (Medifuge, Heraeus Sepatech, Berlin, Germany) for 15 min at 1750g, 4°C. To determine serum cortisol, insulin, testosterone and immunoglobulin A (IgA)

concentration, blood was collected into room temperature serum vacutainers with clot-activator then sat for 30 min at room temperature before centrifugation at 1200 g for 30 min. For each parameter a 1 mL of supernatant was transferred to an Eppendorf tube and immediately frozen at -80°C until analysis. For differential cell counts and neutrophil superoxide anion (O_2^-) production stimulated by phorbol myristate acetate (PMA), blood was collected into LH vacutainers and analysed immediately. Blood sampling times are given in Fig. 4.1.

Analyses

Blood analysis. Cortisol was assayed by radioimmunoassay kit (Active Cortisol RIA DSL-2100, Diagnostic Systems Laboratories, Webster, TX) and Bioplex 200 system (Bio-Rad, Hercules, CA) with intra- and inter-assay CVs of 8.4% and 9.1%, respectively. IL-6 and IL-10 were assayed via Bioplex 200. Testosterone and sex-hormone binding globulin (SHBG) were quantified by competitive chemiluminescence immunoassay/sandwich principle method (Roche/Hitachi Testosterone and SHBG kits, Roche, Auckland, New Zealand) and analyser (Elecsys 2010, Roche, Auckland, New Zealand) with intra- and inter-assay CV of 1.1% and 1.7%, respectively, for testosterone, and 2.1% and 2.7%, respectively, for SHBG. Immunoglobulin-A concentration was determined by rate nephelometry using a Beckman Coulter IMMAGE, with an intra-batch CV of <1.0% and inter-batch CV of 3.5% (LabPlus, Auckland City Hospital, New Zealand). A solid-phase enzyme immunometric assay kit (Milenia Biotec GmbH, Giessen, Germany) measured using the Bio-Rad plate reader was used to determine elastase from polymorphonuclear granulocytes. Intra- and inter-assay CV were <5.2% and <6.4%, respectively. The method for neutrophil separation from whole blood and determination of O_2^- production were as described by Pyne et al. (Pyne et al. 2000). Cell concentrations were determined via automated analyser (AcT Cap Pierce

Hematology Analyser, Beckman-Coulter, Inc., Fullerton, CA). CVs are, for haemoglobin and mean cell volume, <1.0%; for white and red blood cell counts and haematocrit, <2.0%. Plasma metabolites were assayed using a standard kit and liquid chromatography tandem mass spectrometry, with full details provided elsewhere (Nelson et al. 2012).

Statistical Analysis. The effect of treatment on outcomes was estimated with mixed modelling (Proc Mixed, SAS Version 9.1, SAS Institute, Cary, NC). Most outcome variables were 100*log-transformed before modelling to reduce non-uniformity of error and to express outcomes as percentages (Hopkins, Marshall, Batterham and Hanin 2009), with the exception of data sets with negative values (neutrophil O_2^- production). Most outcomes and comparisons were generated from fixed effects models based on the interaction between the respective levels of treatment, test day, and order of treatment. Appropriate random effect models for each parameter included between-athlete variation, additional treatment-associated variation, and variation associated with moving between test days. Variability between blocks at baseline in blood measures was identified *a priori* as a potential confounder and included as a covariate. All covariates were first normalised to and expressed as a proportion of the within-subject standard deviation. We utilised a magnitude-based approach to inferences (Rowlands et al. 2008, Hopkins, Marshall, Batterham and Hanin 2009), with the standardised difference (effect size, ES) used to qualify effect magnitude. Descriptive data are presented as raw means and standard deviations. Means derived from the analysis are least-squares means with variation as coefficient of variation (CV). Uncertainty was set to 90% confidence limits (CL). In the text of the results section, qualitative likelihoods for outcomes of small effect size or larger are likely, unless otherwise stated. When considering plasma concentrations of hormones and cytokines, it is important to consider the total plasma volume which is transient and can rapidly change with exercise

(Kargotich, Goodman, Keast and Morton 1998). We noted a substantial (small effect size) impact of LEUPRO ingestion was observed on plasma volume prior to day-2 exercise but, importantly, effects during the 6-day training block were otherwise unremarkable and we have therefore elected to present cell concentration data without adjustment for changes in plasma volume in text and figures.

RESULTS

Plasma neutrophil PMA-stimulated $O_2^{\cdot-}$ production and elastase concentration

Exercise on days 4 and 6 induced small increases in plasma neutrophil elastase content with LEUPRO (day 4 post-exercise minus pre-exercise change, 32% ±90% CL 33%; ES: 0.49; ±0.55; p=0.071: day 6, 55% ±39%; 0.85; ±0.50; p=0.005) and small to moderate increases with CON (day 4, 28% ±32%; 0.42; ±0.44; p=0.109: day 6, 45% ±37%; 0.70; ±0.48; p=0.015); however, the difference between conditions was unclear. LEUPRO ingestion led to moderate (-17 mmol $O_2^{\cdot-}\cdot cell^{-1}$ ±37 mmol $O_2^{\cdot-}\cdot cell^{-1}$; -0.62; ±0.73; p=0.161) and small (-15 mmol $O_2^{\cdot-}\cdot cell^{-1}$ ±36 mmol $O_2^{\cdot-}\cdot cell^{-1}$; -0.56; ±0.75; p=0.212) reductions in PMA-stimulated neutrophil $O_2^{\cdot-}$ production at 60 min and 180 min into recovery on day 1, respectively (Fig. 4.2). Following exercise on day 6 there was a very likely large increase (33 mmol $O_2^{\cdot-}\cdot cell^{-1}$ ±13 mmol $O_2^{\cdot-}\cdot cell^{-1}$; 1.22; ±0.76; p=0.008) in PMA-stimulated $O_2^{\cdot-}$ production with LEUPRO, compared to CON; comparisons were otherwise unclear (Fig. 4.2).

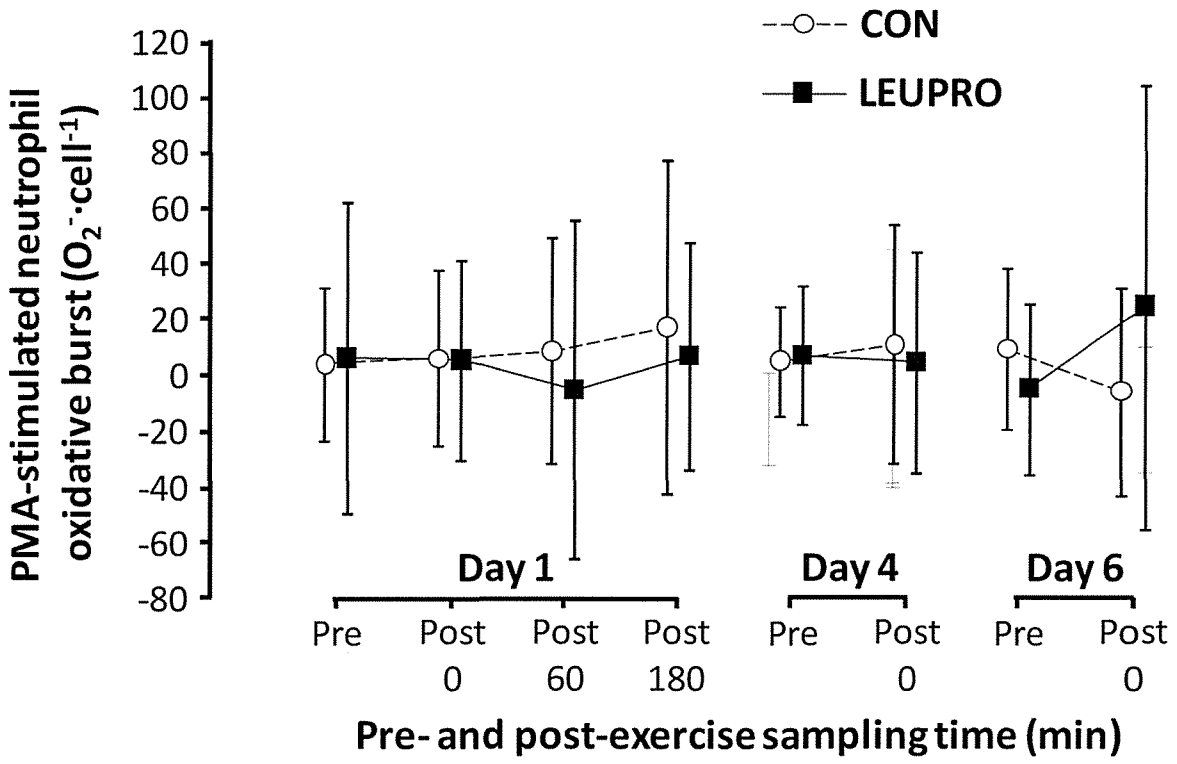


Figure 4.2. Effect of protein-leucine supplementation on phorbol myristate acetate (PMA)-stimulated neutrophil oxidative burst relative to control. Data are the least-squares mean quantity of O_2^{-} produced in PMA-stimulated cells minus production in non-stimulated cells, minus the background. Bars are standard deviations. LEUPRO, protein-leucine supplement. CON, control supplement.

Differential Immune-Cell Counts

Neutrophils. Both conditions exhibited an exercise-induced neutrophilia (Fig. 4.3A). On day 2, LEUPRO supplementation resulted in a small increase (10 ±9%; 0.29 ±0.24; p=0.038) in neutrophil concentration pre-exercise and a possible small increase (9 ±9%; 0.27 ±0.24; p=0.056) following exercise, relative to CON (Fig. 4.3A). However, there were small reductions in neutrophil concentration with LEUPRO ingestion following day-4 exercise (11 ±7%; 0.31 ±0.22; p=0.018) and prior to day-6 exercise (13 ±7%; 0.38 ±0.22; p=0.003), relative to CON supplementation (Fig. 4.3A). The neutrophil concentration before exercise

on day 6 was almost certainly lower than before exercise on days 1, 2 and 4 (-17 to -26% ±6%; -0.50 to -0.62 ±0.20; p=9E-8 to 38E-6) (Fig. 4.3A).

Lymphocytes and neutrophil:lymphocyte ratio. LEUPRO supplementation resulted in small reductions in lymphocyte concentration at 180 min into day-1 recovery (10% ±6%; -0.36 ±0.22; p=0.007), following day-4 exercise (10 ±6%; -0.37 ±0.23; p=0.005) and prior to day-6 exercise (8% ±6%; -0.30 ±0.22; p=0.021), and a possible small reduction following exercise on day 2 (7% ±6%; -0.26 ±0.22; p=0.052); all comparisons were otherwise trivial (Fig. 4.3B). There were small increases in the neutrophil:lymphocyte (N:L) ratio with LEUPRO supplementation at 180 min into day-1 recovery (17% ±9%; 0.32 ±0.16; p=71E-05), following day-2 exercise (18% ±9%; 0.34 ±0.16; p=38E-05) and possibly prior to day-2 exercise (13% ±8%; 0.24 ±0.15; p=0.008); otherwise, effects were trivial (Fig. 4.3C).

Basophils, monocytes, and eosinophils. Overall there was a pattern of reduction in circulating basophils with LEUPRO supplementation (Fig. 4.3D). Small reductions in basophil concentration were likely at 180 min into recovery on day 1 (25 ±13%; -0.33 ±0.20; p=0.007) and pre-exercise on day 4 (24 ±13%; -0.32 ±0.20; p=0.009) and possible following exercise on day 4 (18 ±14%; -0.24 ±0.21; p=0.058) and day 6 (16 ±14%; -0.22 ±0.21; p=0.083). The effect of LEUPRO on monocyte and eosinophil concentrations was unclear (data not shown).

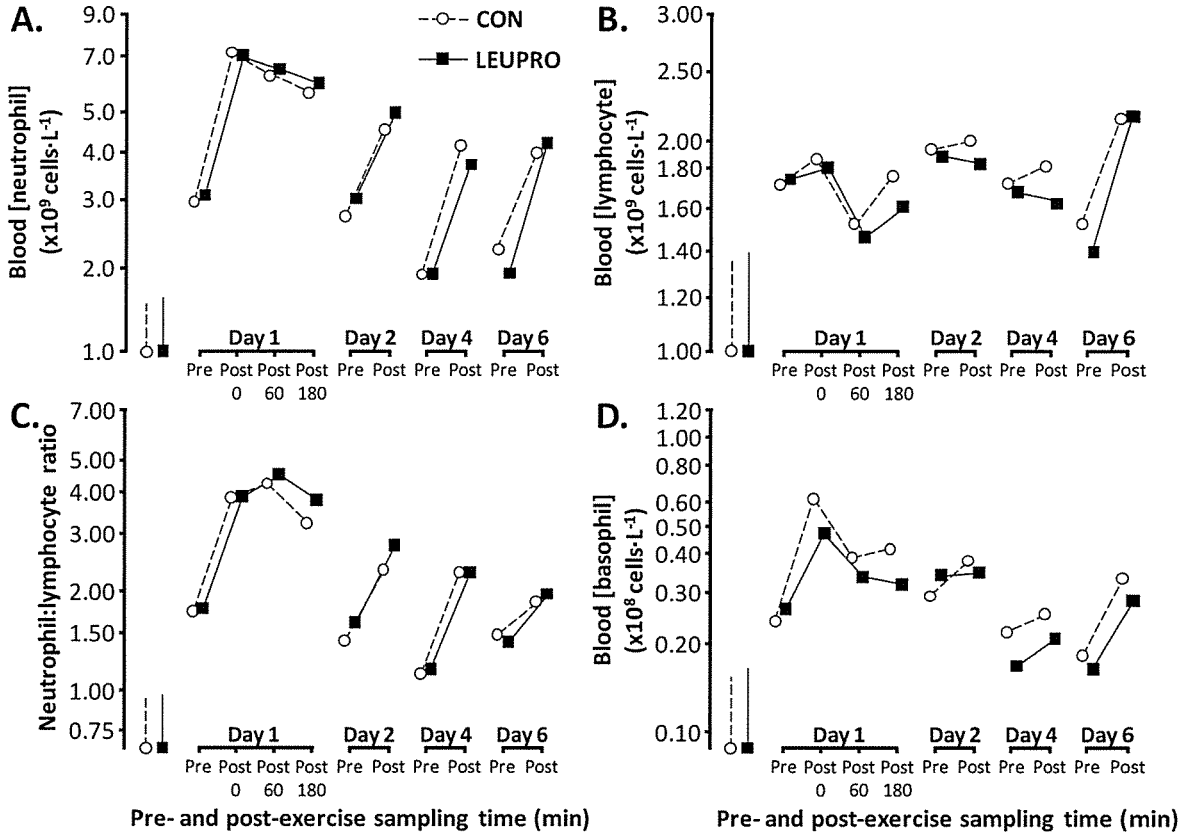


Figure 4.3. Effect of protein-leucine supplementation on the change from baseline in the blood cell concentrations of neutrophils (**A**), lymphocytes (**B**) and basophils (**C**), and the neutrophil-to-lymphocyte ratio (D), before and during recovery (day 1) and pre- and post-exercise over the 6-day block. Data are back log-transformed least-squares mean concentrations plotted on a log scale, with composite between-subject standard deviations (bottom left of each figure). Cell concentration data are not adjusted for plasma volume changes. LEUPRO, protein-leucine supplement. CON, control supplement.

Hormones and cytokines: cortisol, testosterone, immunoglobulin A, interleukin-6 and interleukin-10

Before exercise on day 6, serum cortisol was moderately reduced with LEUPRO ingestion, relative to the CON (Table 4.1). Outcomes for all days for total, free, and bioavailable

testosterone, IL-6, IL-10, and IgA concentration were all inconclusive or trivial (data not shown).

Table 4.1. *Effect of protein-leucine supplementation on the serum concentration of cortisol before and after exercise on days 1, 4 and 6 of the 6-day block.*

Day; Period[a]	LEUPRO-CON Effect (%)[b]; ±90% CL[c]	Effect Size[d]; ±90% CL[e]	p-value	Magnitude-based Inference
		Plasma cortisol concentration		
1; pre	8.2; ±20.2	0.25; ±0.87	0.483	unclear
1; post 180	-1.1; ±18.4	-0.04; ±0.56	0.919	unclear
4; pre	-4.1; ±17.9	-0.14; ±046	0.706	unclear
4; post 0	-5.8; ±15.6	-0.20; ±0.40	0.590	unclear
6; pre	-21.0; ±14.7	-0.77; ±0.18	0.037	moderate ⇩ likely
6; post 0	-4.3; ±17.8	-0.14; ±0.46	0.692	unclear

[a]Day 1, 4 or 6 of the 6-day training block; immediately pre-exercise or minutes post-exercise.
[b]Data are overall mean effect for the protein-leucine minus control difference derived from the statistical analysis and represent the percent-difference for the relevant comparison.
[c]Add this number to or subtract from the mean effect to obtain the upper and lower 90% confidence limits.
[d]Effect size thresholds: <0.2 trivial, <0.6 small, <1.2 moderate, <2.0 large, <4.0 very large, >4.0 extremely large.
[e]Add this number to or subtract from the effect size to obtain the upper and lower 90% confidence limits.
[e]Thresholds for assigning qualitative terms to chances of substantial effects: <0.5%, almost certainly not; <5.0%, very unlikely; <25%, unlikely; <75%, possible; >75%, likely; >95%, very likely; >99.5%, almost certain. An effect is unclear if its confidence interval includes both substantial increases and decreases. Arrow symbols indicate an increase (⇧) or decrease (⇩).
Abbreviations. LEUPRO, protein-leucine, carbohydrate and fat supplement; CON, control carbohydrate and fat supplement.

Changes in blood and plasma volume

With LEUPRO there was a very likely small increase in blood and plasma volume before exercise on day 2, relative to the CON; otherwise all other time points were unremarkable (Table 4.2). The order effect for trial two minus trial one was: for blood volume (as a percent of baseline blood volume), likely trivial (-0.6% ±2.4%; -0.04 ±0.19; p=0.698); for plasma volume (as a percent of total blood volume), unclear (-0.9% ±3.7%; -0.06 ±0.26; p=0.704); and for haematocrit, a possible decrease (-0.004% ±0.008%; -0.21 ±0.39; p=0.365).

Table 4.2. *Effect of protein-leucine supplementation on the change in total blood and plasma volume and haematocrit before and after exercise on days 1, 2, 4 and 6 of the 6-day block.*

Day; Period[a]	LEUPRO-CON Effect (%)[b]; ±90% CL[c]	Effect Size[d]; ±90% CL[e]	p-value	Magnitude-based Inference
		Blood volume		
1; post 0	0.1; ±2.4	0.01; ±0.20	0.923	trivial effect likely
1; post 60	-3E-2; ±2.5	-2E-3; ±0.20	0.984	unclear
1; post 180	0.5; ±2.5	0.04; ±0.20	0.748	trivial effect likely
2; pre	3.8; ±2.5	0.31; ±0.21	0.014	small⇧ likely
2; post 0	1.8; ±2.5	0.14; ±0.20	0.237	trivial effect possible
4; pre	1.3; ±2.5	0.10; ±0.20	0.377	trivial effect likely
4; post 0	0.2; ±2.5	0.02; ±0.20	0.888	trivial effect likely
6; pre	0.6; ±2.5	0.05; ±0.20	0.675	trivial effect likely
6; post 0	-1.6; ±2.5	-0.13; ±0.20	0.284	trivial effect possible
		Plasma volume		
1; post 0	0.3; ±3.8	0.02; ±0.26	0.889	unclear
1; post 60	-0.4; ±3.8	-0.03; ±0.26	0.869	unclear
1; post 180	0.33; ±3.8	0.02; ±0.26	0.887	unclear
2; pre	6.6; ±3.8	0.46; ±0.28	0.006	small⇧ likely
2; post 0	2.8; ±3.8	0.27; ±0.27	0.230	trivial effect possible
4; pre	2.6; ±3.8	0.18; ±0.26	0.259	trivial effect possible
4; post 0	0.2; ±3.8	0.01; ±0.26	0.935	trivial effect likely
6; pre	1.9; ±3.8	0.13; ±0.26	0.415	trivial effect possible
6; post 0	-2.4; ±3.8	-0.17; ±0.26	0.293	trivial effect possible
		Hematocrit		
1; pre	0.001; ±0.007	0.06; ±0.36	0.780	unclear
1; post 0	3E-4; ±0.007	0.01; ±0.36	0.938	unclear
1; post 60	0.003; ±0.007	0.14; ±0.36	0.521	unclear
1; post 180	0.002; ±0.007	0.08; ±0.36	0.698	unclear
2; pre	-0.017; ±0.007	-0.85; ±0.38	2E-05	almost certain small ⇩
2; post 0	-0.005; ±0.007	0.25; ±0.36	0.249	small ⇩ possible
4; pre	-0.006; ±0.007	0.29; ±0.36	0.184	small ⇩ possible
4; post 0	-3E-4; ±0.007	-0.02; ±0.36	0.940	unclear
6; pre	-0.006; ±0.007	-0.31; ±0.36	0.161	small ⇩ possible
6; post 0	0.005; ±0.007	0.27; ±0.36	0.222	small⇧ possible

See Table 4.1 for definitions.

Metabolomics

Full details of metabolomics outcomes, including plasma concentrations of amino acids, acylcarnitines, sphingolipids and glycerophospholipids, during recovery from exercise on day-1 and before and during day-6 exercise are presented elsewhere (Nelson et al. 2012). Qualitative outcomes for selected metabolites with known cytokine production or neutrophil-regulatory function are briefly summarised below.

During day-1 recovery LEUPRO increased mean plasma concentrations of leucine and the BCAAs (extremely large effect-size), glutamine (very large), arginine (large) and glycine (small). Furthermore, small increases in the mean concentrations of plasma fatty acid acylcarnitines C14 and C14:1-OH (myristoylcarnitine and hydroxymyristoleylcarnitine, respectively) were observed. During exercise on day 6, LEUPRO ingestion resulted in a small reduction in the mean plasma concentration of acylcarnitine C16 (palmitoylcarnitine) and a possible small increase in arginine; the effect was possibly trivial for glutamine and glycine plasma concentrations.

DISCUSSION

Earlier we reported increased whole-body leucine turnover and protein synthesis during recovery, and some evidence for attenuated cell-membrane damage and increased extracellular matrix turnover via lower plasma creatine kinase and increased hydroproline-derived protein, respectively, in the current cohort with the ingestion of the leucine-enriched whey-protein supplement post exercise (Nelson et al. 2012). Because the post-exercise immune response is critical to preventing opportunistic infection and an important component of muscle repair and recovery, we investigated the response of immunological, haematological, hormonal and cytokine parameters to post-exercise LEUPRO ingestion. We found that, compared to CON, ingesting LEUPRO had an overall reducing effect on neutrophil, lymphocyte and basophil concentrations but a differential impact on neutrophil function. The latter consisted of reduced day-1 recovery neutrophil O_2^- production but a change to increased production following day-6 exercise, effects that were associated with changes in recovery concentrations of plasma fatty acid or amino acid metabolites, and reduced circulating cortisol prior to exercise on day 6. These data suggest that during a period

of intense exercise, LEUPRO supplementation attenuates serum cortisol but increases circulating neutrophil margination and respiratory burst response to stimulation following exercise-stress, and therefore might impact their cytotoxic and signalling function and immune-cell mediated processes of tissue repair and recovery.

Immunomodulatory plasma metabolites and attenuated cortisol best explain differential effects of LEUPRO on neutrophil O_2^- production

LEUPRO ingestion moderately increased PMA-stimulated neutrophil oxidative burst in response to exercise on day 6. Activation of the superoxide-generating NADPH oxidase complex is critical to neutrophil microbicidal function (Nauseef 2007) and increased release of O_2^- might benefit neutrophil cytotoxic activity. The most likely explanation for increased neutrophil O_2^- was the decrease in pre-exercise serum cortisol. Cortisol can exert an immunosuppressive effect with a lag of several hours (Pedersen and Hoffman-Goetz 2000) and impacts on neutrophil recruitment, degranulation and phagocytic activity (Forslid and Hed 1982); the reduction in pre-exercise cortisol concentration might, therefore, best explain the subsequent increase post-exercise in neutrophil O_2^- production. It should be noted that for all cycling bouts throughout the 6-day period, each participant matched the hour of the day when exercise was undertaken in the second arm of the crossover to the hour it was conducted during the first arm, to minimise the impact of within-individual circadian variation on hormones (cortisol, and testosterone) between training blocks. IL-6 is probably partly responsible for exercise-induced increases in cortisol and IL-10 (Steensberg et al. 2003) yet the trivial effect of LEUPRO supplementation on both IL-6 and IL-10 suggests that these cytokines were not responsible for the reduced cortisol, nor played a direct role in increasing neutrophil O_2^- release. Skeletal muscle IL-6 mRNA is reduced by feeding during exercise and the IL-6 transcriptional rate from nuclei of contracting muscle fibres is

influenced by the muscle glycogen content (Keller et al. 2001, Febbraio et al. 2003). The lack of an effect of protein-leucine feeding on the plasma IL-6 concentration is, therefore, unsurprising given that participants were provided a high-carbohydrate background diet and sports drink during all rides, and that the rate of supplemental carbohydrate intake was designed to maximise the post-exercise recovery glycogen resynthesis rate (Nelson et al. 2012). Additionally, LEUPRO could have modulated other unmeasured hormones or cytokines, for instance, the interleukin-1 receptor antagonist (IL-1ra), IL-8, and tumour necrosis factor alpha (Rusu et al. 2010) or IL-12 and IL-23 (Säemann et al. 2009). To the best of our knowledge, the only recent comparable investigation found that in male runners the reduction from baseline in bacterially-stimulated neutrophil elastase release during recovery from hard running (2 h of 75% VO_2max) was prevented with post-exercise ingestion of a carbohydrate beverage (1.2 $g \cdot kg^{-1}$ body mass), and that adding soya protein (0.4 $g \cdot kg^{-1}$) had no substantial impact (Costa, Walters, Bilzon and Walsh 2011). We did not assess neutrophil elastase release but did find that the elastase concentration in unstimulated neutrophils was not substantially altered by LEUPRO relative to CON.

Metabolomics analysis revealed changes in plasma metabolites with known neutrophil regulatory functions that could have contributed to the increased O_2^- production following day-6 exercise and the reduction during day-1 recovery. Firstly, we reported previously that LEUPRO supplementation reduced the plasma concentration of palmitoylcarnitine during day-6 exercise (Nelson et al. 2012). Protein kinase C (PKC) activates the NADPH oxidase enzyme complex in human neutrophils (Cox et al. 1985) and palmitoylcarnitine competitively inhibits PKC (McIntyre, Reinhold, Prescott and Zimmerman 1987). Reduced plasma palmitoylcarnitine during day-6 exercise might, therefore, release the inhibition of PKC-

mediated NADPH oxidase reactive oxygen species production resulting in increased neutrophil O_2- production, sampled post-exercise.

Secondly, we also reported that LEUPRO supplementation elevated day-1 recovery plasma concentrations of amino acids (leucine, glutamine, arginine and glycine) and acylcarnitines (C14, myristoylcarnitine; and C14:1-OH, hydroxymyristoleylcarnitine) (Nelson et al. 2012). Because the supplement was provided daily, a chronic increase in plasma leucine, glutamine and arginine concentrations during recovery might have contributed to the day-6 increase in neutrophil O_2^- production. Additionally, *in vitro* culturing of neutrophils with whey-protein extract (WPE) and WPE-derived bioactive peptides had a delayed NADPH-oxidase priming effect that increased stimulated-neutrophil chemotaxis, O_2^- production and degranulation (Rusu et al. 2009, Rusu et al. 2010). PMA-stimulated neutrophil oxidative burst is significantly decreased *in vitro* and *in vivo* in transplant patients using the mTOR inhibitor rapamycin (Gee, Trull, Charman and Alexander 2003) indicating involvement of the mTOR pathway in superoxide production, and leucine is the most potent nutritional stimulant of mTOR activity (Anthony et al. 2000). Glutamine supplementation has been shown to prevent exercise-induced neutrophils apoptosis by modulating pro- and anti-apoptotic gene expression (Lagranha et al. 2004) and delaying spontaneous neutrophil apoptosis (Pithon-Curi et al. 2002) in rodents and might therefore benefit neutrophil survival, increasing the relative maturity of circulating neutrophils. Furthermore, both glutamine and arginine modulate PMA-stimulated rat neutrophil O_2^- release by a common pathway of metabolism involving nitric oxide and polyamines, the active metabolites of arginine (Moinard et al. 2002). Glycine is known to inhibit the release of reactive oxygen species from cultured, PMA-stimulation neutrophils in a dose-dependent manner (Giambelluca and Gende 2009). However, given that the observed reductions in cultured neutrophil O_2^- production ranged

from 30 to 75% at glycine concentrations of 0.1-50 mM (Giambelluca and Gende 2009) the small increase in mean plasma glycine concentration of 12 μM during LEUPRO-supplemented day-1 recovery would most likely had a small to trivial impact on circulating neutrophil O_2^- production (Nelson et al. 2012) and would not explain the acute reduction observed at a time when other stimulatory plasma metabolites (i.e. leucine, glutamine and arginine) were increased.

An increased plasma concentration of myristic acid with the LEUPRO supplement might best explain the acute reduction in day-1 recovery neutrophil O_2^- release. Myristic acid is a more potent stimulator of neutrophil O_2^- release than PMA itself (Tada et al. 2009) and an increase in the downstream metabolites of myristic acid (e.g., myristoylcarnitine and hydroxymyristoleylcarnitine) is indicative of enhanced myristic acid cellular transport and metabolism. It follows that with LEUPRO ingestion during day-1 recovery, the stimulation of circulating neutrophils by the oxidative burst assay would be superimposed on cells already stimulated by increased plasma concentrations of myristic acid resulting in a greater mean O_2^- background value (and hence the reduction versus CON at a time when other stimulatory metabolites were elevated).

LEUPRO-altered plasma leucocyte trafficking favoured neutrophil dominance

A typical exercise-induced response of neutrophilia, lymphocytosis and recovery lymphopenia were observed possibly mediated by previously well-described exercise-stress associated increases in cortisol, catecholamines and growth hormone (reviewed in (Pedersen and Hoffman-Goetz 2000)). Reduced cortisol could account for the exercise-induced lymphocytosis seen on day 6, however, without clear effects of cortisol on other days we can only speculate regarding the effects of treatment. LEUPRO-associated expansion of blood

plasma volume overnight on day 1 (discussed below) could have influenced immune cell responses by blunting exercise-associated increases in catecholamines; norepinephrine in particular mediates neutrophil recruitment (Kappel, Poulsen, Galbo and Pedersen 1998).

Opposing changes in acute neutrophil and lymphocyte concentrations established a shift favouring neutrophil dominance (N:L ratio). Some evidence for a decline in neutrophil concentration in the circulation on day 4 (post-exercise) and day-6 (pre-exercise) would suggest greater margination with LEUPRO ingestion by the fourth day of the training block. In contrast, Costa et al. (Costa, Walters, Bilzon and Walsh 2011) reported no apparent differences in immune cell concentrations, despite differences in plasma glucose, insulin and cortisol between treatment groups, and the fat content was held constant between the control and LEUPRO supplements, implicating the leucine component of LEUPRO in the changes in cell concentration observed in the current study. Neutrophil-derived cytotoxic agents, including O_2^-, might be the primary factor contributing to post-exercise muscle membrane damage (Butterfield, Best and Merrick 2006). If so, LEUPRO-associated modification of neutrophil superoxide generating capacity, and neutrophil redistribution from the circulation to exercised muscle, has the potential to impact on the post-exercise subsequent muscle injury repair processes partly explaining the observed reductions in plasma creatine kinase (Nelson et al. 2012).

Blood and plasma volume changes

Ingestion of the LEUPRO supplement was associated with a mean plasma volume increase of ~7% of baseline by the start of exercise on day 2, ~12 h since the first beverage was ingested. Plasma volume increases of ~3.5% have been reported in young and old men ingesting bolus protein-carbohydrate following cycling, relative to isocaloric carbohydrate-only (Okazaki et

al. 2009, Okazaki et al. 2009, Goto et al. 2010) and the observed two-fold greater mean increase in plasma volume in the present study might be due to the greater total dose of protein (20 $g \cdot h^{-1}$ for 3 h during day-1 recovery versus a single bolus of ~10 g (Okazaki et al. 2009, Okazaki et al. 2009) or ~20 g immediately post-exercise (Goto et al. 2010)) and the stimulatory effect of leucine on cellular protein synthesis (Anthony et al. 2000). An increase in plasma volume following post-exercise protein-carbohydrate ingestion appears be acutely driven by increased plasma albumin concentration, promoting a fluid shift from the interstitium to the vascular compartment, most pronounced 5-23 h post-exercise (Okazaki et al. 2009). Albumin protein synthesis is enhanced by glucose and amino acids (De Feo, Horber and Haymond 1992) and exercise (Yang, Mack, Wolfe and Nadel 1998), and increases in a dose-responsive manner to protein ingestion (Moore et al. 2009). Previously, a nearly identical plasma volume increase as the current study (6.5%) via short-term heat acclimation resulted in ~7% increase in VO_2max and time-trial performance (Lorenzo, Halliwill, Sawka and Minson 2010). In the current cohort, subsequent performance was not substantially affected by protein-leucine supplementation (Nelson et al. 2012).

Conclusion

Ingestion of a LEUPRO supplement enhanced neutrophil O_2^- release and altered leucocyte trafficking, which was associated with lower cortisol and independent of IL-6. The enhanced neutrophil response might relate to altered plasma concentrations of important signalling and substrate amino acids that may act synergistically by enhancing cell survival to maturity, lifting inhibition of superoxide production and priming neutrophils to respond to stimuli. Evidence of greater neutrophil margination with LEUPRO is in contrast to similar recent work that found no substantial effect of protein-carbohydrate coingestion following endurance exercise on immune cell concentrations, implicating a role for the leucine

component of the supplement. While the performance outcomes were non-remarkable, a possible immunomodulatory impact of a post-exercise protein-leucine supplement was identified upon neutrophils that might augment their function during a period of intensified training.

CHAPTER 5 STUDY 2A

RESEARCH REPORT

Skeletal muscle fractional protein synthetic and mTOR-pathway signalling responses to the ingestion of low and high saturating doses of a leucine-enriched protein-carbohydrate supplement following high-intensity endurance exercise

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ABSTRACT

Purpose. Dietary protein and leucine intake following strenuous exercise increases the skeletal muscle-protein fractional synthesis rate (FSR) but an optimal dose to saturate FSR and the dose-impact upon intracellular signalling are unknown following endurance exercise. **Methods.** In a crossover study design twelve trained men completed 100 min of high-intensity cycling, then ingested high-dose (70/15/180/30 g protein/leucine/carbohydrate/fat), low-dose (23/5/180/30 g) or control (0/0/274/30 g) beverages in 4 servings during the first 90 min of a 240-min monitored recovery period. $^{13}C_6$-phenylalanine was infused, blood was sampled every 30-60 min, and *vastus lateralis* muscle biopsies were taken 30-min and 240-min into recovery to determine myofibrillar FSR and mammalian target of rapamycin (mTOR)-pathway intracellular signalling activity. **Results.** The increase in myofibrillar FSR with high-dose feeding (mean ± SD; 0.103%·h^{-1} ± 0.027%·h^{-1}) versus control (0.069%·h^{-1} ± 0.014%·h^{-1}) was almost certainly large (1.51-fold ×/÷ 90%CL 1.12-fold, p=1E-05). The mean increase in FSR with the high-dose relative to low-dose feeding (0.092%·h^{-1} ± 0.017%·h^{-1}) was small effect size (13% ×/÷12%, p=0.07), but trivial relative to a 25% threshold for bioequivalence based on pharmacokinetic criteria. Mean concentrations of plasma essential amino acids (EAAs) and leucine over the 240-min recovery were greater with high-dose feeding versus low-dose (EAA, 1.73-fold ×/÷1.24, p=29E-6: and leucine, 2.18-fold ×/÷1.32, p=43E-7) and control (EAA, 2.80-fold ×/÷1.24, p=2E-13: and leucine, 7.97-fold ×/÷1.33, p=1E-26). High-dose resulted in small increases in recovery serum insulin concentration versus low-dose (1.44 ×/÷1.18, p=45E-5) and control (1.57 ×/÷1.18, 14E-6), respectively. Western blot analysis showed that as protein-leucine dose increased from low to high, so too did phosphorylation at 30-min post-exercise of mTOR (2.21-fold ×/÷1.59, p=8E-3) and p70S6K (3.51-fold ×/÷1.93, p=3E-3) and at 240-min of rpS6 (4.85-fold ×/÷1.37, p=2E-13), 4E-BP1-α (1.99-fold ×/÷1.63, p=0.022), but not phosphorylation of eEF2 (0.88-fold and

1.07-fold ×/÷1.25, p=0.334 and 0.750). The effect of protein-leucine dose on AMPK-α phosphorylation and total SIRT1 protein was mostly inconsequential, except for a small increase with high versus low at 30-min post-exercise (1.32-fold ×/÷1.40, p=0.179: and 1.38-fold ×/÷1.33, p=0.060, respectively). At 240 min into recovery, moderate correlations were observed between myofibrillar FSR and p70S6K (r 0.41 ±90%CL 0.29, p=0.029) and rpS6 phosphorylation (0.52 ±0.21, p=0.026), hyper-phosphorylated 4E-BP1γ (0.41 ±0.25, p=0.148) and the mean 240-min recovery plasma leucine (0.42 ±0.23, p=0.011) and EAA (0.30 ±0.26, p=0.076) concentrations. There were moderate positive correlations between the mean recovery plasma leucine concentration and phosphorylation of mTOR, p70S6K and rpS6 at 30 min; and at 240 min, large positive correlation with 4E-BP1γ and rpS6 and moderate negative correlation with phosphorylated 4E-BP1$^{Thr37/46}$. Linear regression revealed that mean recovery plasma leucine concentration and rpS6 and p70S6K phosphorylation at 240 min were the best predictors of myofibrillar FSR, and leucine was the best predictor of both rpS6 and p70S6K. We estimate from linear modelling that to increase 4-h recovery myofibrillar FSR on average by 0.010 %·h^{-1} following a 100-min bout of intense endurance cycling, a relative mean increase in recovery mean plasma leucine concentration of ~210 μM was required. This linear leucine dose-related increase came from the ingestion of approximately 16.3 g of milk proteins and 3.5 g free leucine during the first 90-min post exercise. **Conclusion.** The ingestion of a supplement containing 23 g of milk proteins, 5 g of leucine, carbohydrate and fat following intense endurance exercise augmented anabolic signalling via the mTOR pathway in a dose-sensitive manner, but the corresponding magnitude of the effect of the 3-fold increase in ingested protein-leucine on myofibrillar protein FSR was trivial when expressed in terms of pharmacokinetics. Depending on an individual athlete's requirements, a low protein-leucine dose may be sufficient for near-maximal myofibril protein turnover and adaptive remodelling of the sarcomere complex,

which could be important when restricting caloric intake is also an important consideration relative to the larger dose of recovery nutrition. Otherwise, a high-dose equivalent may ensure saturation of skeletal myofibril protein turnover. Chronic supplementation trials applying graded protein doses with an integrated systems-based analysis that includes both protein and end-point functional outcomes appear to be required to provide a more complete picture of what the best post-exercise protein (+leucine) feeding dose is to optimise skeletal muscle protein and tissue remodelling to augment training adaptation and performance.

INTRODUCTION

The coingestion of protein and leucine with carbohydrate and fat following endurance exercise can substantially improve subsequent high-intensity endurance performance under nitrogen-stressed conditions typical of heavy training loadings (Rowlands et al. 2008, Thomson, Ali and Rowlands 2011), and dietary protein intake may play an important role in promoting aspects of skeletal muscle adaptation to exercise (Hawley, Tipton and Millard-Stafford 2006, D'Antona et al. 2010). Proposed mechanisms for the protein-nutrition mediated enhanced recovery and adaptive response include amino acid induced regulation of mRNA translation and muscle fractional protein synthetic rates (FSR) (Koopman et al. 2005, Kimball and Jefferson 2006, Moore et al. 2009, Safdar et al. 2009, Nielsen et al. 2010) and an overrepresented transcriptome involving expression of genes involved in skeletal muscle development, immunity and defense, and energy metabolism (Rowlands et al. 2011). However, there are limited data available regarding the effect of post-exercise protein ingestion on gene expression and the control of mRNA translation within trained skeletal muscle recovering from intense endurance exercise; the rate of protein synthesis is important

because it influences skeletal-muscle protein turnover and might, therefore, determine the rate of cellular functional adaptation to the prior endurance exercise stress.

Current evidence suggests that only modest protein ingestion is needed to maximally stimulate muscle protein synthesis. In young men fed egg protein (0, 5, 10, 20, or 40 g) after a single bout of resistance training, the mixed-muscle protein FSR appeared to plateau between 20-40 g (Moore et al. 2009). When taken with other data in the literature (e.g.(Miller et al. 2003, Cuthbertson et al. 2005)) it appears that a protein containing approximately 8.5 g of essential amino acids (EAA) leads to saturation of mixed muscle FSR following a single bout of resistance exercise. Acute endurance exercise has been demonstrated to increase mitochondrial FSR in untrained men prior to and following 10 weeks of training (Wilkinson et al. 2008). In endurance trained male cyclists, Breen et al. (Breen et al. 2011) recently found that ingesting a modest dose of carbohydrate (50 g) and protein (20 g) following 90 min of cycling increased myofibrillar FSR at 4-h post-exercise by ~35%, but had a trivial impact on mitochondrial FSR, relative to carbohydrate (50 g) only ingestion. The mitochondrial data are counter instinctive, but may not be surprising because metabolic gene expression peaked 8-12 h post exercise (Yang, Creer, Jemiolo and Trappe 2005), and the biogenesis of new mitochondrial proteins occurs over days to weeks (Hood 2001).

Intake of essential amino acids is necessary to elevate protein synthesis (Tipton et al. 1999), but leucine is the key amino acid stimulating protein synthesis (Buse and Reid 1975, Anthony, Anthony and Layman 1999, Crozier et al. 2005). Koopman et al. (2005) reported a moderate increase in mixed-muscle FSR in young men after resistance exercise with the addition of leucine to protein and carbohydrate. In addition to regenerating glycogen (Ivy

2004), carbohydrate ingestion post-exercise might also reduce muscle-protein breakdown via insulin action leading to improved net protein balance (Levenhagen et al. 2002, Miller et al. 2003). It follows that protein, leucine, and carbohydrate coingestion could benefit recovery and adaptation, specifically in the immediate hours post-exercise by capitalizing on exercise-induced increases in blood flow, glucose and amino acid uptake, and glycogen synthase activity (Levenhagen et al. 2001, Miller et al. 2003, Ivy 2004). Despite the recent work, the effect of post-exercise protein-leucine feeding on myofibrillar and mitochondrial FSR following endurance exercise is under-characterised, and the effect of dose has not been established.

Therefore, the objective of the study was to compare the effect of three doses of protein-leucine (lzero, 0/0; low, 23.3/5; and high, 70/15 g of protein and leucine, respectively) co-ingested with isocaloric carbohydrate and fat during the first 90-min post-endurance exercise on the signalling activity of proteins controlling translation initiation and elongation, and on myofibrillar and mitochondrial FSR. We hypothesised that the quantity of protein-leucine in the low-dose was the minimum required to saturate mitochondrial and myofibrillar FSR, leading to no substantial difference in FSR compared to the high-dose condition.

METHODS

Participants. Twelve endurance-trained male cyclists; aged 30 ± 7 y (mean ± SD), standing 179 ± 5 cm, and weighing 78.1 ± 7.8 kg, completed the study. Maximal oxygen uptake (VO_2max) was 60.4 ± 6.2 $mL \cdot kg^{-1} \cdot min^{-1}$ with a corresponding peak power output (W_{max}) of 323 ± 32 W. All participants were informed of the purpose and risks associated with

procedures and signed a consent form before study participation. The study was approved by the Central Regional Ethics Committee, New Zealand.

Design. The research design was a single-blind, randomized crossover comprising three experimental periods during which exercise and diet were controlled. The intervention consisted of the ingestion of post-exercise low-dose and high-dose protein-leucine beverages, and a zero protein-leucine beverage (control), isocaloric to the high-dose condition. Outcome measures were obtained from blood and skeletal muscle tissue collected following a bout of intense cycling.

Preliminary Testing. Two weeks prior to the first experimental period, cyclists completed a standard test to determine maximal oxygen uptake (VO_2max) and peak aerobic power output (Wmax) using an electromagnetically-braked cycle ergometer (Velotron, Version 1.9 Software, Racer Mate, Seattle, USA) as described previously (Nelson et al. 2012). Expired breath was analysed using an on-line system (Moxus, AEI Technologies, Pittsburgh, PA). Wmax was calculated (Rowlands et al. 2007) and values used to establish individual cyclist workloads employed during the experimental period. The next day participants completed a familiarization ride of the experimental exercise protocol.

Diet and activity prior to the experiment period. Physical activity and diet were standardised for 5-days prior to the experimental period, and repeated prior to the second and third experimental periods (Fig. 1). Exercise was controlled on day -2 (between 1500 and 2000 h) and consisted of a 90-min ride on the Velotron comprising a warm-up (10 min at 30% W_{max},

8 min at 40% W_{max}), intervals (4 x 5 min at 70% W_{max}) interspersed with three blocks of 3 x 2-min hard intervals at 85%, 80%, and 75% W_{max}, respectively, and recovery periods at 50% W_{max}, and a cool-down for 5 min at 40% W_{max}. Following this ride and for the remainder of day -2, day -1 and the experimental day (day 1), participants were provided with a pre-weighed controlled diet providing sufficient energy to balance individual estimated caloric requirements based on the Harris-Benedict equation and an activity factor of 1.6; 14.9 ± 1.5 $MJ \cdot d^{-1}$; 58% carbohydrate, 13% protein and 29% fat). On day 1, participants ingested their final meal 3-h prior to reporting to the laboratory.

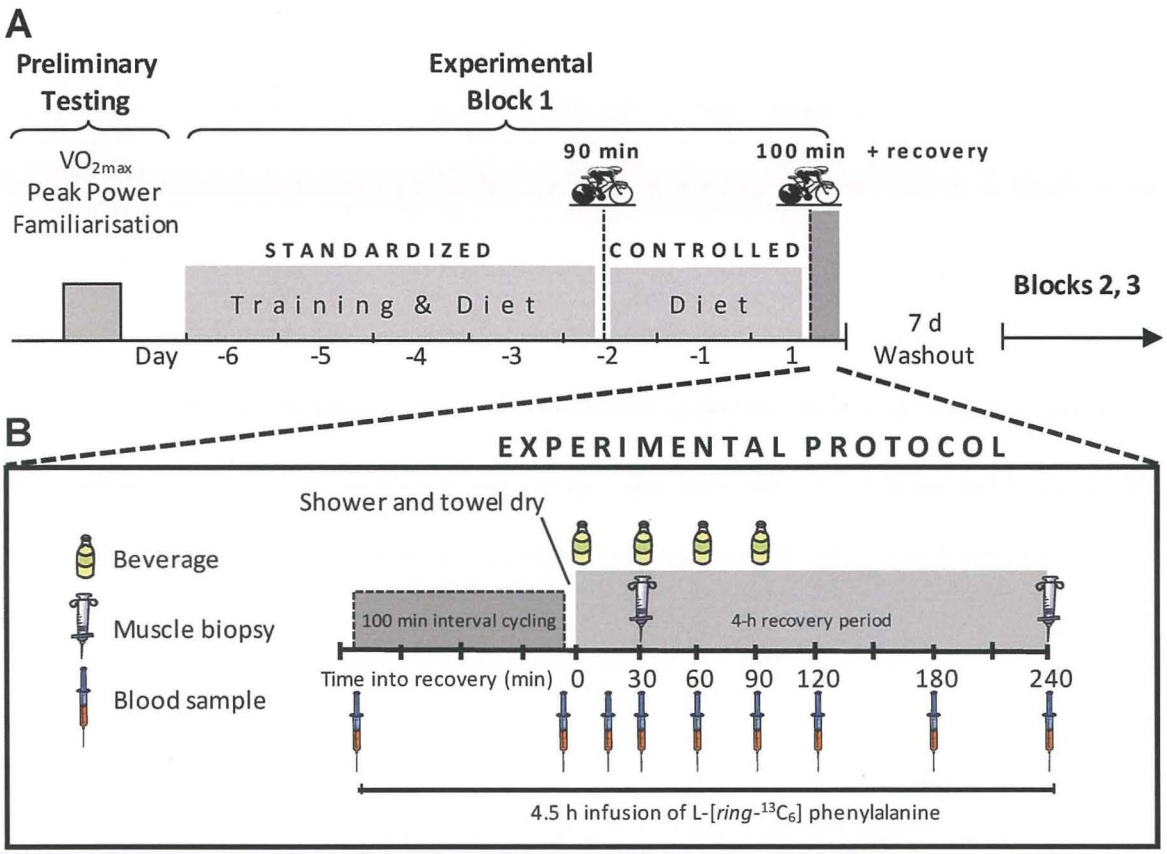


Figure 5.1. Experimental design. Shown are preliminary visits, and standardized (days -6 to -2) and controlled (days -2 to 1) training and diet during a 6-day experimental block (**A**), with

the experimental protocol further outlined, including high-intensity cycling, recovery supplementation, and blood and biopsy collection periods (**B**).

Experimental protocols

Experimental period. Participants arrived at the laboratory in the afternoon (between 1300 and 1700 h) and a 20-gauge catheter was positioned in the antecubital vein of each arm and fitted with a 3-way stopcock. One catheter was kept patent with 0.9% isotonic saline and used for blood sampling. The second catheter was attached to an infusion syringe and pump (74900 Series, Cole-Parmer Instrument Company, Vernon Hills, IL) via a filter and extension line. A baseline blood sample was taken prior to commencing a primed constant infusion of L-[*ring*-$^{13}C_6$] phenylalanine (99% enriched; Cambridge Isotopes, Andover, MA) at a rate of 0.5 $\mu mol \cdot kg^{-1} \cdot min^{-1}$ (prime; 2 $\mu mol \cdot kg^{-1} \cdot min^{-1}$) beginning 10 min into exercise. The exercise protocol totalled 100 min and comprised a warm-up as above, hard intervals of 8 x 2-min at 90% W_{max}, 2 x 5 min at 70% W_{max}, 2 x 2 min at 80% W_{max} and 3 x 1 min at 100% W_{max}) interspersed with recovery intervals at 50% W_{max}, and a cool-down for 8 min at 40% W_{max}. During exercise participants consumed an artificially sweetened electrolyte solution *ad libitum* to maintain hydration and were fan cooled to minimize thermal distress. The volume of electrolyte solution consumed during the first experimental protocol was recorded and repeated during subsequent visits. Following exercise, participants were allowed to quickly shower and towel dry, and the first serving of intervention or control nutrition was ingested exactly 10 min after cessation of exercise and subsequently every 30 min over the first 90 min of the post-exercise recovery period (4 serves). Participant's rested in the supine position for the duration of the recovery period, defined as the time since ingestion of the first serving of intervention nutrition. Muscle biopsies were taken at 30 min into recovery (i.e. 40 min

post-exercise) and at 240 min into recovery from the *vastus lateralis* as described previously (Fu et al. 2009), blotted to remove excess blood, fat and connective tissue and immediately frozen in liquid nitrogen, then stored at -80°C until analysis.

Intervention and control nutrition. The interventions consisted of milk-like beverages containing milk protein concentrate (MPC 470, Fonterra, Auckland, New Zealand) and whey protein isolate (WPI 894, Fonterra, Auckland, New Zealand) (2:1 w/w), L-leucine, maltodextrin and fructose (1:1 w/w), and freeze dried canola oil. The responses to two protein-leucine doses were evaluated. The control beverage was isocaloric with the high-dose and contained zero protein and leucine. Beverages were made up to 1200 mL using water, and split into 4 equal servings. Total 90-min intake of protein, leucine, carbohydrate and fat, were respectively; 23.3/5/180/30 g (low-dose); 70/15/180/30 g (high-dose); 0/0/ 274/30 g (control). All beverages also contained 1.4 g NaCl, 14.4 g vanilla essence, and 3.6 of emulsifier (Paalsgard 0096, Paalsgard A/S, Denmark) per 1200 mL (see Supplementary Data 5.1 Final Ingredients for Manufacture of Supplement at Massey.xlsx). Low-dose protein-leucine represented the estimated minimum quantity to maximally stimulate the skeletal muscle fractional protein synthesis rate. The high-dose protein-leucine was equivalent to that used in a recent study associated with enhanced cycling performance (Thomson, Ali and Rowlands 2011). L-[*ring*-$^{13}C_6$] phenylalanine was added to low-dose and high-dose beverages (0.0199 g and 0.0598 g, respectively) to maintain steady state enrichment.

Sampling and analysis of blood glucose, lactate, insulin and amino acids. Blood samples for plasma phenylalanine enrichment, glucose, lactate and amino acid concentrations, and serum insulin, were taken prior to infusion priming, immediately following exercise (10 min prior to

ingestion of the first intervention beverage) and at 15, 30, 60, 90, 120, 180, and 240 min into recovery from exercise. Blood for glucose and lactate was collected into a chilled EDTA evacuated tubes and centrifuged for 15 min at 1750 g, 4°C; blood for insulin sat in a serum evacuated tubes for 30 min, and was then centrifuged for 30 min at 2000 g. Blood for plasma phenylalanine enrichment was placed in LH evacuated tubes and centrifuged for 10 min at 2500g, 4°C. Perchloric acid extraction of plasma for amino acid analysis was performed as described by Moore *et al.* (Moore et al. 2005). 1 mL samples of supernatant were aspirated to Eppendorf tubes, immediately frozen in liquid nitrogen, and then stored at -80°C until analysis. Glucose and lactate concentrations were determined using an automated analyzer (Bayer RapidLab 800, Bayer HealthCare, Tarrytown, NY). Insulin was assayed via a solid phase two-site enzyme immunoassay kit (Insulin ELISA IS130D 96 Tests, Calbiotech Inc., Spring Valley, CA). Plasma amino acid concentrations were measured by HPLC (Moore et al. 2005).

Analyses

Western Blots. For AMPKα^{Thr172}, AMPKα, mTOR; mTOR^{Ser2448}, p70S6K, p70S6K^{Thr389}, 4E-BP1, and 4E-BP1$^{Thr37/46}$ (Cell Signaling Technology, Beverly, MA) muscle tissue (~40-50 mg) was homogenized in ice-cold homogenization buffer (50 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, 10 mM ß-glycerophosphate, 50 mM NaF, 0.5 mM sodium orthovanadate (all Sigma Aldrich, Poole, UK) and a complete protease inhibitor cocktail tablet (Roche, West Sussex, UK). Homogenates were centrifuged at 22000 g for 10 min at 4°C, before recovery of supernatants representing the sarcoplasmic protein pool. For SIRT1 (AbCam, Cambridge, UK); eEF2 (Novus Biologicals Littleton, CO, USA); 4E-BP1, P-eEF2^{Thr56}, P-rps6$^{Ser240/244}$ (Cell Signaling Technology, Beverly, MA); and α-tubulin (Sigma,

St. Louis, MO, USA), muscle samples (15-20 mg) were homogenized in radioimmunoprecipitation assay buffer (15 mM Tris–HCl pH 8.0, 167 mM NaCl, 1% triton X-100, 0.1% SDS, 0.5% sodium deoxycholate) supplemented with 0.2 mM sodium orthovanadate, and Complete Mini–EDTA free Protease Inhibitor (Roche Diagnostics, West Sussex, UK) immediately before use. Cellular debris was pelleted at 600g x 15 mins at 4°C and supernatant collected.

Protein content of all fractions was determined using the bicinchoninic acid assay (Merck, Darmstadt, Germany, and Thermo Fisher Scientific, Ontario, Canada). Western blotting was performed as previously described (Burd et al. 2010, Breen et al. 2011). Briefly, equal aliquots of protein were separated on sodium dodecyl sulfate polyacrylamide gels and transferred onto 0.2 μm polyvinylidine diflouride membranes (GE-Lifesciences, Uppsala, Sweden) or Protran nitrocellulose membrane (Whatman, Dassel, Germany). Membranes were blocked with either 5% low fat milk, or 1% casein, and incubated overnight at room temperature or 4°C with primary antibody. Membranes were washed with TBST and incubated for 1 hr at room temperature with secondary antibody (Invitrogen Carlsbad, CA, USA, AbCam, and New England Biolabs, UK). Proteins were detected via chemiluminescence (Invitrogen, and GE-Lifesciences) and quantified by densitometry (ImageJ v1.34s 281 software, rsbweb.nih.gov/ij/; and Chemidoc XRS system, Bio-Rad, Hemel Hempstead, UK). Samples for each subject were run in duplicate on the same gel and phosphoproteins quantified relative to α-tubulin or respective total protein abundance.

Plasma and intracellular phenylalanine enrichments. Myofibrillar and mitochondrial protein fractions were isolated from a piece of wet muscle (~80 mg), as described in detail previously

(Coffey et al. 2011). Plasma and intracellular amino acids were isolated, derivatized and analysed for L-[*ring*-$^{13}C_6$] phenylalanine enrichment as described by Moore et al. (Moore et al. 2009). Briefly, plasma amino acids were purified using cation exchange chromatography (Dowex 50WX8-200 resin; Sigma–Aldrich Ltd), converted to N-acetyl-n-propyl ester derivatives, and analysed by gas chromatography combustion-isotope ratio mass spectrometry (GC-C-IRMS: Hewlett Packard 6890; IRMS model Delta Plus XP, Thermo Finnigan, Waltham, MA, USA) while intracellular amino acids were isolated from a separate piece of muscle (~15 mg) and converted to their heptafluorobutyric derivatives before analysis by GC–MS (models 6890 GC and 5973 MS; Hewlett-Packard, Palo Alto, CA). Plasma and intracellular phenylalanine enrichments were determined using electron-impact ionization by monitoring ions 316 and 322 (m+0 and m+6, respectively).

Muscle fractional protein synthesis calculations. The myofibrillar and mitochondrial protein FSR was calculated using the standard precursor-product method, as follows:

$$FSR\ (\%\cdot h^{-1}) = \Delta E_p / E_m \times 1 / t \times 100$$

Where ΔE_p is the change in protein-bound L-[*ring*-$^{13}C_6$] phenylalanine enrichment between the 30 min and 240 min biopsies, E_m is the average enrichment of intracellular phenylalanine across the two biopsies, and t is the time between biopsies.

Statistical analysis. The effect of treatment and time on all dependent variables was estimated from mixed models (Proc Mixed, SAS Version 9.1; SAS Institute, Cary, NC). Where appropriate data were log transformed prior to analysis. Uncertainty was presented as 90% confidence limits and P-value; inference was by effect size (standardised difference for mean

effect comparisons, the correlation coefficient for correlations) magnitude (Hopkins, Marshall, Batterham and Hanin 2009) and by a pharmacokinetic threshold of 25% and the 90% confidence interval for FSR outcome. Inclusion filter criteria for the correlation and regression of plasma amino acid concentrations with phosphoproteins and FSR was a small correlation ($r \geq 0.1$). Thresholds for assigning qualitative terms to chances of substantial ($r > 0.1$) effects: <0.5%, almost certainly not; <5.0%, very unlikely; <25%, unlikely; <75%, possible; >75%, likely; >95%, very likely; >99.5%, almost certain. Lower correlations required greater certainty to be included as a likely-to-almost certainly clear outcome, where: r 0.1<0.3, 99% confidence (clinical type 1 error ≤0.5%); r 0.3<0.5, 95% confidence (2.5%); r ≥0.5, 90% confidence (5%). Therefore, a correlation was unclear if the likelihood for an opposing correlation overlapped into the defined type 1 error probability. Effect size thresholds for correlations were: <0.1 trivial, <0.3 small, <0.5 moderate, <0.7 large, <0.9 very large, <1.0 almost perfect, 1.0 perfect (Hopkins, Marshall, Batterham and Hanin 2009).

RESULTS

Skeletal muscle myofibrillar fractional protein synthetic rate

There was a large increase in myofibrillar FSR with high-dose feeding (mean FSR%·h^{-1} ± SD; 0.103%·h^{-1} ± 0.027) versus control (0.069%·h^{-1} ± 0.014), and a small (statistically non-significant) increase versus low-dose (0.092%·h^{-1} ± 0.017); low-dose feeding resulted in a moderate increase in myofibrillar FSR versus control (Fig. 5.2; Table 5.1A/B).

Table 5.1A. *The effect of protein-leucine dose on myofibrillar protein synthesis using 0.2 x SD in control (4.69%) as the threshold for substantiveness.*

Parameter[a]	Mean fold-effect[b]; ×/÷90% CL[c]	Effect Size[d]; ±90% CL[c]	P-value	Outcome[e]
		Myofibrillar protein synthesis rate		
L - C	1.33; ×/÷1.12	1.16; ±0.59	4.6E-05	moderate⇧ almost certain
H - C	1.51; ×/÷1.12	1.66; ±0.70	1E-05	large⇧ almost certain
H - L	1.13; ×/÷1.12	0.51; ±0.49	0.073	small⇧ likely

[a]Abbreviations. L-C, low-dose minus control; H-C, high-dose minus control; H-L, high-dose minus low-dose.
[b]Data are overall mean effects for the sampling period derived from the statistical analysis and represent the fold-difference for the relevant comparison.
[c]Multiple and divide or add and subtract this number by the mean effect to obtain the upper and lower 90% confidence limits.
[d]Effect size thresholds: <0.2 trivial, <0.6 small, <1.2 moderate, <2.0 large, <4.0 very large, >4.0 extremely large.
[e]Thresholds for assigning qualitative terms to chances of substantial effects: <0.5%, almost certainly not; <5.0%, very unlikely; <25%, unlikely; <75%, possible; >75%, likely; >95%, very likely; >99.5%, almost certain. An effect is unclear if its confidence interval includes both substantial increases and decreases. Arrow symbols indicate an increase (⇧) or decrease (⇩).

Table 5.1B. *The effect of protein-leucine dose on myofibrillar protein synthesis using the pharmacokinetic 25% difference as the threshold for substantiveness.*

Parameter[a]	Mean fold-effect[b]; ×/÷90% CL[c]	Effect Size[d]; ±90% CL[c]	P-value	Outcome[e]
		Myofibrillar protein synthesis rate		
L - C	1.33; ×/÷1.12	1.16; ±0.59	4.6E-05	moderate⇧ likely
H - C	1.51; ×/÷1.12	1.66; ±0.70	1E-05	large⇧ almost certain
H - L	1.13; ×/÷1.12	0.51; ±0.49	0.073	trivial effect likely

Abbreviations. L-C, low-dose minus control; H-C, high-dose minus control; H-L, high-dose minus low-dose.

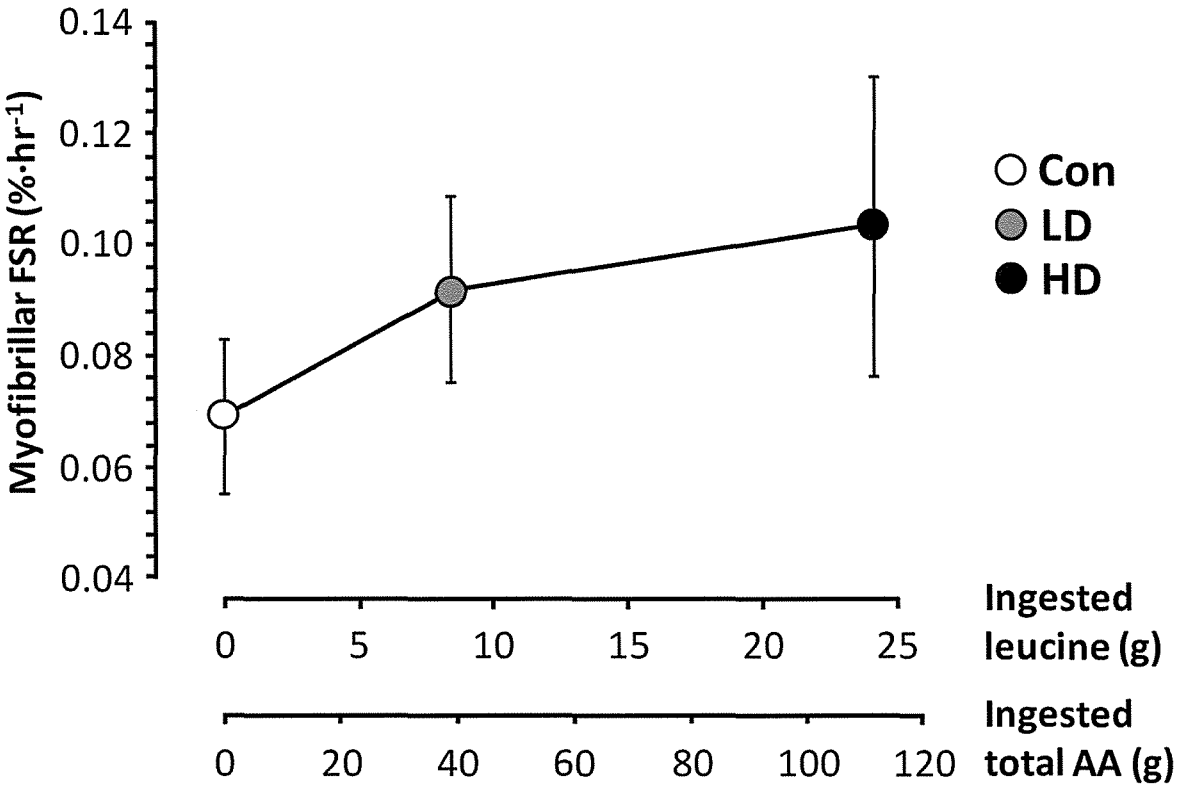
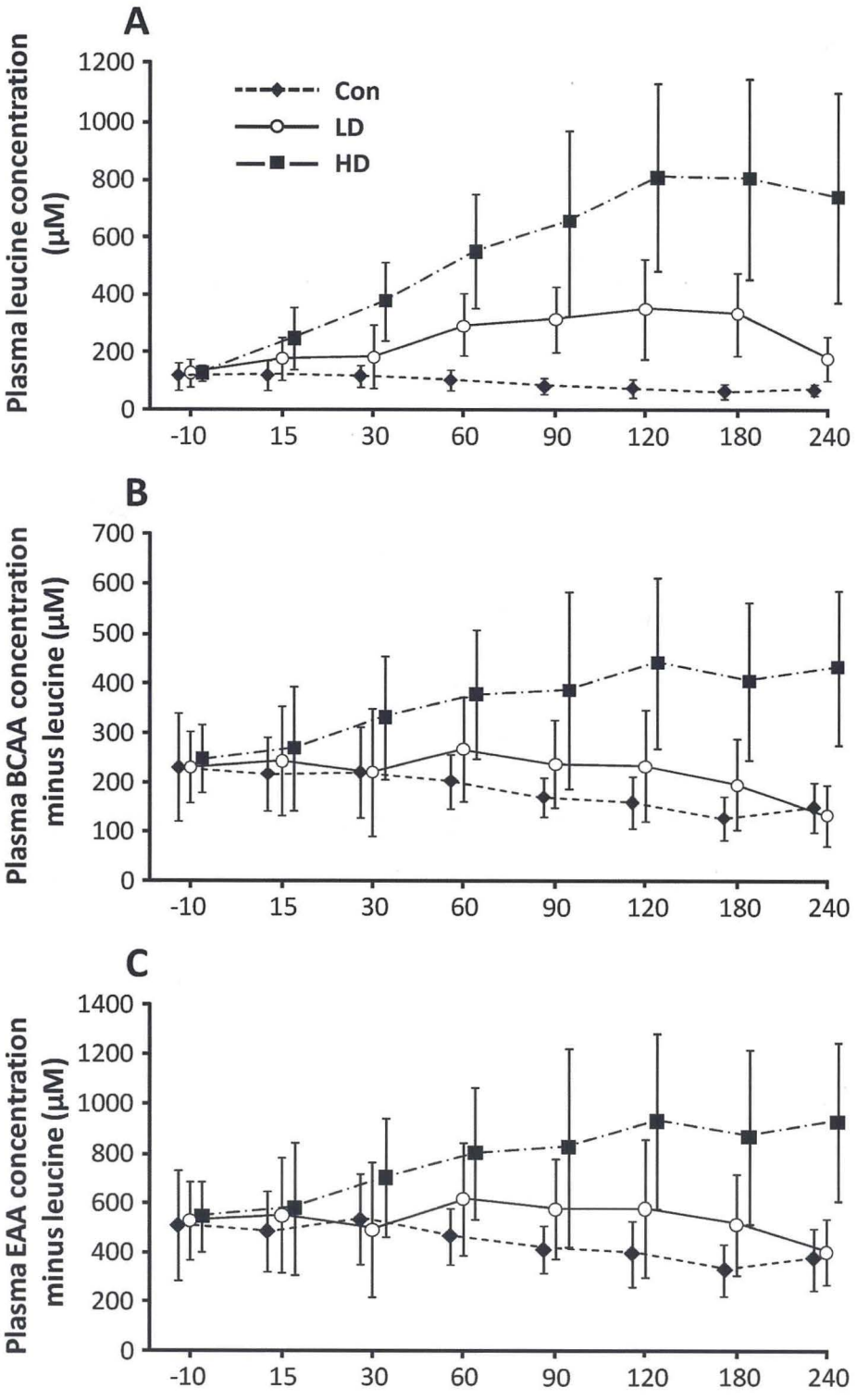


Figure 5.2. Effect of protein-leucine dose on myofibrillar FSR. The x-axes are the total ingested quantity of leucine, or total amino acid (AA) equivalent derived from ingested protein and free leucine. Con, control; LD, low-dose protein-leucine; HD, high-dose protein-leucine. Data are means and standard deviation.

There was insufficient tissue to complete the analysis of mitochondrial FSR. Pre-study, it was estimated that 70 mg of muscle was needed for mitochondrial fraction extraction, but post-study 100 mg was required (Stuart Phillips, pers. comms). From n=1, mitochondrial protein synthesis rates were: control, 0.85%·h^{-1}; low-dose, 0.47%·h^{-1}; high-dose, 0.85%·h^{-1}.

Plasma amino acid concentrations

High-dose resulted in moderate-to-large (effect size) increases in plasma concentrations of leucine, branch-chain, and essential amino acid concentrations (1.73-fold to 2.18-fold; ×/÷1.24 to 1.32, $p<0.001$) versus low-dose, and very large-to-extremely large increases versus control (2.80-fold to 7.97-fold; ×/÷1.30 to 1.40, $p<0.001$). Low dose protein-leucine led to moderate-to-very large increases versus control (1.62-fold to 3.65-fold; ×/÷1.29 to 1.39, $p<0.001$) (Fig. 5.3A-C). There were overall small increases in total plasma amino acid concentration with high-dose versus low-dose (1.29-fold; ×/÷1.30-fold, $p=0.060$) and control (1.35-fold; ×/÷1.31-fold, $p=0.029$); however, there was no clear difference between low-dose and control for total plasma amino acid concentration (1.05-fold; ×/÷1.30-fold, $p=0.701$) (Fig. 5.3D).


A
Plasma leucine concentration (μM)
1200
1000
800
600
400
200
0
Con
LD
HD
-10
15
30
60
90
120
180
240
B
Plasma BCAA concentration minus leucine (μM)
700
600
500
400
300
200
100
0
-10
15
30
60
90
120
180
240
C
Plasma EAA concentration minus leucine (μM)
1400
1200
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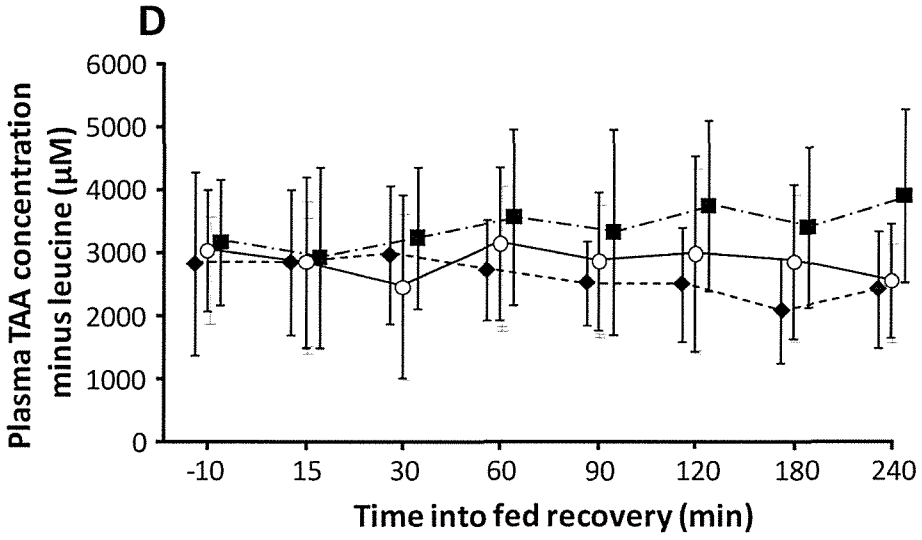


Figure 5.3. Effect of low-dose (LD), high-dose (HD) and control (Con) recovery supplements on plasma concentrations of **A)** leucine and, excluding the leucine concentration, the **B)** branch-chain amino acid (BCAA), **C)** essential amino acid (EAA) and **D)** total amino acid (TAA) concentrations. Negative numbers represent time before ingestion of the first recovery supplement beverage. Values are raw means and bars SD.

Western blotting

Table 5.2 and Fig. 5.4A-H summarise the results. The effect of treatments on AMPKα phosphorylation was mostly unclear, except for a likely small increase in high-dose versus low-dose at 30 min. High-dose protein-leucine moderately increased 30-min mTOR^{Ser2448} phosphorylation versus control and low-dose feeding, but comparisons at 240 min were unclear. There were possible small reductions in 4E-BP1$^{Thr37/46}$ phosphorylation with low-dose versus control (at 240 min) and high-dose versus control (at 30 and 240 min). However, there was greater hyper-phosphorylation of 4E-BP1 to its gamma isoform. At 30 min there was a possible small increase in 4E-BP1α with high-dose versus control feeding, and at 240

min very likely moderate and small increases with high-dose versus control and versus low-dose, respectively. There were small-to-moderate increases in p70S6K^{Thr389} and rps6$^{Ser240/244}$ phosphorylation at 30 min and 240 min with low-dose and high-dose versus control. High-dose almost certainly moderately increased p70S6K^{Thr389} phosphorylation relative to low-dose at 30 min, but at 240 min there was no clear outcome; high-dose feeding had a likely trivial effect on rps6$^{Ser240/244}$ phosphorylation at 30 min versus low-dose, but led to an almost certain small increase in phosphorylation at 240 min. While there was no clear effect of treatments on eEF2^{Thr56} phosphorylation at 30 min, at 240 min there were possible and likely small increases in phosphorylation with high-dose and low-dose feeding versus control, respectively. There was a likely small increase in total SIRT1 protein with high-dose versus low-dose at 30 min, otherwise effects were unclear or trivial.

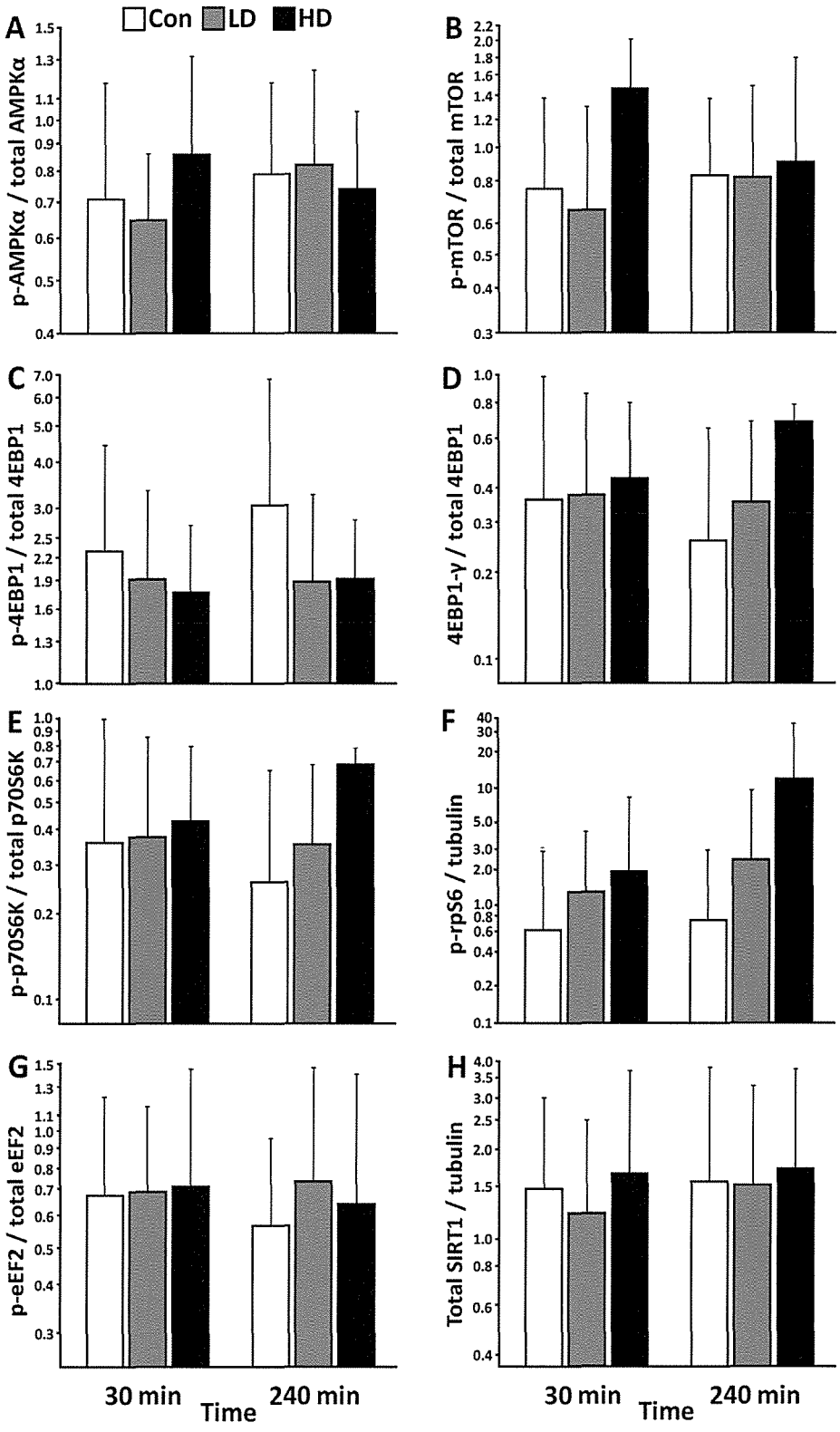


Figure 5.4. Effect of low-dose (LD), high-dose (HD) and control (Con) recovery supplements on phosphorylation of **A**) AMPKα^{Thr172} to total AMPKα, **B**), mTOR^{Ser2448} to total mTOR, **C**) 4E-BP1$^{Thr37/46}$ to total 4E-BP1, **D**) hyper-phosphorylated 4E-BP1 (4E-BP1γ

to total 4E-BP1), **E)** p70S6K^{Thr389} to total p70S6K, **F)** rpS6$^{Ser240/244}$ to tubulin, **G)** eEF2^{Thr56} to total eEF2, and **H)** total SIRT1 protein, quantified by chemiluminesence and densitometry. Values are log-transformed means ± SD.

Table 5.2. *Effect of protein-leucine dose by way of the low-dose, high-dose and control recovery supplements on the phosphorylation of AMPKα^{Thr172}, mTOR^{Ser2448}, 4E-BP1$^{Thr37/46}$, 4E-BP1α to total 4E-BP1, p70S6K^{Thr389}, rpS6$^{Ser240/244}$, eEF2^{Thr56}; and of total SIRT1 protein content relative to tubulin.*

Parameter	Mean fold-effect; ×/÷90% CL	P-value	Outcome
	phospho-AMPKα^{Thr172}:total AMPKα		
30 min			
L - C	0.87; ×/÷1.40	0.501	unclear
H - C	1.15; ×/÷1.40	0.488	unclear
H - L	1.32; ×/÷1.40	0.179	small⇧ likely
240 min			
L - C	1.05; ×/÷1.40	0.803	unclear
H - C	0.95; ×/÷1.40	0.813	unclear
H - L	0.91; ×/÷1.40	0.628	trivial effect possibly
240 min – 30 min			
L - C	1.20; ×/÷1.62	0.514	unclear
H - C	0.83; ×/÷1.62	0.511	unclear
H - L	0.69; ×/÷1.62	0.197	small⇩ possibly
	phospho-mTOR^{Ser2448}:total mTOR		
30 min			
L - C	0.85; ×/÷1.59	0.564	unclear
H - C	1.89; ×/÷1.59	0.028	moderate⇧ very likely
H - L	2.21; ×/÷1.59	0.008	moderate⇧ very likely
240 min			
L - C	0.97; ×/÷1.59	0.908	unclear
H - C	1.07; ×/÷1.59	0.816	unclear
H - L	1.10; ×/÷1.59	0.728	unclear
240 min – 30 min			
L - C	1.14; ×/÷1.93	0.743	unclear
H - C	0.56; ×/÷1.93	0.149	moderate⇩ likely
H - L	0.50; ×/÷1.93	0.081	moderate⇩ likely

Table 5.2. *Western blotting continued*

Parameter	Mean fold-effect; ×/÷90% CL	P-value	Outcome
	phospho-4E-BP1$^{Thr37/46}$:total 4E-BP1		
30 min			
L - C	0.87; ×/÷1.52	0.580	unclear
H - C	0.77; ×/÷1.52	0.286	small⇩ possibly
H - L	0.88; ×/÷1.52	0.601	unclear
240 min			
L - C	0.63; ×/÷1.52	0.070	small⇩ possibly
H - C	0.64; ×/÷1.52	0.080	small⇩ possibly
H - L	1.02; ×/÷1.52	0.946	unclear
240 min – 30 min			
L - C	0.72; ×/÷1.80	0.353	small⇩ possibly
H - C	0.84; ×/÷1.80	0.605	unclear
H - L	1.16; ×/÷1.80	0.676	unclear
	4E-BP1γ:total 4E-BP1		
30 min			
L - C	1.24; ×/÷1.69	0.502	unclear
H - C	1.46; ×/÷1.72	0.247	small⇧ possibly
H - L	1.18; ×/÷1.63	0.569	unclear
240 min			
L - C	1.26; ×/÷1.60	0.422	trivial effect possibly not
H - C	2.50; ×/÷1.63	0.003	moderate⇧ very likely
H - L	1.99; ×/÷1.63	0. 022	small⇧ very likely
240 min – 30 min			
L - C	1.02; ×/÷2.03	0.969	unclear
H - C	1.72; ×/÷2.06	0.217	small⇧ likely
H - L	1.69; ×/÷1.99	0.206	small⇧ likely
	phospho-p70S6K^{Thr389}:total p70S6K		
30 min			
L - C	1.90; ×/÷1.93	0.108	small⇧ likely
H - C	6.67; ×/÷1.93	5.2E-05	moderate⇧ almost certain
H - L	3.51; ×/÷1.93	0.003	moderate⇧ almost certain
240 min			
L - C	1.75; ×/÷1.93	0.159	moderate⇧ likely

Table 5.2. *Western blotting continued*

Parameter	Mean fold-effect; ×/÷90% CL	P-value	Outcome
240 min			
L - C	1.75; ×/÷1.93	0.159	moderate⇧ likely
H - C	1.98; ×/÷1.93	0.087	moderate⇧ likely
H - L	1.13; ×/÷1.93	0. 744	unclear
240 min – 30 min			
L - C	0.92; ×/÷2.52	0.878	unclear
H - C	0.30; ×/÷2.52	0.034	moderate⇩ very likely
H - L	0.32; ×/÷2.52	0.048	moderate⇩ very likely
	phospho-rpS6:tubulin		
30 min			
L - C	2.13; ×/÷1.37	1.1E-04	small⇧ likely
H - C	3.17; ×/÷1.37	1.6E-08	small⇧ almost certain
H - L	1.49; ×/÷1.37	0.039	trivial effect likely
240 min			
L - C	3.33; ×/÷1.37	4E-09	small⇧ almost certain
H - C	16.2; ×/÷1.37	4E-28	moderate⇧ almost certain
H - L	4.85; ×/÷1.37	2E-13	small⇧ almost certain
240 min – 30 min			
L - C	1.56; ×/÷1.56	0.097	trivial effect possible
H - C	5.11; ×/÷1.56	1.5E-08	small⇧ almost certain
H - L	3.27; ×/÷1.56	2.2E-05	small⇧ almost certain
	phospho-eEF2^{Thr56}:total eEF2		
30 min			
L - C	1.02; ×/÷1.25	0.861	unclear
H - C	1.04; ×/÷1.25	0.624	unclear
H - L	1.07; ×/÷1.25	0.750	unclear
240 min			
L - C	1.30; ×/÷1.25	0.049	small⇧ likely
H - C	1.15; ×/÷1.25	0.311	small⇧ possible
H - L	0.88; ×/÷1.25	0.334	trivial effect possible
240 min – 30 min			
L - C	1.27; ×/÷1.37	0.206	small⇧ possible
H - C	1.07; ×/÷1.37	0.716	unclear
H - L	0.84; ×/÷1.37	0.364	small⇩ possible

Table 5.2. *Western blotting continued*

Parameter	Mean fold-effect; ×/÷90% CL	P-value	Outcome
	total SIRT1:tubulin		
30 min			
L - C	0.84; ×/÷1.33	0.317	trivial effect possible
H - C	1.16; ×/÷1.34	0.393	trivial effect possible
H - L	1.38; ×/÷1.33	0.060	small⇧ likely
240 min			
L - C	0.92; ×/÷1.34	0.625	trivial effect likely
H - C	1.06; ×/÷1.33	0.753	trivial effect likely
H - L	1.15; ×/÷1.32	0.401	trivial effect likely
240 min – 30 min			
L - C	1.09; ×/÷1.55	0.720	unclear
H - C	0.91; ×/÷1.51	0.698	unclear
H - L	0.83; ×/÷1.49	0.441	trivial effect possible

Abbreviations: L-C, low-dose minus control; H-C, high-dose minus control; H-L, high-dose minus low-dose.

Plasma glucose, lactate and insulin during recovery

Fig. 5.5 and Table 5.3 summarise the results. High-dose protein-leucine feeding resulted in the largest recovery serum insulin response, while the carbohydrate-only control elicited the smallest insulin response: low-dose resulted in a moderate increase. Correspondingly, high-dose had the lowest overall plasma glucose response, while the control had the highest. The plasma lactate responses to treatments were similar in direction to the changes in plasma glucose concentration.

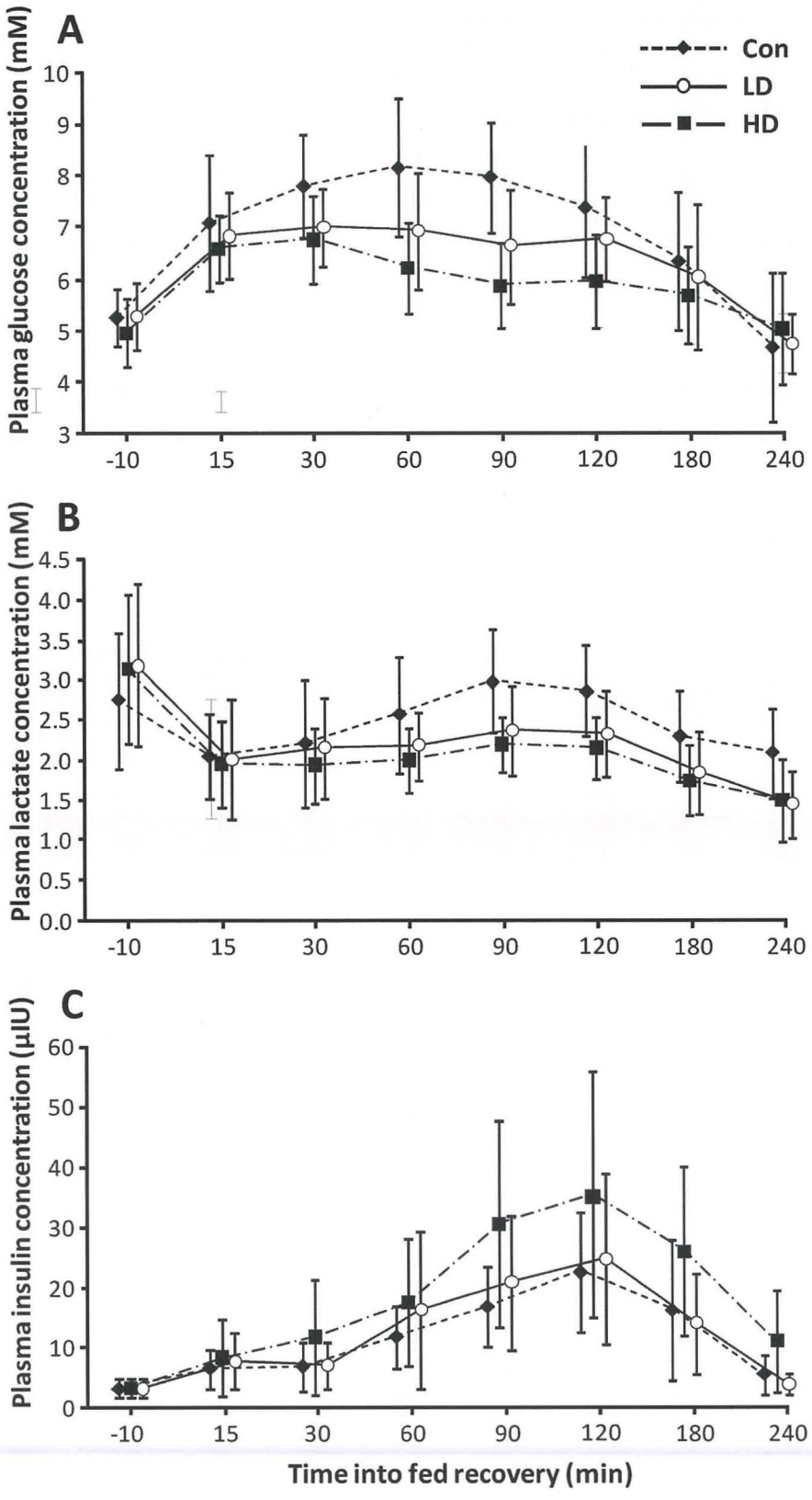


Figure 5.5. Effect of low-dose (LD), high-dose (HD) and control (Con) recovery supplements on **A)** plasma glucose **B)** plasma lactate and **C)** serum insulin concentrations

during recovery from 100 min of high-intensity interval cycling. Negative numbers represent time before ingestion of the first recovery supplement beverage. Data are means and standard deviation.

Table 5.3. *Effect of protein-leucine dose by way of the low-dose, high-dose and control recovery supplements on overall plasma glucose, lactate and serum insulin concentrations during recovery.*

Parameter	Mean fold-effect; ×/÷90% CL	Effect Size; ±90% CL	P-value	Outcome
		Serum insulin concentration		
H - C	1.57; ×/÷1.18	0.37; ±0.14	14E-6	small⇧ very likely
H - L	1.44; ×/÷1.18	0.30; ±0.14	45E-5	small⇧ likely
L - C	1.09; ×/÷1.18	0.07; ±0.14	0.387	trivial effect likely
		Plasma glucose concentration		
H - C	0.76; ×/÷1.06	-0.80; ±0.19	2E-12	moderate ⇩ almost certain
H - L	0.89; ×/÷1.06	-0.36; ±0.18	0.001	small ⇩ likely
L - C	0.86; ×/÷1.06	-0.45; ±0.18	47E-6	small ⇩ very likely
		Plasma lactate concentration		
H - C	0.75; ×/÷1.10	-0.85; ±0.28	5E-7	moderate ⇩ almost certain
H - L	0.92; ×/÷1.10	-0.23; ±0.27	0.16	small ⇩ possibly
L - C	0.81; ×/÷1.10	-0.62; ±0.28	2E-4	moderate ⇩ very likely

Abbreviations. H-C, high-dose minus control; H-L, high-dose minus low-dose; L-C, low-dose minus control.

Correlations between plasma amino acid concentrations, mTOR-pathway phosphoproteins and myofibrillar FSR in response to protein-leucine feeding during recovery from endurance exercise

Table 5.4 shows the outcome of the analysis of correlations between mean plasma leucine, EAA and TAA concentrations, mTOR-pathway phosphoproteins and myofibrillar FSR during recovery. Correlations were ranked and defined as statistically clear or unclear based on the following certainty criteria: r 0.1<0.3 (small), 99% confidence; r 0.3<0.5 (moderate), 95% confidence; r ≥0.5 (large), 90% confidence. All calculations are given in Supplementary

Data 5.2 Correlations and Regressions of PlasmaAA mTOR-pathway phosphoproteins and myofibrillar FSR.xlsx.

Correlations between plasma amino acid concentration and signalling phosphoproteins and myofibrillar FSR. Clear moderate correlations were found between the mean plasma leucine and EAA concentrations and myofibrillar FSR over 240-min period of post-exercise recovery. Upstream, the phosphorylation of p70S6K, rpS6, and the hyper-phosphorylated 4E-BP1γ at 240 min were clearly moderately positively correlated with myofibrillar FSR (Table 5.4).

Correlations between plasma amino acids and signalling phosphoproteins. At 30 min into recovery, there were clear moderate positive correlations between mean plasma leucine concentration and phosphorylation of mTOR and p70S6K phosphorylation, and between EAA concentrations and mTOR phosphorylation; however, correlations at 240 min were unclear. Correlation between 4E-BP1 phosphorylation at Thr37/46 and mean plasma leucine, EAA and TAA concentrations were clear and moderately negative only at 240 min into recovery. On the other hand, at 240 min there were clear large (leucine, EAA) and moderate (TAA) positive correlations with hyper-phosphorylated 4E-BP1γ. RpS6 phosphorylation and mean plasma leucine, EAA and TAA concentrations showed clear large positive correlations at 240 min into recovery, but only clear moderate positive correlation with leucine at 30 min. Correlations between plasma amino acids and phosphorylated eEF2 were unclear, apart from small negative correlations between eEF2 phosphorylation and mean plasma valine concentration at 30 min into recovery, and mean plasma isoleucine concentration at 240 min.

Correlations between total plasma amino acid concentration and phosphorylated AMPKα and total SIRT1 protein did not meet the inclusion threshold criteria (Table 5.4).

We evaluated if the moderate relationship between the phosphoproteins and TAA (i.e. 4E-BP1$^{Thr37/46}$, 4E-BP1γ, rpS6) could be related to individual amino acids other than leucine, isoleucine and valine. Clear negative correlations were found between 4E-BP1$^{Thr37/46}$ and 10 of the 15 mean plasma amino acid concentrations at 240 min, with likely to very likely moderate correlations with glutamine, taurine, tyrosine, cysteine, lysine; and very likely-to-almost certain large correlations with threonine, arginine, alanine, methionine, phenylalanine (Supplementary Data 5.2, Tab 'AA vs PtoT_Thr37&46_4EBP1', cells DI220-DS235). In contrast, clear positive correlations were found between 4E-BP1γ and 10 mean plasma amino acid concentrations at 240 min with moderate correlations for serine, glycine, threonine, arginine, alanine, proline, tyrosine, methionine and phenylalanine, and an almost certain large correlation with lysine (Supplementary Data 5.2, Tab 'AA vs PtoT 'AA vs GAMMAtoT_4EBP1', cells DI220-DS235). Lastly, at 30 min, phosphorylated rpS6 was moderately positively correlated with taurine and threonine, but negatively correlated with asparagine; by 240 min there were 12 amino acids clearly positively correlated with rpS6 phosphorylation, with small (glycine, methionine), moderate (asparagine, taurine, threonine, arginine, alanine, proline, lysine, phenylalanine) and large (tyrosine) correlations (Supplementary Data 5.2, Tab 'AA vs PtoT 'AA vs pRPS6toTUB', cells DI220-DS235).

Linear modelling of the effect of plasma amino acid concentration on FSR and the phosphorylation of proteins regulating translation

Multiple regression linear models were used to describe the effect of linear magnitude increase in recovery mean plasma amino acid concentration on the phosphorylation state of mTOR-pathway phosphoproteins and on myofibrillar FSR, and the phosphoproteins with FSR. Statistical certainty for models was the same as that used for identification clear correlation (r) relationships, with the clearly correlated dependent and independent variables shown in Fig. 5.6. Linear regression scatterplots (Fig. 5.7) show the relationship between plasma leucine concentration, key mTOR-pathway translational control phosphoproteins and FSR.

Myofibrillar FSR. Clear predictors of myofibrillar FSR were p70S6K and rpS6 phosphorylation (Fig. 5.7A, B) and 4E-BP1γ hyper-phosphorylation at 240 min, and the mean plasma leucine (Fig. 5.7C), isoleucine and EAA concentrations over 240-min recovery. An increase in mean recovery plasma leucine concentration of 210 μM (from a baseline of 125 μM, the mean across treatments) is predicted to result in an increase in mean 4-h recovery FSR of $0.010\%\cdot h^{-1}$ in trained men following 100 min of intense endurance cycling (Supplementary Data 5.2, Tab 'AA vs FSR', cells J589-N613).

mTOR Pathway Phosphoproteins. Briefly, the mean plasma leucine concentration clearly predicted p70S6K (Fig. 5.7D) and rpS6 phosphorylation at 30 min into recovery, and phosphorylated rpS6 (Fig. 5.7E) and 4E-BP1γ at 240 min. Mean 240 min recovery EAA and

TAA concentrations both clearly predicted phosphorylation of 4E-BP1γ and rpS6, and 4E-BP1$^{Thr37/46}$.

Table 5.4. *Outcomes for correlation of dependent (plasma amino acid concentration or mTOR-pathway phosphoprotein phosphorylation state) on independent variables (phosphoprotein phosphorylation state or myofibrillar fractional protein synthesis rate, FSR) in the analysis of the impact of protein-leucine dose on skeletal muscle FSR.*

Dependent variable vs independent variable	*Mean independent variable over 30 min recovery*				*Mean independent variable over 240 min recovery*		
	r; ±90% CL[a]	p-value	Correlation outcome[b]		r; ±90% CL	p-value	Correlation outcome
FSR vs [leucine]	0.21; ±0.27	0.223	unclear		0.42; ±0.23	0.011	moderate positive very likely
FSR vs [isoleucine]	-0.01; ±0.28	0.964	unclear		0.30; ±0.26	0.076	moderate positive likely
FSR vs [EAA]	0.01; ±0.28	0.968	unclear		0.34; ±0.25	0.042	moderate positive likely
FSR vs [TAA]	-0.11; ±0.28	0.540	unclear		0.18; ±0.27	0.281	unclear
FSR vs p-AMPKα	0.13; ±0.34	0.552	unclear		-0.17; ±0.34	0.688	unclear
FSR vs p-mTOR	0.29; ±0.32	0.174	unclear		-0.10; ±0.34	0.062	unclear
FSR vs p-p70S6K	0.31; ±0.31	0.134	unclear		0.41; ±0.29	0.029	moderate positive likely
FSR vs p-4E-BP1$^{Thr37/46}$	-0.13; ±0.34	0.559	unclear		-0.31; ±0.31	0.244	unclear
FSR vs 4E-BP1γ:total	0.08; ±0.30	0.660	unclear		0.41; ±0.25	0.148	moderate positive very likely
FSR vs SIRT1:tubulin	0.02; ±0.29	0.927	unclear		0.26; ±0.26	0.391	unclear
FSR vs p-rpS6:tubulin	0.29; ±0.26	0.089	unclear		0.52; ±0.21	0.026	moderate positive almost certain
FSR vs p-eEF2	0.18; ±0.27	0.296	unclear		0.22; ±0.27	0.183	unclear
p-AMPKα vs [leucine]	0.30; ±0.32	0.150	unclear		0.01; ±0.34	0.310	unclear
p-AMPKα vs [EAA]	0.12; ±0.34	0.566	unclear		-0.25; ±0.32	0.232	unclear
p-AMPKα vs [TAA]	0.04; ±0.34	0.870	unclear		-0.31; ±0.31	0.142	unclear
p-mTOR vs [leucine]	0.35; ±0.31	0.097	moderate positive likely		0.26; ±0.32	0.221	unclear
p-mTOR vs [isoleucine]	0.35; ±0.31	0.097	moderate positive likely		0.29; ±0.32	0.167	unclear
p-mTOR vs [EAA]	0.34; ±0.31	0.103	moderate positive likely		0.26; ±0.32	0.211	unclear
p-mTOR vs [TAA]	0.24; ±0.33	0.253	unclear		0.28; ±0.32	0.190	unclear

Table 5.4. *Outcomes for correlations continued*

Dependent variable vs independent variable	*Mean independent variable over 30 min recovery*				*Mean independent variable over 240 min recovery*		
	r; ±90% CL	p-value	Correlation outcome[b]		r; ±90% CL	p-value	Correlation outcome
p-p70S6K vs [leucine]	0.44; ±0.28	0.032	moderate positive very likely		0.26; ±0.32	0.219	unclear
p-p70S6K vs [EAA]	0.21; ±0.33	0.335	unclear		0.26; ±0.32	0.221	unclear
p-p70S6K vs [TAA]	0.00; ±0.34	0.985	unclear		0.16; ±0.34	0.447	unclear
p-4E-BP1$^{Thr37/46}$ vs [leucine]	-0.24; ±0.33	0.254	unclear		-0.35; ±0.31	0.097	moderate negative likely
p-4E-BP1$^{Thr37/46}$ vs [isoleucine]	-0.20; ±0.33	0.357	unclear		-0.35; ±0.31	0.091	moderate negative likely
p-4E-BP1$^{Thr37/46}$ vs [valine]	-0.13; ±0.34	0.534	unclear		-0.35; ±0.31	0.090	moderate negative likely
p-4E-BP1$^{Thr37/46}$ vs [EAA]	-0.21; ±0.33	0.326	unclear		-0.41; ±0.29	0.046	moderate negative likely
p-4E-BP1$^{Thr37/46}$ vs [TAA]	-0.13; ±0.34	0.551	unclear		-0.46; ±0.28	0.023	moderate negative very likely
4E-BP1γ:total vs [leucine]	0.03; ±0.31	0.895	unclear		0.53; ±0.23	1.6E-3	large positive very likely
4E-BP1γ:total vs [isoleucine]	-0.04; ±0.31	0.853	unclear		0.55; ±0.22	1.0E-3	large positive almost certain
4E-BP1γ:total vs [valine]	-0.05; ±0.31	0.775	unclear		0.52; ±0.23	1.9E-3	large positive very likely
4E-BP1γ:total vs [EAA]	-0.05; ±0.31	0.776	unclear		0.55; ±0.22	9.0E-4	large positive almost certain
4E-BP1γ:total vs [TAA]	-0.14; ±0.3	0.448	unclear		0.44; ±0.25	0.011	moderate positive very likely
SIRT1:tubulin vs [leucine]	0.02; ±0.20	0.862	unclear		0.12; ±0.20	0.319	unclear
SIRT1:tubulin vs [EAA]	-0.07; ±0.2	0.555	unclear		0.07; ±0.20	0.557	unclear
SIRT1:tubulin vs [TAA]	-0.18; ±0.2	0.148	unclear		0.03; ±0.20	0.804	unclear
p-rpS6:tubulin vs [leucine]	0.30; ±0.18	0.010	moderate positive very likely		0.65; ±0.11	<1E-4	large positive almost certain
p-rpS6:tubulin vs [isoleucine]	0.09; ±0.19	0.458	unclear		0.54; ±0.14	<1E-4	large positive almost certain
p-rpS6:tubulin vs [valine]	0.01; ±0.20	0.904	unclear		0.52; ±0.14	<1E-4	large positive almost certain

Table 5.4. *Outcomes for correlations continued*

Dependent variable vs independent variable	*Mean independent variable over 30 min recovery*				*Mean independent variable over 240 min recovery*		
	r; ±90% CL[a]	p-value	Correlation outcome[b]		r; ±90% CL	p-value	Correlation outcome
p-rpS6:tubulin vs [EAA]	0.15; ±0.19	0.207	unclear		0.61; ±0.13	<1E-4	large positive almost certain
p-rpS6:tubulin vs [TAA]	0.14; ±0.19	0.248	unclear		0.50; ±0.15	<1E-4	large positive almost certain
p-eEF2 vs [leucine]	-0.12; ±0.19	0.307	unclear		-0.06; ±0.20	0.635	unclear
p-eEF2 vs [isoleucine]	-0.17; ±0.19	0.147	unclear		-0.22; ±0.19	0.069	small negative likely
p-eEF2 vs [valine]	-0.29; ±0.18	0.016	small negative likely		-0.21; ±0.19	0.072	unclear
p-eEF2 vs [EAA]	-0.15; ±0.19	0.207	unclear		-0.14; ±0.19	0.245	unclear
p-eEF2 vs [TAA]	-0.08; ±0.20	0.524	unclear		-0.12; ±0.19	0.302	unclear

Abbreviations: r, correlation coefficient; EAA, essential amino acids; TAA, total amino acids; FSR, fractional synthesis rate.
[a]Add and subtract this number by the mean effect to obtain the upper and lower 90% confidence limits.
[b]Thresholds for assigning qualitative terms to chances of substantial (r>0.1) effects: <0.5%, almost certainly not; <5.0%, very unlikely; <25%, unlikely; <75%, possible; >75%, likely; >95%, very likely; >99.5%, almost certain. Lower correlations required greater certainty to be included as a likely-to-almost certainly clear outcome, where: r 0.1<0.3, 99% confidence (clinical type 1 error ≤0.5%); r 0.3<0.5, 95% confidence (2.5%); r ≥0.5, 90% confidence (5%). Therefore, a correlation was unclear if the likelihood for an opposing correlation overlapped into the defined type 1 error probability. Effect size thresholds were: <0.1 trivial, <0.3 small, <0.5 moderate, <0.7 large, <0.9 very large, <1.0 almost perfect, 1.0 perfect. For brevity, data for correlations involving isoleucine and valine are only shown if at least one of the time-points met inclusion threshold criteria.

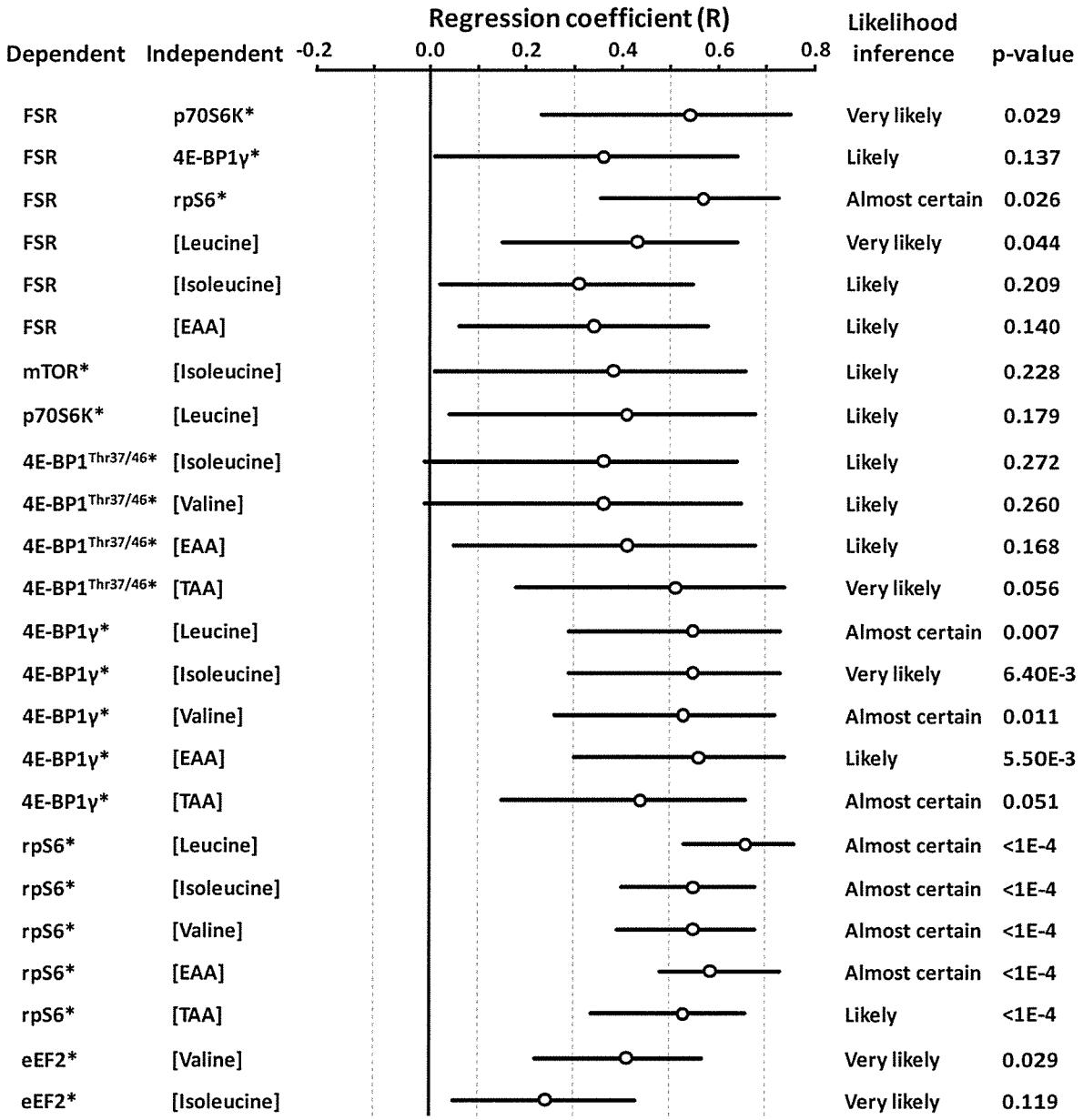
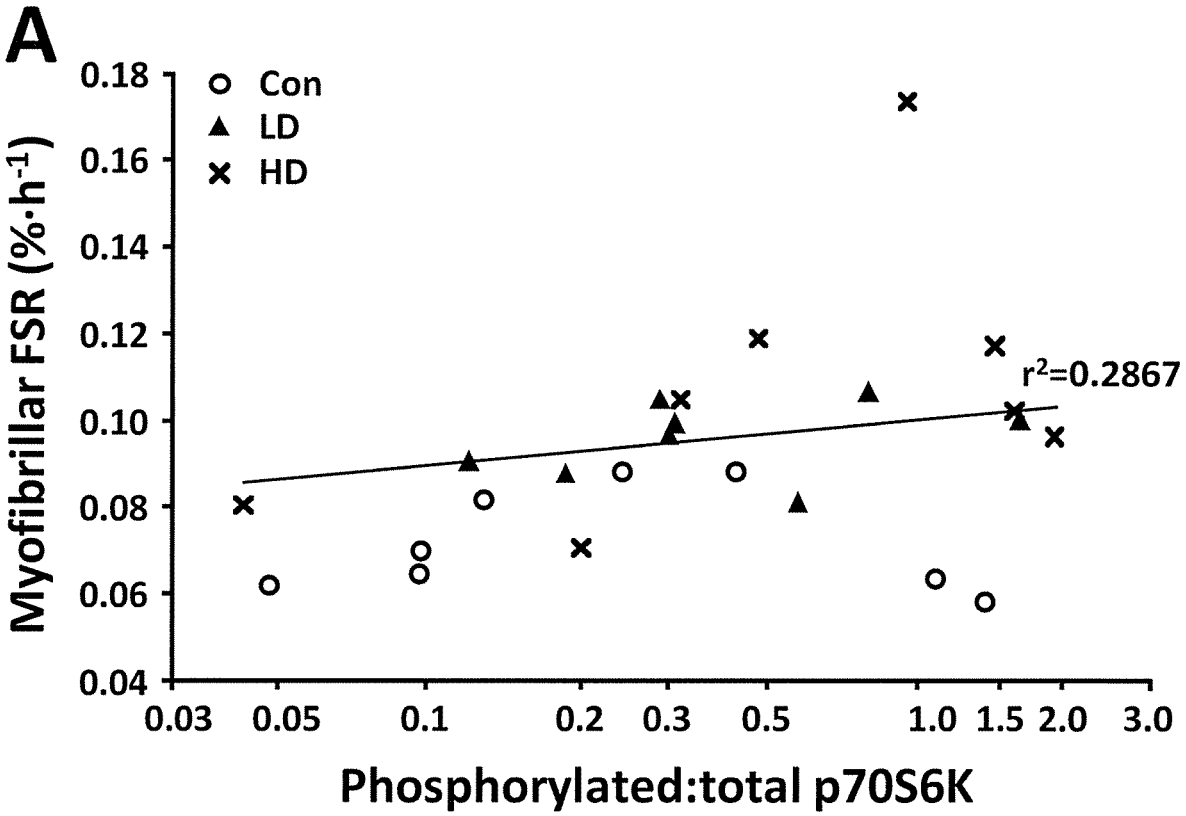
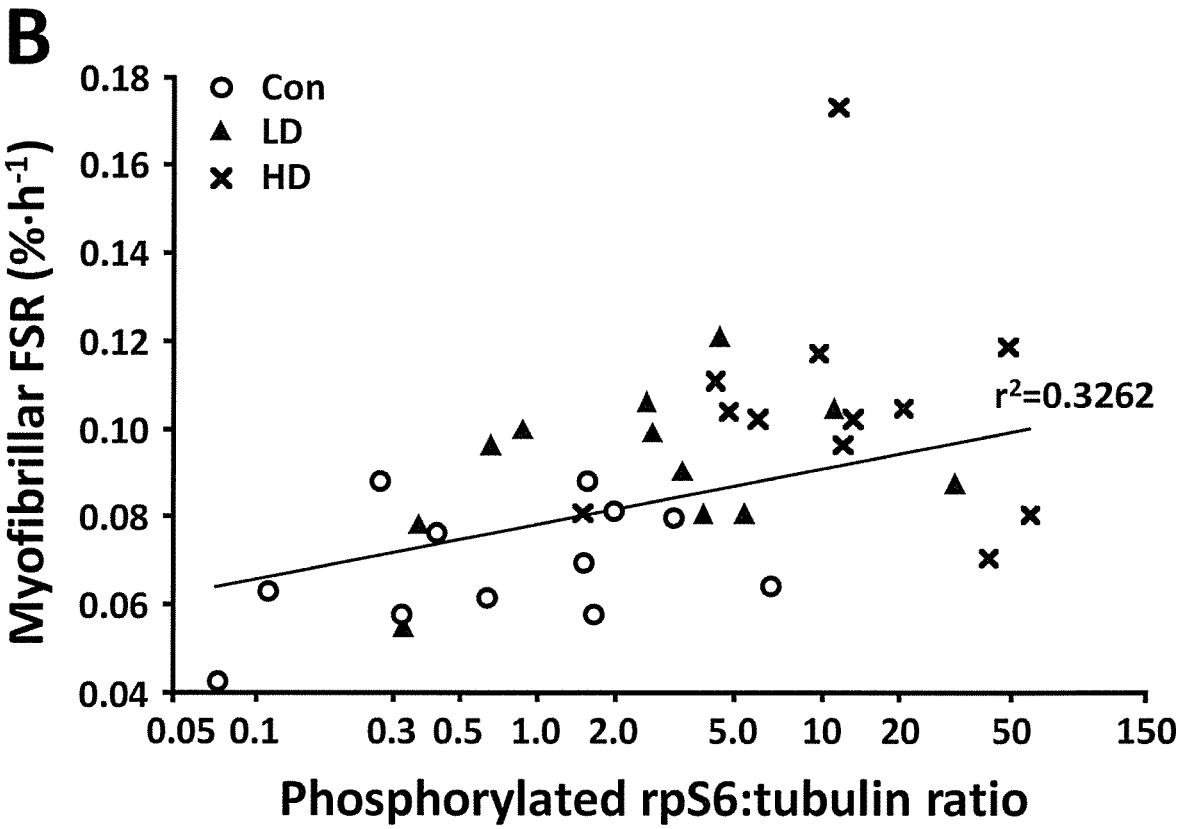
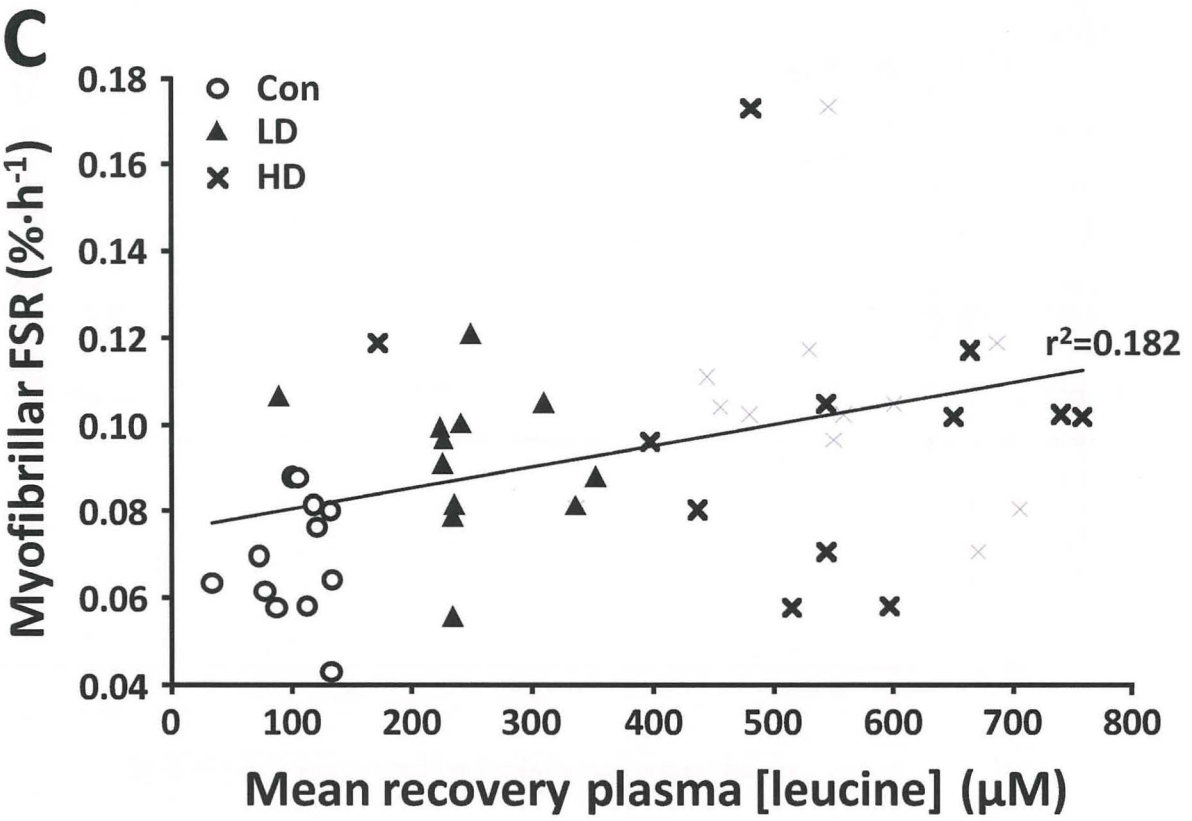


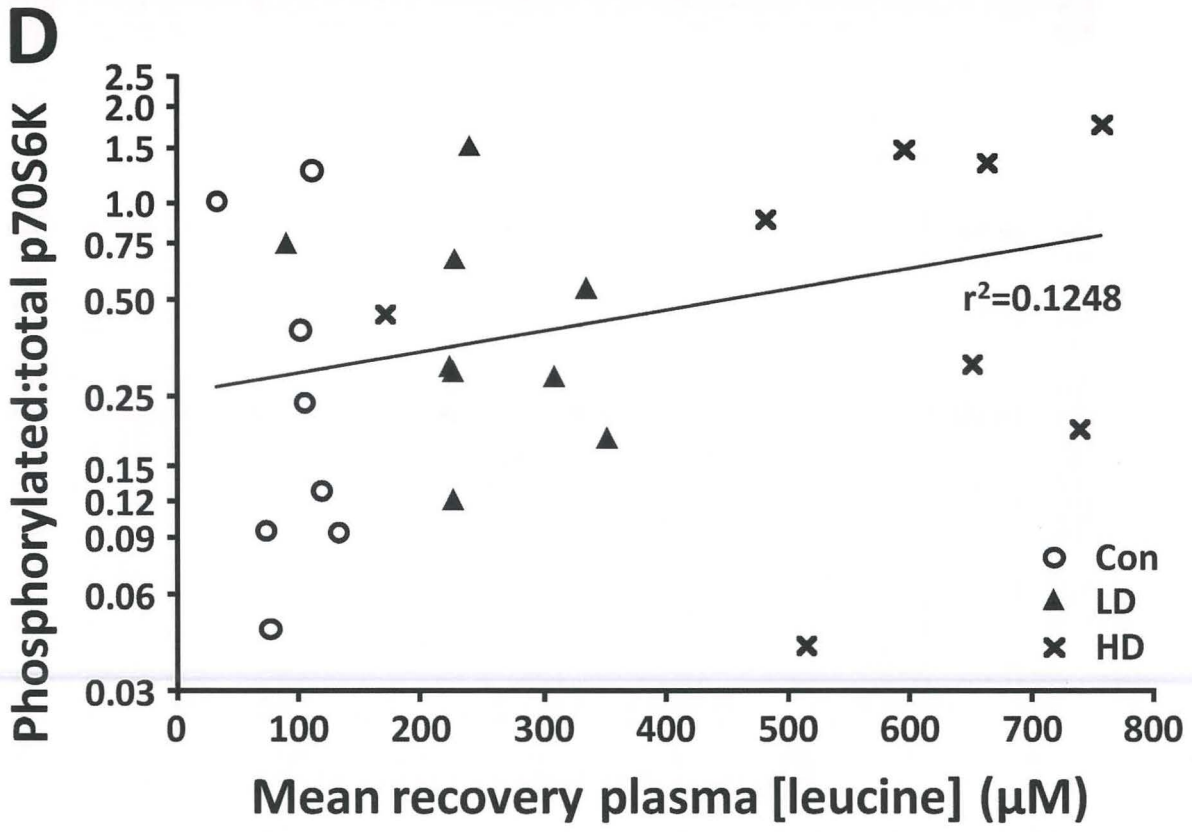
Figure 5.6. Outcome for the regressions of mean plasma amino acid concentration or mTOR-pathway phosphoprotein phosphorylation state on the myofibrillar protein fractional synthetic rate (FSR), and plasma amino acids on phosphoprotein phosphorylation state and myofibrillar FSR. Data are effect size (regression coefficient, R) with 90% confidence interval. Only statistically clear outcomes based on the magnitude-based inference method are presented. Abbreviations: EAA, essential amino acids; TAA, total amino acids. Thresholds for assigning qualitative terms to chances of substantial ($r>0.1$) correlations: <0.5%, almost certainly not;

<5.0%, very unlikely; <25%, unlikely; <75%, possible; >75%, likely; >95%, very likely; >99.5%, almost certain. Lower correlations required greater certainty to be included as a clear outcome (i.e. likely-to-almost certain), where r =0.1<0.3, 99% confidence; r=0.3<0.5, 95% confidence; r >0.5, 90% confidence. Therefore, a correlation was unclear if the likelihood for an opposing correlation overlapped into the lower level of confidence. Effect size thresholds were: <0.1 trivial, <0.3 small, <0.5 moderate, <0.7 large, <0.9 very large, <1.0 almost perfect. For brevity, data for correlations involving isoleucine and valine are only shown if at least one of the time-points met inclusion threshold criteria.*denotes log-transformed variable.


A
Con
LD
HD
Myofibrillar FSR (%·h-1)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
r2=0.2867
0.03 0.05 0.1 0.2 0.3 0.5 1.0 1.5 2.0 3.0
Phosphorylated:total p70S6K


B
Con
LD
HD
Myofibrillar FSR (%·h-1)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
r2=0.3262
0.05 0.1 0.3 0.5 1.0 2.0 5.0 10 20 50 150
Phosphorylated rpS6:tubulin ratio


C
Myofibrillar FSR (%·h-1)
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
Con
LD
HD
r2=0.182
0
100
200
300
400
500
600
700
800
Mean recovery plasma [leucine] (µM)


D
Phosphorylated:total p70S6K
2.5
2.0
1.5
1.0
0.75
0.50
0.25
0.15
0.12
0.09
0.06
0.03
r2=0.1248
Con
LD
HD
0
100
200
300
400
500
600
700
800
Mean recovery plasma [leucine] (µM)

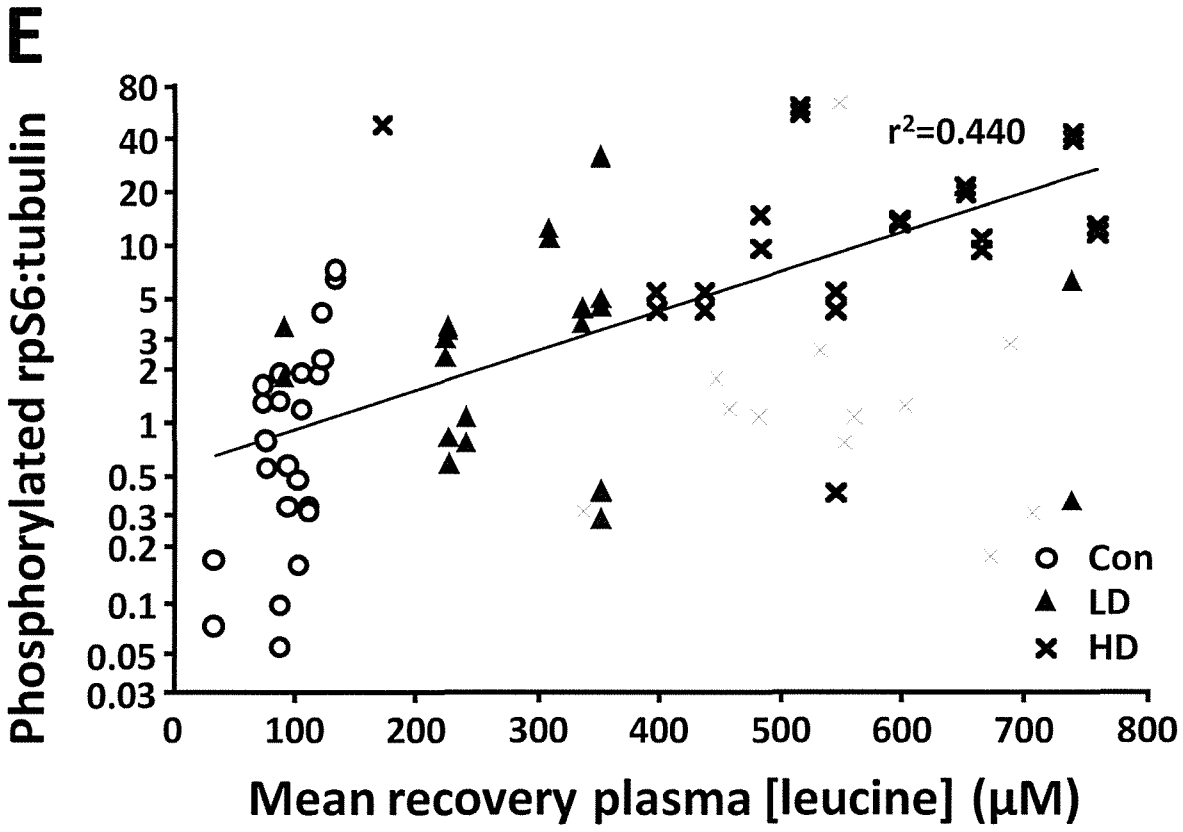


Figure 5.7. Linear regression scatterplot of the dependent variable (y-axis) myofibrillar FSR versus **A)** phosphorylated:total p70S6K and **B)** phosphorylated rpS6:tubulin, **C)** mean recovery plasma leucine concentration; and the dependent variables **D)** phosphorylated:total p70S6K at 30 min and **E)** phosphorylated rpS6:tubulin at 240 min into recovery from 100 min of high-intensity interval cycling versus mean recovery plasma leucine concentration. For A-C, the x-axis; and for D and E, the y-axis; is the log10 of the ratio of phosphoprotein to the reference protein. Lines of best fit represent the model derived from the regression analysis. Con, control; LD, low-dose protein-leucine; HD, high-dose protein-leucine. p70S6K, n=8; FSR and rpS6, n=12.

DISCUSSION

The impact of protein-leucine dose on skeletal muscle myofibrillar protein fractional synthetic rate

The purpose of this document is to report on the effect of ingested protein-leucine dose on skeletal muscle myofibrillar and mitochondrial fractional protein synthesis rate (FSR), and associated dose-regulated signalling control of translation initiation during recovery from high-intensity endurance exercise in well-trained young men. The increases in myofibrillar FSR with low- and high-dose protein-leucine feeding relative to control were large (51%; 90% CI 37-69%) and moderate (33%; 18-49%), respectively. However, while the observed mean increase in myofibrillar FSR with the high-dose supplement was small (13%; 1-26%) relative to control, the magnitude of the increase was statistically trivial (equivalent) relative to the 25% suggested reference standard in bio-equivalence trials in pharmacokinetics. Furthermore, to achieve the 13% increase, an additional 46.6 g of protein and 10 g of free leucine (3-fold increase in dose) was required suggesting that the physiological impact of a large increase in protein and leucine on the rate of adaptive remodelling within the skeletal-muscle contractile proteins may be of only minor physiological consequence. Nevertheless, there was a moderate correlation between plasma leucine concentration and myofibrillar FSR, with the inference from linear regression modelling suggesting that following intense endurance exercise, every 210 μM increase in recovery mean plasma leucine concentration will increase myofibrillar FSR by $0.010\%\cdot h^{-1}$. Such an increase in mean plasma leucine concentration and myofibrillar FSR was attainable through the ingestion of 4.1 g protein and 0.9 g free leucine every 30 min for 90 min following exercise.

Based on the ingested leucine-myofibrilar FSR relationships, the amount of Powerbar ProteinPlus (23 g Trisource™ protein) and ProteinPlus30g (30 g Trisource protein) to achieve a myofibrillar FSR increase of 0.010 $\%\cdot h^{-1}$ is approximately 1.9 and 1.6 bars. Comparatively, to obtain the same myofibrillar FSR to that observed with the current low (0.92 $\%\cdot h^{-1}$) and high dose (0.103 $\%\cdot h^{-1}$) supplement conditions, 4.5 and 12.3 Powerbar ProteinPlus (23 g Trisource protein) and 3.8 and 10.6 ProteinPlus30g (30 g Trisource protein) bars, respectively, would have been ingested revealing the comparative protein-synthetic potency of the current recovery supplement. It has been almost universally reported that soy protein provides inferior muscle protein synthesis outcomes compared to dairy-based proteins (Bos et al. 2003, Luiking, Deutz, Jakel and Soeters 2005, Fouillet et al. 2009, Tang et al. 2009) probably due to differences in amino acid content (e.g. lower leucine) and digestibility. Therefore, replacing the soy component of the Trisource with dairy will likely increase product protein synthetic potency. In terms of study design comparisons to the recently established subsequent performance outcomes, the quantity of total leucine provided over 4 h post-exercise in whole milk proteins in the time-course recovery study by Rowlands et al. (Rowlands et al. 2008) was nearly identical to that provided in the current high dose over 90-min post exercise, whereas the quantity of total leucine provided in the Thomson et al. (Thomson, Ali and Rowlands 2011) study was similar to the low dose; both studies returned clear substantial mean 4.1% and 2.5% enhancements in subsequent performance, respectively. Unfortunately, mitochondrial FSR analysis could not be completed because of insufficient tissue.

The practical objective of the current project was to determine the effect of protein-leucine dose on the myofibrillar protein FSR in men following endurance exercise in order to provide

evidence towards the optimal average dose for protein-leucine based recovery supplements in endurance athletes. Due to ethical limitations on the number of biopsies, we can provide information only on the protein-leucine dose nutritional effect superimposed onto any exercise-induced responses; we were also unable to include a protein only nutrition control. Nevertheless, in order to inform on the protein-leucine nutritional effect versus the exercise and protein-only effects, it is reasonable to compare against standardised effect size in other presently available literature. Without feeding, there was a large increase in post-endurance-exercise mixed-muscle FSR in recreationally active male muscle (Harber et al. 2010) (ES 1.83; ±90%CL 1.47). The addition of protein-carbohydrate post-exercise led to a small increase in mixed-muscle FSR (ES 0.49; ±0.40) relative to fasted (Harber et al. 2010), and a large increase (ES 1.60; ±0.80) versus isocaloric carbohydrate-only in recreationally active men (Howarth, Moreau, Phillips and Gibala 2009), while the increase with isocaloric carbohydrate-matched relative was also large at 1.31 ±0.65 ES (Howarth, Moreau, Phillips and Gibala 2009). With regard to specific protein fractions, Wilkinson et al. (Wilkinson et al. 2008) reported an increase (33%, p=0.12) in myofibrillar FSR which when reanalysed using a threshold for substantial effect of 25% difference between treatment groups (the *a priori* threshold of Wilkinson et al. (Wilkinson et al. 2008)) revealed a likely moderate increase (ES 0.70; ±0.75) in post-exercise myofibrillar FSR in 10-wk endurance-trained skeletal muscle (Supplementary Data 2.4 Wilkinson ES and Inferences.xlsx and Supplementary Data 2.5 Wilkinson ES and Inferences.pptx). More recently, protein-carbohydrate ingested immediately following steady-state endurance cycling (Breen et al. 2011) or co-ingested with additional leucine prior to repeated sprint cycling (Coffey et al. 2011) increased post-exercise myofibrillar FSR versus carbohydrate-only (~54%; ES 1.32; ±0.90) and artificially sweetened water (~48%; ES 0.99; ±0.59), respectively. The effect size in the current study is in-line with the mixed-muscle and myofibrillar FSR data reviewed above and unsurprising considering

the contribution of myofibrillar proteins to total skeletal muscle protein content (Højlund et al. 2007) and the individual rates of synthesis of major proteins (Rooyackers, Adey, Ades and Nair 1996, Hasten, Morris, Ramanadham and Yarasheski 1998). Therefore, it is reasonable to conclude that: a) an acute bout of endurance exercise stimulates a moderate-large increase in myofibrillar FSR; b) post-exercise carbohydrate feeding additively increases FSR by a small ES; c) protein-carbohydrate feeding further increases FSR by a moderate-large ES; and d) high-dose protein-leucine increased FSR relative to carbohydrate by a large ES.

Provision of protein-carbohydrate following endurance or sprint-cycling exercise appears to substantially increases the rate of myofibrillar protein synthesis, which is a marker for the prospects of the myofibril remodelling processes that are directed by the transcriptome and post-translational regulation. However, interpretation of the impact of protein-leucine dose on myofibrillar FSR is difficult given that in the literature it is not clearly established what a substantial or functionally meaningful increase in FSR might be. Until the relationship between chronic nutrient-induced FSR and functional phenotype is established, a pharmacokinetic threshold provides one reference point for the practical interpretation of the current and other comparable data (Haidar et al. 2008). Current European Union (EU) guidelines recommend that for narrow therapeutic index drugs a 90%-111.11%CV acceptance interval is used (http://www.emea.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/01/WC500070039.pdf) (i.e. if the difference and 90% confidence interval lies between a value equivalent to 90-111% of the coefficient of variation (CV) for the outcome it is deemed equivalent. Likewise, to be considered a clear difference the confidence interval should be no less than 5% greater or less than the 90-111% bioequivalent threshold, otherwise the outcome

is unclear). While this provides a rigorous cut-off for substantiveness, the appropriateness with a post-exercise protein feeding-FSR response model is in question, given that the protein nutrition may not be considered a narrow therapeutic index drug. Therefore, we chose to use a flat 25% threshold. Additionally, there are other experimental factors to consider before settling on what is a functionally important change in FSR. Comparatively, post-resistance exercise myofibrillar FSR appears to peak at between 20-30 g whole egg protein (Moore et al. 2009), whereas the current protein-leucine feeding after 100-min of endurance exercise did not show clear sign for plateau in dose response with 25/5 g versus 70/15 g of protein/leucine. While we suggest that the increase may be of trivial biological consequence, and that care must be taken because the current nutrition also included carbohydrate and fat while the study by Moore et al. contained protein only (Moore et al. 2009), interpretation of the confidence interval also indicates that the requirement for protein to maximise muscle-protein synthesis post-endurance exercise could be greater than post-resistance exercise. Additionally, the timing of muscle biopsies requires careful attention before clearer generalised biologically-functional dose-response inferences can be made because there is evidence for early and late transcriptional and protein synthetic responses, which will clearly affect conclusions drawn from sampling proximal or distal to the exercise and nutrition induced signalling. For example, both myofibrillar FSR and muscle gene expression (Breen et al. 2011, Rowlands et al. 2011) are acutely (i.e. within 4 h post) regulated by protein-feeding post-endurance exercise. However, mitochondrial FSR was not substantially altered within a 4-h recovery window by nutrient provision (Breen et al. 2011, Coffey et al. 2011) which could be due to one or more of the later rise of metabolic gene expression in response to endurance exercise (Yang, Creer, Jemiolo and Trappe 2005), an apparent delayed time course (48 h) of the metabolic-mitochondrial transcriptome with nutrient signalling (Rowlands et al. 2011), a relatively low abundance of mitochondrial compared with

myofibrillar proteins, and the time requirement to transport polypeptides and assemble new proteins within the mitochondrion (Hood, Irrcher, Ljubicic and Joseph 2006). Using a 1-legged endurance-kicking model, Miller et al. (Miller et al. 2005) found that myofibrillar and sarcoplasmic FSR peaked 24 h post-exercise and were still elevated above resting values at 48 h post-exercise (72 h for myofibrillar FSR). Comparatively, the myofibrillar FSR response with both the current high-dose and low-dose protein-leucine supplements versus control were greater than that found by Breen et al. (Breen et al. 2011) and Coffey et al. (Coffey et al. 2011) but 0.77-fold lower than that observed by Miller and colleagues (Miller et al. 2005). At least some of the variation in the magnitude of the responses could be accounted for by differences in exercise regimen duration and intensity, the timing and quantity of nutrients provided, and stable-isotope tracer methodologies (Smith, Villareal and Mittendorfer 2007); or could be just due to normal sampling variability with the majority of confidence intervals across studies overlapping, suggesting more highly powered studies are needed to accurately determine the true dose response to protein and protein-leucine feeding, not to mention, better presentation of statistical data and allowance for estimate uncertainty. Furthermore, future workers should consider serial biopsies out to 48 h post-exercise to ensure possible increases in mitochondrial FSR are captured.

Inferences from the mTOR-pathway signalling outcomes

The mTOR intracellular signalling pathway is a key regulator of cellular mRNA translation. We observed clear positive protein-leucine dose response relationships for the phosphorylation of rpS6, and 4E-BP1-γ and 4E-BP1$^{Thr37/46}$ at 240 min; additionally, 30-min rpS6 and p70S6K phosphorylation was increased in a dose-sensitive fashion and mTOR phosphorylation was increased relative to control only with high-dose. The plasma leucine

concentration showed moderate to large correlations with phosphorylation of the mTOR-rpS6 pathway axis. Notwithstanding the statistical uncertainty (confidence interval) and general inferential limitations associated with correlations with low sample sizes, these data revealed that the magnitude of increase in plasma leucine and the translational signalling response through p70S6K moderately aligned with the myofibrillar FSR response.

Recent evidence from C2C12 myotubes cultured with each of the EAAs indicates that only valine and isoleucine do not substantially stimulate phosphorylation of p70S6K and rpS6, with leucine having ~3 times the potency of other EAAs (Atherton et al. 2010). Furthermore, EAA signalling potency appeared to be limited to regulation of mTOR, p70S6K and rpS6 phosphorylation (translation initiation) rather than elongation via eEF2 and eIF2α (Atherton et al. 2010). In agreement with the leucine potency assertion of Atherton et al. (Atherton et al. 2010) we observed that, of the amino acids, leucine had the strongest correlation with almost all mTOR-pathway phosphoproteins (mTOR, p70S6K, 4E-BP1$^{Thr37/46}$, 4E-BP1γ and rpS6). Additionally, we found likely and possible small increases in eEF2 at 240 min with the low and high doses respectively, relative to control, and a small negative correlation between mean recovery plasma isoleucine and valine concentrations and eEF2 phosphorylation. 4E-BP1 de-phosphorylation at Thr$^{37/46}$ and phosphorylation of eEF2 at Thr^{56} may be part of a mechanism by which exercise blunts skeletal muscle protein synthesis (Rose et al. 2008). Importantly, eEF2^{Thr56} phosphorylation only occurred in type I muscle fibres (Rose et al. 2008). Tentatively, it could be that the predominant impact of protein-leucine dose on eEF2 phosphorylation post-endurance exercise is in the type I fibres. However, we suggest that this is a cautious interpretation given the temporal variation in mTOR-pathway phosphoprotein activation post-feeding (Atherton et al. 2010). For instance, an exercise induced rise and

subsequent decline in eEF2 phosphorylation in the control condition may have already occurred by 4-h, and protein-leucine feeding may simply have delay this response.

Our 4E-BP1 hyper-phosphorylation data (the ratio of 4E-BP1-γ to total 4E-BP1) confirms the rat data of Morrison et al. (Morrison, Hara, Ding and Ivy 2008), who observed increased 4E-BP1-γ in rats swum to exhaustion and then fed protein-carbohydrate compared to those that were not fed (~120% greater), or fed only carbohydrate (~33% greater) or protein (~60% greater). However, an unexpected finding was that while there were small increases in 4E-BP1-γ isoform hyper-phosphorylation with the high-dose versus both low-dose and control feeding, 4E-BP1 phosphorylation at $Thr^{37/46}$ was reduced in both protein-leucine conditions relative to the control. This finding is in contrast to the recent work in cultured rat fibroblasts by Dennis, Baum, Kimball and Jefferson (Dennis, Baum, Kimball and Jefferson 2011) showing that leucine and insulin additively increased $Thr^{37/46}$ phosphorylation (Ser^{65} and Thr^{70} were not assessed). Glucose-mediated insulin signalling phosphorylates 4E-BP1 at $Thr^{37/46}$, priming it for subsequent amino acid mediated phosphorylation at Thr^{70} and Ser^{65} by mTOR (Gingras, Raught and Sonenberg 2001), although recent evidence suggests physiological stimuli such as hypoxia may alter the hierarchy of 4E-BP1 phosphorylation (Ayuso et al. 2010). This suggests that phosphorylation of 4E-BP1 in response to amino acids in exercised human skeletal muscle might differ from that in found in cell culture. Furthermore, the significance of reduced $Thr^{37/46}$ phosphorylation to subsequent downstream signalling is not immediately clear, although the extent of inhibition of eIF4E binding to 4E-BP1 and, therefore, stimulation of mRNA translation largely depends on the level of 4E-BP1 hyper-phosphorylation (Gingras et al. 1999, Ayuso et al. 2010).

Conclusions

The ingestion of a protein-leucine rich supplement following intense endurance exercise augments anabolic signalling via the mTOR pathway in a dose-sensitive manner, but the corresponding magnitude of the effect of the 3-fold increase of ingested protein-leucine dose on myofibrillar protein FSR was trivial when expressed in terms of pharmacokinetics. Nevertheless, the clear dose-sensitive regulation of several of the translation-initiation factors downstream of mTOR suggests that protein dose may activate a growth, angiogenic, and metabolic transcriptional program involved in adaptation to endurance exercise. Depending on an individual athlete's requirements, a low protein-leucine dose may be optimal for stimulating myofibril FSR when appropriate caloric intake is also an important consideration to attain or maintain body composition goals. Otherwise, a high-dose equivalent may ensure saturation of FSR while also differentially regulating expression of genes directing or constituent of functional myocellular adaptation to endurance exercise via protein-leucine dose-sensitive transcriptional control. Chronic nutritional intervention trials are recommended to understand how FSR, signalling, and other skeletal-muscle molecular and physiological systems respond to dietary protein and amino acid dose, and how the system translates to functional adaptive remodelling and physical performance. This can be done by taking an integrative approach that incorporates genomics, targeted post-translational regulatory evaluation and proteomics, coupled to muscle function and performance measures in target populations.

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CHAPTER 6 STUDY 2B

MANUSCRIPT DRAFT

The action of protein-leucine feeding and dose on the acute-phase skeletal muscle transcriptome following endurance exercise

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ABSTRACT

Purpose. Protein ingestion following intense cycling up-regulates early-recovery expression of skeletal muscle growth and development, extracellular matrix (ECM) and immunity gene ontology, which could be a mechanism contributing to improved adaptation to endurance exercise with protein and protein-leucine feeding. However, the effect of dose has not been characterized. **Methods.** In a 3-way crossover design, 12 trained men ingested low-dose and high-dose (23.3/5/180/30 g and 70/15/180/30 g protein, leucine, carbohydrate and fat, respectively) or control (274/30 g carbohydrate and fat) supplements over 90 min following 100 min of intense cycling. *Vastus lateralis* biopsies taken 30 and 240 min following ingestion of the first beverage were analysed by Illumina microarray. **Results.** Ingenuity Pathway Analysis (IPA) and Database for Annotation, Visualization and Integrated Discovery (DAVID) bioinformatic approaches revealed that protein-leucine (low-dose versus control, low-con; high-dose versus control, high-con) and dose (high-low) principally regulated cell-cycle control, muscle growth and development, leukocyte trafficking and ECM biology. The top functional modular network in the high-low dose contrast at 30 min was proinflammatory, centred on interleukin *(IL)1β*, and programmed increased leukocyte migration and differentiation. Skeletal muscle growth and development genes *MYOD1*, *CDKN1A*, *GADD45A*, *DUSP1* (low-con) and *MYOG* (high-con) formed an up-regulated backbone among 30-min top IPA gene-networks. By 240 min, an *IL6* centred antiinflammatory and promyogenic transcriptome, associated with inferred inhibition of NF-Kappa-β and SMAD pathway activity, guided decreased leukocyte migration, and increased apoptosis of immune and muscle cells and cell metabolism. Cellular stress-response *(GADD45A/B/G),* cell-cycle control *(CDKN1A)*, fatty acid transport/metabolism (*ABCA1*, *PTGER4*, *PTGS2*, *LDLR*) and intracellular pump/channel genes (*ATP2A1/A2*, *CLIC4*, *SGK1)* were also prominent among top networks. IPA functions and transcription factor analysis

supported top network-derived muscle/immune biology inferences, suggesting high-dose regulated cell-cycle via 30-min inhibition of *MYC* (high-low), *JUN* and *TP63* (high-con), but 240-min activation (high-low); reduced 30-min muscle-cell proliferation and differentiation with 240-min *SMAD4* (TGFβ1/myostatin signalling) inhibition (high-con); and increased 30-min leukocyte migration, differentiation and survival but down-regulated 240-min leukocyte trafficking and immune-related functions, with *NFKB1-RELA* and *STAT3* inhibition (high-con). DAVID analysis validated IPA findings with overrepresented 30-min ECM (high-con, high-low), immunity/inflammation and angiogenesis (high-low) functional annotations. **Conclusion.** Low-dose and high-dose versus control promoted cytostasis and increased cell viability with a myogenic signature, and were indicative of progression through the early-phase regeneration response. Protein-nutrition might impact on post-endurance exercise muscle recovery and adaptive remodelling by regulating dose-responsive inflammatory, myogenic and cell-adhesion/signalling processes within the fed-recovery transcriptome.

INTRODUCTION

Endurance exercise disrupts muscle cell energy homeostasis, generates reactive oxygen species, compromises cell integrity and promotes inflammation. These stimuli are transduced by intracellular signalling pathways to activate and coordinate transcriptional and translational responses that restore cell homeostasis and induce recovery programs of wound-healing and adaptive remodelling of skeletal muscle (Fluck and Hoppeler 2006). Adaptations to chronic endurance training, such as increases in skeletal muscle mitochondrial and capillary density and a transition toward a slow contractile muscle fibre-type, represent the summative functional expression of the transcriptomal response to each acute training bout (Fluck and Hoppeler 2003). Recent transcriptomic analyses of genome-wide microarray data

from human skeletal muscle have begun to unravel the transcriptional programs regulated by endurance-type exercise and training (Mahoney et al. 2005, Timmons et al. 2005, Keller et al. 2007, Stepto et al. 2009, Keller et al. 2011). Bioinformatic interrogation of gene networks and functional processes have revealed that endurance exercise regulates myocellular metabolism, oxidative stress, electrolyte transport, transcription factor binding and mitochondrial biogenesis in the immediate hours to days of recovery (3 h to 48 h post-exercise) (Mahoney et al. 2005, Keller et al. 2007) although the global early recovery transcriptome (<3 h post-exercise) has yet to be investigated.

Dietary protein intake may play an import role in promoting aspects of skeletal muscle adaptation to exercise (Hawley, Tipton and Millard-Stafford 2006, D'Antona et al. 2010). Coingestion of protein and protein and leucine with carbohydrate and fat following endurance exercise improved subsequent high-intensity endurance performance under nitrogen-stressed conditions typical of heavy training loadings (Rowlands et al. 2008, Thomson, Ali and Rowlands 2011). Proposed mechanisms for the protein-nutrition mediated enhanced recovery adaptive response included amino acid induced regulation of mRNA translation and muscle fractional protein synthetic rates (FSR) (Koopman et al. 2005, Kimball and Jefferson 2006, Moore et al. 2009, Safdar et al. 2009, Nielsen et al. 2010) and reduced muscle damage or faster restoration of disruption to myocellular integrity (Saunders, Kane and Todd 2004). Using an unbiased hypothesis-seeking approach, our group determined the transcriptome specifically induced in response to protein coingestion following endurance exercise (Rowlands et al. 2011). Protein feeding up-regulated the expression of several gene ontology that could conceivably be aligned with improved adaptation to endurance exercise, for example, extracellular matrix (ECM), stress response, myocellular growth and development,

and immunity and defense (complement-mediated immunity and macrophage activation) at 3-h, and mitochondria and energy production at 48-h post exercise. Importantly, the ontology associated with myocellular growth, immune interaction and ECM genes are involved in the skeletal muscle wound-healing response, which involves macrophages, inflammatory and growth-related chemokine and cytokine expression, satellite cell activation, and myogenesis (Ten Broek, Grefte and Von den Hoff 2010, Tidball and Villalta 2010). Protein-nutrition mediated processes involved in inflammation and wound healing (e.g. phagocytosis of debris, membrane repair, and reduced fibrosis) could be important mechanisms explaining the apparent improvement in recovery with protein-feeding post exercise. Macrophages, for instance, are crucial for tissue clean-up and for stimulating successful regeneration and myogenesis after exercise (Arnold et al. 2007, Tidball and Villalta 2010). Despite some leads, the immediate early recovery transcriptome response to feeding in the first hour post exercise has not been characterized and, moreover, there are no data currently available regarding the effect of post-exercise protein or protein-leucine dose on the global transcriptional or translational responses within trained skeletal muscle recovering from intense endurance exercise.

Therefore, the objective of the study was to investigate the effect of three doses of protein-leucine recovery nutrition (zero, low and high) ingested in the first 90-min post-endurance exercise on the early recovery transcriptomal profile. Several bioinformatic approaches were utilized to discover biological pathways and metabolic processes significantly affected by protein-leucine feeding and, through the 3-way design, determine if dose thresholds exist for the primary acute-phase biological process associated with muscle repair and regeneration, and adaptive remodelling.

METHODS

The general design of the study, including participants, preliminary testing, diet and activity prior to and during the experimental period, and the nutritional interventions, are as described in Chapter 5.

Analyses

RNA extraction, labelled-cRNA synthesis, hybridization and selection. Muscle tissue for gene microarray analysis was disrupted and homogenized in lysis buffer using FastPrep instrument and lysing tubes containing ceramic beads (MP Biomedicals, Irvine, CA, USA). Total RNA was extracted and purified by RNAdvance tissue kit (Agencourt, Beckman Coulter Genomics, Danvers, MA) and quality checked (Bioanalyzer 2100, Santa Clara, CA; RNA integrity ≥8). All cRNA targets were synthesized, labelled, and purified via automated procedure (Raymond et al. 2006). All samples were analysed with HumanRef-12 v3.0 Expression BeadChips (Illumina, San Diego, CA, USA) which comprise probes to interrogate 48774 transcripts.

Gene array processing and statistical analysis. Scanning was performed using the BeadArray Reader and signal intensity quantified in Genome Studio (Illumina). The microarray output was deposited in NCBI's Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?token=lxmbbqcegquaezo&acc=GSE123456). All arrays were quantile normalized. Homoscedasticity was obtained using the Box-Cox power transformation. A constant was chosen to realize positive values before log2 transformation to normality and to stabilize the variance related to mean expression.

Treatment and time affected expression were estimated using a mixed model analysis of variance (ANOVA) for repeated measures (Partek, St Louis, MO). Model fixed effect parameters were sequence, treatment, time, and appropriate interactions thereof. The ANOVA was further analysed by Global Error Assessment (GEA) leading to a robust analysis and p-value (Mansourian et al. 2004). For the array analysis, we acknowledge that developments in statistical analysis are on-going and that effect size and biological-variance-standardised likelihood-based gene selection criteria may be inferentially superior. In the absence of a satisfactory validated approach we utilised a traditional null-hypothesis based gene selection criteria ($p<0.001$). In addition to the fold change, we calculated the effect size from composite standard deviation obtained from the MSE (see Supplementary Data 6.1 p0.001ANOVAsAllContrastsFoldESCIs.xlsx). The analysis returned sufficient power to detect a large effect size (ES 1.3) at the chosen threshold for significance. Bioinformatic evaluation was based on the effect of protein-leucine dose versus control at 30 min and 240 min post-exercise, and for the 240-30 min time effect.

Bioinformatics. Gene selections were loaded into Ingenuity Pathway Analysis (IPA, Winter Release, http://www.ingenuity.com) software for targeted network and canonical pathway analysis. The IPA analysis criteria comprised Ingenuity Expert Information only, which are associations supported by literature. We filtered by selecting species human and tissue skeletal muscle (relaxed filter). From the analysis, we examined IPA network, function, and transcription factor outcomes and selected the top pathways assigned a focus score of 3.0 or higher, equivalent to 99.9% confidence (focus score is derived from the negative logarithm of the p-value). To provide a bioinformatic validation to the findings in IPA, we used the Database for Annotation, Visualization and Integrated Discovery (DAVID, v6.7,

http://david.abcc.ncifcrf.gov/) to highlight enriched biological themes and functionally related gene groups. A gene list comprising genes significant at $p<0.001$ was uploaded to DAVID; Huang, Sherman and Lempicki (Huang, Sherman and Lempicki 2008) describe a step-by-step procedure to use the DAVID bioinformatic tools. DAVID-derived annotation clusters were filtered using an overall enrichment score of >3.0; the cluster enrichment score is based on the Expression Analysis Systematic Explorer score (EASE score, a modified Fisher Exact Test) of each of terms within a cluster, with a higher score representative of greater enrichment. We then focused on terms within selected annotation clusters with Benjamini-corrected p-value of <0.05.

RESULTS

Nutrient-responsive transcriptome. Protein-leucine ingestion differentially affected 173-479 genes with robust p-value (ROBP)<0.001 (Supplementary Data 6.1). Meanwhile, a biphasic gene expression response was evident for genes involved in inflammation or immune cell function, and skeletal muscle growth and development (Figure 6.1A, B).

IPA outcomes

Ingenuity functions analysis inferences. Ingenuity functions and network analysis provided an objective bioinformatic output from transcriptome interrogation. In response to a single unit of supplement ingestion at 30 min post-exercise, the modular functions analysis suggested increased leukocyte migration with high-dose versus control, while examination of the top networks found both doses of protein-leucine increased cell stability, and initiation of processes involved in skeletal muscle growth, organization, function and development

(Figure 6.2A, B; Supplementary Data 6.2 Muscle Growth & Development and Immune Related Functions.xlsx). The most resounding transcriptome response at 30 min was the majority upregulated proinflammatory immune cell trafficking functions with the high-low dose contrast (Figure 6.2C; Figure 6.3A; Supplementary Data 6.2; Supplementary Data 6.3 IPA Functions.xlsx). In contrast, by 240 min functional modular network analysis indicated progression through the myeloid cell-associated regeneration response, represented by decreased immune-cell migration and growth, antiinflammatory networks, and increased immune and muscle cell apoptosis (Figure 6.2D-F; Figure 6.3B; Supplementary Data 6.3).

Connectivity within and between functional gene modules. A benefit arising from the present modular network construction is the ease of identification of highly-connected functional genes (hub genes), based on both functional positioning within and between other biologically connected modules and network topology (nodal genes with high intergene connectivity are listed in Table 6.1). Accordingly, the central hubs regulating increased leukocyte migration at 30 min with high-dose versus low-dose were interleukin (*IL*)-1 beta (*IL1β*) and cluster of differentiation 44 (*CD44*). These hubs connected immune-cell differentiation and connective tissue remodelling factors to construct a cell-growth regulatory network that included insulin-like growth factor 1 (*IGF1*) and binding protein 3 (*IGFBP3*), and transforming growth factor beta 1 (*TGFB1*) and receptor 2 (*TGFBR2*), ECM function, remodelling, adhesion genes (e.g. decorin, *DCN;* biglycan, *BGN;* versican, *VCAN;* tenascin C, *TNC;* lumican, *LUM;* connective tissue growth factor, *CTGF*), and others involved in macrophage activation and adhesion *(CD86, CD44, CD163, CD14, CD68*) (Figure 6.2C; Figure 6.3). Additional exploration of immune-cell trafficking networks revealed expression consistent with a stimulatory impulse for migration, infiltration, adhesion and activation of

mononucleocytes, neutrophils, and macrophages (Supplementary Data 6.2). Meanwhile, in the high-dose and low-dose versus control contrasts at 30 min, modular hub gene regulation suggested myogenic or satellite cell activation (myocyte differentiation factor 1, *MYOD1*), cell cycle control consistent with cell cycle arrest and increased cell stability via cyclin dependent kinase (*CDKN1A*), growth arrest and DNA-damage-inducible alpha (*GADD45A*), and dual specificity phosphatase *(DUSP1*) (Figure 6.2A, B; Supplementary Data 6.3). Downstream of *MYOD1* were up-regulated cytoskeletal and muscle contractile genes *ACTC1* (actin cardiac muscle 1) and *MYBPH* (myosin binding protein H) in low-con and high-con top networks, and *MYL4* (myosin light chain 4) in the high-con top network; and *MYH1* (myosin 1) which was down-regulated in the high-con contrast (Fig. 6.2A, B).

By 240 min, *IL6* (interleukin-6) was the top gene hub (Figure 6.2D, E). Network connections and the functions analysis suggested progression to an antiinflammatory expression environment and a change in leukocyte function to increased apoptosis and decreased cell viability, which included phagocyte adhesion and reactive oxygen species (ROS) production, and decreased expression of connective tissue genes (Supplementary Data 6.3). Downregulation of cell cycle regulators supported progression through apoptosis and differentiation (e.g. GADD45 family genes *GADD45A/B/G, CDKN1A;* myelocytomatosis viral oncogene homolog, *MYC*). Metabolic gene expression involving increased metabolism of lipids and nucleic acid components, and the synthesis of steroids, was also consistent with cell differentiation (Supplementary Data 6.3).

Upstream regulators. The upstream regulator analysis draws from the interrogated transcriptome and the Ingenuity-curated knowledge database to predict the directional

activation status of regulatory transcription factors, kinases, cytokines and other factors, and is presented in Table 6.2. Noteworthy at 30 min in the high-low contrast was activation of proinflammatory cytokines IL1β, osteopontin (SPP1), chemokine C-C motif ligand 5 (CCL5), cell cycle arrest and growth regulators (CEBPA/B, EGF), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (NFKBIA), and components of the mothers against decapentaplegic homolog (SMAD) signalling pathway; this outcome was consistent with the transitory early phase inflammatory response revealed from the network analysis (Table 6.2).

By 240 min, predicted relative inhibition of the IL6 and IL1β associated inflammatory response was observed in the low-dose and high-dose versus control contrasts (Table 6.2). At 30 min, the analysis predicted inhibition of the cell growth regulators MYC, jun proto-oncogene (JUN) and tumour protein 63 (TP63) with high-con, and MYC with high-low at 30 min, but activation by 240 min in the high-low contrast. Furthermore, there was predicted RORA activation, and inhibition of the SMAD (TGFβ1/myostatin signalling) signalling pathway, NFKB-transcription factor p65 (reticuloendotheliosis viral oncogene homolog A, RELA), and signal-transducer and activator of transcription 3 (STAT3) at 240 min with protein-leucine feeding (Table 6.2). Figure 6.4 shows the downstream gene networks constructed from interrogation of the NFKB-RELA complex and STAT3, highlighting their involvement in cell differentiation and metabolism. Together, this data suggests reduced cell proliferation, organisation of energy metabolism networks, and increased myocellular growth and differentiation (Trenerry, Carey, Ward and Cameron-Smith 2007, Mauro et al. 2011).

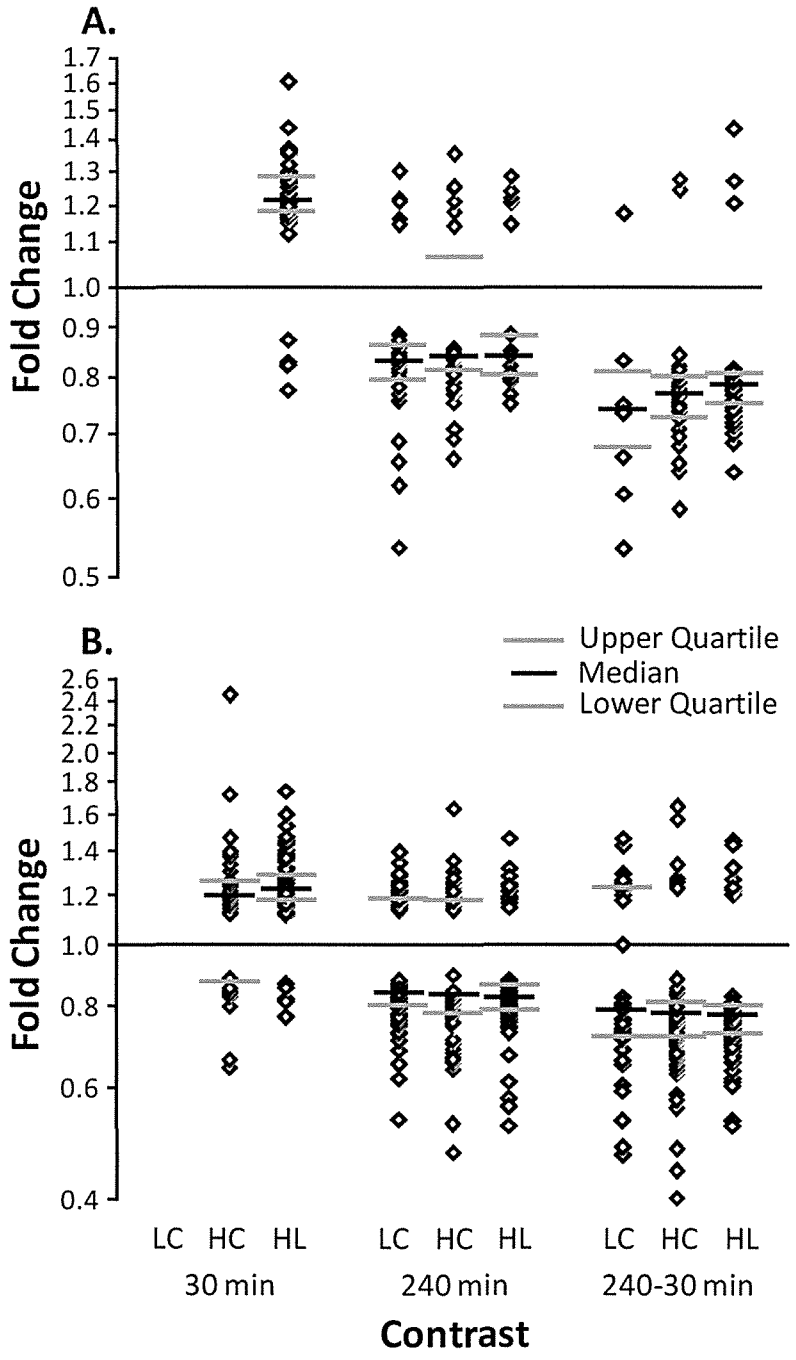
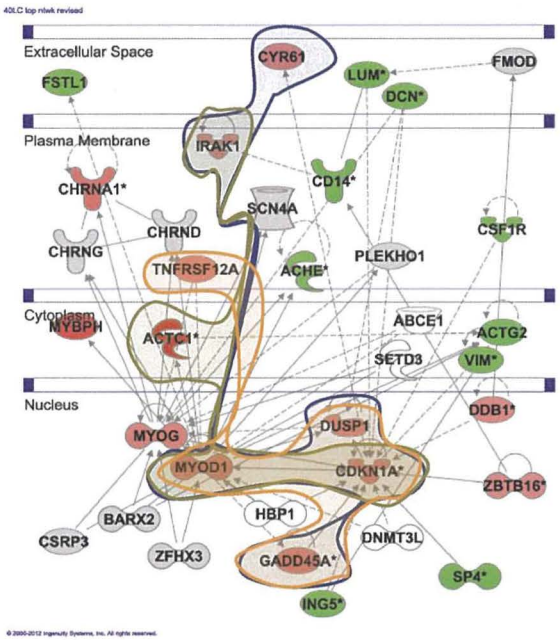


Figure 6.1. Summary of fold-change in the expression of genes involved in **A)** inflammation and activation, adhesion, migration, and infiltration of immune cells, and **B)** cell growth, organization, proliferation, development, and cell death in skeletal muscle. Data were obtained from the gene lists comprising the top 10 functional annotations across the 9 differential protein-leucine dose and time contrasts. LC, low-dose minus control contrast. HC, high-dose minus control contrast. HL, high-dose minus low-dose contrast.

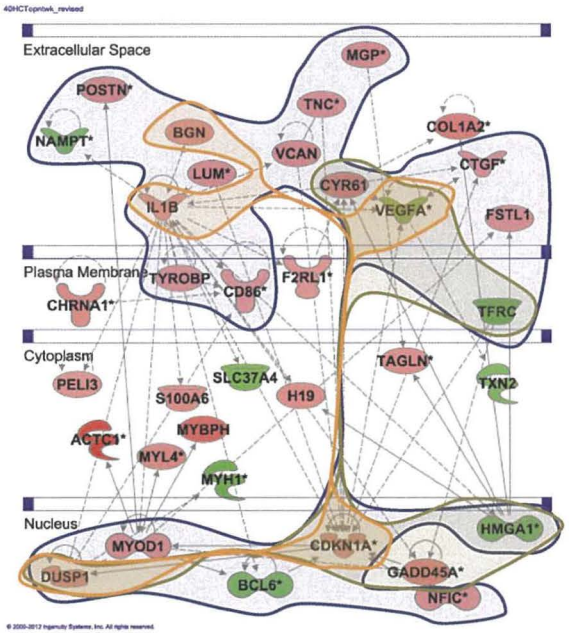
Table 6.1. *Nodal genes presented in the top IPA networks in Figure 6.2*	
Comparison	Nodal genes (↑/↓regulated, gene, (number of nodes))
30 min into recovery	
LC	↑***MYOD1*** **(19),** ↑***MYOG*** **(16),** ↑***CDKN1A*** **(15),** ↓*ACTG2* (5),↓*CD14* (5)
HC	↑***IL1B*** **(17),** ↑***CDKN1A*** **(12),** ↑***MYOD1*** **(10),** ↑*CYR61* (6), ↓*HMGA1* (6), ↑*DUSP1* (5), ↑*GADD45A* (5), ↓*VEGFA* (5)
HL	↑***IL1B*** **(19),** ↑***TGFBR2*** **(13),** ↑***CD44*** **(10),** ↑*IGF1* (8), ↑*DCN* (5), ↑*BGN* (5)
240 min into recovery	
LC	↓***IL6*** **(20),** ↓*VCAM1* (8), ↓*PTGS2* (7), ↓*EGR1* (7), ↓*ACHE* (6),↓*ABCA1* (5)
HC	↓ ***IL6*** **(22),** ↓***MYC*** **(17),** ↓***CDKN1A*** **(12),** ↓***EGR1*** **(10),** ↓*GADD45A* (8), ↓*VCAM1* (7), ↓*CXCL2* (5), ↓*IRF1* (5), ↓*KLF2* (5), ↓*SOCS3* (5)
HL	↓***CDKN1A*** **(11),** ↑***NFKBIA*** **(10),** ↓*ATP2A2* (6), ↑*DDIT3* (6)
240 min minus 30 min comparison	
LC	↓***MYC*** **(20),** ↓***IL6*** **(13),** ↓***CDKN1A*** **(12),** ↓***FOS*** **(11),** ↓*EGR1* (7), ↓*MYOD1* (7), ↓*GADD45A* (6), ↓*KLF2* (5), ↓*KLF4* (5)
HC	↓***MYC*** **(27),** ↓***CDKN1A*** **(13),** ↓*GADD45A* (5), ↓*GADD45B* (5), ↓*ID2* (5), ↓*KLF2* (5), *SMAD3* (5), ↓*TNC* (5)
HL	↑***NFKBIA*** **(16),** ↓*DMD* (8), ↓*IGF1* (8), ↓*BGN* (5), ↓*CD44* (5)
Nodal genes were defined as those with 5 or more connections to other genes in the same network; genes in bold were defined as top nodal genes and have 10 or more connections. LC, low-dose versus control; HC, high-dose versus control; HL, high-dose versus low-dose.	

30 min

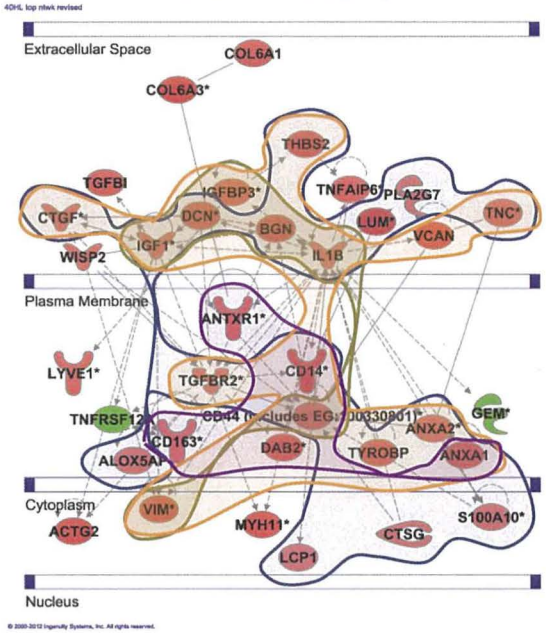
A. Low dose minus control contrast.
Top Functions (highlight): increased cell viability (blue), cytostasis, transactivation; decreased cell death of muscle cells (tan), proliferation of connective tissue (orange).



B. High dose minus control contrast.
Increased differentiation of cells (blue); decreased G1 phase cell cycle (tan), apoptosis of connective tissue (orange), binding of cells.



C. High dose minus low dose contrast.
Increased leukocyte migration (blue), recruitment of phagocytes, movement of mononuclear leukocytes, engulfment of cells (purple), adhesion of connective tissue, differentiation of cells (orange); decreased apoptosis of connective tissue (tan).



240 min

D Low dose minus control contrast.
Decreased leukocyte migration (blue), phagocyte and neutrophil migration, granulocyte accumulation (tan), cell differentiation, inflammatory response (orange).

E. High dose minus control contrast.
Increased quantity of neutrophils, quantity of blood cells (blue); decreased cell viability (tan), formation of lesion (orange).

F. High dose minus low dose contrast.
Increased Apoptosis of hematopoietic cell lines (blue), cell death of muscle cells (tan), quantity of Ca2+ (orange); decreased cell viability.

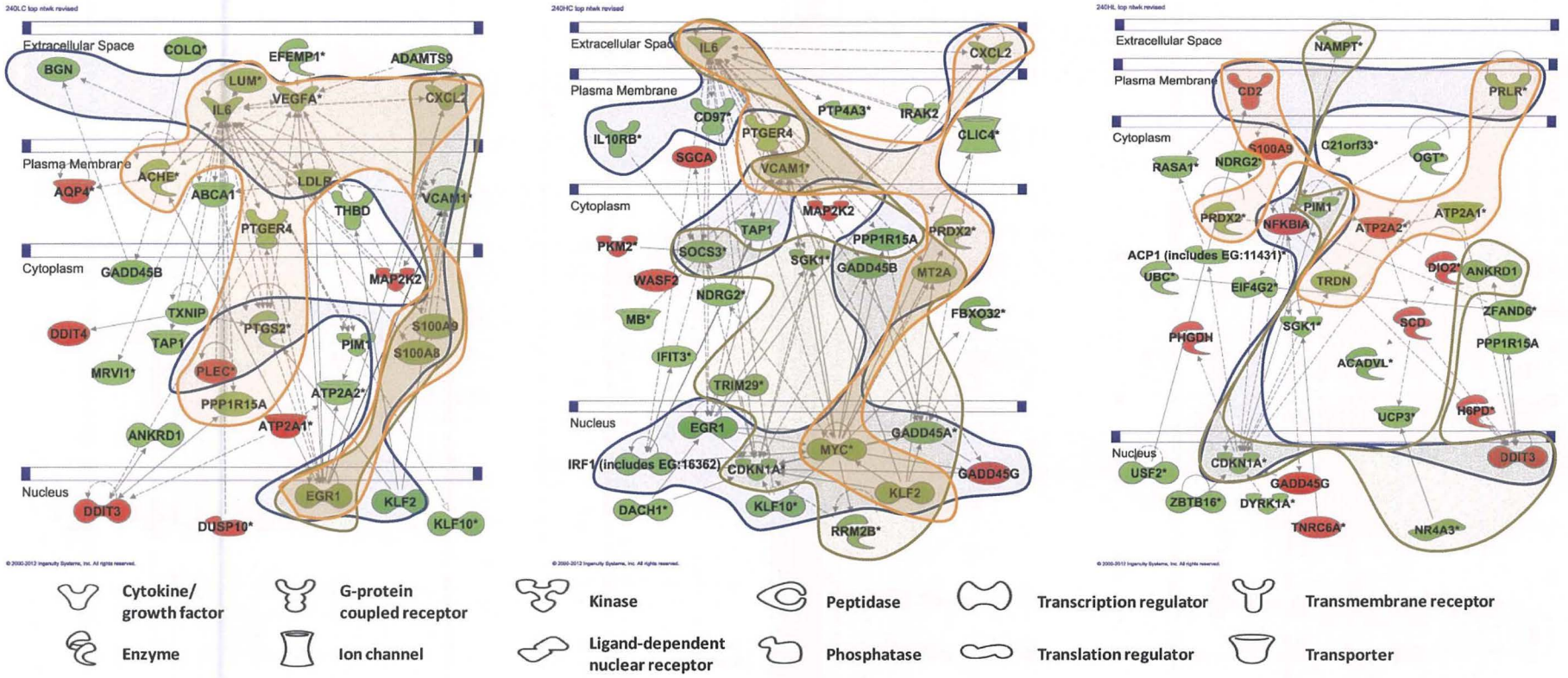


Figure 6.2A-F. The top ranked networks for the response to leucine-protein dose at 30 min (plates **A-C**) and 240-min (plates **D-F**) into recovery. Shown are the symbol and direction of gene expression as labelled. The top ranked molecular functions modules for each network were chosen based on biological interest and top ranked Z-Score statistic >2.0 and P<0.05 (detailed in OSM4), and were identified in the plates by coloured

module shading, with respective labelling. Symbol colour indicates the direction of gene regulation: green, down-regulated; red, up-regulated; grey, within global gene selection, but not significantly affected within contrast. Genes denoted with an asterisk returned multiple transcripts from the array analysis with ROBP<0.001. For gene abbreviations see Supplementary Data 6.2, tab 'Abbreviations list'.

Table 6.2. *Summary of predicted activation state of upstream transcriptome regulatory factors affected by protein-leucine dose at 30-min and 240-min post intense endurance exercise.*

Contrast	Upstream regulator	Molecular function	Predicted activation state	Regulation z-score	p-value of overlap	Contrast	Upstream regulator	Molecular function	Predicted activation state	Regulation z-score	p-value of overlap
			30 min						*240 min*		
Low-Con	NCOR1	transcription regulator	Inhibited	-2.1	6.3E-05	Low-Con	IL1B	cytokine	Inhibited	-3.1	3.6E-13
	MYOD1	transcription regulator	Activated	2.3	1.4E-07		TNF	cytokine	Inhibited	-2.8	4.4E-12
High-Con	SMAD3	transcription regulator	Activated	2.2	6.0E-13		CREB1	transcription regulator	Inhibited	-2.6	9.8E-12
	TGFB3	growth factor	Activated	2.1	2.3E-11		IL6	cytokine	Inhibited	-3.0	6.6E-09
	TP53	transcription regulator	Activated	2.0	2.2E-05		AGER	transmembrane receptor	Inhibited	-2.8	1.6E-08
	BDNF	growth factor	Activated	2.2	3.3E-05		CHUK	kinase	Inhibited	-2.4	4.7E-08
	HTT	transcription regulator ligand-dependent	Activated	2.0	1.0E-04		EGFR	kinase	Inhibited	-2.2	1.5E-07
	NR3C2	nuclear receptor	Activated	2.0	6.5E-03		CREM	other	Inhibited	-2.3	2.1E-07
	SMAD7	transcription regulator	Inhibited	-3.4	2.5E-11		STAT3	transcription regulator	Inhibited	-3.0	2.4E-07
	FBN1	other	Inhibited	-2.2	7.8E-08		PDGFB	growth factor	Inhibited	-2.4	5.0E-07
	MYC	transcription regulator	Inhibited	-2.9	1.3E-07		NFKBIA	other	Inhibited	-2.6	6.3E-07
High-Low	CEBPA	transcription regulator	Activated	3.7	2.5E-08		C3	peptidase	Inhibited	-2.2	1.3E-06
	SMAD3	transcription regulator	Activated	3.1	4.8E-08		MYD88	other	Inhibited	-2.4	1.9E-06
	IL1B	cytokine	Activated	2.9	2.9E-09		RAC1	enzyme	Inhibited	-2.2	2.0E-06
	CD38	enzyme	Activated	2.6	5.2E-04		IKBKB	kinase	Inhibited	-2.5	3.3E-06
	NFKBIA	other	Activated	2.6	8.7E-08		PRKCA	kinase	Inhibited	-2.8	5.4E-06
	EGF	growth factor	Activated	2.5	1.2E-12		FN1	enzyme	Inhibited	-2.4	6.1E-06
	TGFB3	growth factor	Activated	2.4	1.5E-06		NFKB1	transcription regulator	Inhibited	-2.3	6.3E-06
	MTPN	transcription regulator	Activated	2.3	2.1E-07		EDN1	cytokine	Inhibited	-2.3	9.4E-06
	MYD88	other	Activated	2.3	1.9E-03		ERBB2	kinase	Inhibited	-2.3	1.0E-05
	IKBKB	kinase	Activated	2.3	8.3E-07		RELA	transcription regulator	Inhibited	-3.2	1.1E-05
	HTT	transcription regulator	Activated	2.2	5.8E-05		EGR1	transcription regulator	Inhibited	-2.2	1.4E-05
	CEBPB	transcription regulator	Activated	2.2	4.1E-13		F2	peptidase	Inhibited	-3.0	1.9E-05
	SPI1	transcription regulator	Activated	2.2	7.7E-04		PTGS2	enzyme	Inhibited	-2.1	2.0E-05
	KITLG	growth factor	Activated	2.2	1.7E-02		VEGFA	growth factor	Inhibited	-2.6	3.3E-05
	MAP2K1	kinase	Activated	2.2	2.1E-05		PRKCE	kinase	Inhibited	-2.4	3.7E-05
	SPP1	cytokine	Activated	2.2	3.0E-04		TLR9	transmembrane receptor	Inhibited	-2.6	5.7E-05
	TLR9	transmembrane receptor	Activated	2.2	1.8E-03						
	EDN1	Cytokine	Activated	2.2	8.9E-04						

Table 6.2 continued. *Summary of predicted activation state of upstream transcriptome regulatory factors affected by protein-leucine dose at 30-min and 240-min post intense endurance exercise.*

Contrast	Upstream regulator	Molecular function	Predicted activation state	Regulation z-score	p-value of overlap	Contrast	Upstream regulator	Molecular function	Predicted activation state	Regulation z-score	p-value of overlap
			30 min						*240 min*		
High-Low	CHUK	kinase	Activated	2.2	4.4E-05	High-Con	IL6	cytokine	Inhibited	-2.8	1.7E-08
	IKBKG	kinase	Activated	2.1	9.8E-06		SMAD1	transcription regulator	Inhibited	-2.4	7.2E-08
	CCL5	cytokine	Activated	2.0	1.5E-03		TNF	cytokine	Inhibited	-3.3	7.4E-08
	SRF	transcription regulator	Activated	2.0	1.8E-03		NFKBIA	other	Inhibited	-2.2	9.8E-08
							CREB1	transcription regulator	Inhibited	-3.1	9.5E-07
							PRKCA	kinase	Inhibited	-2.4	1.1E-06
							IL1B	cytokine	Inhibited	-2.8	1.3E-06
							STAT3	transcription regulator	Inhibited	-2.5	1.5E-06
							ERBB2	kinase	Inhibited	-2.3	3.8E-06
							CTNNB1	transcription regulator	Inhibited	-2.4	7.2E-06
							RAC1	enzyme	Inhibited	-2.2	9.1E-06
							SMAD4	transcription regulator	Inhibited	-2.5	9.3E-06
							MAP2K1	kinase	Inhibited	-2.1	9.9E-06
							VEGFA	growth factor	Inhibited	-2.6	1.1E-05
							NFKB1	transcription regulator	Inhibited	-2.8	1.7E-05
							SMAD3	transcription regulator	Inhibited	-2.3	2.2E-05
							F2R	G-protein coupled receptor	Inhibited	-2.0	3.5E-05
							PTK2	kinase	Inhibited	-2.0	4.1E-05
							FGF2	growth factor	Inhibited	-2.0	4.5E-05
							EGFR	kinase	Inhibited	-2.5	5.0E-05
							PRKCE	kinase	Inhibited	-2.3	6.6E-05
							RELA	transcription regulator	Inhibited	-2.9	8.8E-05
						High-Low	DMD	other	Inhibited	-2.0	2.2E-05
							FOXO1	transcription regulator	Inhibited	-2.0	8.1E-04
							MYC	transcription regulator	Activated	2.1	2.4E-06

The activation status of the upstream regulator is predicted from the Z-Score, which is the mean effect of all known gene associations derived from the Ingenuity Pathway Analysis database (Spring release 2012) that are affected by the regulator differentially expressed either up or down within the transcriptome. Regulators with a Z-Score >2.0 from the analysis, which represents 95% confidence, are included within the table.

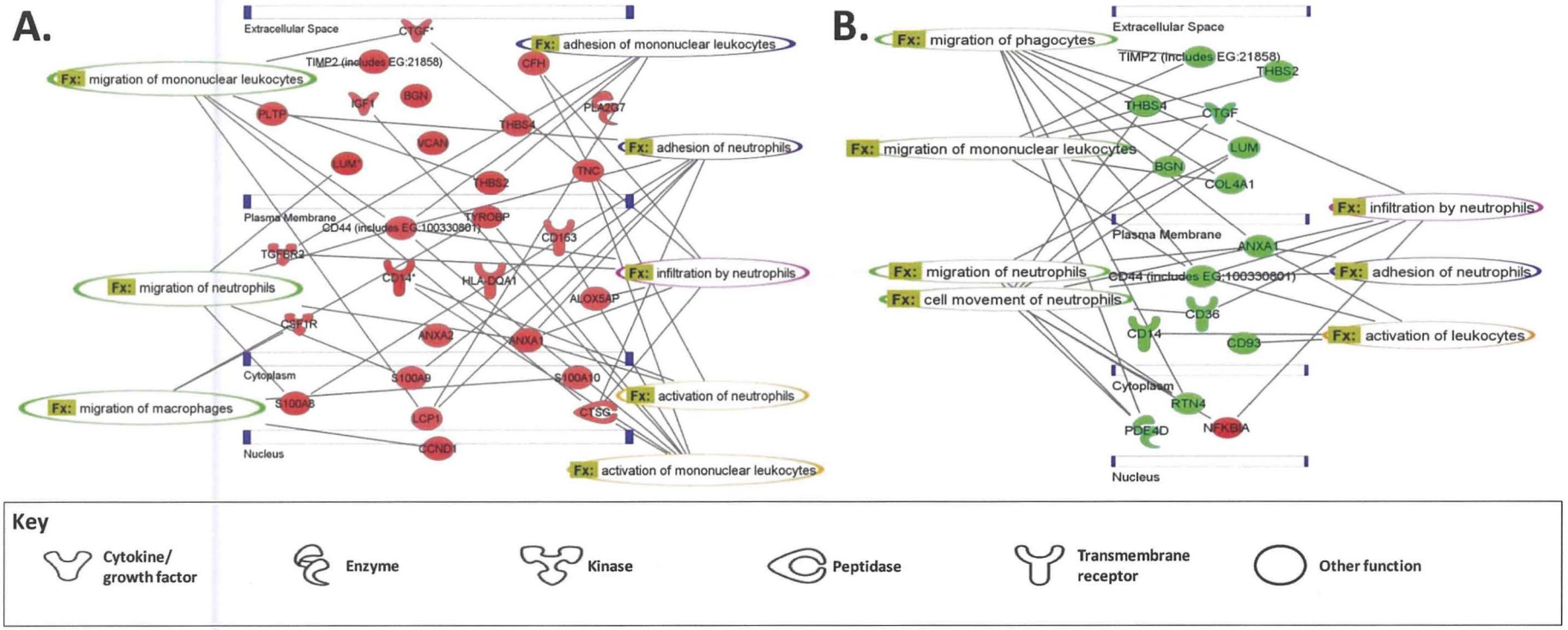


Figure 6.3. Gene networks constructed from interrogation of differentially affected genes categorised within the immune cell trafficking IPA functions for the high-low protein-leucine dose contrast at **A)** 30 min and **B)** 240-30 min. Shown are relevant functional annotations linked to genes. Colour indicates the direction of gene-regulation. Green, down-regulated. Red, up-regulated. Genes denoted with an asterisk have multiple transcripts.

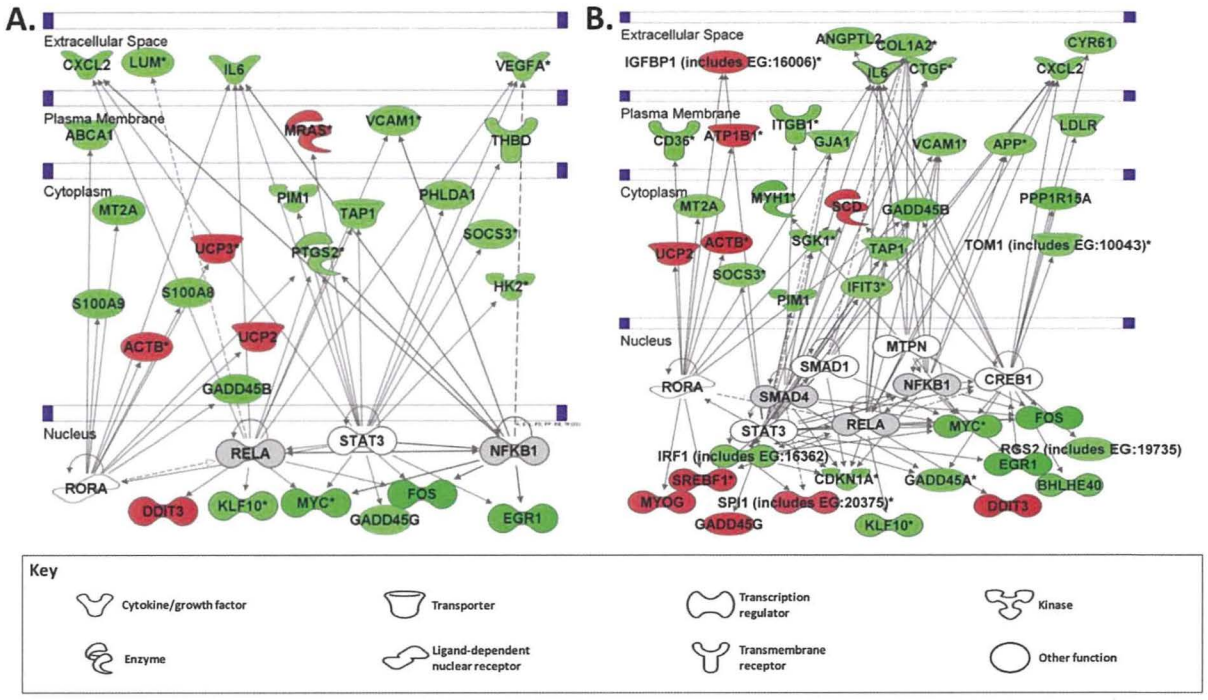


Figure 6.4. Downstream gene networks constructed from interrogation of the nuclear factor kappa-B (NFKB)-transcription factor p65 (RELA) complex and signal-transducer and activator of transcription 3 (STAT3) transcription factors whose activity is predicted to be regulated by protein-leucine feeding versus control. Shown are the contrasts at 240 min post-exercise for **A)** low-control and **B)** high-control. Colour indicates the direction of gene-regulation. Green, down-regulated. Red, up-regulated. Grey, within global gene selection, but not significantly affected within contrast. Clear, not present in the array. Genes denoted with an asterisk have multiple transcripts.

DAVID clustering analysis

The top ranked clusters obtained from the DAVID analysis validated the inflammatory response findings of the IPA interrogation (Table 6.3). At 30 min into recovery, with low-dose protein-leucine versus control there were no gene clusters with an EASE score >3.0

(greatest EASE score = 2.7) but, with high-dose feeding relative to control, ECM functional annotations were overrepresented. Top-ranked clusters at 30 min for high-dose versus low-dose were for ECM, immunity (inflammatory response, cell movement, scavenger receptor activity) and angiogenesis functions. An interesting feature of the DAVID analysis was that by 240 min into recovery there were comparatively fewer gene clusters significantly expressed. For the high-dose protein-leucine versus low-dose comparison at 240 min there were no gene clusters with an EASE score >3.0. Low-dose relative to control feeding resulted in only three overrepresented gene clusters relating to functions of skeletal muscle structure and immune cell movement at 240 min, and with high-dose feeding relative to control the only gene cluster with an EASE score >3.0 (skeletal muscle contraction) did not contain any functional annotation with a Benjamini-corrected p-value <0.05. Subsequently, the 240 min minus 30 min comparisons appeared to be dominated by gene clusters for functions overrepresented at 30 min. High-dose feeding versus control gene clusters were overrepresented by ECM, angiogenesis and cell movement functional annotations, although low-dose versus control yielded only one gene cluster with an EASE score >3.0 (containing functions relating to skeletal muscle fibre components). Not surprisingly, high-dose versus low-dose feeding was also characterised by clusters with functional annotations related to the ECM and angiogenesis, as well as growth-factor binding.

Table 6.3. *Top gene clusters obtained from DAVID clustering analysis of the biological processes at 30 min and 240 min into recovery from exercise differentially affected by the addition of low-dose and high-dose protein-leucine to post-exercise carbohydrate-lipid nutrition.*

Contrast	Function annotation; EASE score	Terms within cluster
	30 min into recovery	
L-C	ECM; 2.7	Secreted, disulfide bond, signal peptide, extracellular region part, glycoprotein, glycosylation site:N-linked (GlcNAc...)
L-C	Muscle function; 2.4	Contractile fibre part, structural constituent of muscle, sarcomere, I band, myofibril, actin cytoskeleton
L-C	Muscle function; 2.3	Contractile fibre part, smooth muscle contractile fibre, muscle protein, smooth muscle contraction, muscle contraction, muscle system process, actin cytoskeleton
L-C	ECM; 2.1	ECM, organization, proteoglycan, compositionally biased region:Cys-rich
H-C	ECM; 6.2	VWF type C repeat
H-C	GAG binding; 5.6	Glycosaminoglycan binding, heparin binding
H-C	ECM; 4.7	Trimer, ECM, hydroxylysine, ECM-receptor interaction, hydroxyproline, collagen, fibrillar collagen, pyroglutamic acid, focal adhesion, growth factor binding, hydroxylation, platelet-derived growth factor binding, collagen fibril organization, cell attachment site motif, collagen VI, disulfide bond, VWF A domain-containing protein 3, pyrrolidone carboxylic acid
H-C	Cell adhesion; 3.8	Cell adhesion
H-L	ECM; 22.1	Signal peptide, secreted, extracellular region, disulfide bond, glycoprotein
H-L	ECM; 15.0	ECM, ECM organization, collagen
H-L	Cell adhesion; 12.2	Cell adhesion
H-L	Inflammatory response; 10.9	Inflammatory response
	240 min into recovery	
L-C	Muscle function; 5.2	Contractile fibre, myofibril, sarcomere, striated muscle contraction, actin cytoskeleton
L-C	Muscle function; 3.8	Cytoskeletal protein binding, actin cytoskeleton, actin binding
L-C	Immune cell movement; 3.1	Leukocyte migration, cell motion, cell migration
L-C	GAG binding; 2.9	Polysaccharide binding, pattern binding, heparin binding, glycosaminoglycan binding, carbohydrate binding
H-C	Muscle function; 3.2	Muscle contraction, muscle system process, striated muscle contraction
H-C	Cell movement; 2.8	Cell migration, cell motion, cell motility, localization of cell, leukocyte migration
H-C	Angiogenesis; 2.8	Blood vessel development, vasculature development, angiogenesis, blood vessel morphogenesis
H-C	mRNA metabolism; 2.4	Nuclear-transcribed mRNA catabolic process, nonsense-mediated mRNA decay, RNA catabolic process, nuclear-transcribed mRNA catabolic process, nonsense-mediated decay, mRNA metabolic process
H-L	Muscle function; 2.9	Structural constituent of muscle, contractile fibre part, actin cytoskeleton, myofibril, striated muscle contraction, muscle contraction, sarcomere, muscle system process, muscle myosin complex, myosin II complex, actin binding, viral myocarditis, thick filament, tight junction, myosin filament, cytoskeletal protein binding, heavy chain of myosin, myosin motor region, myosin N-terminal SH3-like, myosin tail, motor protein, I band, motor activity, calmodulin-binding region, IQ domain, cytoskeletal part
H-L	Ribosome function; 2.8	Cytosolic part, translational elongation, ribonucleoprotein, ribosome, translation, protein biosynthesis, structural molecule activity, ribosomal protein, structural constituent of ribosome, RNA binding, cytosolic large and small ribosomal subunits
H-L	Organelle function; 2.5	Non-membrane-bounded organelle, cytoskeleton
H-L	Ubiquitination; 2.2	Cross-link:Glycyl lysine isopeptide (Lys-Gly) (interchain with G-Cter in ubiquitin), isopeptide bond, ubl conjugation

Table 6.3 continued. *Top gene clusters obtained from DAVID clustering analysis of the biological processes at 30 min and 240 min into recovery from exercise differentially affected by the addition of low-dose and high-dose protein-leucine to post-exercise carbohydrate-lipid nutrition.*

Contrast	Function annotation; EASE score	Terms within cluster
	240-30 min	
L-C	Muscle function; 3.3	Contractile fibre part, sarcomere, myofibril
L-C	GAG binding; 2.4	GAG binding, heparin-binding, heparin binding, polysaccharide binding, pattern binding, thrombospondin, type 1 repeat, carbohydrate binding
L-C	Muscle function; 2.4	Myosin complex, thick filament, myosin filament
L-C	Cellular response; 2.2	Response to extracellular stimulus, response to nutrient, response to nutrient levels
H-C	ECM; 7.4	Polysaccharide binding, glycosaminoglycan binding, ECM-receptor interaction, heparin-binding
H-C	ECM; 6.7	Domain: VWF type C, trimer, growth factor binding, thrombospondin, type 1 repeat
H-C	ECM; 5.8	ECM, secreted, signal
H-C	ECM; 4.1	ECM-receptor interaction, trimer, focal adhesion, extracellular structure organization, fibrillar collagen, pyroglutamic acid, hydroxylation, collagen alpha 1(I) chain, hydroxylysine, triple helix, hydroxyproline, pyrrolidone carboxylic acid, platelet-derived growth factor binding
H-L	ECM; 14	ECM, secreted, signal, disulfide bond, glycoprotein
H-L	ECM; 8.4	ECM, secreted, collagen, trimer, ECM-receptor interaction, collagen triple helix repeat, hydroxylation, focal adhesion, hydroxylysine, hydroxyproline, growth factor binding, platelet-derived growth factor binding, fibrillar collagen, pyroglutamic acid, collagen fibril organization, collagen alpha 1(I) chain
H-L	Cell adhesion; 8.1	Cell adhesion
H-L	GAG binding; 6.9	GAG binding, polysaccharide binding, heparin-binding

Shown are the top 4 clusters for each comparison. Function annotation is an author-designated label representative of the DAVID-derived terms found within a given cluster. VWF, von Willebrand factor. GAG, glycosaminoglycan.

DISCUSSION

The most striking outcome of the Ingenuity Pathway Analysis and secondary unbiased interrogation via DAVID was that protein-leucine feeding regulated a proinflammatory transcriptome associated with increased leukocyte migration most evident with the high-dose at 30 min into recovery, that reverting to an anti-inflammatory promyogenic molecular programme by 240 min. Ingestion of a single low-dose beverage (6 g of protein and 1.25 g of additional leucine) was sufficient to elicit a differential transcriptomal response at 30 min relative to the control, and is testament to the potency of the amino acid signalling upon the post-exercise transcriptome. However, the relatively smaller number of significantly expressed genes, networks and functions at 30 min compared to the high-dose transcriptome suggests that consuming more protein-leucine accentuates gene expression networks that play an important role in regeneration and adaptive remodelling of exercised human skeletal muscle. These transcriptome data provide mRNA-level inferences that protein-leucine feeding enhanced early-phase skeletal muscle regeneration and renewal to trauma, which combined with increased protein synthesis could, in part, explain reported improvement in performance following 3-days of ingesting similar quantities of protein and leucine after intense endurance cycling (Thomson, Ali and Rowlands 2011).

Protein-leucine feeding following intense endurance exercise stimulates a temporally biphasic dose-dependent skeletal muscle transcriptome regulating proinflammatory leukocyte migration, cell stability, regeneration and growth.

The high-low dose feeding contrast produced a proinflammatory transcriptome at 30 min centred on the cytokine IL1β and representing increased leukocyte invasion, while both doses of protein-leucine induced expression suggesting increased cell stability and differentiation.

By 240 min, gene functions analysis predicted attenuation of the initial proinflammatory impulse and progression through the skeletal muscle regeneration via decreased leukocyte migration replaced with increased apoptosis of myeloid and muscle cells, and promyogenic networks. The classical inflammatory response of skeletal muscle tissue to change-of-use or injury involves the release and increased concentration of growth factors and cytokines, an increased migration and activation of phagocytes (neutrophils and M1-phenotype macrophages) and other leukocytes, fibroblast production of ECM components that are degraded as regeneration proceeds (Pizza, Peterson, Baas and Koh 2005, Burks and Cohn 2011), and the activation, proliferation, and differentiation of satellite cells to either fuse with existing fibres or with other myogenic cells to generate new fibres (Tidball and Villalta 2010). This early response initiates removal of tissue debris, contributes to exercise-induced membrane and oxidative damage, and stimulates gene expression to modulate regeneration (Burks and Cohn 2011). Here we present new evidence that myeloid-cell associated muscle regeneration processes may be upregulated by post-exercise protein-leucine feeding, and that this effect is dose modulated.

First, an example of activation of M1 macrophage resolved from increased CD68 expression with high-dose feeding. CD68 is a functionally important glycoprotein involved in phagocytosis and cytokine release (Zouaoui Boudjeltia et al. 2004). M1 macrophages (and neutrophils) can increase muscle damage via ROS including nitric oxide (Villalta et al. 2009); a transcriptome responding to increased production of reactive oxygen species response was inferred in the high-low dose contrast. Increased nitric oxide may also promote greater leukocyte adhesion (Kubes, Suzuki and Granger 1991), another step in the classical muscle regeneration response (Tidball and Villalta 2010). Increased *CD14* expression, a toll receptor complex component, and activation of toll receptor 9 was predicted from upstream regulatory

analysis, suggesting activation of NFKB, cytokine secretion (CC and CXC chemokines) and the inflammatory response (Boyd et al. 2006). Increased *S100A10* supports increased macrophage migration with high protein-leucine dose (O'Connell et al. 2010). Unfortunately, muscle sample damage by freeze artefact prevented subsequent immunohistochemical quantification of macrophage and neutrophil antigen content within the skeletal muscle. Nevertheless, future validation work should also determine the physiological processes regulated by amino acids directing acute phase neutrophil and M1 macrophage activation including production of proinflammatory cytokines and stimulation of phagocytosis involved in the early regeneration response. For example, increased neutrophil invasion (Figure 6.3A) and myeloperoxidase release (Supplementary Data 6.3) may have induced cell membrane damage and oxidation of low-density lipoproteins (LDL) (Zouaoui Boudjeltia et al. 2004). The binding of modified LDL to CD68 activates phagocytosis and muscle cell lysis by M1 macrophages (Nguyen and Tidball 2003). Coupled with increased cytokine production, the interaction of neutrophils and macrophages regulated by feeding in the first few hours post exercise may be one of several important regulatory processes in muscle recovery from intense exercise (Pizza, Koh, McGregor and Brooks 2002, Pizza, Peterson, Baas and Koh 2005, Arnold et al. 2007).

Second, the 240-min IL6-centred antiinflammatory transcriptome, and inhibition of IL6, transforming factor alpha (TNFα), and NFKB activity by both protein-leucine doses (Table 6.2; Figure 6.4) may contribute to progression towards muscle regeneration by shifting macrophages from the M1 to M2 phenotype (Tidball and Villalta 2010) which is also coupled to a promyogenic response involving satellite cells (Serrano et al. 2008). *CD163* was up-regulated with high-dose protein-leucine, and the functional protein is an M2c macrophage-specific receptor for complexes of haemoglobin and haptaglobin. Internalisation and

breakdown of the ligated complex can contribute to lowering extracellular haemoglobin and associated free radical production and cellular damage, while also promoting an antiinflammatory cytokine release (Moestrup and Møller 2004). Activation of NFKB signalling in the high-low contrast at 30 min suggests control of myogenic differentiation. Activation of classical NFKB signalling causes migration of the p65 (RELA)-p50 (NFKB1) dimers to the nucleus to regulate cyclinD1 causing inhibition of differentiation and expression of proinflammatory cytokines IL6, TNFα, and IL1 (Bakkar and Guttridge 2010). However, increased *MYOD1* expression and activation suggests initiation of a myogenic differentiation molecular programme: MYOD stimulates cell-cycle re-entry by inducing NFKB complex relocalization from the nucleus to the cytoplasm (Parker et al. 2012); indeed, by 240 min the NFKB pathway was inhibited with both protein-leucine doses along with networks and functions associated with downstream targets IL6 and VEGFA. Rowlands et al. (Rowlands et al. 2011) also reported increased expression of myogenic differentiation factors (*MYOD1, myogenic factor 5, MYOG*) with milk protein ingestion following intense cycling in trained men. In murine skeletal muscle, increased inflammatory cell content and satellite cell activation displaying strong staining for MYOD, preceded extensive myofibrillar regeneration in response to injury (Yan et al. 2003). These data present a complex picture of acute-phase nutritional regulation of myogenesis, but support the notion that recovery protein feeding regulates satellite cell activity involved in skeletal muscle regeneration after intense exercise. Because it was not possible to decipher the cell types from which the nutrition-responsive transcriptome was most active, future workers should consider in situ methods to quantify functional mRNA and protein expression to better define the specific cellular response to amino acids in regenerating skeletal muscle.

The biphasic transcriptome for increased then decreased cellular stability and viability through cell cycle arrest and cell apoptosis, defined in part through the *CDKN1A* (*p21*), *MYC*, and *GADD45A* network hubs (Figure 6.2), is consistent with phagocyte invasion prior to activation of targeted cell destruction and debris removal (Burks and Cohn 2011). The interaction of apoptotic leukocytes with macrophages leads to the clearance of cellular debris by the macrophages, while concomitantly silencing the proinflammatory/phagocytoxic action of the macrophages (Ariel and Serhan 2012). *GADD45A* responds to cell stress and is an important regulator of anabolic signalling and energy homeostasis (Ebert et al. 2012). P21 induces cell cycle arrest (Lokireddy et al. 2011). MYC has a widespread impact on the transcriptome including regulation of cell growth arrest and adhesion, metabolism, ribosome biogenesis, protein synthesis, and mitochondrial function (Dang et al. 2006). Therefore, the data suggest that one of the early responses to post-exercise protein feeding in trained skeletal muscle is cell stabilisation, which may assist in maintenance of homeostasis and protein synthetic machinery prior to activation of the restorative programme.

The TGFβ-SMAD signalling pathway was predicted to be activated at 30 min, and then relatively inhibited by 240 min with the high-dose contrast only (Table 6.2). Early SMAD pathway activation and extensive upregulation of ECM component expression would normally contribute towards scar formation (Li et al. 2004) negatively affecting skeletal muscle regeneration by inhibiting satellite cell proliferation and myofibre fusion in adult muscle (Allen and Boxhorn 1987). However, SMAD pathway inhibition by 240 min suggests any profibrotic response was more likely transient and guiding an impulse of ECM protein deposition (e.g. collagen, proteoglycans, laminin) in the high-dose condition (e.g. Figure 6.2A, B, orange overlay; 6.2C, tan overlay) to support basement membrane and other ECM remodelling (Chen and Li 2009). Accordingly, the biphasic expression of *CTGF* and other

fibrotic regulators, e.g., the proteoglycan DCN which antagonises TBFβ and IGF1, may have invoked accelerated muscle regenerative by modulating inflammatory cytokines and chemokines (Pelosi et al. 2007, Brandan, Cabello-Verrugio and Vial 2008) and contributed to relative dampening of protein synthesis through regulation of mTORC1 through IGF1-AKT. Earlier evidence of a role for protein feeding in ECM turnover was by way of regulated expression of matrix metallopeptidases and inhibitors (increased *MMP9*, *MMP13*, *MMP19*; decreased *TIMP1*, *TIMP2*) (Rowlands et al. 2011), which control basement membrane degradation facilitating the recruitment of myogenic, myeloid, vascular, and fibroblastic cells to damaged muscle (Mann et al. 2011), and ECM remodelling and the interface for leukocytes, cytokines, growth factors (Chen and Li 2009).

A final consideration is that the present nutrition responsive myeloid-cell associated transcriptome programme for tissue regeneration occurred after normal non-injurious contractile activity in trained muscle. Passive stretches and isometric contractions elevated neutrophils without causing injury and offered protection from damage caused by subsequent lengthening contractions (Pizza, Koh, McGregor and Brooks 2002). Therefore, intense concentric cycling exercise in trained muscle, while causing fatigue and free-radical associated damage unlikely caused substantial microstructural damage associated with heavy eccentric damage models used to study the inflammatory regeneration response (e.g. (Paulsen et al. 2010)). Furthermore, training muscle is in a state of constant remodelling implying that the skeletal muscle of athletes is (a) likely to exhibit a relatively modulated or moderated inflammatory and regeneration response, (b) is in a state of constant flux between damage and regeneration events, and (c) is integrating regeneration responses with the molecular programme necessary for increased homeostatic scope required for improved contractile function. Because myeloid-associated regeneration processes responding to heavy exercise

occur over several days to weeks (Pizza, Koh, McGregor and Brooks 2002, Paulsen et al. 2010, Tidball and Villalta 2010), the collection of tissue samples for several days following a bout of strenuous exercise is required to gain a thorough appreciation of nutrition-mediated regeneration. Furthermore, likely cumulative effects with repeated peritraining feeding warrant examination of the skeletal-muscle performance phenotype responding to chronic feeding.

Conclusion.

New evidence is presented for a post-exercise protein-leucine nutrition dose sensitive transcriptome associated with known skeletal muscle regeneration biology. While aspects of the regenerative molecular programme were apparent with the low-dose protein-leucine feeding, the proinflammatory transcriptome was engaged primarily with high-dose feeding; this was despite near saturation of myofibrillar protein synthesis with low-dose nutrition comprising 23 g of whey protein and 5 g of leucine. As such, a higher dose of protein-leucine could be mechanistically instrumental in accelerated restoration and supercompensation of contractile function. Further research is warranted to define the effects of post-exercise amino acid exposure and dose on function and role of myeloid cells, satellite cells and myofibres on the integrative complex response guiding adaptive regeneration.

CHAPTER 7 GENERAL DISCUSSION

INTRODUCTION

Investigations of the effects of post-endurance exercise protein and carbohydrate coingestion on performance have predominantly utilised testing protocols consisting of glycogen-depleting initial exercise and a total recovery period prior to performance-testing exercise of <20 h, which establish the rate of muscle glycogen resynthesis as the most important mechanism determining the recovery of exercise function. As such, when ingestion of carbohydrate during recovery is suboptimal (<1.2 $g \cdot kg^{-1} \cdot h^{-1}$), additional protein might enhance the rate of glycogen resynthesis sufficiently to have a meaningful impact on the performance of subsequent intense exercise. Yet, studies conducted to test the performance phenomenon have been, overall, equivocal. Furthermore, the mixed effects on performance are of little relevance to athletic practice, in that endurance athletes typically consume carbohydrate at a greater rate than is provided by many protein-carbohydrate interventions and carbohydrate-matched controls, or the interventions are compared to non-caloric placebo conditions (Williams, Raven, Fogt and Ivy 2003, Karp et al. 2006, Betts, Williams, Duffy and Gunner 2007, Pritchett et al. 2009, Lunn et al. 2012). Mixed outcomes for the effect of post-exercise protein consumed with suboptimal carbohydrate on the performance of a second bout of intense exercise conducted within ~20 h of initial feeding are unsurprising. Differences in the energy content of protein-containing treatments versus controls (Williams, Raven, Fogt and Ivy 2003, Millard-Stafford et al. 2005, Betts, Williams, Duffy and Gunner 2007), variability in the extent of the recovery period from 2 h (Millard-Stafford et al. 2005) to nearly 20 h (Rowlands et al. 2007, Pritchett et al. 2009) and an unknown effect of the impact of other nutritional factors such as micronutrients in milk (Ferguson-Stegall et al. 2011) or additional electrolyes with protein-carbohydrate (Williams, Raven, Fogt and Ivy 2003) have probably contributed to the equivocal performance outcomes. However, of the studies in men investigating performance of a second bout of endurance-type exercise within

~20 h of initial exercise, several (Williams, Raven, Fogt and Ivy 2003) though not all (Millard-Stafford et al. 2005, Luden, Saunders and Todd 2007, Rowlands et al. 2007, Rowlands et al. 2008, Pritchett et al. 2009, Goh et al. 2012) have reported reductions in indirect plasma markers of muscle damage (CK) and muscle soreness with protein-carbohydrate recovery feeding (despite a lack of a substantial effect on subsequent performance), providing some suggestion of an attenuation in muscle structural or membrane damage with dietary protein in a short timeframe. With regards to the lack of a performance effect, it is possible that longer than 20 h is required for recovery to normal or supercompensated function following intense cycling exercise (Parra et al. 2000).

Functional recovery of muscle from an acute bout of intense endurance exercise requires cellular restoration of homeostasis, and the repair of muscle damage via recycling of cellular constituents and the synthesis and accumulation of new and adaptive proteins (Seene, Alev, Kaasik and Pehme 2007, Egan et al. 2011, Seene, Kaasik and Alev 2011). Inflammation appears to contribute to muscle repair and subsequent remodelling by stimulating resident and infiltrating immune cells (monocytes, macrophages and neutrophils) to cleanup cellular debris in muscle, stabilize membrane structure, and the release of soluble factors to promote myogenesis and inflammatory resolution (Tidball and Villalta 2010, Bosurgi, Manfredi and Rovere-Querini 2011). Skeletal muscle adaptation to endurance training ensures that future bouts are better tolerated, which necessitates transcriptome-guided rearrangement of the contractile apparatus, mitochondria and other organelles, and extracellular changes that include remodelling of the extracellular matrix (ECM) and changes at the neuromuscular junction (Fluck and Hoppeler 2003, Hood, Irrcher, Ljubicic and Joseph 2006, Seene, Kaasik and Umnova 2009, Egan et al. 2011). Consuming sufficient post-exercise carbohydrate is the key nutrient for endurance athletes in order to ensure maximal resynthesis of muscle

glycogen. However, only when protein is ingested does post-exercise net protein balance (the rate of protein synthesis minus the rate of breakdown) become positive (Bowtell et al. 2000, Levenhagen et al. 2002), which is likely to be indicative of sufficient amino acid substrate for the tissue needs. Leucine is the key amino acid stimulating enhanced translational signalling via the mTOR-pathway, thereby increasing the rate of tissue protein synthesis (Anthony et al. 2000, Crozier et al. 2005, Atherton et al. 2010) which is likely to be an important mechanism impacting on muscle repair and adaptation following endurance exercise (Rodriguez 2009). Rowlands et al. (Rowlands et al. 2008) found that ingesting a high-protein and carbohydrate diet with some fat in the immediate hours after endurance exercise did not substantially improve a bout of high-intensity repeated sprint cycling undertaken the next day (~15-19 h later) relative to a isocaloric low-protein, fat and higher-carbohydrate diet, but was associated with reductions in the plasma CK concentration and a 4.1% improvement in the performance of another identical test ~60 h following the initial exercise bout. Subsequently, Thomson et al. (Thomson, Ali and Rowlands 2011) found that ingesting a protein, leucine, carbohydrate and fat recovery diet after endurance exercise over three consecutive days again reduced plasma CK, and improved performance of the same repeated sprint cycling test undertaken ~36-39 h after previous exercise by 2.5%, relative to isocaloric carbohydrate and fat feeding. Associated with the protein-containing conditions was positive nitrogen balance, which might also be indicative of sufficient provision of dietary amino acids for whole-body tissue requirements, while in the comparative control conditions nitrogen balance was negative to neutral across the experimental periods (Rowlands et al. 2008, Thomson, Ali and Rowlands 2011). This raised some suspicion that during short periods of intense endurance training, consuming dietary protein in the first few hours after exercise might only be of substantial benefit to subsequent exercise performance when overall protein or amino acid provision is insufficient (as estimated by nitrogen balance) for the whole-body requirement.

Therefore, using a crossover design the effect of ingesting post-exercise protein, leucine, carbohydrate and fat feeding was compared to isocaloric carbohydrate and fat on whole-body glucose and protein metabolism, plasma and urinary metabolomics, nitrogen balance, CK, immunological parameters and subsequent performance during an extended training block consisting of six days of intense cycling (study 1; Chapters 3 and 4). Performance was assayed during (day 4) and at the end (day 6) of each training block, while other rides consisted of initial glycogen-depleting protocols on days 1 and 2, recovery rides on days 3 and 5, and additional prolonged steady-state riding prior to performance tests. The purpose of this study was not just to confirm a benefit of protein-feeding to performance during an intense multiday training regimen, but also to investigate putative mechanisms of a predicted delayed performance benefit that might relate to enhanced amino acid turnover, protein synthesis and reduced muscle membrane damage, and immune cell function and systemic inflammation. Therefore, the nutritional intervention and control were provided as beverages in the post-exercise recovery period for 3 h (after intense rides on days 1, 2 and 4) and 1 h (after less intense rides on days 3 and 5) to provide a strong nutritional signal.

In the second study (Chapters 5 and 6), the effect of low-dose and high-dose protein and leucine, carbohydrate and fat supplements, and a carbohydrate and fat control isocaloric to the high-dose supplement, were compared to determine the effect of dose on putative mechanisms defining muscle tissue recovery: myofibrillar FSR, translational control and other signalling via the mTOR pathway, and the global expression picture via the muscle-tissue transcriptome, at 30-min and 240-min into recovery from an acute bout of intense endurance cycling. The first main experimental purpose with the congruent hypothesis was

that the low-dose protein-leucine feeding would be sufficient to saturate FSR, but that a dose-response would be evident in mTOR-pathway phosphoprotein phosphorylation. The second main study purpose was to explore the primary acute-phase biology responding to protein-leucine dose via interrogation of the transcriptome. Previous work by our group (Rowlands et al. 2008, Thomson, Ali and Rowlands 2011) had established that post-endurance exercise protein-feeding enriched the muscle-tissue transcriptome at 3 h into recovery for muscle development, ECM signalling and structure, and immunity and defense ontology; however, there was no available evidence for a dose-response effect of protein-feeding on the muscle transcriptome. Therefore, a second hypothesis was that protein-leucine dose thresholds would be identified for key biology previously shown to be upregulated at 3 h post-endurance exercise and aligned to skeletal muscle repair and remodelling, including cell homeostasis, muscle growth and development, ECM, and immune and inflammatory response biology. The intention was to also determine the mitochondrial FSR response, but sufficient muscle tissue was only obtained from one participant across the three treatment conditions.

The collective evidence obtained from these two studies was intended to provide a substantial contribution toward our understanding of the molecular and metabolic mechanisms guiding and regulating skeletal muscle repair and adaptation in response to acute post-exercise protein-leucine supplementation, and to determine the nature and magnitude of the effect of protein-leucine feeding on high-intensity endurance exercise performance.

PERFORMANCE OUTCOME

Trivial impact of post-exercise protein-leucine, carbohydrate and fat feeding on repeated-sprint cycling performance during a six-day intense cycling model

The primary outcome from study 1 was that, contrary to our hypothesis, a prolonged, repeat-feeding protocol providing a large dose of micellar whey protein with added free-leucine, sufficient carbohydrate to saturate glycogen resynthesis, and a small amount of fat, did not substantially improve repeated-sprint cycling performance on day 4 and day 6 of the six-day cycling regimen, relative to carbohydrate and fat. This was in contrast to the two earlier investigations by our group which found delayed improvements in repeated-sprint mean power of 2.5% (±99%CI 2.6%) with protein, leucine, carbohydrate and fat feeding and 4.1% (±95%CI 4.1%) with a high-protein, carbohydrate and fat recovery diet, relative to carbohydrate and fat and low-protein carbohydrate and fat control conditions (Rowlands et al. 2011, Thomson, Ali and Rowlands 2011). Furthermore, these earlier studies also found that associated with the protein conditions were reductions in the muscle membrane damage marker CK suggestive of improved myocytes membrane integrity or repair, which was supported in the current investigation. Thus, the performance outcome was even more surprising.

As noted in Chapter 3, it is important to consider that in the earlier work by our group, the observed mean improvements to repeated-sprint mean power was small (2.5%; 99%CL: ±2.6%) (Thomson, Ali and Rowlands 2011)) to moderate (4.1%; 95%CL: ±4.1% (Rowlands et al. 2008)), but with uncertainty of the magnitude of the true (population) effect (indicated by the confidence interval) that allows for the effect on performance to overlap a trivial effect-size. Therefore, it is possible that the true effect of post-exercise high-protein or

protein-leucine feeding on the performance of subsequent exercise, conducted some 3-5 days following initial exercise, is indeed trivial, or that one or more physiological mechanism covariates were in play to attenuate the response seen in the other studies (Rowlands et al. 2008, Thomson, Ali and Rowlands 2011).

Post-exercise protein-leucine supplementation might benefit subsequent performance only under conditions of negative to neutral nitrogen balance

Nitrogen balance, the sum of nitrogen intake from dietary protein less measured and estimated losses, is a relatively simple method to estimate dietary sufficiency of amino acids for whole-body tissue metabolic processes, or determine periods when excessive catabolism of body proteins to provide amino acids for the tissue processes might be occurring, which might be important covariates impacting on performance during intense endurance training. Rowlands et al. (Rowlands et al. 2008) found that following the initial intense exercise bout and supplementation on day 1, overnight nitrogen balance was positive in the protein-enriched condition (249 ± 70 mg nitrogen (N)·kg fat free mass $(FFM)^{-1}$, but negative (-48 ± 26 mg N.kg FFM(-1)) in the low-protein control, but importantly that the 60-h total nitrogen balance in the control condition was approximately -125 mg N·kg FFM^{-1} (versus a gain of ~225 mg N·kg FFM^{-1} with the high-protein intervention). Thomson et al. (Thomson, Ali and Rowlands 2011) estimated that across four days of urine collection for nitrogen balance following initial exercise, mean daily nitrogen balance was substantially negative (ES -0.46 to -0.51) in both the protein-leucine and control conditions on all days except day two in the protein-leucine condition and on day three with the control condition, where the changes from net neutral balance were trivial (ES 0.18 and ES -0.01, respectively). On days three and four there were small-sized differences in daily nitrogen balance between conditions, with 24

h net balance more negative in the protein-leucine than the control condition on day three (-18 mg $N \cdot kg^{-1}$ ±90%CL 20 mg $N \cdot kg^{-1}$; ES -0.40 ±0.45) but less negative on day four (20 mg $N \cdot kg^{-1}$±29 mg $N \cdot kg^{-1}$; ES 0.45 ±0.65) (Thomson, Ali and Rowlands 2011). The small between-day variations in nitrogen balance could be explained by sampling variation, or by the study design. On each arm of the crossover study design, the tested intervention (protein-leucine or control feeding) was provided immediately following afternoon (1600-1800 h) exercise bouts on the first three days of the five day training block, with the alternate supplement having been provided that morning to balance mean daily macronutrient intake (the background diets being identical). A time-lag between ingesting the protein-leucine supplement in the morning versus following late afternoon exercise (~10 h) could result in nitrogen provided by the morning protein-leucine feeding being sampled in the urine that day, whereas the nitrogen from the post-exercise feeding (in the alternate crossover arm) might be sampled in the urine of the next day. However, the differences are small, and the mean nitrogen balance across the four-day nitrogen balance urine collection period was mildly negative (-23 ± 39 mg $N \cdot kg^{-1}$) (Thomson, Ali and Rowlands 2011).

In the current study 1 (Chapter 3) it was found that nitrogen balance was approximately neutral in the control condition over days 1-5 of the experimental period. However, with the control condition almost no protein was consumed by participants over the period encompassing day-1 post-exercise recovery and overnight, with an estimated nitrogen loss of -90 ± 43 $mg \cdot kg^{-1}$. Importantly, and in contrast to the earlier studies (Rowlands et al. 2008, Thomson, Ali and Rowlands 2011), when this large nitrogen loss was excluded, there was a large positive nitrogen balance (ES 0.90 ±0.52) over days 2-5 with the control supplement (Fig. 3.5) and with the difference relative to the protein-leucine condition inconclusive on

days two and three and trivial on days four and five. This was despite total daily protein intake being greater in the protein-leucine condition (1.9 $g \cdot kg^{-1}$) than the control (1.5 $g \cdot kg^{-1}$), suggesting that the protein intake in the background diet was sufficient to establish a net protein gain over days 2-5.

In Chapter 3 it was discussed that a habitually lower protein intake amongst the tested cohort than that provided by the experimental diet would lead to a transient period of enhanced nitrogen retention until they adapted to the relatively greater nitrogen intake (Quevedo et al. 1994) which might partly explain the differences in nitrogen balance estimates between study 1 and that of Thomson et al. (Thomson, Ali and Rowlands 2011), who provided a slightly greater dietary protein intake overall (1.6 $g \cdot kg^{-1} \cdot d^{-1}$). Thomson et al. (Thomson, Ali and Rowlands 2011) estimated via 3-day diet diary that their cohort mean habitual intake was 2.0 g protein$\cdot kg^{-1} \cdot d^{-1}$; the step down in protein intake provided by the experimental diet would have most likely led to a temporary period of reduced nitrogen retention efficiency until participants had adapted to the lower overall intake. Given the similarity of that cohort (Thomson, Ali and Rowlands 2011) to our own, a difference in habitual protein intake of more than 0.5 $g \cdot kg^{-1} \cdot d^{-1}$ (that being the difference between the intake estimated by 3-day diet diary by Thomson et al. and the experimental diet in study 1) is unlikely. Nevertheless, a high-protein diet could actually be beneficial during some intense periods of training; increased dietary protein versus normal (3.0 versus 1.5 $g \cdot kg^{-1} \cdot d^{-1}$) possibly attenuated (4.3% ×/÷90% confidence limit 5.4%) a decrement in time trial performance after a block of high-intensity training (Witard et al. 2010).

As discussed in Chapter 2, the digestion characteristics of dietary proteins can impact on the tissue utilization of their derived amino acids at rest and following exercise (e.g. (Lacroix et al. 2006, Pennings et al. 2011)). The quality of the proteins in the diets (mostly non-vegetable based versus vegetarian (Fouillet et al. 2009)) and the timing of protein intake (spread evenly across the day versus provided at the opposite end of the day to exercise (Jordan et al. 2010)) might, therefore, have impacted on the relative efficiency of retention of the background-diet derived nitrogen. Additionally, all three protein-rich nutritional interventions were composed of different combinations of proteins; whey and soy protein isolates, calcium caseinate and milk protein concentrate in bars and milk-like beverages (Rowlands et al. 2008); free-leucine, milk protein concentrate and whey protein isolate (Thomson, Ali and Rowlands 2011); and free-leucine with a micellar whey protein isolate in study 1. While not measured in any of the studies, it is plausible that the most rapidly absorbed supplement was the protein-leucine beverage in the study 1, given that the other protein-rich interventions contained some solid food items, and that the other interventions contained mixtures of slower-digestion proteins including casein and milk protein concentrate (Lacroix et al. 2006, Fouillet et al. 2009, Pennings et al. 2011). The accumulated evidence is that peripheral tissue (e.g. muscle) utilization of dietary amino acids is improved after exercise, and that rapidly digested amino acids provide the most rapid stimulatory effect on muscle amino acid metabolism. Despite the supplement in the current investigation being superior in terms of the total quantity of nutrients provided, and perhaps also absorption and tissue utilization, no substantial impact on performance was realized when nitrogen balance was positive in both the protein-leucine and control conditions.

The nitrogen balance method tends to underestimate nitrogen losses through incomplete sample collection or unmeasured losses (breath ammonia, and skin, hair, nail, semen and

mucous). However, ~85% of losses are accounted for in the sweat and urine (Tarnopolsky, MacDougall and Atkinson 1988) which were both collected, and appropriate adjustment for non-measured nitrogen losses was made (Supplementary Data 3.7); given the size of the mean gain (111 ± 86 mg nitrogen·kg^{-1}, equivalent to ~50 g of body proteins) it is unlikely that mean nitrogen balance in the current study was not at least somewhat positive. Furthermore, while the variation in estimates for nitrogen balance in the studies of Rowlands et al. (Rowlands et al. 2008) and Thomson et al. (Thomson, Ali and Rowlands 2011) indicate that selected participants were in positive nitrogen balance at some stage of the respective three-day and four-day nitrogen-measurement periods, the values are most likely to be overestimates, rather than underestimates.

Nitrogen balance is the gross turnover of body proteins by measured nitrogen losses in urine and sweat, with some correction for non-measured losses (e.g. faeces) and estimation of dietary-protein derived nitrogen intake. Given that a negative daily nitrogen balance represents a greater net loss of body nitrogen (protein) than gain (i.e. total intake) it could also represent a short period of a relative amino acid insufficiency for the tissue metabolic needs during that time. Post-exercise protein and protein-leucine supplementation appears to provide a delayed benefit to subsequent performance over low-protein, high-carbohydrate feeding when mean daily nitrogen balance is approximately neutral to negative (Rowlands et al. 2008, Thomson, Ali and Rowlands 2011) but not when nitrogen balance in the comparative condition is positive (study 1). Furthermore, the largest benefit was observed when the net nitrogen balance difference between protein-rich and control conditions across the experimental period is greatest (Rowlands et al. 2008); it may not be a coincidence that this investigation is also the shortest duration of the series of work. Dietary protein intake impacts on whole-body and muscle protein turnover at rest and after exercise (Bolster et al.

2005, Gaine et al. 2006, Pikosky et al. 2006) but the interaction of exercise, a large dose of protein or protein-leucine immediately post-exercise and the subsequent utilization of dietary protein ingested later is not well understood. A longer experimental period might, therefore, allow more time for any possible small effect of dietary proteins on whole-body and muscle protein metabolism or other mechanisms related to repair and adaptation to become a substantial determinant of subsequent exercise performance.

While the nitrogen balance method is relatively inexpensive and the drawbacks are well understood, future investigations should utilise more recently applied methods that will prove to be more informative of whole-body changes in individual amino acids in man, such as variations of the indicator amino acid method (Zello, Wykes, Ball and Pencharz 1995, Kriengsinyos, Wykes, Ball and Pencharz 2002, Kurpad, Regan, Raj and Gnanou 2006, Hayamizu, Kato and Hattori 2011). Interestingly, reanalysis of the population dietary protein requirement suggests that the current recommendations derived from nitrogen balance studies have underestimated daily protein needs by as much as 40-50% (Humayun, Elango, Ball and Pencharz 2007). Use of this method to estimate the requirements of athletic populations might be also warranted. Furthermore, this method could prove useful in simultaneously determining the metabolic availability of amino acids for protein synthesis and estimating total amino acid requirements (Humayun et al. 2007). Consideration should also be made for the differences in the dietary carbohydrate content required to balance the diets energetically. It is plausible that the background diet providing more carbohydrate in the control condition might have had a greater protein-sparing effect relative to the protein-leucine condition, thereby contributing to the positive nitrogen balance outcomes despite lower daily protein intake. However, it is not possible to conclusively state that the dietary carbohydrate difference played any meaningful part in the trivial performance outcome.

MECHANISMS OF REPAIR AND RECOVERY

The leucine synthetic and oxidative capacity of whole-body tissues is increased during acute post-exercise recovery and could explain acute plasma volume changes, but is not an important mechanism impacting on subsequent performance in the short-term

Investigation of whole-body tissue glucose and protein metabolism responses to protein-leucine supplementation was conducted based on prior evidence of higher blood lactate and glucose concentrations during repeated-sprint cycling with the protein-enriched supplement (Rowlands et al. 2008), and the prevailing hypothesis that increased tissue protein synthesis (inferred from positive nitrogen balance (Rowlands et al. 2008) and the evidence of others e.g. (Levenhagen et al. 2002)) might be an important recovery mechanism associated with a delayed performance benefit. Because the primary aim of the investigation was to investigate performance, we elected to investigate whole-body leucine turnover rather than muscle protein metabolism via biopsy which could have increased variation in the performance measure, masking a predicted substantial improvement. While the effect of protein-leucine supplement on whole-body glucose turnover was, overall, trivial, the changes in whole-body leucine turnover (from which an estimate of whole-body protein metabolism is inferred) were indicative of increased whole-body protein synthesis and turnover. Interpolating the whole-body turnover data to the muscle-tissue should be avoided, but given the increase in myofibrillar FSR observed with low-dose and high-dose versus control feeding in Chapter 5, it is most likely that myofibrillar protein synthesis was increased by the protein-leucine supplement provided in study 1. However, this in itself does not appear to be an important mechanism mediating the earlier observations of a delayed performance benefit associated with post-exercise protein-rich and protein-leucine feeding, given the trivial effect of protein-leucine on performance (Chapter 3).

Prior to the start of day-2 exercise it was noted that plasma volume had expanded by 6.6% (±90%CL 3.8%) with the protein-leucine feeding, relative to the control supplement. Enhanced blood albumin synthesis might be a mechanism explaining this increase. Acute and long-term increases in plasma volume of approximately half the magnitude (~3.5%) have been observed by others in response to post-endurance exercise protein-carbohydrate supplementation (Okazaki et al. 2009, Okazaki et al. 2009, Goto et al. 2010). Albumin synthesis following resistance exercise increases in a dose-responsive manner to protein, peaking with ~20 g of whole egg protein (Moore et al. 2009) and the apparent two-fold greater plasma volume increase observed in study 1 might be due to the much larger dose of protein with added leucine provided (~20 g protein and ~7 g leucine per hour for 3 h versus a single bolus of ~10 g (Okazaki et al. 2009, Okazaki et al. 2009) or ~20 g (Goto et al. 2010) of protein). Interestingly, in the current study the substantial increase was negated by day-2 exercise, and effects on blood and plasma volume were otherwise trivial or unclear, suggesting a transient response to the feeding following day-1 recovery. It might be that day-3 and day-5 supplementation (which was for only 1 h as cycling was shorter and less intense than on other days) was insufficient to substantially increase albumin synthesis after day 1 of the training block, perhaps because of an interaction with the exercise regimen or an impact of dietary protein intake. Therefore, other workers might wish to investigate whether substantial short or long-term changes in blood albumin content and plasma volume are still observed when dietary protein intake is elevated.

Ingestion of 24 g of whey protein and 5 g of free leucine stimulates near-maximal myofibrillar FSR during recovery from intense endurance cycling

Chapter 3 discussed the recent interest in quantifying the effect of post-endurance exercise protein-carbohydrate consumption on the rate of skeletal muscle protein fractional synthesis rate (FSR) during recovery. Only recently have investigators used primed continuous stable-isotope infusions and muscle biopsies to quantify the post-endurance exercise FSR response of mixed-muscle (Howarth, Moreau, Phillips and Gibala 2009, Harber et al. 2010, Lunn et al. 2012), myofibrillar and mitochondrial (Breen et al. 2011) protein fractions to the ingestion of protein-carbohydrate beverages during recovery. Preliminary evidence is that the effect of protein feeding on mitochondrial FSR is trivial (Breen et al. 2011), at least during an acute 4-h recovery period. On the contrary, over the same recovery period myofibrillar FSR appears to have a large effect-sized increase (Breen et al. 2011) and probably constitutes the bulk of the increase observed in mixed-muscle FSRs with protein-carbohydrate interventions (Chapter 2).

In Chapter 5, the myofibrillar FSR response to three levels of protein-leucine dose (zero, low-dose, and high-dose) following intense endurance cycling was investigated. As expected, protein-leucine feeding increased myofibrillar FSR relative to the carbohydrate-fat control. We had hypothesised that the low-dose protein-leucine feeding would be sufficient to saturate muscle FSR, based on earlier observations of fed-recovery mixed-muscle FSR peaking with ~20-25 g of egg white protein after resistance training (Moore et al. 2009) and other observations in rested and exercised rat and human muscle (e.g. (Anthony, Anthony and Layman 1999, Tipton et al. 1999, Anthony et al. 2000, Crozier et al. 2005, Cuthbertson et al. 2005)) giving a collective insight into the minimum dose of essential amino acid, and most

importantly leucine, required to maximally stimulate muscle FSR. In contrast to our hypothesis, it was found that the difference in myofibrillar FSR between the low-dose and high-dose conditions was a likely small effect-sized increase. However, the inferential confidence interval approach our group has used with previous (amongst other interventions) protein-carbohydrate performance studies (Rowlands et al. 2007, Rowlands et al. 2008, Rowlands and Wadsworth 2010, Thomson, Ali and Rowlands 2011), the standardised reference value (smallest important change) for the mean effect is estimated from the value of 0.2 times the standard deviation observed in the control condition (Cohen 1994, Batterham and Hopkins 2006, Hopkins, Marshall, Batterham and Hanin 2009). While this is an appropriate and indeed recommended method for determining performance (Hopkins, Hawley and Burke 1999) and other biological variables (Nakagawa and Cuthill 2007), we questioned if this was an appropriate approach for muscle FSR. To the best of our knowledge, there is no available evidence with regards to FSR for what a meaningful or important change of any size actually is. Upon careful consideration, and given the lack of available data, we elected to use a pharmacokinetic threshold approach commonly used in drug trials, in which a change of 25% from the reference condition is deemed the threshold value. The 90% confidence interval is used to determine the likelihood that the true effect overlaps a value of <25% of the threshold; if the overlap is ≤5%, then the effect is different, and the size of the mean effect difference in FSR and likelihood of overlap reported.

In order to answer the important question of what is a meaningful (functionally important or biologically relevant) change in the global muscle-tissue or fraction rate of protein synthesis, further research is needed. Briefly, a study design or approach (and not limited to post-endurance exercise) to investigate the question posed would involve measurement of the acute change in post-exercise muscle FSR during a chronic training plus recovery feeding

study, and coupled to a measure of performance. While measurement of the impact of protein feeding following endurance exercise (summarised in Chapter 5; and Chapter 5 FSR outcomes) has already been completed, and others have concurrently assayed performance (Lunn et al. 2012), none of these investigations have been in the setting of a chronic training and feeding investigation. A practical design would therefore at the least require post-exercise fed-recovery FSR to be determined following the last acute bout of the chronic training, with a measure of change in a functional outcome determined pre- and post-training; the most appropriate functional outcome measures being muscular size with resistance-type training studies, and maximal aerobic power (VO_2max) with endurance-type training. If only a single measure of the muscle FSR response is taken, the recovery period following the last training session may be the most appropriate time to determine the muscle FSR response to protein feeding, as any change in protein metabolism as a result of the training or level of total dietary protein. If cost and ethical issues were of no concern, pre-training and during-training measures could also be taken. Regression of the total FSR difference (mean FSR change over the acute recovery period multiplied by the number of training bouts) upon the change in the performance measure would then obtain the $\%\cdot h^{-1}$ change in FSR during recovery that is related to a meaningful change in performance. Alternatively, a long-term incorporation method such as ^2H_2O could be used to give a mean protein synthesis rate over the entire training study. In fact, Robinson et al. (Robinson et al. 2011) used the ^2H_2O incorporation method to measure protein synthesis during a chronic endurance-exercise training study with a post-exercise protein-carbohydrate intervention. However, while aerobic power was greater following training there was no substantial effect of the intervention on long-term muscle protein synthesis versus an isocaloric carbohydrate control, which could be due to aspects of the free-living study design (i.e. a difference in dietary protein intake between treatments) (Robinson et al. 2011).

Several studies have described the impact of dosed protein-feeding on muscle protein synthesis in human skeletal muscle. Moore et al. (Moore et al. 2009) gave whole egg protein (0, 5, 10, 20 or 40 g as a single bolus dose) to young men following a bout of resistance training. Mixed-muscle FSR with the 20 g dose was not significantly different to that of the 40 g dose (Moore et al. 2009) and reanalysis of their data confirms a very likely trivial mean change (Supplementary Data 2.2) indicating that somewhere between 20-40 g of high-quality protein can maximally stimulate muscle FSR following resistance training. Pennings et al. (Pennings et al. 2012) fed older adults 10, 20 or 35 g of whey protein and found that the rates of whole-body and mixed-muscle protein synthesis were highest with the 35 g dose. In a parallel trials design, 40 young men completed unilateral knee-extensor resistance exercise before ingesting 25 g of whey protein (with a 3 g total leucine dose), 6.25 g whey protein (0.75 g of leucine), 6.25 g whey protein supplemented with leucine to a 3 g leucine dose, 6.25 g whey protein supplemented with leucine to a 5 g leucine dose, or 6.25 g whey protein supplemented with leucine, isoleucine, and valine to a 5 g leucine dose (Churchward-Venne et al. 2014). The primary outcome was that 6.25 g of whey protein supplemented with leucine up to a 5 g dose was as effective as 25 g whey protein in stimulating increased muscle protein synthesis (Churchward-Venne et al. 2014). In another parallel trials study with 48 resistance-trained young men, it was found that ingestion of whey protein isolate after unilateral resistance exercise increased post-exercise myofibrillar FSR above that of a placebo by 49% with a 20 g dose, and by 56% with a 40 g dose; a 10 g dose did not substantially increase FSR relative to placebo (Witard et al. 2014). Taken together with the present post-endurance exercise myofibrillar FSR data showing equivalency of the high-dose and low-dose, which provided ~23 g of protein and 5 g of leucine, a protein or protein-leucine intake of approximately 20-25 g might maximally stimulate post-exercise protein synthesis.

Unfortunately, muscle samples provided insufficient tissue for a sufficiently powered complete dataset to determine the mitochondrial FSR response to protein-leucine feeding (Chapter 5). Breen et al. (Breen et al. 2011) found no substantial effect of protein-carbohydrate ingestion on mitochondrial FSR during a 4-h recovery from endurance cycling. However, changes in the mitochondrial FSR might occur outside of an ~4 h immediate recovery window (Breen et al. 2011).

Leucine was the amino acid most strongly correlated with myofibrillar FSR and increased phosphorylation of mTOR-pathway phosphoproteins. There is some suggestion that the extracellular amino acid concentration regulates muscle protein synthesis, rather than the intramuscular concentration (Bohe, Low, Wolfe and Rennie 2003) though this is not universally accepted and there is evidence to the contrary (Biolo et al. 1995) and recent evidence suggests that the two are related via amino-acid cotransporters that shuttle amino acids across the plasma membrane (Nicklin et al. 2009). Leucine is predominantly transported into the cell by the bidirectional SLC7A5/SLC3A2 transporter that simultaneously exports glutamine (Nicklin et al. 2009), and the intracellular leucine concentration is sensed by the leucyl-tRNA synthetase (LRS), directly binding to and activating the RagD GTPase in an amino acid-dependent manner, which then activates mTORC1 (Han et al. 2012). To the best of our knowledge, no other authors have regressed FSR upon the plasma leucine concentration to estimate the change in leucine concentration required to stimulate an increase in FSR. It was estimated that every increase in the mean recovery plasma leucine concentration of 210 μM, attainable by ingesting 4.1 g protein and 0.9 g free leucine every 30 min for 90 min following exercise, would stimulate a myofibrillar FSR increase of $0.010\%\cdot h^{-1}$. While this regression estimate is untested, and would most likely

be specific to the conditions of this study, similar comparisons could easily be made by other workers measuring myofibrillar FSR and plasma leucine to determine if, under different experimental conditions, such a hypothesised estimate holds true.

During acute post-exercise recovery, transcriptome analysis infers that protein-leucine feeding upregulates muscle-tissue extracellular matrix gene expression; metabolomics analysis supports increased collagen turnover and saturation of BCAA metabolism

There has been recent suggestion that a high intake of dietary branch-chain amino acids or leucine could be toxic, particularly if leucine is consumed out of proportion with isoleucine and valine, (Hutson, Sweatt and LaNoue 2005) and with an estimate for the daily upper tolerable limit for leucine ingestion (whole-body tissue oxidative capacity) in men at rest recently established at ~550 $mg \cdot kg^{-1} \cdot d^{-1}$ (Elango et al. 2010). We found that, despite ingesting within a 3 h recovery period a quantity of leucine (protein-bound plus free-leucine, ~14.7 g) one-third of that estimated as the maximum oxidisable in day at rest (42 g for a 77 kg individual, the mean mass of the cohort in study 1), there was no observable peak in leucine oxidation. Tracer infusion methodological considerations aside (as discussed in Chapter 3), a high capacity to oxidise leucine post-exercise might be at least in part the result of a training-induced higher enzymatic capability of the branch-chain oxo-acid dehydrogenase (BCOADH), a rate limiting enzyme in BCAA oxidation (Bowtell et al. 1998). However, using a metabolomics analysis of blood and urine samples we found evidence of saturation of BCAA metabolism (Supplementary Data 3.9) based on accumulating concentrations of BCAA metabolites acylcarnitine C3 (propionylcarnitine), C4 (isobutyrylcarnitine) and C5 (isovalerylcarnitine) in the plasma, and increased leucine, isoleucine, valine, and the valine and thymine catabolite β-aminoisobutyrate in the urine.

Thus, there was some evidence that large doses of leucine-enriched protein might have implications for BCAA metabolic pathways, but whether chronic intakes at a similar rate have any short or long-term health implications or (in the context of the present body of work) performance effects, remains to be determined. Nevertheless, it is hard to fathom ingestion of sufficient dietary protein to provide the predicted upper leucine limit (e.g. approximately 350 g of a high-quality whey protein isolate with 12% leucine amino acid content), unless by individuals consuming free amino acid products sold as sports supplements; the latter could arise given that provision of additional leucine in the diet might be a method to overcome age-related declines in amino acid metabolism and muscle mass (Timmerman and Volpi 2008). In any case, habitual exercise training would appear to be a countermeasure to an excessive intake of leucine or BCAAs (Chapter 3).

Metabolomics analysis of blood and urine metabolites also provided evidence that the protein-leucine supplement might augment connective tissue protein turnover. At the transcript level, alterations in ECM gene expression are associated with the extent of adaptation in response to endurance exercise training (Timmons et al. 2005). In an electrical muscle-stimulation model, sequenced events of gastrocnemius muscle ECM remodelling and fibrotic regulation act to protect the muscle-tissue against future injury (Mackey et al. 2011). In an endurance-like exercise model, Miller et al. (Miller et al. 2005) showed that patellar tendon and muscle collagen-protein synthesis is increased in the fed state in response to 1 h of single-leg kicking at 67% of maximal workload, and peaked 24 h post-exercise with tendon collagen synthesis remaining elevated at 72 h post-exercise. However, the serum concentration of procollagen type I N-terminal propeptide, obtained from patellar and muscle tissue-fluid, was not substantially altered from rest by single-leg kicking exercise (Miller et al. 2005). Altogether, the data suggest that remodelling of the ECM is a normal aspect of

muscle repair and adaptation following exercise, and at the mRNA level ECM-related biology might be predictive of the adaptive capacity. During day-1 recovery, protein-leucine feeding was associated with a moderate increase in plasma proline, probably the result of the ingested supplemental-protein derived proline, but also small-sized increases in urinary proline, glycylproline, and prolyl-hydroxyproline (Supplementary Data 3.9). Prolyl-hydroxyproline is derived from collagen, and an increase in the urinary concentration is indicative of greater collagen breakdown liberating the prolyl-hydroxyproline which is then excreted. Unfortunately we did not measure the blood concentration of prolyl-hydroxyproline, which would have provided corroborative evidence of an increase in turnover and excretion. However, as it stands, the increased urinary concentration along with proline and its other metabolites does suggest greater turnover of collagen and its substrates during protein-leucine fed recovery.

Ingenuity®-based analysis (IPA) infers clear dose-responsive ECM-related gene and transcription factor networks, principally 30-min upregulated collagen fibril-assembly and ECM-adhesion and remodelling genes (e.g. *COL6A1, COL6A3, DCN*, *BGN*, *LUM*, *VCAN*, *WISP1*, and *THBS2*). IPA also suggested that genes for some of the main components of the basal membrane (satellite cells lie between the basal membrane and the sarcolemma of the myofibre) such as collagen IV, laminin and heparin sulfate proteoglycans (HSPGs, e.g. syndecan aka CD138) were upregulated with both low-dose and high-dose protein-leucine feeding in the (Chapter 6). Laminin connects collagen with satellite cell surface integrins to anchor the basal membrane to the cytoskeleton, influencing cellular migration, shape and cell-cell signalling (Colognato and Yurchenco 2000) and laminin β1 may play an important role in adaptive strengthening of the muscle ECM to protect against future exercise-induced damage (Mackey et al. 2011). Furthermore, syndecans 'trap' growth factors to co-localize

them with surface receptors e.g. fibroblast and hepatocyte growth factors FGF and HGF (Lopes, Dietrich and Nader 2006) thereby increasing their growth stimulatory effect without an increase in their synthesis or concentration. IPA functions analysis inferred a down regulation of connective tissue functions at 240 min. In order to remodel the connective tissue network, fibroblasts secrete growth factors and extracellular components with both scaffolding and signalling roles (e.g. fibronectin, collagens, proteoglycans) to induce cell migration and proliferation and provide an anchorage site (Serrano and Muñoz-Cánoves 2010). Uncontrolled deposition of ECM components by fibroblasts can lead to the formation of permanent collagenous networks that can impair muscle contractile function (Serrano and Muñoz-Cánoves 2010, Serrano et al. 2011). In support of the IPA-derived transcriptomal evidence, the primary feature of the secondary DAVID analysis was that ECM-related functional clusters for collagen organization, signal peptide secretion, adhesion and binding, and ECM substrates (e.g. hydroxyproline, hydroxylysine) were greatly overrepresented at 30 min into recovery, but were not significantly overrepresented at 240 min (Table 6.3).

The overall evidence is that a transient impulse of ECM-related mRNA transcription is followed by a rapid downregulation, possibly to prevent excessive ECM protein synthesis and deposition. On the other hand, DAVID analysis also revealed overrepresented gene clusters for the inflammatory response, and cell movement and scavenger receptor activity within ECM functions; the ECM plays an important role in satellite cell function and tissue inflammatory regulation by providing binding and sequestering sites for myogenic and inflammatory cytokines, anchors for cell adhesion, and regulating movement of cells (Serrano et al. 2011). ECM proteins such as DCN might even provide a level of feedback regulation upon protein synthesis by acting as an IGF1 antagonist, thereby regulating IGF1-AKT-mTORC1 signalling. Thus, the programme for ECM remodelling is initiated at the

mRNA level, but ECM-related functions also overlap with inflammatory and myogenic biology that might be critical to the muscle repair response. Confirmatory work is required to determine if the magnitude and direction of change in the encoded-protein concentrations matches that of the mRNAs.

Circulating neutrophil concentrations and responsiveness to stimulation are augmented by post-exercise protein-leucine feeding

Providing exogenous amino acids immediately post-exercise might ameliorating the exercise-induced immunosuppression (Calder and Kew 2002, Gleeson, Nieman and Pedersen 2004). Nutritional stimuli from key fuel and substrate amino acids activate immune cell functional processes through intracellular signalling pathways that detect (Säemann et al. 2009, Powell and Delgoffe 2010, Dodd and Tee 2012). A high intake of dietary protein during a period of intense training can ameliorate training-induced impairments in T-lymphocyte mobilization, thereby improving immune cell surveillance, and was associated with less self-reported symptoms of upper respiratory tract infections (URTIs) (Witard et al. 2013). mTOR pathway activity modulates many immune cell functions (Powell and Delgoffe 2010) and could be a mechanism by which BCAAs exert immunomodulatory effects in inflammatory cells.

There is evidence that during a period of intense training, protein-leucine supplementation can alter neutrophil superoxide release in response to phorbol myristic acid (PMA) stimulation, and that neutrophil margination might also be affected by supplementation. The likely moderate and small reductions in neutrophil responsiveness to PMA stimulation at 60 min and 180 min into day-1 recovery with protein-leucine feeding might partly be

attributable to increased plasma concentrations of myristic acid, which stimulates neutrophil O_2^- production (Tada et al. 2009) and thereby reducing the PMA-stimulation of neutrophil O_2- production via the neutrophil oxidative burst assay. Interestingly, isovalerylcarnitine increases apoptosis but also cell killing among U937 leukemic cells, a cell model used to investigate monocyte and macrophage function (Ferrara, Bertelli and Falchi 2005), and activates calpains in a variety of rat tissues including skeletal muscle, and in human neutrophils (Pontremoli et al. 1987, Pontremoli et al. 1990) which could increase neutrophil adhesion and oxidative burst activity (Wiemer et al. 2010). The large-sized increase in isovalerylcarnitine observed during day-1 recovery might, therefore, be another protein-leucine supplement derived mechanism increasing neutrophil stimulation prior to the oxidative burst assay.

Contrastingly, there was a clear, large-sized increase in neutrophil superoxide production in response to PMA following day-6 exercise (33 mmol $O_2^-\cdot cell^{-1}$ ±13 mmol $O_2^-\cdot cell^{-1}$) that is most likely related to a pre-exercise cortisol reduction. Alternatively a reduction in the plasma concentration of the NADPH-oxidase inhibitor palmitoylcarnitine during day-6 exercise might have contributed to the observed O_2- increase with the protein-leucine supplement. Furthermore, there was some evidence for reductions in circulating neutrophil concentration following day-4 exercise and prior to day-6 exercise that could be due to increased margination of neutrophils. Inferences from the microarrays were that a key feature of the protein-leucine post-exercise recovery transcriptome is a strong signal to recruit infiltrator immune cells such as neutrophils via upregulation of genes that increase immune-cell migration and margination.

The effect of protein-leucine supplementation appears to be on top of the well-established effects of high-carbohydrate supplementation in the peri-exercise period. For instance, pre-exercise carbohydrate status influences the plasma cytokine response during prolonged exercise (Bishop et al. 2001), while ingesting carbohydrate before and after (64 $g \cdot L^{-1}$; 5 $mL \cdot kg^{-1}$) and during (2 $mL \cdot kg^{-1}$) 2 h of cycling at 75% VO_2max prevents a post-exercise decline in mitogen-stimulated neutrophil elastase release (Bishop, Walsh and Scanlon 2003). Future work will be required to establish if changes in circulating neutrophil function are carried over to muscle-infiltrating neutrophils, which may be important regulators of muscle repair and adaptation after exercise (Tidball 2005, Tidball and Wehling-Henricks 2007, Tidball and Villalta 2010). Changes in circulating neutrophil responsiveness to stimulation might be important for innate immunity as a result of injury or infection. However, neutrophils also play an important part in muscle recovery and regeneration following injury (Tidball 2005, Tidball and Villalta 2010).

The biphasic and promyogenic inflammasomal response to post-exercise protein-leucine might be regulated by mTOR pathway activity, with IL1β, IL6, NFKB and STAT3 the key transcriptional effectors

Dose-dependent signalling via the mTOR pathway might explain the temporal change in the direction (proinflammatory versus antiinflammatory) of the inflammasome seen with protein-leucine feeding. mTOR pathway signalling is correlated with, but does not quantitatively predict, the myofibrillar FSR response to post-exercise protein-leucine feeding. However, in its role as a master-switch controlling cellular growth and metabolism, mTOR-pathway signalling regulates other processes that are also likely to contribute to the inflammatory response. mTOR has been shown to alter polymerase I expression (Mayer, Zhao, Yuan and

Grummt 2004) and cell cycle activity (Nader, McLoughlin and Esser 2005), and appears to be integral to the regulation of immune cell function and tissue inflammation (Lee et al. 2007, Weichhart et al. 2008, Thomson, Turnquist and Raimondi 2009). Because mTOR pathway signalling is dose-responsive, effectors downstream from mTOR might be able to exert a fine level of control of inflammatory transcriptional responses under conditions of nutrient availability or excess.

The impact of feeding low-dose and high-dose protein-leucine nutrition on protein translation regulation via mTOR-pathway phosphoprotein activity was investigated in Chapter 5. The only substantial change in AMPKα phosphorylation was a small increase at 30 min with high-dose protein-leucine feeding, and similarly phosphorylation of $mTOR^{Ser2448}$ was moderately increased only at 30 min with the high-dose. $4E\text{-}BP1^{Thr37/46}$ phosphorylation was reduced at 30 min and 240 min with high-dose protein-leucine, while the low-dose only reduced 4E-BP1 phosphorylation at 240 min, relative to control; increased hyper-phosphorylation of 4E-BP1 to its gamma isoform mirrored the decline in 4E-BP1 phosphorylation. The effect of protein-leucine dose was most evident in phosphorylated $p70S6K^{Thr389}$ and $rps6^{Ser240/244}$ at 30 min and 240 min with low-dose and high-dose feeding. However, phosphorylation of eEF2 did not appear to have dose-dependent effects. As muscle tissue was only taken at 30 min and 240 min post-feeding, it is possible that other important but rapid (<30 min) or intermediate (30-240 min) changes in the phosphorylation state of mTOR and/or its downstream effectors were missed, given that others have showed temporal variation in mTOR-pathway phosphoprotein activation post-feeding (Atherton et al. 2010). For instance, when viewed in the context of the downstream changes, the pattern of mTOR phosphorylation suggests that with low-dose feeding an increase in phosphorylated mTOR probably occurred between 30 min and 240 min as subsequent supplemental beverages were

ingested and plasma leucine and essential amino acid concentrations increased. Experimental error might also be a contributing factor. Nevertheless, these data suggest that the phosphorylation of the mTOR-pathway phosphoproteins is protein-leucine dose-dependent. Furthermore, a large protein-leucine dose appears to be a requirement to induce an early, moderate-sized increase in mTOR phosphorylation, which was relatively delayed in the low-dose condition. There was a likely small increase in AMPKα phosphorylation at 30 min, but otherwise effects were unremarkable. Again, it is possible earlier or subsequent changes occurred but were missed by sampling.

The current investigation extends on the findings of other investigations of the impact of post-endurance exercise protein-feeding on mTOR-pathway phosphorylation in human muscle, by providing the first post-exercise evidence of dose-dependent phosphorylation activity. In endurance trained men, Ivy et al. found that ingestion protein-carbohydrate during recovery from 45 min of intense cycling increased phosphorylation of AKT, mTOR and rpS6, relative to a non-caloric placebo (Ivy et al. 2008). In trained male and female triathletes, Kammer et al. observed increased AKT and mTOR phosphorylation with milk and cereal ingestion following 2 h of moderate-intensity cycling, relative to a carbohydrate-matched control (Kammer et al. 2009). However, a criticism of these two investigations is that the comparative conditions were not energy-matched to the intervention. Protein translation has a high energy cost to the cell, and translation initiation signalling via mTOR is linked to the cellular energy status via AMPK (Dennis et al. 2001, Inoki, Zhu and Guan 2003, Hahn-Windgassen et al. 2005). The small increase in phosphorylated AMPKα at 30 min with high-dose protein-leucine relative to low-dose feeding is difficult to reconcile. AKT phosphorylation is increased by protein-feeding (Ivy et al. 2008, Kammer et al. 2009) and activated AKT functions as a negative regulator of AMPK. Thomson et al. found a reduction

in AMPK phosphorylation at 3 h post-exercise with protein-rich feeding (Thomson, Ali and Rowlands 2011), although the 4-h data from the current study 2 did not corroborate a protein-leucine induced change, which might be transient or tightly temporally regulated.

Earlier, Morrison et al. (Morrison, Hara, Ding and Ivy 2008) found that in rats swum to exhaustion then fed protein-carbohydrate, 4E-BP1-γ was increased by 33-120% relative to rats that were unfed, or fed only carbohydrate or protein. Interestingly, the concurrent reduction in 4E-BP1$^{Thr37/46}$ phosphorylation is in contrast to work in cultured rat fibroblasts (Dennis, Baum, Kimball and Jefferson 2011) showing that leucine and insulin additively increased Thr$^{37/46}$ phosphorylation, although 4E-BP1^{Ser65} and 4E-BP1^{Thr70} were not assessed. It is possible that the effects of prior intense exercise could alter 4E-BP1 phosphorylation (Ayuso et al. 2010) but this difference also emphasises the importance of *in vivo* human studies. 4E-BP1$^{Thr37/46}$ dephosphorylation and eEF2^{Thr56} phosphorylation might be a mechanism by which exercise impairs skeletal muscle protein synthesis, and eEF2^{Thr56} phosphorylation appears to be muscle fibre-type (type I) dependent (Rose et al. 2008). Future workers might consider isolating type I and type II fibres to determine if post-exercise protein or protein-leucine intracellular signalling effects are specific to type I fibres following endurance exercise. Breen et al. (Breen et al. 2011) found that while ingesting 20 g of protein and 50 g of carbohydrate in the first hour after 90 min of intense cycling did not substantially alter the p-eEF2^{Thr56}:total eEF2 ratio at 4 h relative to immediately post-exercise, the carbohydrate-matched control condition at 4 h was associated with a 40% increase from immediately post-exercise. In contrast, in the present study 4-h eEF2 phosphorylation was likely greater with the low-dose and possibly with the high-dose versus control feeding. Interestingly, Breen et al. (Breen et al. 2011) also found that mTOR phosphorylation was increased at 4 h with the protein-carbohydrate condition, compared with no clear change in

the current study 2; differences in the rate and quantity of protein (and leucine) provided could have contributed to this signalling difference.

Insulin signalling is also important to mTOR activation. Insulin stimulates mTOR and protein synthesis via AKT phosphorylation, although at normal cell amino acid concentrations, the effect on protein synthesis is minimal (Dennis, Baum, Kimball and Jefferson 2011). Amino acids signal mTORC1 through the Rag protein complex, while insulin activation of mTORC1 is via another regulatory protein Rheb (Dennis, Baum, Kimball and Jefferson 2011). The concentration of plasma insulin was greatest with protein-leucine feeding, while the difference between low-dose and control feeding was trivial despite the low-dose providing one third less total carbohydrate, reflecting the synergistic stimulatory action of carbohydrates and insulinotropic amino acids (e.g. leucine, arginine, glutamine, alanine, homocysteine (Newsholme and Krause 2012)) and their relative rate of ingestion in the respective supplements.

Expression of the inflammatory and myogenic regulators NFKB, STAT3, IL6 and IL1β is regulated by protein-leucine feeding in skeletal muscle

Skeletal muscle regeneration from injury is characterized by three overlapping stages of an initial neutrophil and macrophage-dominated inflammatory response, satellite cell activation and subsequent myofibre maturation and muscle remodelling (Ciciliot and Schiaffino 2010). mRNA-level evidence from the microarrays supports an increased signal to or amongst classically-activated (M1) macrophage at 30 min into recovery with protein-leucine feeding, but by 240 min there is a shift toward antiinflammatory biology and an alternatively activated

M2 phenotype for muscle-tissue macrophage. This phenotype shift from the M1 to the pro-repair, antiinflammatory M2c phenotype results in macrophage stimulation of satellite cell proliferation (Villalta et al. 2009) linking immune-cell function and inflammation to myogenesis. Prominent among IPA top-ranked genes upregulated at 30 min were genes related to myogenesis (*MYOG*, *MYOD* and its downstream transcription target *CDKN1A,* aka *p21*) that couldmight be regulated in part via early phase interactions between the initial inflammatory-response cells that infiltrate or are residents of the muscle. A reduction in IL6 expression is a marker of M1 phase macrophage deactivation (Villalta et al. 2009) and in contrast M1 macrophage secrete proinflammatory mediators such as TNFα and IL1β when activated (Olefsky JM 2010). Of course, changes in the transcriptome must be viewed with some caution given that the muscle biopsies contain multiple cell types (muscle, neural, endothelial, satellite cells) and that changes in expression levels do not fully determine the final or functional protein levels (Schwanhäusser et al. 2011). For instance, in study 1, there were only trivial effects of protein-leucine feeding on IL6 (Chapter 3) although the times samples were taken, normal sampling variation might explain the large variability in measured values that could have contributed to the outcome.

It has been suggested that activation of mTOR in myeloid cells is a mechanism to limit the proinflammatory response to pathogens (Säemann et al. 2009) and there is increasing evidence that mTOR has a potent antiinflammatory role in tissue-resident macrophages (Zhong et al. 2012). In microglial cells, the resident macrophages in the brain and spinal cord, resveratrol inhibition of lipopolysaccharide (LPS)-induced proinflammatory enzyme and cytokine production was via mTOR-dependent phosphorylative inhibition of NFKB, CREB and MAPKs (Zhong et al. 2012). Furthermore, mTOR activation in monocytes and macrophages limits caspase-1 and NFKB activity but enhances activation of interferon

regulatory element 5 (IRF-5) and IRF-7, producing bioactive IL1β and repressing IL12/23, but also stimulating STAT3 to produce IL10 (Schmitz et al. 2008, Weichhart et al. 2008). Inhibition of mTOR by rapamycin promotes NFKB-dependent proinflammatory cytokine production and inhibition of IL-10 release by STAT3 in bacterially-stimulated monocytes and macrophage (Weichhart et al. 2008). Furthermore, monocyte and macrophage TSC2 deletion diminishes NFKB activity, but increases that of STAT3 and reverses the proinflammatory shift (Weichhart et al. 2008). Taken together, the available evidence indicates a widespread and potent role for mTOR in regulating the function of multiple immune cell types, and the overall inflammatory response. High-dose feeding upregulated IL1β at 30 min, and downregulated IL6 at 240 min, and both cytokines were members of the top-ranked low-dose and high-dose versus control comparisons at those times. Additionally, IPA transcription factor analysis predicted inhibition of IL1β, IL6, STAT3 and NFKB transcription factor networks at 240 min into recovery with both low-dose and high-dose protein-leucine feeding (Table 6.2; and Figure 6.4).

STAT3 also interacts with MYOD to regulate the development of muscle cells (Kataoka et al. 2003). STAT3 signalling induces IL6 following muscle-lengthening contractions and stimulates proliferation of c-MYC+ satellite cells (Toth et al. 2011). Phosphorylated STAT3 has been observed in muscle stem cells 4 h after 300 muscle-lengthening contractions, and the downstream genes *cyclin D1* and *SOCS3* are upregulated at 24 h, and proliferation of stem cells peaked at 72 h following the lengthening contractions (McKay et al. 2009). Resistance exercise has been shown to phosphorylate $STAT3^{Tyr705}$, localize it to the nucleus, and increase downstream transcription of *c-MYC* and *c-FOS* (Trenerry, Carey, Ward and Cameron-Smith 2007). C-MYC is involved in regulation of cell cycle, metabolism, ribosome biogenesis, protein synthesis, and mitochondrial function (Jimenez et al. 2010) and is

predicted to be inhibited at 30 min with high-dose feeding versus control, but activated compared to the low-dose at 240 min (Table 6.2). *SOCS3*, a well-established negative regulator of STAT signalling (Trenerry, Carey, Ward and Cameron-Smith 2007), was down-regulated at 240 min with high-dose relative to control feeding. Taken altogether, activation of mTOR by increased concentrations of amino acids associated with post-exercise feeding might promote an antiinflammatory change that is also promyogenic in the skeletal muscle transcriptome by 240 min into recovery, via regulation of the expression of IL1β, IL6, STAT3 and NFKB.

Insulin-induction of AKT/p70S6K and p38-MAPK activity induces myoblast growth arrest (including *p21* expression) and myogenesis, concurrent with *NFKB* downregulation and NFKB translocation to the nucleus to initiate myogenic-gene transcription (Conejo et al. 2002). In the present analysis, we saw 30-min upregulation of *p21* and predicted NFKB inhibition at 240 min with protein-leucine feeding. Insulin receptor binding and rapid signalling might drive the early proinflammatory muscle transcriptome, whereas amino acid induced mTOR-pathway signalling, which requires an increase in the intracellular leucine concentration to activate mTORC1, might be responsible for the relatively delayed antiinflammatory signal observed at 240 min into post-exercise recovery with protein-leucine feeding. Interestingly, in response to excess amino acids STAT3 is phosphorylated by mTOR at serine 727, inhibiting amino acid induced insulin signalling in hepatic cells (Kim, Yoon and Chen 2009). Although the data of Kim et al. (Kim, Yoon and Chen 2009) are from liver cells, and mTOR phosphorylation of STAT3 might not occur in muscle or immune cells, it does at least allow the possibility for increased STAT3 phosphorylation when concentrations of insulinotropic amino acids (such as leucine) are high, which could dampen an insulin-

stimulated proinflammatory response at that time. More research is required to elucidate the role of STAT3 in muscle tissue, and until then any inferences are speculative at best.

Are reactive oxygen species involved in high-dose protein-leucine regulation of NFKB?

Reactive oxygen species might also be important signalling molecules involved in the inflammasomal response to protein-leucine feeding. IPA functions analysis inferred that ROS production increased at 240 min with the high-low dose contrast. In skeletal muscle, nitric oxide is synthesized from arginine by the nitric oxide synthases (NOS) 1, 2 and 3 (Moylan and Reid 2007), while superoxide is predominantly produced from complexes I and III of the mitochondrial electron transport chain (Barja 1999). Superoxide is also produced from NADPH oxidases located in the sarcolemma, transverse tubules and sarcoplasmic reticulum (Powers and Jackson 2008). ROS act as signal transducers from the cytoplasm to the nucleus, promoting gene expression; NFKB is one important redox-regulated transcription factor (Ji, Gomez-Cabrera and Vina 2007, Kramer and Goodyear 2007). Increased cytosolic ROS activate IKK which phosphorylates IkB, initiating its degradation and thereby releasing the NFKB complex to dimerize and translocate to the nucleus, but the oxidized NFKB-complex shows reduced DNA binding capability and activity (Kabe et al. 2005). Therefore, a secondary effect of protein-leucine feeding upon NFKB regulation might be via increased ROS production with high-dose feeding. Interestingly, the p70S6K arm of the mTOR pathway is one of several intracellular signalling conduits involved in mitochondrial protection and inhibition of apoptosis in response to elevated ROS (Juhaszova et al. 2004) and increased p70S6K phosphorylation at 240 min with high-dose feeding could, therefore, have a protective effect when ROS are elevated, or modify ROS signalling to NFKB.

A proposed muscle-tissue model for the impact of post-endurance exercise protein-leucine, carbohydrate and fat feeding on mechanisms of cellular repair and adaptation

All numbers in text related to Figure 7.1. (1) Exercise induces mechanical strain and is associated with muscle cell metabolic changes such as increased reactive oxygen and nitrogen species (RONS), that altogether can damage proteins, myofibrils, mitochondria, cell membranes, and intra- and extracellular scaffold and matrix glycoproteins (Faulkner, Brooks and Opiteck 1993, Tee, Bosch and Lambert 2007, Powers, Talbert and Adhihetty 2011). (2) RONS also regulate muscle inflammation via phagocyte infiltration (Aoi et al. 2004) and transduce signals through the cytosol to the nucleus to regulate the cellular response to the exercise stress (Hughes, Murphy and Ledgerwood 2005), which can trigger apoptosis (Brancaccio, Lippi and Maffulli 2010) and might signal translocation of NFKB to the nucleus (Kabe et al. 2005). At 240 min into recovery, transcriptome inference suggested reactive oxygen species were increased with high-dose feeding. (3) Cell-cycle arrest may act to impair further damage to genome integrity until cellular homeostasis is restored, and regulate muscle-cell proliferation, differentiation and development. Low-dose and high-dose protein-leucine feeding regulates *CDKN1A*, *GADD45A* and *DUSP1* expression consistent with cell cycle arrest and increased genome stability, although how this signal is transduced was not determined in the current studies. (4) Early-phase immune cells (neutrophils, recruited monocytes and tissue-resident macrophages) attracted and activated by cytokine and chemokine signalling and ROS infiltrate skeletal muscle to phagocytose cellular debris, stabilise cell membranes, and direct subsequent inflammation and myogenesis. The accumulation of phagocytes at 30 min and the transition from proinflammatory M1 scavenger to antiinflammatory M2 macrophages at 240 min is expedited by protein-leucine feeding and dose. Phagocyte accumulation at 30 min might be as a result of rapid and proinflammatory insulin signalling via the insulin receptor. An M1 to M2 transition might instead be regulated

by amino acid induced by (5) mTOR-pathway signalling and inhibition of the classical NFKB pathway, possibly transduced via p70S6K in response to rapid insulin signalling and (6) a downregulation of IL1β and IL6 coupled and altered NFKB and STAT3 activity. Changes in mTOR phosphorylation could regulate STAT3 transcription factor activity and might act as a feedback loop upon amino acid stimulated insulin signalling; changes in STAT3 phosphorylation and thus activity are inferred from the changes in mTOR phosphorylation and expression of STAT3 downstream gene targets (McKay et al. 2009) (Toth et al. 2011) (Trenerry, Carey, Ward and Cameron-Smith 2007). Evidence of a reduction in post-exercise plasma creatine kinase with protein and protein-leucine recovery feeding might be related to the membrane-stabilising role of M2 macrophage. (7) An increased myofibrillar FSR associated with protein feeding might accelerate the replacement of damaged proteins and accumulation of new adaptive myofibril proteins.

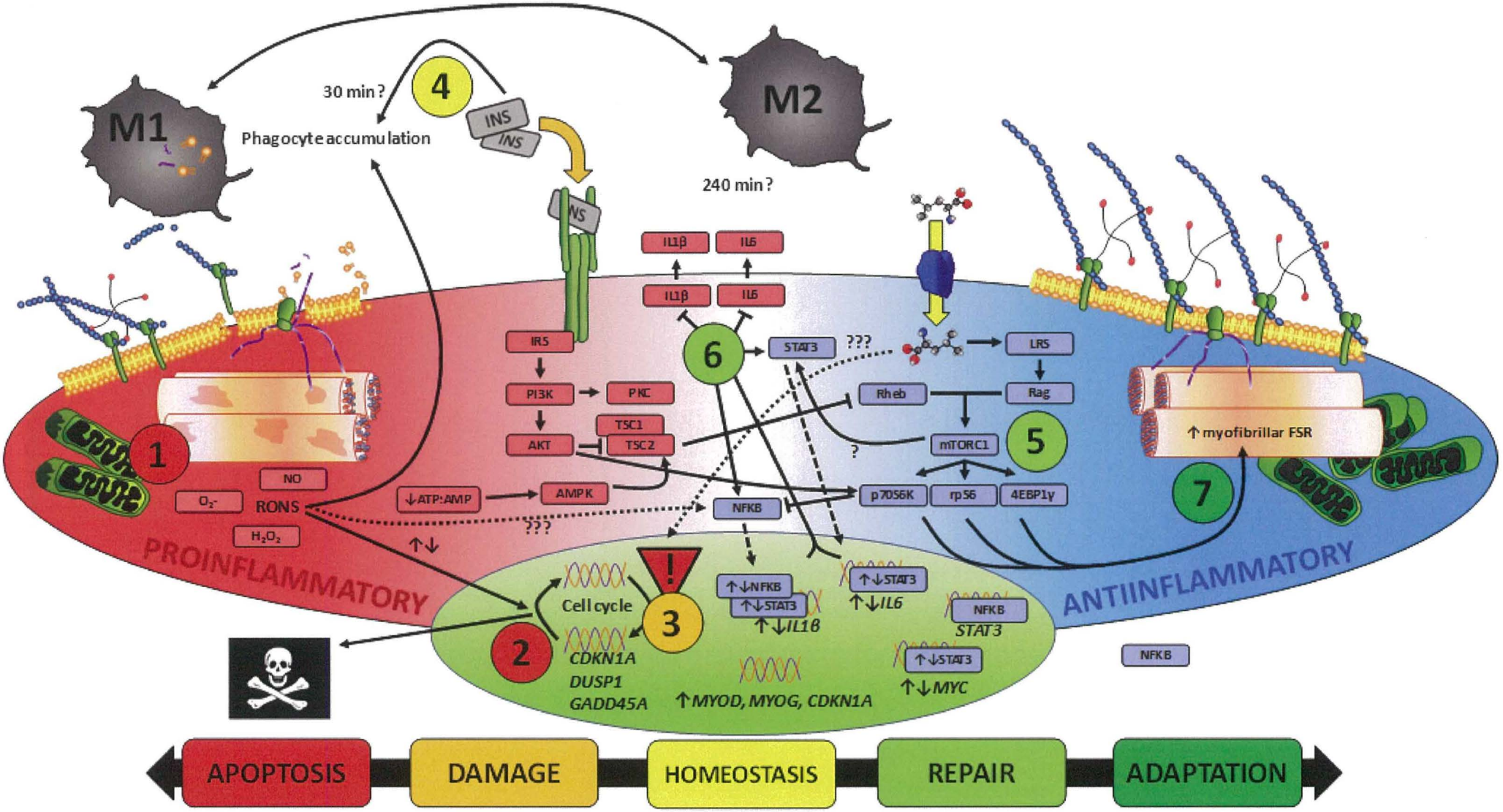


Figure 7.1. A skeletal muscle model for the impact of post-endurance exercise protein-leucine, carbohydrate and fat feeding on mechanisms of cellular repair and adaptation in a generalized muscle-tissue cell. Abbreviations: IRS, insulin receptor substrate. O_2-, superoxide ion; NO, nitric oxide; H_2O_2, hydrogen

peroxide; PI3K, phosphoinositol-3 kinase. PKC, protein kinase C. AKT, protein kinase B. TSC1/2, tuberous sclerosis complex protein 1 and protein 2. AMPK, adenosine monophosphate-activated protein kinase; Rheb, ras-homolog enriched in brain. LRS, leucyl-tRNA synthetase. Rag, RagGTPase complex. mTORC1, mammalian target of rapamycin complex 1. P70S6K, IL6, interleukin 6; IL1β, interleukin 1-beta; NFKB, nuclear factor of kappa beta; STAT3, signal transducer and activator of transcription 3; CDKN1A, cyclin dependent kinase inhibitor 1A; DUSP1, dual specificity phosphatase 1; GADD45A, growth-arrest and DNA damage inducible 45A.

CONCLUSIONS

Post-exercise protein and leucine coingestion differentially affected the expression of 200-500 genes of the muscle-tissue transcriptome across the dose and time comparisons, and enhanced the rate of protein synthesis in the muscle myofibrillar protein fraction. Amino acid-induced and dose-responsive intracellular signalling via the mTOR pathway was a likely key regulator of translation initiation of protein synthesis, but might also contribute to the pattern of gene-network expression observed during recovery. The protein-leucine fed recovery-transcriptome inferred a transient proinflammatory response with and impulse of ECM-deposition, and then antiinflammatory and promyogenic gene networks became prominent by the end of a four hour recovery period post-exercise. Upregulated expression of nutrition-responsive skeletal muscle growth and development, ECM and inflammatory genes suggested a moderated wound-healing response to exercise-induced muscle micro-trauma and, as such, might be a mechanism by which post-exercise protein-leucine feeding could improve muscle repair or stimulate adaptation. Based on the dose-responsive plasma concentrations, predicted mTOR-pathway activity, and the transcriptomal interrogation, crosstalk between insulin and mTOR signalling pathways might play an important role in control of the inflammatory response in the immediate hours after endurance exercise by regulating NFKB, STAT3, IL1β and IL6 expression and activity. Leucine, the insulinotropic and essential branch-chain amino acid, has the most potent effect on mTOR signalling of any amino acid, and showed moderate to large positive correlation with the phosphorylation of mTOR, p70S6K and rps6, and myofibrillar FSR, during recovery from exercise. Therefore, coingesting protein with leucine, carbohydrate and fat has the potential to augment skeletal muscle gene transcription, protein translation and inflammation via amino acid and insulin responsive intracellular signalling pathways, and could impact on the subsequent performance of intense endurance exercise. Nevertheless, despite attenuating creatine kinase

and altering circulating neutrophil superoxide production, the effect of ingesting post-exercise protein, leucine, carbohydrate and fat on subsequent performance was trivial during a six-day cycling protocol relative to isocaloric carbohydrate and fat. This could be the result of a background diet providing sufficient high-quality protein to establish positive nitrogen balance throughout days 2-5 of the training block, indicative of sufficient dietary amino acids for the sum metabolic needs. Thus, the quantity and type of dietary proteins ingested in the daily diet may be an important consideration for athletes during periods of intense training. Taken together with the available evidence, short-term protein-leucine supplementation might only be of an added benefit to high-carbohydrate feeding during times of inadequate dietary protein, despite apparent beneficial effects on acute recovery muscle physiology.

DELIMITATIONS AND PERSPECTIVES

Participation in the two investigations outlined in this thesis was limited to healthy, trained men aged 18-50 years of age. Mean age for study 1 (Chapters 3 and 4) was 35 ± 10 y and the range was in fact 18-50 y; and for study 2 (Chapters 5 and 6) the mean age was 30 ± 7 y and range 20-46 y (see Supplementary Data 7.1 Study 1 Descriptive Data.xlsx; and Supplementary Data 7.2 Study 2 Descriptive Data.xlsx). Age might be particularly pertinent for muscle protein turnover, which appears to decline with age in resting muscle (Short et al. 2004) although aerobic exercise training appears to increase rested fasted muscle protein synthesis in older muscle above the rate seen in sedentary young muscle (Robinson et al. 2011).

For both investigations, participants were predominantly cyclists (road and mountain bike) with several triathletes and multisport competitors who met the cycle training requirements. The minimum aerobic power requirement for study 1 was 55 $mL \cdot kg^{-1} \cdot min^{-1}$ and in regular training of >8 h cycling per week for the most recent 4-6 months, and preferably with high-intensity training or competition cycling in that time. For the second study the inclusion criteria for training was the same, but with a reduced aerobic power requirement (50 $mL \cdot kg^{-1} \cdot min^{-1}$). Supplementation in the first study was prescribed on the basis of peak power output, with riders producing the greatest power output consuming the most supplement (Supplementary Data 7.1, Tabs 'Day 1', 'Days 2-6' and 'Control all days'). The rationale for this was to balance estimated daily energy expenditure (exercise workloads during the six-day riding protocol were programmed at fixed percentages of peak power) but under the additional constraint that the background diet was controlled and with daily protein intake set at 1.5 $g \cdot kg^{-1} \cdot d^{-1}$ in the control condition but 1.9 $g \cdot kg^{-1} \cdot d^{-1}$. With each individual eating the

same food and drink on either arm of the crossover, the supplement provided the protein difference between the control and intervention total daily protein intake. On a gram per kilogram of body mass basis, the most powerful riders received the most protein and leucine. However, the dose of protein and leucine was prescribed at a rate we believed would be sufficient to saturate mechanisms that could impact performance, such as post-exercise muscle FSR (although this variable was not determined in study 1). Had the intervention nutrition been prescribed based on a set protein-leucine intake per kilogram of body mass, there would have been an energy balance differential that could have influenced performance instead.

Protein-leucine nutrition in the second study was prescribed at a set rate for all participants. Stature could influence the quantity of protein required to stimulate whole-body and muscle protein synthesis, and gene expression. Characteristics of participants in the second study (Supplementary Data 7.2) were: body mass, 78.09 ± 7.81 kg and range 68.04-88.45 kg; peak power output, 323 ±32 W and range 267-366 W; peak power output per kilogram of body mass, 4.16 ±0.48 $W \cdot kg^{-1}$ and range 3.93-4.93 $W \cdot kg^{-1}$. The supplemental intake therefore differed per kilogram of body mass (Supplementary Data 7.2). Every effort was made to select a homogenous cohort of participants, but at least some of the variation in FSR response might be attributable to factors such as body mass. In saying that, the outcomes of the protein translation signalling were clear and substantial effects.

In study 1, nitrogen balance was not substantially different between treatments on days 2-5 of the six-day cycling protocol, with an overall net-protein gain across the six days in both conditions despite a substantial net loss on day-1 and overnight of day-1 in the control

condition. Previously, dietary protein intakes of 1.6 $g \cdot kg^{-1} \cdot d^{-1}$ had yielded net negative nitrogen balance in a similar cohort of riders during a shorter investigation (Thomson, Ali and Rowlands 2011). However, there are well-known limitations in the nitrogen balance method that include an underestimation of nitrogen losses from non-sampled sites (e.g. skin, faeces). While we applied appropriate correction for these losses (see Supplementary Data 3.7) the assessment of nitrogen balance may still underestimate losses and, therefore, accentuate a net positive balance. Despite this, the size of the positive protein gain over days 2-5(Figure 3.5A) would tend to suggest that even with a small overestimation of nitrogen balance on each day of the training block, the overall outcome would still be a mild net positive nitrogen gain overall in the control (and protein-leucine) condition. Therefore, our hypothesis that protein or protein-leucine post-exercise supplementation might only be effective as an ergogenic aid in short (2-6 day) timeframes under conditions of net negative-to-neutral nitrogen balance holds firm.

Muscle tissue contains multiple cell lineages including myocytes, satellite cells, fibroblasts, and resident immune cells. The transcriptomal response to exercise and protein-leucine nutrition represents a mean global muscle-tissue response and, therefore, extrapolation to a single cell type is not appropriate. In saying that, cell-types differentiate themselves by the expression of different functional proteins; for instance, macrophages express high levels of the fat receptor CD32, and satellite cells express high levels of (at various stages of proliferation and differentiation) MYOD1 and MYOG. Therefore, clear and consistent changes in the expression of genes coding for these specific protein markers and with corresponding changes in the expression of up or downstream functionally-related genes in a global muscle-tissue sample may be interpretable as a programme for a cell-type specific response. Furthermore, changes within biologically-conserved signalling pathways acting as

major regulators of cell growth, differentiation and fate, may be characteristic responses amongst most cells types. A final caveat is that changes in gene expression do not necessarily indicate functional protein changes in those gene-products, nor a similar magnitude of protein response to that of the mRNA transcript, owing to post-translational regulation. It is probably reasonable to suggest that the direction of change of the protein-product is in line with that of the mRNA e.g. an upregulation of the mRNA results in some increase in the protein-product, but whether that increase is of a meaningful magnitude cannot be resolved in the present analysis. Additionally, many proteins are stored in membrane-bound vesicles and released to the cell membrane upon stimulation, and therefore their mRNAs may not reflect the functional protein levels. However, because we also measured translation initiation signalling via mTOR-pathway activity and the global myofibrillar FSR, changes in protein synthesis would at least support any increased transcript abundance, and presumably increase a differential between the protein-products of temporally-aligned upregulated and downregulated transcripts which could be important for functional change within a cell.

The study design included a week of lead-in training and diet prior to each experimental visit. An interaction between repeated exercise bouts and the habitual diet during lead-in could result in a shift out of quiescence for some of the muscle satellite cell population. Therefore, biopsied *vastus lateralis* muscle samples would likely contain a mixture of quiescent, activated, proliferated, differentiated and self-renewing satellite cells. However, this constant state of turnover among muscle satellite cells is likely the case in endurance athletes training regularly and intensely anyway and, therefore, represents a realistic snapshot of muscle-tissue cellular turnover.

RECOMMENDATIONS FOR FUTURE RESEARCH

It is possible that post-exercise protein ingestion may only be of substantial benefit to the performance of intense endurance exercise under conditions of neutral-to-negative nitrogen balance, at least during a short-term period of several days of intense training. Therefore, the impact of post-exercise ingestion of protein or protein-leucine, carbohydrate and fat on subsequent performance under conditions of positive, neutral and negative nitrogen balance deserves further investigation. Providing post-exercise protein, leucine and carbohydrate at a time of dietary inadequacy could take advantage of a plausible enhanced sensitivity to amino acid or insulin stimulation, or amplify signalling or protein synthesis. Thus, measuring mTOR pathway signalling and muscle protein synthesis could also be outcome measures. Net positive to negative nitrogen balance could be established by appropriately high, moderate and low dietary protein intakes in the background diet, or alternatively, the background dietary protein content could be maintained and more protein provided during recovery either at a higher rate of provision within a predetermined recovery time, or at a constant rate but with a longer recovery to spread the feeding over. In order to specifically determine the effect of protein in the diet on tissue (whole-body or muscle) protein metabolism, it might be possible to construct a diet of intrinsically-labelled foods, which would presumably mean a diet based heavily around foods made from intrinsically-labelled wheat (e.g. (Bos et al. 2005)) and labelled dairy-based products (e.g. (Koopman et al. 2009)) and with other low-protein foods (vegetables, fruit) to ensure sufficient carbohydrate for a practical endurance exercise study. Otherwise, it might be feasible to add a crystalline free amino acid tracer to mixed-meals if the total protein content and amino acid composition of each meal is known, though considerable piloting would need to be done to establish if this could be a valid method.

At the muscle level, mechanisms contributing to a proposed neutral-to-negative nitrogen performance enhancement effect most likely include an elevated post-exercise FSR and initiation of an accelerated acute recovery and adaptation transcriptome, and regulated via activation of key intracellular signalling pathways by post-exercise increases in insulin and amino acids. Accordingly, skeletal muscle FSR and the transcriptome for important repair and recovery biology should be outcome measures and, if practical, assayed concurrently with performance measures to establish the strength of any relationship. These processes are protein-leucine dose-responsive, but total dietary protein and the types of proteins ingested affect muscle protein metabolism at rest and following endurance exercise, and might also impact cellular sensitivity to the post-exercise nutrition. Therefore, future research should also aim to determine the impact of manipulating the background-diet protein quantity on fed-recovery skeletal muscle FSR and repair and recovery biology of the transcriptome. To do so, stable-isotope tracer infusions and muscle biopsies could be integrated into a low/moderate/high dietary protein performance-study design (or subtle variation thereof), such as that intimated. A practical method could be to utilize the single-biopsy method and primed continuous tracer infusion during an intense training block to provide a snapshot of the muscle FSR response, but with a consideration to limit a possible impact of the biopsy procedure on performance variability.

To briefly outline a potential study scenario, in a three-way crossover design a multiday laboratory-based model of a period of intense training, similar to that outlined in Chapter 3 or by Thomson et al. (Thomson, Ali and Rowlands 2011), could be used to assay the impact of several intervention conditions (e.g. a low, moderate and high quantity of background-diet protein with all treatments receiving an identical post-exercise protein-leucine-carbohydrate supplement) on performance. Diet and exercise would be controlled in the days prior to the

experimental protocol. During the multiday exercise regimen minor outcome measures could be sampled (for instance, blood for circulating hormones, cytokines, miRNAs, immune-cells, and metabolomics, and urine for metabolomics from which nitrogen losses could be estimated). To determine an impact of diet on the supplement on muscle FSR during the training block, the single-biopsy method is preferred as no baseline biopsy is required; one muscle sample could be taken at the designated end of supplemented recovery from a mid-block performance test (e.g. the current study 1) and the impact of several days of a low/moderate/high protein diet could be assessed; a snapshot of the transcriptome would also be available from the muscle sample. This approach would minimize any effect of the biopsy procedure on variability of sensitive exercise performance tests.

However, this methodology would necessitate the use of at least three tracer isotopomers, for instance, a carbon, nitrogen or hydrogen labelled leucine and/or phenylalanine tracer, as the single-biopsy method is suitable only for tracer-naïve participants. It should be noted that further measurement of muscle FSR following the final performance bout could be achieved using standard serial biopsies. Additionally, if there were concerns over the impact of the continuous infusion method of mid-week and end-block performance tests (owing to the presence of an indwelling intravenous infusion line while performing repeated maximal-effort cycling sprints) then a flooding-dose method could be used, although this might limit the extent of the recovery period over which FSR could be measured, providing only an early-recovery FSR. To overcome this, immediately post-exercise an additional primed continuous infusion of a fourth tracer could be applied throughout all study arms to assess longer-duration recovery FSR responses. Alternatively, a previously-used tracer (from a previous study arm) could be applied, although this approach would effectively reduce the

sample size for the late-recovery FSR response as this would only be feasible for the second and subsequent crossover arms.

Upregulation of the expression of key ECM genes appears to be related to an individual's responsiveness to endurance training (Timmons et al. 2005), and acute endurance-like exercise increases collagen protein synthesis in the patellar tendon for at least 72 h post-exercise (Miller et al. 2005) indicating that ECM remodelling is a key factor determining successful adaptation to endurance training. Changes in collagen synthesis could be investigated in leg tendons in response to exercise and feeding, though the invasiveness might preclude this method from being used during a performance study at present.

Deuterated water administration (^2H_2O) appeals as a simple method to measure longer-term rates of protein and DNA synthesis in humans (Gasier, Fluckey and Previs 2010). Only one study has investigated the effect of chronic protein-carbohydrate supplementation following endurance exercise on muscle protein synthesis; it was found that post-exercise protein did not substantially alter longer-term rates of muscle protein synthesis in older individuals (Robinson et al. 2011). This method might be useful for chronic studies and in conjunction with other methods to assess acute (e.g. 0-4 h) recovery and 24 h FSR responses to enable temporal variations to be determined.

Future work may take advantage of other emerging technologies and methods (e.g. see Chapter 2). The assessment of a wide array of muscle proteins, or the full muscle proteome; changes within fibre-types or specific to myocells or other cell lineages such as muscle-

resident fibroblasts and immune cells (e.g. using laser dissecting microscope to separate cell types); and consequences to the tissue or systemic metabolome of protein and protein-leucine supplementation under a mix of low to high dietary protein intakes would greatly enhance our understanding of the interaction of nutrition-induced molecular and early adaptive remodelling and the temporal sequence of events in exercised muscle. There is already evidence that type I and type II fibres differ in their synthetic rate at rest and with feeding. The rates of synthesis of key proteins involved in endurance adaptation, including mitochondrial components or cytoskeletal proteins, should be determined. Will the coupling of nutrient-responsive gene expression changes in the observed biology with an increased acute recovery protein synthetic rate result in a corresponding increase in functional proteins? Medium-term (4.5-8 wk) training combined with post-bout supplementation has been shown to increase VO_2max and lean body mass relative to carbohydrate and/or placebo in untrained young males and females (Ferguson-Stegall et al. 2011), and in moderately active older men with endurance adaptations of the cardiovascular system (plasma volume expansion) (Okazaki et al. 2009). It is, however, difficult to reconcile these findings with the observed lack of a substantial difference in long-term protein synthesis with post-exercise protein-carbohydrate ingestion (Robinson et al. 2011) apart from perhaps methodological limitations with the later study, and that the participants were older individuals (range 37-64 y; mean 50 ± 8y).

Given the logistical complexity and time and financial cost of stable isotope tracer enrichment of dietary and supplemental foods, prolonged infusions, repeated muscle biopsies and subsequent analyses - and notwithstanding the burden upon participants – such investigation may best be achieved in small steps. However, utilizing methods in exercise science as they are mainstreamed from other biological, chemical and physical sciences will

see costs reduce, and improving collaboration will increase access to technology and expertise.

Based on the current evidence (Study 3), approximately the same total protein is required to maximally stimulate myofibrillar FSR following endurance exercise as following resistance exercise; a caveat is that confirmation of the findings of Moore et al. (Moore et al. 2009) and of the current investigation is warranted. Subsequent research should determine an optimal dose-response for myofibrillar FSR and perhaps also the peak activation and timecourse of nutrient-responsive gene expression changes following endurance-type exercise. For instance, knowledge of peak activation of effectors from amongst the mTOR signalling pathway (e.g. rpS6, p70S6K, 4EBP1γ) may be useful to design repeat-feeding strategies in order to optimize the nutritional signal that appears to stimulate both transcription and translation. The impact of the so-called 'muscle-full effect' (Atherton et al. 2010) should be investigated after endurance exercise also.

The use of the tracer methods to assess individual amino acid requirements would provide some methodological and analysis advantages over the nitrogen balance method (Hayamizu, Kato and Hattori 2011); the oral indicator amino acid oxidation method might be preferential to the intravenous method and direct amino acid oxidation as there appear to be trivial differences between oral and IV methods in estimation of amino acid requirement, with the advantages of being less invasive, less technically demanding, and allows the study of a range of protein intakes in the same individual (Zello, Wykes, Ball and Pencharz 1995, Kriengsinyos, Wykes, Ball and Pencharz 2002, Humayun, Elango, Ball and Pencharz 2007, Elango, Humayun, Ball and Pencharz 2010) and can assess requirement for amino acids that

cannot be studied by direct oxidation because of their particular metabolism (Lazaris-Brunner, Rafii, Ball and Pencharz 1998, Wilson, Rafii, Ball and Pencharz 2000, Elango, Ball and Pencharz 2008). Furthermore, this approach can be used to determine the metabolic availability of individual amino acids in the test protein supplement (Humayun et al. 2007, Elango, Ball and Pencharz 2009). Large-scale plasma and urine metabolomics to assess nitrogen turnover and excretion is another alternative to the nitrogen balance method.

The temporal pattern of change in gene expression during recovery in response to exercise, nutrition and insulin and other hormones also deserves investigation. Endurance exercise stimulates time-dependent changes in a range of muscle mRNAs related to inflammation, muscle growth and protein turnover (e.g. IL-6, IL-8, myostatin, TNF-a, MuRF-1, atrogin-1, FOXOA3 (Louis et al. 2007)) while insulin treatment mediates time-dependent transcriptional patterns among functionally related gene clusters in rat skeletal muscle (Di Camillo et al. 2012) and inflammatory and proangiogenic biology in cultured myotubes from type II diabetic patients (Hansen et al. 2004).

Finally, the present body of work provides transcriptome-level evidence that inflammation and the immune response are key facets of post-exercise recovery. Protein-leucine feeding has effects on circulating neutrophil functional activity and concentrations (Chapter 4) and the skeletal muscle tissue inflammasome (Chapter 6). Additional work should look at quantitative antibody staining of exercised muscle tissue with panels of antibodies for markers of neutrophil or macrophage cells and secreted molecules, such as, anti-elastase, anti-myeloperoxidase and anti CD11b for neutrophils. The relationship between neutrophil function and muscle morphological changes should be investigated, and an impact of protein-

rich feeding could be investigated to determine if changes in circulating neutrophil function and gene biology contribute to functional differences at the muscle. Muscle myogenesis is related to the immune and inflammatory response. If quantitative investigation of the post-exercise muscle-tissue immune cell response to protein ingestion is conducted, a concurrent investigation of satellite cell activity using appropriate markers would also be advantageous to elucidate the functional change in muscle and the temporal response, given that progression of muscle repair and adaptation requires both.

APPENDICES

APPENDIX A RESEARCH PROPOSAL

RESEARCH PROPOSAL FOR PhD

Provisional Registration Date: 25/03/2008

Expected date of completion: end of 2011

The effects of leucine-enriched protein-carbohydrate supplementation following high-intensity endurance exercise: subsequent performance effects, and molecular and cellular mechanisms

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ABSTRACT

Background:

Chronic training results in long-term muscle adaptation in response to the cumulative effects of repeated bouts of exercise and can be viewed as increases in exercise-induced proteins specific to the type of exercise undertaken (Hawley et al. 2006). Optimal adaptation to repeated, high-intensity training likely requires "an optimal diet"; a diet that provides sufficient energy and nutrition to replenish muscle energy stores and facilitate recovery and adaptation (Coyle 2000, Hawley et al. 2006). A key mechanism for optimal recovery and adaptation is probably the net rate of muscle protein synthesis immediately post-exercise; nutritional interventions may enhance recovery if they increase protein synthesis and alleviate protein breakdown. Essential amino acids directly stimulate protein synthesis in a dose-dependent manner (Bohe et al. 2001), and act through signalling pathways such as mTOR, the mammalian target of rapamycin (Deldicque et al. 2005). Branched-chain amino acids, particularly leucine, are the most important amino acid nutritional signals affecting translational up-regulation (Bolster et al. 2004, Crozier et al. 2005, Kimball and Jefferson 2004, Koopman et al. 2005). Studies have suggested ~8.5-10g of EAA may saturate protein synthesis (Cuthbertson et al. 2005, Miller et al. 2003, Moore et al. 2009) but only a small body of research has looked at post-endurance exercise amino acid supplementation. mTOR is a key signal transduction protein involved in integrating environmental stimuli and subsequently regulating gene transcription and translation. Further research is need on how nutrient signals interact with exercise signals at a molecular level during the recovery period from high-intensity endurance-exercise, and linking these signals with greater protein synthesis and better subsequent performance. Recently, Rowlands et al. (Rowlands et al. 2008) found a post-exercise protein-enriched feeding intervention had no clear effect on 15 h next morning high-intensity cycling performance, but a clear-cut substantial effect 45 h following (60 h after the first exercise). This delayed effect possibly relates to the time-course for repair and protein expression (Rowlands et al. 2008). There is evidence indicating leucine-enriched and/or whole-protein supplementation upregulates genes associated with adaptation to endurance exercise and increases activity of the mTOR pathway, which in part controls protein synthesis. However there are no direct measures of protein synthesis in response to the added leucine conditions, the dose that can optimise protein synthesis is not known, and linking the upregulation of genes with greater protein accretion and enhanced subsequent performance has yet to be established.

Aims and Objectives:

1. Determine the magnitude of the effect of a leucine-enriched protein-carbohydrate recovery supplement (LeuPlus) ingested following several days of prolonged high-intensity intermittent exercise on subsequent performance several days later, relative to a control iso-caloric high-carbohydrate supplement.

2. Non-invasively study the mechanism of action of the LeuPlus supplement via measures of carbohydrate metabolism and protein turnover, a marker of muscle cell-membrane damage, the degree of the systemic immune and inflammatory response, and perceptions of wellbeing and tiredness.

3. Determine the dose of LeuPlus that elicits the peak fractional protein synthetic rate outcome.

4. Determine the effect of dose on the transcriptomal profile, on the signal transduction pathways linked to regulation of translation and cellular anabolism, on the expression of transcriptional control factors important in the regulation of mitochondrial biogenesis and other processes involved in adaptation to endurance exercise, and on muscle glycogen restoration.

Methods:

The PhD will consist of two laboratory intervention studies; data collection for the first being completed, and the second in preparation.

Study 1: In a double-blind placebo controlled crossover, 12 trained male cyclists completed a 6-day intense ergometer cycling regimen. This consisted of two strenuous rides (day 1; 3hr, day 2; 2.5hr), two light recovery rides (days 3 and 5; 1hr) and two 90 min rides followed by repeated sprint performance test on days 4 and 6. Participants received an isocaloric supplement in half hourly feedings immediately post-exercise comprising protein, free leucine, carbohydrate, and fat (LeuPlus) or carbohydrate and fat only (Control). Performance was assessed using the magnitude of effect (change) in repeated sprint time. 1-^{13}C-leucine, [6,6-$^{2}H_2$]-glucose stable isotope infusions were used to determine whole body protein turnover, and plasma glucose metabolism. Blood samples were taken at various time points for analysis of the effect of supplement on metabolomic profile, as well as assessing testosterone, cortisol, insulin, glucose, muscle damage and selected markers of systemic immune function and inflammatory response. Sweat and urine were collected for metabolomics and to assess nitrogen balance.

Study 2: In a single blind 3-way crossover study, 12 male endurance cyclists/recreational fitness enthusiasts will perform a single 2 h bout of high-intensity cycling followed by the ingestion of low, high and control levels of protein, leucine, carbohydrate and fat. During recovery, *ring*-$^{13}C_6$-phenylalanine will be infused and biopsies of the V. lateralis at 1 and 4 h post exercise collected for measurement of muscle protein myofibrilar and mitochondrial fractional synthetic rate. The expression of candidate genes for control of mitochondrial biogenesis and other targeted processes indentified in the recent gene ontology analysis (regulation of myofibrilar and intra and extracellular matrix proteins, stress response, ubiquitin proteosome, immunoattractive protein expression, chemokines such as MCP-1), and ion and solute transporters) will be quantified using RT-PCR, the pattern of global gene expression determined by Illumina microarray, and the activity of the akt-mTOR-p70s6k-4EBP1 pathway assessed by Western blot.

Literature Review to be completed encompassing LeuPlus and protein+CHO use after exercise as a means for enhancing recovery in athletes. It will focus on endurance exercise but may include research from resistance exercise studies also. It will highlight the importance of post-exercise recovery to subsequently adaptation, outline current knowledge in the field, and identify areas that need further work such as finding an optimal dose, and linking signalling and protein synthesis to performance.

Expected Outcome: The findings from the first study should provide relevant information for expanding the area of knowledge around leucine, protein and their combined ingestion with carbohydrate and fat following high-intensity endurance exercise. We expect an improvement in subsequent performance following LeuPlus feeding relative to an isocaloric carbohydrate+fat control. We aim to provide evidence for possible mechanisms of better performance from enhanced immune function, reduced muscle damage and a decreased stress response, and possibly altered fuel substrate metabolism. The second study will develop the cellular and molecular investigation further, and provide valuable data on the dose of leucine-enriched protein necessary to saturate fractional protein synthesis following high-intensity endurance exercise, and shed light on the effect of dose on specific transcriptomal and signal transduction pathways likely involved in the cellular response to exercise and nutrients and adaptation to exercise.

CONTENTS

PURPOSE

Main Research Questions

i) Does the ingestion of a LeuPlus following high-intensity endurance exercise improve recovery of subsequent performance capability?

ii) If there is a performance benefit, are there associated changes in muscle damage, immune and inflammatory systems that may explain a mechanism for enhanced performance?

iii) How does the metabolomic profile change in response to the ingestion of LeuPlus following exercise?

iv) What is the optimum (minimum) dose of LeuPlus required to maximally activate muscle fractional protein synthesis following a single bout of high-intensity endurance exercise?

v) Do different doses of LeuPlus produce different signalling and transcriptomal responses in candidate signal transduction pathways and genes involved in recovery and adaptation to endurance exercise? How does the effect of different doses of LeuPlus alter the signal transduction pathways linked to regulation of translation and cellular anabolism, and the transcriptomal profile with regard to genes involved in the adaptation to endurance exercise, in endurance-trained males?

Secondary Research Questions

i) If there is a benefit of subsequent performance following high-intensity endurance exercise, what is the magnitude of effect?

ii) Is there a beneficial effect of post exercise LeuPlus coingestion on markers of muscle damage, systemic immune response and inflammation, compared to control, over a 6 day intervention? Do these changes suggest a possible mechanism to explain any enhanced performance?

iii) If muscle mitochondrial and myofibrillar protein synthesis shows evidence of saturation at both the low and high protein+leucine doses, are there corresponding changes in our candidate signalling pathways and transcriptomal control factors that can explain this result? Are there other potential factors involved that need investigating?

v) To what extent is the expression of candidate genes changed in response to 2 doses of a LeuPlus- post-exercise recovery supplement, relative to a control?

iv) To what extent are the activities of signalling proteins changed in response to 2 doses of a LeuPlus post-exercise recovery supplement, relative to a control

v) Does the low LeuPlus supplement produce the same degree of expression of candidate genes compared to high LeuPlus supplement? Is this transcriptomal profile different to the zero protein control condition?

BACKGROUND

Chronic training results in long-term muscle adaptation in response to the cumulative effects of repeated bouts of exercise (Hawley et al. 2006) and can be viewed as increases in exercise-induced proteins specific to the type of exercise undertaken, that occur via a coordinated series of events in response to single and repeated exercise bouts (Hansen et al. 2004, Hawley et al. 2006)..As such, it is important to understand the acute cellular, metabolic and molecular changes that occur during and after exercise in order to conduct research or plan to maximize

training adaptation and recovery (Hawley et al. 2006). At the onset of and during endurance exercise, enzymes involved in energy production and muscle contraction activate rapidly, leading to intensity-dependent changes in metabolite concentrations such as [ADP] and [AMP] and other associated events such as a drop in blood and muscle pH, increased muscle lactate concentration, substrate depletion, and impaired oxygen flux (Hawley et al. 2006). Such metabolic disturbances result in the activation of signal transduction pathways that mediate various cellular processes responsible for metabolic and growth responses in the face of stressors; these include AMPK, MAPK, Akt/PKB and mTOR transduction pathways (Hawley 2002, Hawley et al. 2006). Because of the wide array of metabolic and morphological adaptations to endurance exercise, multiple factors probably work together during adaptation, rather than one single system (Hawley 2002). Chronic endurance training is associated with an increase in mitochondrial electron transport chain enzyme activity, and mitochondrial protein concentration. There is also a shift toward a greater reliance on fat as substrate over carbohydrate during exercise. Optimal adaptation to repeated, high-intensity training likely requires "an optimal diet"; a diet that provides sufficient nutrition to facilitate recovery and adaptation, and sufficient energy to replenish muscle energy stores (Coyle 2000, Hawley et al. 2006). One of the key mechanisms likely to be involved in recovery and adaptation is the net rate of protein synthesis immediately post-exercise; a greater, more prolonged protein synthetic response in skeletal muscle should lead to faster adaptation by faster production of new muscle tissue proteins, and the repair of proteins used to produce force and energy. Rat wheel-running studies indicate chronic aerobic training increases skeletal muscle protein synthesis rates (Munoz et al. 1994, Reynolds et al. 2004). Short et al. (Short et al. 2004) completed a study of seventy-eight healthy, untrained men and women aged 19-87yrs, and found mixed muscle protein synthesis increase 22% following 4 months of bicycle training of ~ 45min at 80% peak heart rate, 3-4x per week, versus control (stretching) activity; methodological limitations meant this increase was likely underestimated and thus may have been even higher. It is likely that chronic endurance training increases resting protein synthesis rates. Protein synthetic responses to acute bouts of endurance exercise have also been investigated. Sheffield-Moore et al. (Sheffield-Moore et al. 2004) used a short (45min treadmill walk at 40% VO2peak) aerobic exercise bout and found increased protein synthesis, as well as breakdown, post-exercise, with a net negative rate of protein synthesis. Earlier studies (Carraro et al. 1990, Tipton et al. 1996) had longer and more intense exercise and different modalities (combined resistance exercise and swimming, and 4hr treadmill walking respectively), and unsurprisingly outcomes were equivocal; Tipton et al. found mixed muscle FSR increase ~41% above resting but without statistical significance (Tipton et al. 1996); Carraro et al. reported increased mixed muscle FSR post-exercise but also increased muscle breakdown (Carraro et al. 1990). Despite differences in methodology, exercise mode, intensity and duration, and subject cohort, acute aerobic exercise does appear to cause moderate, transient increases in muscle protein synthesis in the absence of nutrition, post-exercise (Sheffield-Moore et al. 2004). The effects on muscle protein breakdown are less clear but acute aerobic exercise bouts without nutritional intervention probably result in a temporary increase in protein breakdown, leading to a short period of enhanced protein turnover and a net negative protein gain (Sheffield-Moore et al. 2004). Therefore nutritional interventions may enhance recovery if they act to further enhance protein synthesis and alleviate protein breakdown leading to protein accretion.

The main rationale for the use of amino acid supplementation is that the use of a supplement will increase net muscle protein synthesis more than if regular food was ingested alone

(Paddon-Jones et al. 2005). Supplementation should create a stronger anabolic signal than a regular meal, but not be so energy and nutrient dense as to inhibit a subsequent anabolic response from daily food intake or reduce an individual's ability to consume regular meals. The general responses to feeding on human muscle protein synthesis were succinctly summarized by Rasmussen & Phillips (Rasmussen and Phillips 2003); muscle protein synthesis increases 30-100% from oral or intravenous feeding, with amino acid stimulation a major component; the effect of insulin on protein synthesis is dependent on amino acid availability; and the stimulation of protein synthesis contributes far more to the anabolic response than the inhibition of protein breakdown. Also, the response is transient (lasting a few hours following a meal) and breakdown exceeds synthesis in a fasted state (Rasmussen and Phillips 2003). Unfortunately there is only a small body of research looking at amino acid post-endurance exercise supplementation. Recent work has shown that essential amino acids (EAAs) are effective at stimulating muscle protein synthesis (Cuthbertson et al. 2005, Deldicque et al. 2005b, Fujita et al. 2007, Koopman et al. 2006, Miller et al. 2003). EAAs directly stimulate protein synthesis of the main intracellular fractions (myofibrillar, sacroplasmic, mitochondrial) in a dose-dependent manner(Bohe et al. 2001), and act through nutrient, cellular stress and energy sensing signal transducers such as mTOR, the mammalian target of rapamycin (Deldicque et al. 2005). Upon investigation into different EAAs and their effects on molecular signals and protein synthesis, it was found that the branched-chain amino acids (BCAAs), in particular leucine, are the most important amino acid nutritional signals affecting translational up-regulation (Bolster et al. 2004, Crozier et al. 2005, Kimball and Jefferson 2004, Kimball and Jefferson 2006, Koopman et al. 2006). EAAs or BCAAs alone as supplementation are likely not optimal for promoting enhanced net protein gain however; it has been shown that branched-chain amino acids (BCAAs) can enhance translation but at the same time inhibit proteolysis, resulting in a deprivation of amino acids needed for protein formation, if fed alone (Wolfe 2002). Therefore optimal supplementation will need to include both EAA and non-EAA amino acid components, as well as consideration for carbohydrate intake for glycogen restoration post-endurance exercise, and carbohydrate and fat for energy intake and palatability of an oral supplement.

The benefit of post-exercise intake of carbohydrate to restore muscle glycogen is well established (reviewed in (Millard-Stafford et al. 2008)). The effect of protein-carbohydrate mixtures on glycogen resynthesis has been investigated, with most but not all studies showing no additional benefit to muscle glycogen resynthesis rates with mixed protein-carbohydrate supplementation over carbohydrate alone (Berardi et al. 2006, Tarnopolsky et al. 1997, van Loon et al. 2000, Zawadzki et al. 1992). It may be that there is no additional glycogen resynthesis benefit when adding protein if carbohydrate intake is above a saturation point of around $1.2g.kg^{-1}.h^{-1}$, but at lower carbohydrate intakes protein coingestion may increase glycogen resynthesis via enhanced insulin secretion leading to greater glucose uptake and use by skeletal muscle. The response of muscle protein synthesis to the combined ingestion of carbohydrate and essential amino acids is, at least, the sum of the individual anabolic effects of the carbohydrate (which stimulates the insulin response) plus that of the EAAs (Miller et al. 2003), and in young individuals may be greater than the sum of their independent effects (Volpi et al. 2000). van Loon et al. (van Loon et al. 2000) showed that plasma leucine, phenylalanine and tyrosine concentrations are positively correlated with an insulin response at rest, and that amino acid and protein hydrolysate mixtures of $0.4g.kg^{-1}.h^{-1}$ can be used with carbohydrate doses of $0.8g.kg^{-1}.h^{-1}$ to produce up to a 100% greater insulinotropic effect than $0.8g.kg^{-1}.h^{-1}$ carbohydrate alone. This indicates amino acids themselves have a stimulatory effect on insulin release. However, insulin acts at low (~5mU) concentrations to reduce

protein breakdown – greater concentrations do not result in any further benefit (Bell et al. 2005, Miller et al. 2003). It does appear that insulin increases the potential for accelerated muscle protein synthesis post-exercise, but only in the presence of adequate amounts of amino acids (Bell et al. 2005, Miller et al. 2003, Rasmussen and Phillips 2003). Timing of the insulin response may be critical; Miller et al. (Miller et al. 2003) suggest that the peak insulin effect may need to be coupled with the peak amino acid effect to maximize amino acid uptake; amino acids ingested in a more slowly absorbed form (intact protein) are likely to have a greater interactive effect with insulin, as the peak actions of each overlap for a greater period of time. Total energy intake post-exercise may not be as important as amino acid content; in a study of 10 subjects performing 60 min of cycling at 60% VO_{2max} and supplemented with either 0/8/3 g of protein:carbohydrate:fat (SUPP), or 10/8/3 g (SUPP+PRO), or no nutrition (NO), leg and whole-body protein synthesis was increased 6-fold and 15% respectively in the SUPP+PRO condition; Levenhagen et al. proposed that the availability of amino acids may be more important than the availability of energy for post-exercise repair and protein synthesis (Levenhagen et al. 2002). Whether this holds true for longer (>60min) exercise, at greater intensities, or multi-day events remains to be investigated. Moore et al. (Moore et al. 2009) investigated the effect of egg protein doses of 0, 5, 10, 20 and 40g on mixed muscle protein fractional synthetic rate (FSR) in young men after a bout of resistance exercise, and found a possible peak in the FSR at the 20g dose; this may not be the peak dose following endurance exercise and with coingestion of carbohydrate however. Other studies have suggested estimated ~8.5-10g of EAA may be all that is required to maximise protein synthesis (Cuthbertson et al. 2005, Miller et al. 2003), which corresponds with ~8.6g EAA in 20g whole egg protein as used by Moore et al. (Moore et al. 2009). The metabolic requirements of high-intensity endurance exercise are very different to resistance exercise, and a dose of 8-10g EAA may or may not be sufficient, but these studies give an indication of the degree of responsiveness of muscle post-exercise to protein intake and a useful starting point for dose-response studies using prolonged endurance exercise models.

Endurance exercise creates a metabolic disturbance with subsequent release of hormonal effector molecules and metabolites of energy production and muscle contraction that are sensed by skeletal muscle, resulting in changes in transcription and translation that lead to adaptation with repeated stimulation. mTOR is a putative energy sensor and key signal transduction protein involved in integrating environmental stimuli such as nutrients, growth factor levels and exercise effects, by exert control on regulators of gene transcription and translation. Energy stress, via AMPK dependent regulation, and possibly other stress response molecules such as REDD1/REDD2 can inhibit mTOR function, while growth factors, amino acid availability and mechanical strain can activate mTOR (Miyazaki and Esser 2009). mTOR activation is only transiently upregulated after low-force contractions, analogous to aerobic exercise, in muscle (Miyazaki and Esser 2009). Amino acid deprivation in mammals results in reduced mTOR signalling and decreased protein synthesis, a state that can be easily and quickly reversed with amino acid feeding (Fox et al. 1998, Hara et al. 1998). Fujita et al. found that a leucine-enriched essential amino acid-carbohydrate (EAC) mixture reduced AMPK phosphorylation, increased PKB and mTOR phosphorylation as well as their downstream effectors S6K1 and 4E-BP1, and enhanced protein synthesis, relative to the no-nutrition control group (Fujita et al. 2007). Eukaryotic elongation factor 2 (eEF2) phosphorylation was reduced in the EAC group, suggesting protein synthesis increases were due to both enhanced translation initiation and also signalling promoting translation elongation (Fujita et al. 2007). Further research is need on how nutrient signals interact with

exercise signals at a molecular level during the recovery period from high-intensity endurance-exercise. For instance, it is plausible that activation of AMPK in skeletal muscle by exercise will contribute to mTOR inactivation and diminished protein synthesis, but whether this inhibition extends for a period after exercise and/or in the face of a strong nutritional signal remains to be investigated. Leucine likely exerts its stimulatory effect on muscle protein synthesis by enhancing mTORC1 activation and its subsequent downstream effectors of gene transcription, increasing mRNA binding during translation (Suryawan et al. 2008).

There is a gap in the literature surrounding combined protein-carbohydrate post-endurance exercise nutrition, the responses to such interventions at the molecular and cellular level, and correlations with performance. Furthermore, there have been no performance studies to date adding fat to supplementation; fats are important as fuels, signalling molecules, and molecular substrates, crucial for example in mitochondrial biogenesis with adaptation to endurance training (Hawley et al. 2006). While there may be molecular changes in skeletal muscle signalling and enhanced protein synthesis with protein-carbohydrate interventions, does this in fact result in better recovery and enhanced subsequent performance? Recent performance studies investigating protein-carbohydrate recovery beverages and subsequent exercise have produced mainly unclear effects (Betts et al. 2005, Millard-Stafford et al. 2005, Niles et al. 2001)) although two have found improvements in time-to-fatigue tests following glycogen-depleting endurance exercise (Niles et al. 2001, Williams et al. 2003). Recent work by Rowlands et al. (Rowlands et al. 2008)found a post-exercise protein-enriched feeding intervention had no clear effect on 15 h next morning high-intensity cycling performance, but a clear-cut substantial effect at 45 h following (60 h after the first exercise). This delayed effect possibly relates to the time-course for repair and protein expression and interaction with rest days (Rowlands et al. 2008). Mechanisms for the enhanced performance may include superior glucose homeostasis during repeated-sprint exercise suggesting indirectly conserved or enhanced carbohydrate metabolism, and a reduction in muscle damage as evidenced by reduced CK levels on the fourth day (Rowlands et al. 2008). In further work it was found a leucine-enriched high protein-carbohydrate supplement ingested along with other high-carbohydrate foods (simulating normal athlete practice) in the first 90min following high-intensity cycling exercise over 2 days and a rest day, resulted in a likely substantial enhancement of repeated-sprint cycling performance on the fourth day (Thompson, J., Rowlands D., 2009, unpublished data). In a specific 6-day high-intensity cycling model, a leucine-enriched high protein-carbohydrate supplement had a most likely negligible effect on performance on the fourth and sixth days of a 6 day block (Rowlands et al. In Prep 2009). Differences in the type of proteins used in the studies (intact protein isolate; (Rowlands et al. 2008, Rowlands et al. 2007); micelle-encapsulated whey-protein isolate; (Rowlands et al. In Prep 2009), including a full rest day before the performance test (Thompson, J., Rowlands D., 2009, unpublished data) as opposed to very light aerobic exercise (Rowlands et al. In Prep 2009), possible impairment of supplement absorption due to the quality of the fat used (Rowlands et al. In Prep 2009), and finally the confounding and contributing influence of energy and protein balance differences between studies, may account for the differences in the outcomes. While there is sufficient evidence indicating leucine-enriched and/or whole-protein feedings upregulate genes associated with adaptation to endurance exercise and to increased activity of the mTOR pathway, which in part controls protein synthesis rates, there are no direct measure of protein synthesis in response to the added leucine conditions, the dose that can saturate protein synthesis is not known, and linking the levels of organizational change to performance (i.e. upregulated genes; greater

protein accretion and reduced muscle damage; enhanced performance) has yet to be established.

FOCUS

Efforts to examine the effects of nutrition and its interaction with the post-exercise muscle tissue have highlighted the advantages of protein plus carbohydrate coingestion following endurance exercise. With the finding that essential amino acids only are necessary to upregulate protein synthesis, and that branched chain amino acids, in particular leucine, can strongly stimulate the mTOR pathway, subsequent research has looked at the response to and possible benefits of post-exercise nutrition with enriched EAA/BCAA/leucine. Most of this work has to date looked at resistance-training exercise models, and so there is a gap in the literature regarding the effects of similarly designed interventions, post-endurance-exercise. Work in endurance models has so far found equivocal effects for protein-carbohydrate coingestion on subsequent performance.

The first of the two proposed studies will extend on previous work by (Rowlands et al. 2008) and parallel with unpublished work by Jasmine Thompson, looking at the effect of a leucine-enriched protein-carbohydrate endurance exercise recovery supplement on subsequent high-intensity endurance performance. It will advance prior work by using modern stable isotope methods and candidate immune, inflammatory, and hormonal parameters, to more thoroughly investigate a possible mechanism of delayed, enhanced performance seen by Rowlands et al. (Rowlands et al. 2008). The second study will be the first that we know of to look at the effect of two different doses of leucine-enriched-protein+carbohydrate (LeuPlus) on muscle FSR following high-intensity endurance exercise, relative to a zero nutritional (exercise-only) response, with a hypothesis that the lower nutrition condition is optimal for maximizing muscle FSR. It will use a synthesis of a close to market formulation and similar to that used by Jasmine Thompson, and an intake of nutrients that is practical and relevant to what an endurance athlete or fitness enthusiast might consume post-exercise. This study will investigate candidate genes likely involved in the process of adaptation to endurance exercise, along with relevant signalling pathways and translational control factors believed to be involved during recovery with concurrent nutrition.

Proposed are two studies.

1. In the first study I will investigate the effect of post-exercise LeuPlus coingestion compared with carbohydrate+fat only on subsequent performance and markers of muscle damage, systemic immune response and inflammation, selected hormonal parameters, and the metabolomic profile. Using modern stable isotope methods, I will quantify the effect on whole body protein turnover, leucine disposal, and substrate oxidation.

2. Secondly I aim to quantify the effect of 3 different recovery supplements; control (carbohydrate-fat only), low LeuPlus, and high LeuPlus, on the expression of candidate genes, signal transduction pathways and transcriptomal control factors involved in nutrient-stimulated recovery from and adaptation to prolonged high-intensity exercise.

Benefits of Undertaking this Research

The proposed research will advance the state of knowledge regarding the protein requirements of endurance athletes. It will generate information to critically analyse whether it is better to prescribe less total protein, and more specific amino acids such as leucine that may produce a greater anabolic response. It is possible that the present recommendations of 1.2-1.6 $g \cdot kg^{-1} \cdot d^{-1}$ may be adequate for N balance, but not optimal for performance. Evidence suggests that the addition of leucine to protein-carbohydrate supplements may enhance the recovery and repair processes and aid adaptation to endurance training. An optimal dose of leucine+protein has yet to be determined in endurance athletes, and this research will provide some evidence towards the optimal best dose.

METHODS

Study 1: Effect of protein recovery formulation on subsequent performance and physiological outcomes during one-week of high-intensity cycling

Data Collection and Collaboration

The data collection for this study was conducted June-December 2008 at Massey University Wellington with my supervisor Dr David Rowlands, Miss Lara Jackson, Mr Jim Clarke, Miss Marjolein Ros, and Mr Dan Wadsworth. Sample analysis is presently underway by collaborators at McMaster University and by Nestle Research Center well as in-house at Massey University. I was responsible for the recruitment and organization of the participants, VO_2max testing and familiarization rides, controlled diets, cannulation and blood sampling and handling, data collection and staffing.

Statement of the Problem

Rowlands et al. (Rowlands et al. 2008)found that a post-exercise protein-carbohydrate feeding intervention had no clear effect on 15-h next morning high-intensity cycling performance, but a clear-cut substantial effect at 45-h following (60-h after the first exercise). This delayed effect possibly relates to the time-course for repair and protein expression and possibly to an interaction with rest days (Rowlands et al. 2008). Possible mechanisms for the enhanced performance include superior glucose homeostasis during repeated-sprint exercise suggesting indirectly conserved or enhanced carbohydrate metabolism, and a reduction in muscle damage as evidenced by reduced CK levels on the fourth day; however, there was little firm evidence for a mechanism for the delayed improved performance (Rowlands et al. 2008). Further work should look at the magnitude of effect protein-enriched nutritional interventions can have on subsequent performance accounting for a possible delay in effect of at least 45 h, and look at plausible mechanisms of action such as via carbohydrate metabolism, protein synthesis and turnover, cell damage markers, and systemic immune and inflammatory responses.

Aims

1. Extend the investigation of the delayed effect of a potentially marketable protein-enriched recovery supplement on high-intensity performance to a one-week period.

2. Determine the nature and quantify the systemic immune and inflammatory responses over the week of high-intensity exercise.

3. Trace the change in circulatory markers of physical and oxidative skeletal muscle damage.

4. Determine the effect of the protein-enriched recovery supplement on the oxidation of ingested carbohydrate during exercise and endogenous carbohydrate and fat oxidation.

Hypotheses

1. The delayed benefit of the protein-enriched recovery supplement ("LeuPlus") to high-intensity cycling performance will be substantial from Day 4 of the testing protocol.

2. One-week on the protein-enriched recovery supplement will modify carbohydrate and fat metabolism, but have little effect on the oxidation of ingested carbohydrate during exercise.

3. The elevation in markers of skeletal muscle damage will be attenuated by the protein-enriched supplement.

4. The immune and inflammatory responses will be attenuated in the protein-enriched condition.

5. The LeuPlus supplement will increase leucine oxidation during exercise.

Study Design

This was a placebo-controlled, double-blind, randomized crossover trial of twelve endurance-trained male cyclists (mean ± SD: age, 35 ± 10 y; height, 182 ± 5 cm; body mass 76.9 ± 6.5 kg) completed the study. Maximal oxygen uptake (VO_{2max}) was 64.8 ± 6.8 $mL.kg^{-1}.min^{-1}$; 5.0 ± 0.6 $L.min^{-1}$) and the corresponding peak power output (W_{max}) was 355 ± 36 W. All participants had been training for minimum 2 y, with a weekly average of ≥10 h spent training over the 6 months prior to the study, which included high-intensity training and competition. There were two experimental blocks spaced 14 days apart, the details of which are illustrated in Fig. 1 and described below.

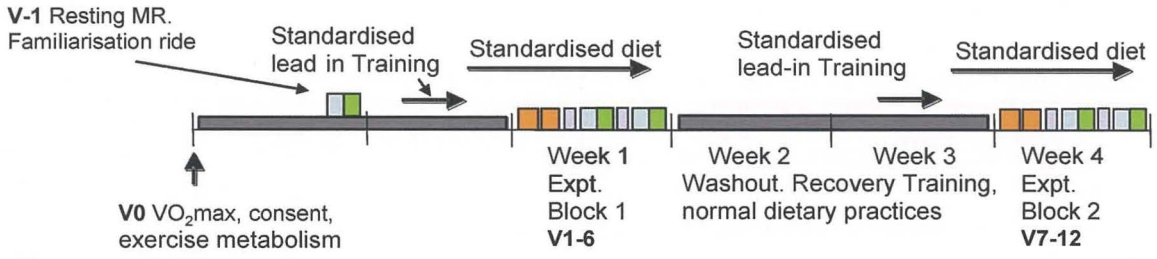


Figure 1: Experimental design and procedures

One to two weeks before the start of the first block, cyclists reported to the laboratory for a test to determine exercise metabolic rate, VO_{2max} and W_{max} (Visit -1, V-1) and in a subsequent visit (V0) a fasted basal metabolic rate measurement followed by a practice ride using the experimental exercise protocol. At V-1, cyclists were given instructions and advice to eliminate foods containing high levels of ^{13}C, such as processed foods with a high sugar content, and corn-derived products, for 7-d before the first testing block. They were given a training diary and diet diary to record their training and diet for 7-d and 2-d prior to the first testing block, respectively. Training was kept light for 3-d before the first testing block. The

training and diet regimen was repeated preceding the second block to standardize pre-conditioning. Participants completed an activity diary prior to testing, estimating their daily activities over the time they were outside the laboratory during the testing block. They were required to complete the same or similar activities in the second block. During the experimental period participants consumed a standard diet based on energy expenditure and macronutrient prescriptions.

Subjects arrived at the lab at 3PM for V1, the first day of the testing block, and had cannulae placed in the antecubital vein of each forearm; one for a primed, constant infusion of 1-^{13}C-leucine, the other for blood sampling. A baseline blood sample was taken pre-exercise. Participants then performed an intense 3-h ride consisting of 30 high-intensity intervals, representative of a 120-km race. During all rides subjects received an 8% CHO sports drink at a rate of 800mL per hour (for a model 75kg, 360W cyclist) as half-hourly serves. Soreness and perception scales were completed during exercise. The infusion was started at t= 90 min into exercise, and continued until the end of the 3-h recovery period. Blood samples were taken at t= 88 min exercise, and t= 0, 30, 60, 90, 120 and 180 min post-exercise. Expired breath gas samples were collected for $^{13}CO_2$ enrichment at pre-exercise (rest background), at 88 min exercise prior to infusion (exercise background check), and recovery t= 0, 30, 60, 90, 120 and 180 min post-exercise. Expired breath gas was collected into Douglas Bags at 88 min exercise and recovery t= 2-10, 22-30, 52-60, 82-90, 112-120 and 172-180 min, for indirect calorimetry. Subjects received post-exercise recovery supplements (control or intervention) at recovery t= 0, 30, 60, 90, 120 and 150 min. Subjects received a small pasta meal after the final breath and blood collection and left the lab.

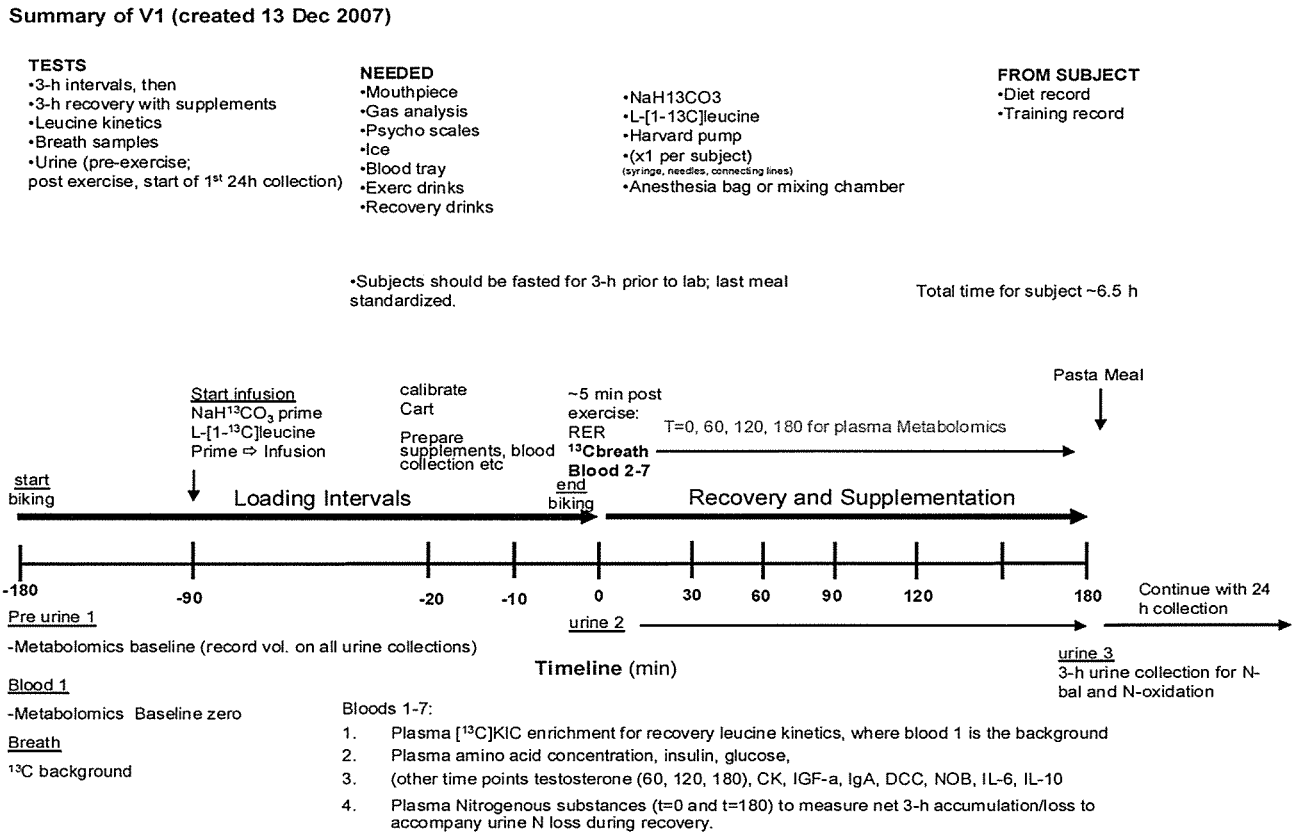


Figure 2: Summary of Day V1

Day V2 began the next morning, subjects arrived overnight-fasted and received a small standard breakfast (toast and 250 mL water). Subjects completed a second loading day consisting of 2.5 h of intervals. Soreness and perception scales during exercise. Blood samples pre- and post-exercise only. Sweat will be collected during exercise for exercising nitrogen loss rates (urea, creatinine, NH_3). Intervention supplementation began immediately post exercise and continued for 3 h. Pasta meal as for day 1.

V3 was an afternoon recovery ride of 1-h at 30% W_{max}. No blood or breath samples were taken. Intervention supplementation began immediately post exercise and continued for 1 h.

V4 was the first performance ride day. Subjects arrived overnight-fasted and received a standard breakfast. Subjects completed 90 min cycling at 50% then the performance test; 10 sprints at maximal intensity (~1.5-2min) to complete a pre-set quantity of calories determined by Wmax, interspersed with recovery periods where they burn the same number of calories (~5-6min). Blood was collected pre and post exercise. Breath was collected at t= 55-60, 70-75, 85-90 min during 90 min pre-performance ride. Soreness and perception scales during exercise. Intervention supplementation began immediately post exercise and continued for 3 h. Pasta meal as for day 1.

V5 was as per V3.

V6 was the second performance ride day. Subjects arrived in the morning fasted and were double-cannulated. Into one arm, 1-^{13}C-Leucine, [6,6-$^{2}H_2$]glucose and $NaH^{13}CO_3$ priming followed by 90 min continuous infusion of the leucine and glucose tracers at rest, with blood sampling from the other arm. Participants completed the same ride as V4. Soreness and perception scales during exercise. Sweat was collected during the 90 min pre-performance ride, and breath collection as per V4. Blood was taken pre-exercise, at t= 60, 75, 90 min during exercise, and post-exercise. End of block 1.

Block 2 began following a two-week washout period, with a repeat of 1-week training prior to the start of block 2, and diet repeated for the 2-days prior to V7-12.

Summary of V6 (updated 1 Jan 2007)

TESTS
- 1.5-h bike ride, then
- Rept Sprint test
- Glucose turnover
- Leucine kinetics
- Breath samples
- Urine (pre/post rest; pre/post 90 min exercise)

NEEDED
- HR monitor
- Mouthpiece
- Psycho scales
- Ice
- Blood tray
- Exerc drinks

- NaH13CO3
- L-[1-13C]leucine
- [6,6-2H]glucose
- Harvard pump
- (x2 per subject) (syringe, needles, connecting lines)
- Anesthesia bag

FROM SUBJECT
- Diet record
- 24-h urine container

TO SUBJECT
- Parking pass

NOTES
- Subjects should be fasted for 12-h
- Check workload

Total time for subject ~4.75 h

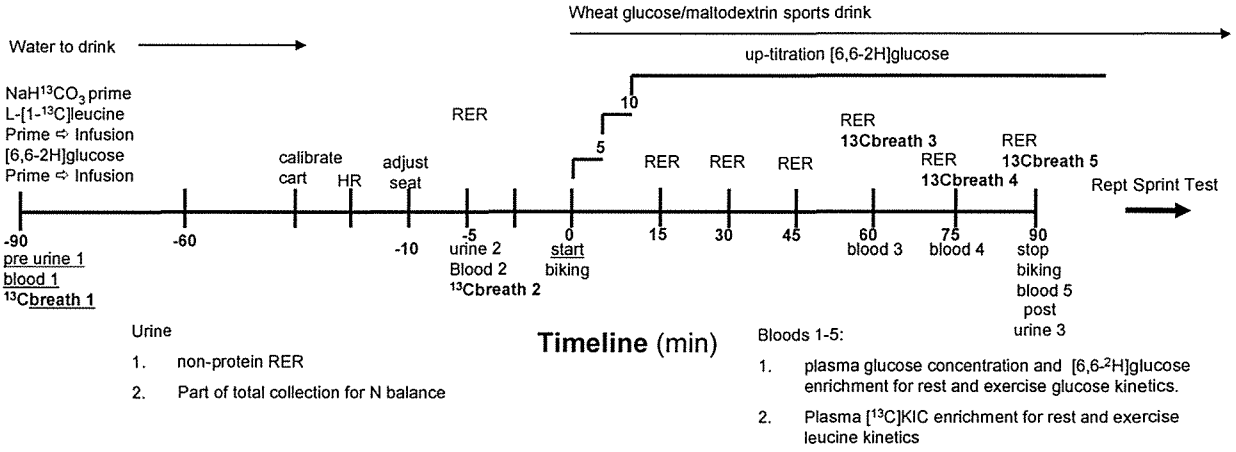


Figure 3: Summary of V6

Urine samples:

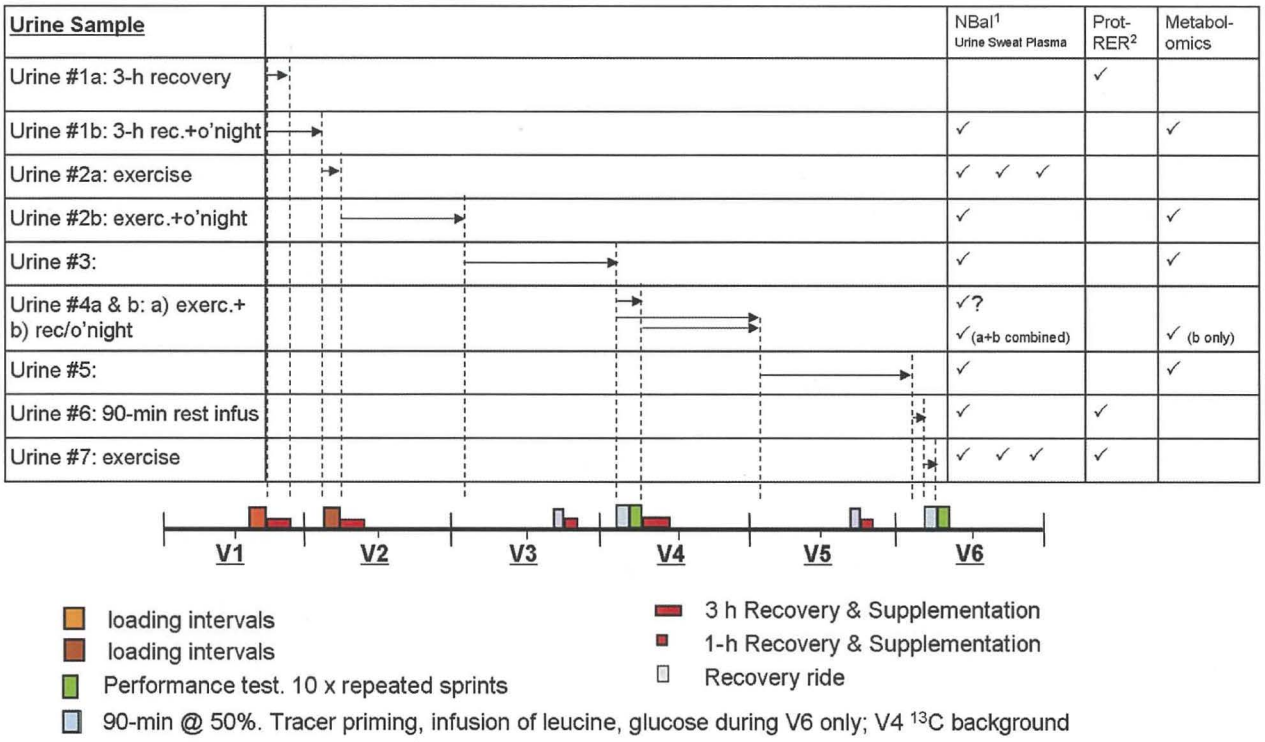
Urine was collected throughout experiment during defined exercise and recovery periods in approximately 24 h accumulated units and nitrogenous products quantified for N balance, protein-RER, and metabolomics. In addition, the accumulation of nitrogenous products in sweat and plasma is to be quantified during specific exercise as follows:

- V1; During the 3-h recovery on V1, total urine was collected during the 3 h recovery period and assayed for total urea+creatinine+NH3 nitrogen excretion (also assayed for body-water urea+creatinine+NH3 accumulation, UC) to account for protein metabolism in VO_2 and VCO_2 calculations = protein RER, while also providing an index of the effect of treatment on net N excretion during the 3 h recovery period. The *volume of the accumulated 3 h urine sample was measured* and 1.5 ml sample collected and stored for later UC analysis. The *remaining sample* was *added* to the remaining collection period for urine sample #1, which continues until the beginning of exercise at 0600 or 0700 (standardized within subject) on day V2.

- V2; Emptied bladder at the end of exercise (not collected). All remaining urine collected into total container #2. Continued collection until and including void upon waking on the morning of V3.

- V3-5; see summary below.

- V6; urine was collected during the 90-min ride and the sprints for exercise urea+creatinine+NH3 nitrogen excretion (also assayed for body-water urea+creatinine+NH3 accumulation, UC) to account for protein metabolism in VO_2 and VCO_2 calculations = protein RER, while also providing an index of the effect of treatment on net N excretion during exercise.



[1]NBal: assay urea, creatinine, NH3
[2]Prot-RER: assay urea, creatinine, NH3 accumulation in the urine only at rest, but in urine, sweat, and plasma during exercise

Figure 4: Summary of urine collection

Duration

Subjects were involved in the study for 5-6 weeks each inclusive of preliminary and familiarization trials, both arms of the cross over and washout. Sample collection was done over 22 weeks during 2008, and an additional 10 months is required for analysis.

Methods

Supplement Formulation:

1. **Intervention:** The leucine-enriched-high-protein, high carbohydrate, moderate fat formulation contained whey/casein protein (11.3g) + leucine (3.8g), carbohydrate (45.0g), fat (7.5g); 1270 kJ; ~302kcal. Total nutrition over the 3 hr recovery period (6 serves): 67.8g whey; 22.8g leucine; 270g CHO; 45g fat; ~7620kJ; 1814 kcal.

2. **Control:** The isocaloric carbohydrate+fat only formulation contained carbohydrate (60.4g), fat (7.5g); 1270 kJ; ~302 kcal. Total nutrition over the 3 hr recovery period (6 serves): 6.6g whey; 362.4g CHO; 45g fat; ~7620kJ; 1814 kcal.

Outcomes

This project expanded on recent work by (Rowlands et al. 2008), looking out longer than previous work to 4-d and 6-d for delayed performance effects, and will be the first to quantify any potential performance effect of leucine-enriched protein taken in the immediate post-exercise recovery period, relative to an isocaloric carbohydrate-only control. Further, this study looked at potential mechanisms for any potential beneficial performance effect. We assessed whole body protein turnover (synthesis, breakdown and oxidation) using 1-^{13}C-leucine infusion during recovery from exercise, as well as the rate of appearance and turnover of leucine. The metabolic response to the multi-day use of the supplement was investigated via stable isotope and indirect calorimetry to look at the oxidative and non-oxidative disposal of leucine, glucose and muscle glycogen oxidation by [6,6-^{2}H]-glucose infusion, and fat oxidation. We also examined the systemic immune and inflammatory response by a muscle damage marker (creatine kinase, CK), immune and inflammatory markers (IgA, cortisol, neutrophil oxidative burst (NOB) assay, differential cell count (DCC)). Leuplus resulted in a possible trivial increase in mean power on V4, and otherwise inconclusive outcomes for mean power and fatigue. Leuplus supplementation resulted in a very large increase in superoxide anion production (ie. representing oxidative burst activity) on visit 6 for the post-exercise minus pre-exercise comparison of 46 mmol $O_2^{\cdot}$·cell x 10^{-1} (90%CL: ±25 mmol $O_2^{\cdot}$ ·cell x 10^{-1}). There were also very large sized increases in superoxide anion production following exercise on V6 relative to following exercise on V1 and V2 of 36 mmol $O_2^{\cdot}$·cell x 10^{-1} (±6 mmol $O_2^{\cdot}$·cell x 10^{-1}) and 41 mmol $O_2^{\cdot}$·cell x 10^{-1} (±11 mmol $O_2^{\cdot}$·cell x 10^{-1}), respectively. The effect of Leuplus on NOB on V1, V2 and V4 was inconclusive. Both conditions displayed a rise in neutrophil concentration consistent with high-intensity exercise-induced neutrophilia. LeuPlus lead to substantial 10-12% (± 2-3%) increases in neutrophil concentration on V2 before and after exercise. This increase was reversed on V4 and V6, with neutrophil concentration being 17-21% (± 6%) lower pre-exercise in V6 compared with V1, V2 and V4. However, the lower pre-exercise neutrophil count on V6 was attenuated by exercise, with there being no clear difference following exercise, and the post-pre gain in Leuplus relative to high-carbohydrate of 22% (± 9%) being similar to the pre-exercise reduction. Cortisol showed a moderate-sized reduction of 20% (± 19%) pre-exercise on V6 with LeuPlus, and lower pre-V6 than pre-V1 or pre-V4 (27% ± 23% and 18% ± 26% respectively). All other cortisol effects were inconclusive. IgA analysis was unclear. Blood glucose was moderately reduced (12% ± 7%) at all post-30, post-60, post-90, and post-180min on V1 with the Leuplus condition. The postV6-postV1 and preV6-preV1 LeuPlus-Control difference was 27-29% (± 12%) lower for LeuPlus, and postV4-postV1 and preV4-preV1 LeuPlus-Control difference was 26-30% (± 12-13%) less.

METHODS

Study 2: ***Acute phase fractional muscle protein synthetic, transcriptomal, and signalling responses, to the ingestion of low and high saturating doses of a leucine-enriched protein-carbohydrate supplement following high-intensity endurance exercise***

Statement of the Problem

A recent dose-response study by (Moore et al. 2009) suggests that a single dose of 20g (whole-egg) protein, ~8.6g essential amino acids, may maximally activate muscle protein synthesis in the post-exercise period, in a resistance-training model. However, dose-response studies to protein/protein+carbohydrate in endurance models are lacking, and due to the different metabolic requirements of resistance training and high-intensity endurance exercise, it is plausible that differences exist in the dose required to maximize protein synthesis. BCAA's, in particular leucine, may enhance rates of protein synthesis by acting on the mTOR translational control pathway. This pathway acts as an integrator of nutritional signals to activate cellular machinery and, following exercise that may disturb homeostasis and induce muscle damage, and is likely an important biological pathway stimulated by nutritional signals to maximally activate, in order to enhance the recovery process. Leucine-enriched protein may be more efficient at inducing a nutrient signal to stimulate muscle protein synthesis.

Aims

1. Determine the dose of LeuPlus that elicits the peak fractional protein synthetic rate outcome.

2. Determine the effect of dose on the transcriptomal profile, which may yield useful information as to dose-response thresholds for the activation/deactivation of particular biological processes important for recovery and/or training adaptation.

3. Establish the effect of dose on the signal transduction pathways linked to regulation of translation and cellular anabolism.

4. Determine the effect of dose on the expression of transcriptional control factors important in the regulation of mitochondrial biogenesis (and possibly also other processes indentified in the current DNA array, such as, regulation of myofibrilar and matrix proteins, ubiquitin proteosome, and ion and solute transporters).

Hypotheses

1. The magnitude of muscle mitochondrial and myofibrilar protein synthesis will be the same between the Low LeuPlus dose and the High protein+leucine intervention, with evidence of saturation at both the Low and High protein+leucine doses.

2. Bioinformatics analysis based on the DNA array will confirm that gene clusters involved in processes involving transcriptional regulation and protein turnover, cell structure and growth, and muscle contraction, mitochondria and energy-substrate metabolism are over-expressed in a dose-dependent manner with the ingestion of protein-rich recovery supplements with added leucine following endurance exercise.

3. The phosphorylation of key signalling proteins involved in the control of ribosomal activity and translation rate linked to amino acid and insulin signalling will be greater with the Med and Hi doses of LeuPlus, relative to No Food or Low controls.

4. The expression pattern of transcription factors involved in the control of mitochondrial genes will be substantially altered to favour increased biogenesis in the Med and Hi LeuPlus conditions, in a positive dose-response fashion

Duration

Preliminary and pilot work, data collection and analysis will be done over 12-15 months starting late-June 2009.

Study Design

12 trained male endurance cyclists (road, mountain, triathletes) OR recreational fitness enthusiasts will be recruited to participate in this study. A triple-crossover design will be used with a 1-2 week washout period between each experimental block. This study will replicate a recovery supplement formulation used in by Jasmine Thompson and a close-to-market formulation based on products designed by the funder, in a low vs high dose response study, relative to a zero-nutrition control.

In their first visit participants' VO_{2max} will be determined; screening of participants will be based on high aerobic fitness (pre-testing maximal oxygen capacity of 55 $mL \cdot kg^{-1} \cdot min^{-1}$ or greater), training history, and general health. From Day -7 participants will record their training volumes and intensities, and from Day -2 their daily dietary intakes, for repeating before the second block and third blocks of the trial. On Visit 1, subjects will arrive at the lab approximately 210 min prior to the start of exercise, be cannulated for a blood sample, and consume a standardised low protein meal. They will then be cannulated in the other arm and a constant primed infusion of $^{13}C_6$-Phe set to run for 560 min. During this time, a baseline biopsy will be taken at 130 min before recovery, then a 2 h high-intensity cycling protocol started. Blood samples will be taken periodically during the resting period before cycling, and during recovery. A second biopsy will be taken at ~40 min recovery, and a third at the end of recovery ~250min post-exercise). Supplement will be ingested at 30min intervals for 90min from the start of recovery (4 serves total). Visit 1 is summarized below in *Figure 5*.

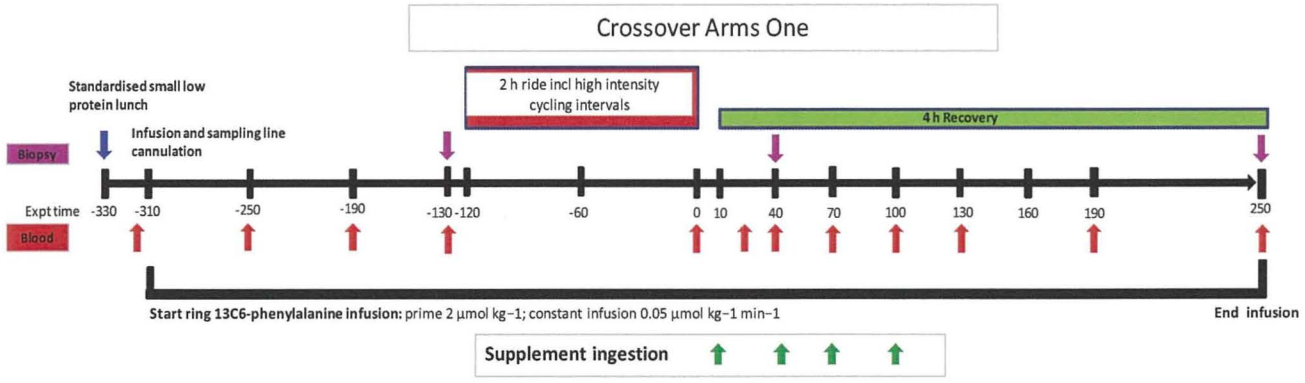


Figure 5: Crossover arm one for Study II

Crossover arms 2 and 3 will require a shorter infusion, and no baseline pre-exercise biopsy, but maintain the standardised meal 330 min prior to recovery. All other measures will be as per crossover 1. This is summarized below in *Figure 6*. The total number of biopsies is 7.

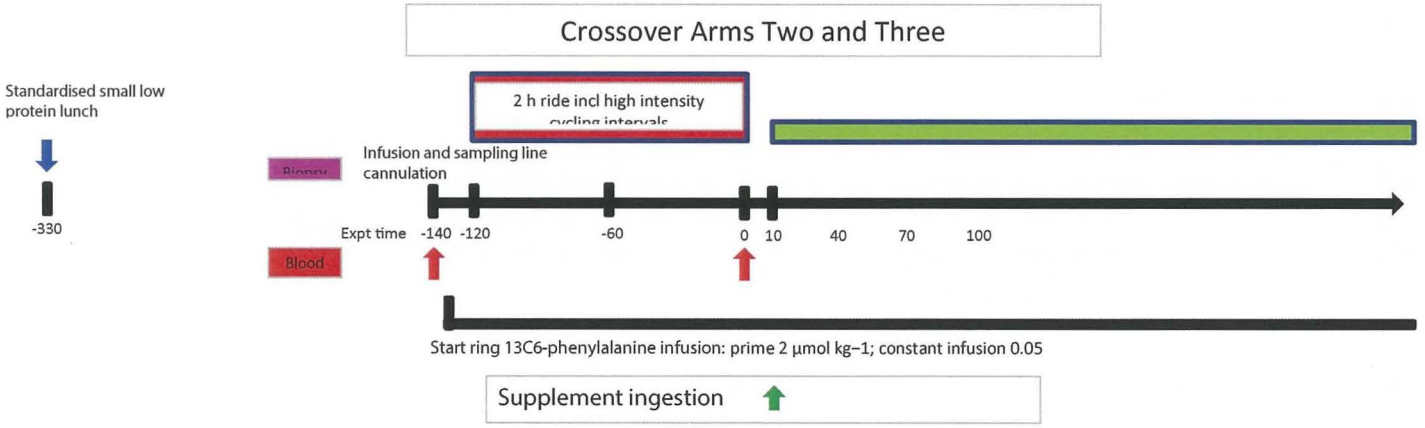


Figure 6: Crossover arms two and three for Study II

Methods

One of three iso-caloric nutritional interventions: Average nutrients ingested via oral route at 0, 30, 60, 90min (4 serves) over a 90min post-exercise intervention recovery period following a single bout of high-intensity aerobic exercise. Sample collection will occur at Massey University; signalling proteins will be analysed at Massey University/McMaster University; plasma glucose and insulin (if required) at Massey University; isotope analysis and amino acid profile will be done at McMaster University; DNA array, RT-PCR, and bioinformatics at NRC, Lausanne, Switzerland.

Experimental Conditions:

1) **Low protein + free leucine:**

Per hour essential amino acid content same as the lowest single egg-protein dose demonstrated recently to elicit maximal mixed-muscle FSR (Moore et al. AJCN, 2009) combined with relatively lowest dose to optimize post-exercise glycogen resynthesis (1.2 g CHO/kg). Beverage composition per serve (every 30 min over 90 min): whole protein (5.75 g), leucine (1.875 g), carbohydrate (22.5 g),fat (1.875 g) energy ~637 kJ/154 kcal. Total nutrition over the 90-min recovery period (4 serves): 23 g protein, 7.5 g leucine, 90 g CHO, 7.5 g fat; ~2241 kJ/~537 kcals. This dose is equivalent to one Powerbar ProteinPlus bar + 750 ml of 7% sports drink.

2) **High protein + free leucine**

Modelled on the protein+leucine dose used in Jasmine Thomson's study that demonstrated a likely substantial performance effect. Based on Moore et al. (2009), this dose will super-saturate FSR yield the same FSR outcome as the Low dose. Beverage composition per serve: whole protein (11.5 g), free leucine (3.75 g), carbohydrate (45 g), fat (7.5 g), energy 684

kJ/163 kcal. Total nutrition over the 90-min recovery period (4 serves): 46 g protein, 15 g leucine, 180 g CHO, 30 g fat; 5097 kJ/1217 kcals.

3) **Control**

Zero protein+leucine, isocalorically balanced. Per serve: carbohydrate (61.9 g), fat (7.5 g); 420 kJ/100 kcal. Total nutrition over 90 min: 248 g CHO, 30 g fat, 5097 kJ/1217 kcals.

Controlled Diet

To standardise diet, participants will receive a normalised diet based on body weight and an acitivty-correction factor for the two days leading up to each experimental day.

Muscle biopsies

Needle biopsy samples will be taken from the *Vastus lateralis* approximately 15-20 cm proximal to the lateral knee joint under local anesthesia using a Bergström needle with manual suction.

Protein Fractional Synthetic Rate

We will measure the mixed muscle mitochondrial and myofibrilar fractions, using the modern stable isotope ring-$^{13}C_6$-Phe infusion method.

Signalling proteins

The expression and phosphorylation of candidate signalling proteins will be assayed by Western Blot technique. We will look at candidate signalling proteins important for exercise and nutritive signalling, such as but not limited to; mTOR, AKT/PKB, AMPK, $p70^{s6k}$.

Reverse transcriptase real time polymerase chain reaction (RT-PCR)

The expression of candidate genes will be assessed via mRNA quantification by RT-PCR (real time reverse transcriptase polymerase chain reaction). mRNAs to be analysed may include; mitochondrial (NRF2, TFAM, PGC 1α, PDK4), transcription factors (FOXO1, NFκB), cell growth, development & maintenance (IGF1).

Transcriptomal profile

Analysis of candidate transcription factors controlling mitochondrial biogenesis, and also possibly, expression of endurance-type contractile proteins, matrix proteins, and possibly solute and ion transporters.

Outcomes

We expect LeuPlus will substantially elevate protein synthesis and increase the phosphorylation of specific signalling proteins involved in control of translation and increase the cellular RNA/protein ratio – indicating capacity for muscle protein synthesis, which will both correlate with the nutrient-induced increase in protein synthesis. From the findings of this study and relevant literature, we hope to develop a molecular model for the action of the LeuPlus supplement. Finally, we aim to produce mechanistic evidence to support marketing of LeuPlus as a proven effective recovery product.

Statistical Analysis

The sample numbers to be used in each study will be derived by a combination of two methods. Initially we will establish numbers of participants that are realistic in terms of recruiting participants, study resources, and smallest acceptable sample sizes for metabolism and physiology studies. Secondly we will use a published spreadsheet (Hopkins 2006) to estimate sample size based on acceptable uncertainty defined by error rates. Type 1 error was set to 0.5%, and Type 2 error set at 25%.

I will report the size of the effect and associated 90 or 95% confidence limits rather than null-hypothesis testing. I will report the probabilities that effects are negative, trivial, or positive based on the analysis of the effects and confidence limits and the smallest standardised change (0.2 multiplied by the standard deviation for control condition) using published spreadsheets or coded outputs from SAS. Qualitative interpretations of practical importance are defined by the confidence limits and the smallest important or non-trivial effects. The magnitude of the difference in physiological/gene effects will be expressed as shifts relative to the control sample and Cohen Effect Sizes.

TIMETABLE

A provisional timeline for the completion of my PhD is shown in the figure below.

Figure 1: Proposed Timeline

2009	
March	Planning for Study 2. Analysis of data from Study 1 and report to funder due 30 March. PhD presentation for 1 year registration. Writing methods and results for scientific paper based on study 1. Prepare abstract for IBEC.
April	Planning and possible pilot trials for second study. Work on PhD thesis literature review. Methods and results for paper from study 1.
May	Pilots for second study. Work on PhD thesis literature review. Prepare poster for IBEC. ACSM & IBEC last week of May.
June	First weeks of June at McMaster University. Planning and recruitment for second study upon return.
July	Recruitment and possible start for second study.
August	Study 2 data collection. Work on PhD thesis literature review.

September	Study 2 data collection. Work on PhD thesis literature review.
October	Study 2 data collection.
November	Study 2 data collection.
December	Wrap-up of second study. Begin data analysis and write up
2010	
January	Data analysis and write up.
February	Data analysis and write up.
March	Data analysis and write up.
April	Data analysis and write up.
May	Data analysis and write up.
June	Write up papers for studies and Submit Papers to Journals. Convert papers to Thesis Chapters and Appendices as I go. Paper drafts to supervisors as they are written.
July	Write up papers for studies and Submit Papers to Journals.
August	Write up papers for studies and Submit Papers to Journals.
September	Work on Thesis General Discussion.
October	Write up papers for studies and Submit Papers to Journals.
November	Submit final draft to supervisors.
December	Work on any changes to thesis.
2011	
January	Submit thesis for examination.
February	Submit thesis for examination.
March	Exam preparation
April	Oral examination?
May	Graduation?
June	
July	
August	
September	
October	
November	
December	Graduation?

RESEARCH ETHICS

Ethical approval will be obtained from the applicable research ethics bodies for the location of proposed research (Central Health and Disability Ethics Committee). The potential risks and management of these risks involved in this series of studies is detailed below:

All participants will be screened via a health questionnaire for pre-existing conditions to ensure participants are physically healthy and able to take part in the study. This series of studies will limit participants to athletes in regular training, and individuals who are neither disabled nor elderly and at increased risk of injury or discomfort during the exercise and performance portions of the research. Some fatigue during exercise and performance trials is expected, however this will be of a similar level to that normally experienced in subject's own endurance training and competition. There is some discomfort and minor risk of infection associated with blood catheter insertion and muscle biopsies procedures. Discomfort will be minimised by having subjects lie prone on a hospital bed, and the blood and biopsy procedures will be performed by trained phlebotomists and a medic with experience in the procedures. Amount of blood and muscle tissue samples taken will pose no risk of adverse health effects. Risk of infection will be minimised by following sterile procedural guidelines.

Social or psychological risks will be minimised by ensuring privacy and confidentiality of participants throughout data collection and data storage periods. Initially by obtaining informed consent and communicating to participants their rights to discontinue or withdraw from the studies at any time. Also by providing adequate change and shower facilities, minimising the number of observers while subjects are being examined and tests conducted, and secure storage of data and any identifying information.

Economic risk to participants will be reduced by reimbursement of participants for travel and time where necessary.

POTENTIAL JOURNALS FOR PUBLICATION

Journals

Suggested journals for submission of results generated from this series of studies has been compiled based on relevance to the scientific questions we will be answering, and the journal impact factor (average article citation frequency). This list is by no means complete. Relevant journals are presented in Table 1, below.

Table 1: Potential Journals for Publications and Their Impact Factors

IF	Journal Name	Scope of Journal	Fee
7	PLoS Biology	Biological Science	3000
2.9	Acta Physiologica	Physiology and biological pharmacology	
4.6	American Journal of Physiology	Activation of gene expression; hormonal or metabolite control of metabolism	$50 + $70/p + $350/colour

1.9	British Journal of Sports Medicine	All aspects of sports medicine, including exercise physiology	
1.1	Applied Physiology, Nutrition and Metabolism	The application of physiology, nutrition and metabolism to the study of human health, physical activity and fitness	
1.6	European Journal of Applied Physiology	Research that contributes to our understanding of the function of the healthy human body under a variety of environmental and exercise conditions	€ 950.00 for colour
2.2	European Journal of Clinical Nutrition	All aspects of human nutrition: including metabolic studies; relations of function to nutritional status	
1.0	International Journal of Sport Nutrition & Exercise Metabolism	Insights into sport nutrition and exercise metabolism, including the application of the principles of biochemistry, physiology, and nutrition to sport and exercise.	
1.4	International Journal of Sports Medicine	Developments in sports medicine and exercise science including nutrition, physiology & biochemistry	of € 160/p after 3rd p
3.0	Journal of Applied Physiology	Physiology, especially studies emphasizing adaptive and integrative mechanisms including adaptations to exercise especially techniques such as molecular and cellular biology	$50 + $70/p + $350/colour
3.7	Journal of Nutrition	Original nutrition research, including; energy & macronutrient metabolism; nutrient-gene interactions; proteins and amino acids, especially mechanistic studies	$75 + $75/p + $120/p >7p + $400/colour
4.3	Journal of Physiology	New physiological principles or mechanisms	$$/colour
2.8	Medicine and Science in Sports and Exercise	Current topics in sports medicine and exercise science	$US50 + $55-$70/p + $100-$800 /colour
2.2	Scandinavian Journal of Medicine & Science in Sports	Original investigations on physiologic aspects of sports	
3.3	Sports Medicine	Original manuscripts on clinically relevant sports science	

Conferences

I will be attending the ACSM Conference in Seattle in May and IBEC Conference in Toronto in May/June 2009, where I will present a poster on the first study.

APPENDIX B CONFERENCE ABSTRACTS AND POSTER

Abstract for: Institute of Food, Nutrition and Human Health (Massey University) Presentation

EFFECTS OF A LEUCINE-ENRICHED PROTEIN SUPPLEMENT ON PERFORMANCE AND METABOLIC RESPONSES FOLLOWING INTENSE CYCLING

Presenting Author A.R. Nelson[1], *S.M. Phillips*[2], *T. Stellingwerff*[4], *S. Bruce*[4], *I. Breton*[4], *A. Thorimbert*[4], *P.A. Guy*[4], *J. Clarke*[1], *M.A. Tarnopolsky*[3], *and D.S. Rowlands*[1]

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Department of Pediatrics and Medicine, McMaster University, Hamilton, Canada[3]
Physical Performance and Mobility, Nestle Research Center, Lausanne, Switzerland[4]

The emphasis on recovery from endurance exercise has been focused on carbohydrate ingestion, with little attention given to protein. Only with post-exercise protein ingestion is the muscle protein synthesis rate increased sufficiently to establish positive net protein balance, which could support protein accretion and the repair and adaptive remodelling of damaged protein structures or cell contents. In recent investigations in well-trained males we observed a moderate 4.1% enhancement of repeated-sprint cycling performance when fed a high protein-carbohydrate versus low protein recovery diet, or a small 2.5% improvement in performance with a leucine-enriched protein-carbohydrate post-exercise recovery supplement, during respective 4-day and 5-day investigations. The effect of post-exercise protein ingestion on metabolic processes that might support recovery during several days of intense endurance exercise are largely unknown, and performance findings require confirmation,. Therefore we investigated the impact that a high protein, high leucine, carbohydrate and fat supplement would have on performance, whole-body leucine turnover, nitrogen balance, and metabolism during a 6-day period of intensified training. Twelve male cyclists completed a blinded, randomized crossover trial consisting of high-intensity intervals (days 1, 2), recovery rides (days 3, 4) and performance tests (days 5, 6). Subjects ingested beverages containing leucine, micellar whey-protein, carbohydrate and fat (LEUPRO; respectively 6.7/20/44/22 $g \cdot h^{-1}$) or isocaloric carbohydrate and fat (CON; respectively 120/22 $g \cdot h^{-1}$) in the immediate 1-3 h following exercise. Protein intake was clamped at 1.9 and 1.5 $g \cdot kg^{-1} \cdot d^{-1}$; remaining food was carbohydrate-rich and intake balanced to estimated energy expenditure. The effect of LEUPRO supplementation on mechanistic outcomes (whole-body leucine turnover, nitrogen balance, metabolomics) during the recovery from exercise over the 6-day training block will be discussed; performance outcomes will also be discussed in the context of these findings and those of our recent and similar investigations.

Abstract for: Sport and Exercise Science New Zealand Conference 2010

EFFECTS OF A LEUCINE-ENRICHED PROTEIN SUPPLEMENT ON PERFORMANCE AND METABOLIC RESPONSES FOLLOWING INTENSE CYCLING

Presenting Author A.R. Nelson[1], *Co-Authors S.M. Phillips*[2], *T. Stellingwerff*[4], *S. Bruce*[4], *J. Clark*[1], *M.A. Tarnopolsky*[3], *and D.S. Rowlands*[1]

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The emphasis on recovery from endurance exercise has been focused on carbohydrate ingestion, with little attention given to protein. Only with post-exercise protein ingestion is the muscle protein synthesis rate increased sufficiently to establish positive net protein balance, which could support protein accretion and the repair and adaptive remodelling of damaged protein structures or cell contents. In recent investigations in well-trained males we observed a moderate 4.1% enhancement of repeated-sprint cycling performance when fed a high protein-carbohydrate versus low protein recovery diet, or a small 2.5% improvement in performance with a leucine-enriched protein-carbohydrate post-exercise recovery supplement, during respective 4-day and 5-day investigations. However, these performance findings require confirmation, and effects on metabolic processes that might support recovery during several days of exercise are largely unknown. Therefore the aim of the current study was to determine the impact that a high protein, high leucine, carbohydrate-containing supplement would have on performance, whole-body leucine turnover, nitrogen balance, and metabolism during a 6-day period of intensified training. Twelve male cyclists completed a blinded, randomized crossover trial consisting of high-intensity intervals (days 1, 2), recovery rides (days 3, 4) and performance tests (days 5, 6). Subjects ingested beverages containing leucine, micellar whey-protein, carbohydrate and fat (LEUPRO; respectively 6.7/20/44/22 $g \cdot h^{-1}$) or isocaloric carbohydrate and fat (CON; respectively 120/22 $g \cdot h^{-1}$) in the immediate 1-3 h following exercise. Protein intake was clamped at 1.9 and 1.5 $g \cdot kg^{-1} \cdot d^{-1}$; remaining food was carbohydrate-rich and intake balanced to estimated energy expenditure. During recovery from exercise on day 1, LEUPRO increased whole-body leucine flux (5.2-fold; 90%CL x/÷1.1), oxidation (5.6; x/÷1.1), non-oxidative disposal (4.8; x/÷1.1) and plasma essential amino acids (2.2; x/÷1.1). LEUPRO altered the plasma metabolome during recovery on day 1, with increases in the plasma concentrations of acylcarnitines C3 (1.51; x/÷1.0), C3:1 (23%; x/÷27%) and C4 (20%; x/÷15%), as well as an increase in plasma acylcarnitine C5 (3.0; x/÷1.1) which represents a mixture of the branched-chain amino acid metabolites isovalerylcarnitine, 2-methylbutyrylcarnitine, and pivaloylcarnitine. During exercise on day 6, LEUPRO increased non-oxidative leucine disposal (14%; ±13%). While we found that with LEUPRO supplementation serum creatine kinase was reduced by 21-25% (90%CL ±14%) on day 4 and day 6, surprisingly the effect on sprint mean power was inconclusive (day 4/6: 0.4% 99%CL ±2.4%/-0.3% ±2.4%). Mean six-day nitrogen balance was positive in both conditions, a profile that differs to that found in our previous studies. To conclude, a leucine-enriched micellar whey-protein supplement increased whole-body leucine turnover and incorporation into tissue, but exceeded the tissue capacity to metabolise branched-chain amino acids and led to an accumulation of their metabolites during the immediate few hours

of recovery from endurance exercise. Despite these effects, the supplement did not benefit subsequent performance, which might be related to positive nitrogen balance.

Abstract for: ACSM Oral Presentation 2011

Effects of Leucine-enriched Protein Supplementation on Subsequent Performance and Metabolism following High-Intensity Cycling

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Recently, we observed that the addition of leucine with protein to high-carbohydrate recovery feeding ingested following intense cycling over 3 days led to a small enhancement in subsequent performance. It is also established that post-exercise protein feeding, both with and without added leucine, attenuates muscle damage and soreness, and can increase muscle-protein synthesis, but the metabolic consequences are largely unknown. **PURPOSE:** To investigate further putative mechanisms and the effects on metabolism of a post-exercise leucine-protein supplement and confirm a benefit to subsequent performance. **METHODS:** In a double-blind randomized crossover, 12 male cyclists ingested either a leucine/protein/carbohydrate/fat supplement (LEUPRO; respectively 7.5/20/89/22 $g \cdot h^{-1}$) or isocaloric carbohydrate/fat control (CON: 119/22 $g \cdot h^{-1}$) for 1-3 h post-exercise during a 6-day training block. Protein intake was clamped at 1.9 (LEUPRO) and 1.5 $g \cdot kg^{-1} \cdot d^{-1}$ (CON). Using stable isotope methodology and LC-MS based metabolomics we determined the impact of LEUPRO on leucine turnover and amino acid metabolism. **RESULTS:** Following exercise, LEUPRO increased branch-chain amino acids (BCAA) in plasma (2.6-fold; 90%CL x/÷1.1) and urine (2.8-fold; x/÷1.2) and products of their metabolism: plasma acylcarnitine C5 (3.0-fold; x/÷0.9) and urinary β-aminoisobutyrate (3.4-fold; x/÷1.4). LEUPRO also increased whole-body leucine oxidation (5.6-fold; x/÷1.1) and synthesis (4.8-fold; x/÷1.1), and only with LEUPRO was recovery leucine balance (mean ± SD: 580 ± 215 $\mu mol \cdot kg^{-1} \cdot h^{-1}$, control; -43 ± 24 $\mu mol \cdot kg^{-1} \cdot h^{-1}$) and day-1 nitrogen balance (17 ± 20 $mg \cdot kg^{-1}$, control; -90 ± 44 $mg \cdot kg^{-1}$) positive. However, subsequent day 2-5 nitrogen balance was positive in CON (111 ± 86 $mg \cdot kg^{-1}$; LEUPRO: 130 ± 110 $mg \cdot kg^{-1}$). LEUPRO reduced serum creatine kinase by 21-25% (90%CL ±14%). Despite these effects, the impact of LEUPRO on sprint power was trivial (day 4: 0.4% ±1.0%; day 6: -0.3% ±1.0%). **CONCLUSIONS:** Despite saturation of post-exercise BCAA metabolism and apparent attenuated tissue damage, a leucine-protein supplement had a trivial effect on performance compared to control, possibly due to both treatment groups being in positive nitrogen balance. Supported by a grant from Nestec Ltd., Vevey, Switzerland.

Category
604 protein and amino acid metabolism

Leucine-enriched Protein-carbohydrate Feeding After High-intensity Cycling Enhances Neutrophil Oxidative Burst

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University of Guelph, Ontario, Canada

INTRODUCTION

- Neutrophils are the first cells recruited from the blood stream to sites of infection and act as a first line of defence for the innate immune system
- When stimulated neutrophils produce a range of toxic reactive oxygen species (oxidative burst; Figure 1) and a suite of proteolytic enzymes, which interact to kill infectious agents[1]
- Neutrophils also release cytokines that influence both T-cell and B-cell activities, covering both the efferent and afferent limbs of the immune response[1] (Figure 2)

Figure 1: The production of ROS by neutrophils[2]

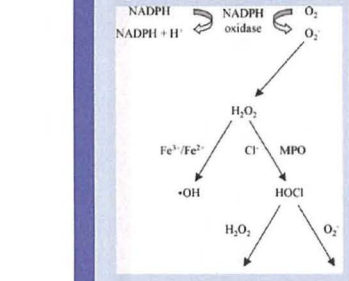
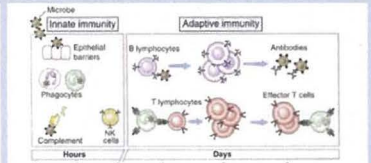


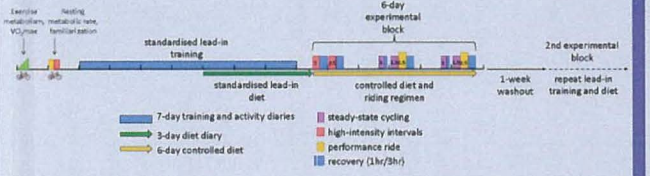
Figure 2: The principal mechanisms of innate and adaptive immunity[3]



- High-intensity prolonged strenuous exercise decreases neutrophil function and creates an 'open window' for opportunistic infections[4]
- Post exercise carbohydrate consumption is likely to improve neutrophil function e.g. oxidative burst[5]
- Glutamine and leucine may provide essential substrate for leukocytes[5]
- Athletes in competition or heavy training may benefit from the ingestion of a leucine-enriched carbohydrate supplement during recovery to improve neutrophil function and reduce susceptibility to infection
- We hypothesised that the leucine-enriched protein-carbohydrate recovery supplement would attenuate the exercise-induced immunodepressive effects on neutrophil function, specifically neutrophil oxidative burst, relative to a carbohydrate-only beverage

METHODS

- Twelve endurance trained male cyclists; double-blinded, randomised, crossover design.
- Riding protocol consisted of four high-intensity rides and two recovery rides over two 6-day experimental blocks, with a one-week washout between; diet and training were standardised leading into each experimental block.
- Each experimental block comprised: visit one, 3-h loading intervals; visit two, 2.5-h loading intervals; visits three and five, 60 min at 30% of peak aerobic power; visits four and six, 90-min at 50% of peak aerobic power followed by a repeated sprint performance test.



- Immediately post-exercise, cyclists ingested isocaloric beverages composed of (for a model 75kg rider with peak power of 360W; per serve):
  Leucine-enriched high protein-carbohydrate: whey protein (10.1g), leucine (3.8g), carbohydrate (45g), and fat (11.0g)
  High-carbohydrate: carbohydrate (60.35g) and fat (11.0g)
- Beverages were ingested every 30min, for 3-h on days 1, 2 and 4 and for 1-h on days 3 and 5
- Blood samples taken on days 1, 2, 4 and 6 for neutrophil oxidative burst, differential cell count, creatine kinase, cortisol, IL6, IL10, and IgA.
- Neutrophil oxidative burst was assessed by analysing the production of superoxide anion (O_2^-), measured by the spectral wavelength of the superoxide dismutase inhibitable reduction of cytochrome-C.

OXIDATIVE BURST, CREATINE KINASE, CORTISOL, IL6, IL10

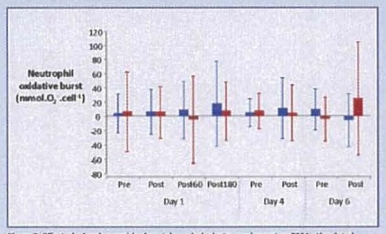


Figure 3: Effect of a leucine-enriched protein-carbohydrate supplement on PMA-stimulated neutrophil oxidative burst. Data are the least-squares mean quantity of O_2 produced in PMA-stimulated cells minus production in non-stimulated cells, minus the background. Bars are standard deviations.

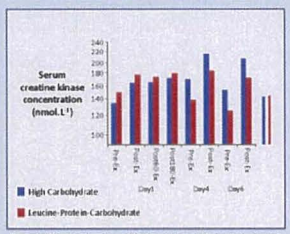


Figure 4: Effect of a leucine-enriched protein-carbohydrate supplement on plasma creatine kinase concentration. Data are back log-transformed least-squares mean concentrations plotted on a log scale with geometric CV error bars for high carbohydrate (blue) and leucine-enriched protein-carbohydrate (red).

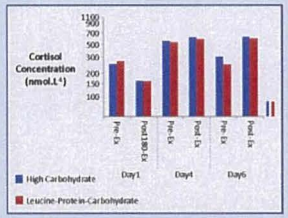


Figure 5: Effect of a leucine-enriched protein-carbohydrate supplement on serum cortisol. Data are back log-transformed least-squares mean concentrations plotted on a log scale with geometric CV error bars for high carbohydrate (blue) and leucine-enriched protein-carbohydrate (red).

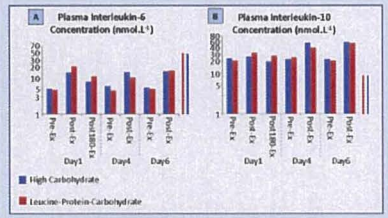


Figure 6: Effect of a leucine-enriched protein-carbohydrate supplement on plasma Interleukin-6 (A) and Interleukin-10 (B) concentrations. Data are back log-transformed least-squares mean concentrations plotted on a log scale with geometric CV error bars for high carbohydrate (blue) and leucine-enriched protein-carbohydrate (red).

DIFFERENTIAL CELL COUNT

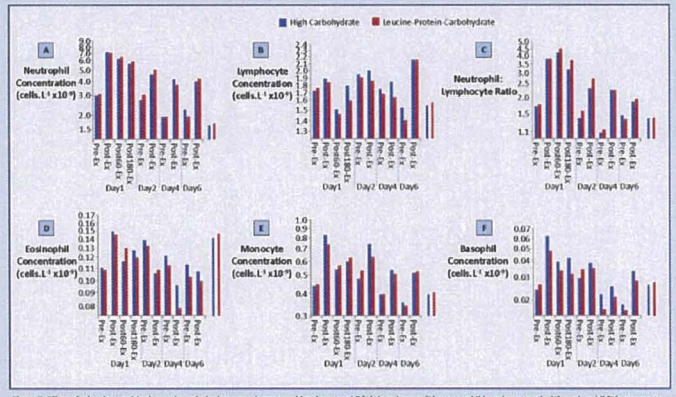


Figure 7: Effect of a leucine-enriched protein-carbohydrate supplement on blood neutrophil (A), lymphocyte (B), neutrophil:lymphocyte ratio (C), eosinophil (D), monocyte (E) and basophil (F) concentrations. Data are back log-transformed least-squares mean concentrations plotted on a log scale with geometric error bars for high carbohydrate (blue) and leucine-enriched protein-carbohydrate (red).

STATISTICAL SUMMARY

Effect of Leucine-Enriched Protein-Carbohydrate Feeding on Selected Blood Parameters

Parameter	Day; Sample	Effect (nmol.O_2^-.cell)[a] +/- 90% CL[b]	Threshold for Substantial Effect (nmol.O_2^-.cell)[c]	Effect Size[d]; ±90% CL[e]	Qualitative Inference[f]
Oxidative burst	1; pre	-3.14; ±19.67	±5.45	-0.11; ±0.71	unclear
	1; post0	-5.44; ±19.52	±5.45	-0.20; ±0.71	unclear
	1; post 60	**-17.0; ±18.98**	**±5.45**	**-0.62; ±0.75**	**likely moderate ↓**
	1; post 180	**-16.8; ±20.47**	**±5.45**	**-0.59; ±0.75**	**likely small ↓**
	4; pre	-4.52; ±20.65	±5.45	-0.16; ±0.75	unclear
	4; post	-7.65; ±20.63	±5.45	-0.28; ±0.75	unclear
	6; pre	-12.2; ±20.52	±5.45	-0.44; ±0.75	unclear
	6; post	**28.90; ±20.36**	**±5.45**	**1.22; ±0.76**	**very likely large ↑**

Parameter	Day; Sample	Fold Effect[g]; ÷/× 90% CL[h]	Threshold for Substantial Effect (Factor)	Effect Size; ±90% CL	Qualitative Inference
DCC - Neutrophils	1; pre	1.06; ×/÷1.08	1.07	0.18; ±0.23	possibly trivial
	1; post 0	**0.88; ×/÷1.08**	**1.07**	**-0.06; ±0.22**	**likely trivial**
	1; post 60	**1.02; ×/÷1.08**	**1.07**	**0.08; ±0.23**	**likely trivial**
	1; post180	1.05; ×/÷1.08	1.07	0.15; ±0.23	possibly trivial
	2; pre	**1.10; ×/÷1.08**	**1.07**	**0.28; ±0.24**	**likely small ↑**
	2; post	1.10; ×/÷1.08	1.07	0.27; ±0.24	possibly small↑
	4; pre	**1.00; ×/÷1.08**	**1.07**	**0.01; ±0.22**	**likely trivial**
	4; post	**0.88; ×/÷1.08**	**1.07**	**-0.31; ±0.22**	**likely small ↓**
	6; pre	**0.86; ×/÷1.08**	**1.07**	**-0.38; ±0.22**	**likely small ↓**
	6; post	1.06; ×/÷1.08	1.07	0.16; ±0.23	possibly trivial
DCC - Lymphocytes	**1; pre**	**1.02; ×/÷1.07**	**1.05**	**0.03; ±0.29**	**likely trivial**
	1; post 0	**0.97; ×/÷1.07**	**1.05**	**-0.10; ±0.22**	**likely trivial**
	1; post60	0.97; ×/÷1.07	1.05	-0.12; ±0.23	possibly trivial
	1; post 180	**0.90; ×/÷1.07**	**1.05**	**-0.34; ±0.22**	**likely small ↓**
	2; pre	**0.88; ×/÷1.06**	**1.05**	**-0.38; ±0.22**	**likely small ↓**
	2; post	0.92; ×/÷1.06	1.05	-0.26; ±0.22	possibly small↓
	4; pre	0.97; ×/÷1.07	1.05	-0.12; ±0.22	possibly trivial
	4; post	**0.90; ×/÷1.07**	**1.05**	**-0.37; ±0.22**	**likely small ↓**
	6; pre	**0.92; ×/÷1.06**	**1.05**	**-0.30; ±0.22**	**likely small ↓**
	6; post	**1.00; ×/÷1.06**	**1.05**	**0.00; ±0.22**	**likely trivial**
Creatine kinase	1; pre	1.11; ×/÷1.15	1.05	0.40; ±0.53	possibly small↑
	1; post0	1.09; ×/÷1.15	1.05	0.32; ±0.53	possibly small↑
	1; post60	1.06; ×/÷1.15	1.05	0.23; ±0.54	unclear
	1; post180	1.06; ×/÷1.15	1.05	0.22; ±0.52	unclear
	4; pre	**0.81; ×/÷1.15**	**1.05**	**-0.68; ±0.47**	**very likely moderate ↓**
	4; post	**0.85; ×/÷1.15**	**1.05**	**-0.54; ±0.47**	**likely small ↓**
	6; pre	**0.81; ×/÷1.15**	**1.05**	**-0.69; ±0.47**	**very likely moderate ↓**
	6; post	**0.82; ×/÷1.15**	**1.05**	**-0.63; ±0.47**	**likely moderate ↓**
Cortisol	1; pre	1.08; ×/÷1.20	1.06	0.23; ±0.60	unclear
	1; post180	0.99; ×/÷1.20	1.06	-0.04; ±0.60	unclear
	4; pre	0.96; ×/÷1.20	1.06	-0.14; ±0.61	unclear
	4; post	0.84; ×/÷1.20	1.06	-0.39; ±0.61	unclear
	6; pre	**0.79; ×/÷1.20**	**1.06**	**-0.73; ±0.62**	**likely moderate ↓**
	6; post	0.96; ×/÷1.20	1.06	-0.14; ±0.61	unclear
Interleukin-6	**All days/samples**	**0.74; ×/÷1.44 to 1.52; ×/÷1.44**	**0.11-1.89**	**-0.07; ±0.08 to 0.08; ±0.08**	**likely to almost certainly trivial**
Interleukin-10	**All days/samples**	**0.81; ×/÷1.314 to 1.41; ×/÷1.314**	**0.38-1.62**	**-0.07; ±0.09 to 0.11; ±0.09**	**likely to almost certainly trivial**

[a] Data are difference in units (nmol.O_2^-.cell^{-1}) of oxidative burst in the leucine rich protein-carbohydrate condition minus the high carbohydrate condition.
[b] Add and subtract this number by the mean effect to obtain the upper and lower confidents limits.
[c] Threshold for a substantial is the value for the smallest Cohen effect size.
[d] Effect size thresholds: <0.2 trivial, 0.2-0.6 small, 0.6-1.2 moderate, 1.2-2.0 large 2.0-4.0 very large, >4.0 enormous.
[e] Add and subtract this number by the mean effect to obtain the upper and lower confidents limits.
[f] Thresholds for assigning qualitative terms to chances of substantial effects were as follows: <1.0%, almost certainly not; <5.0%, very unlikely; <25%, unlikely; <75%, possible; >75%, likely; >95%, very likely; >99%, almost certain. An effect is unclear if its confidence interval includes both substantial increases and decreases. Cell text style represents the certainty of a substantial outcome: **bold** is almost certain, very likely, or likely; *italic* is possible; and regular is unclear. Arrow symbols indicate an increase (↑) or decrease (↓)
[g] Data are fold (factorial) difference in the relevant parameter in the leucine-rich protein-carbohydrate condition minus the high carbohydrate condition.
[h] Multiply and divide this number by the mean effect to obtain the upper and lower confidents limits.

SUMMARY

- The leucine-enriched protein-carbohydrate supplement had no effect on the exercise-induced neutrophilia.
- The likely small/moderate decreases in superoxide anion production (neutrophil oxidative burst) on day 1 and very likely large increase on day 6 demonstrates a delayed response to the effect of the leucine-enriched protein-carbohydrate supplement.
- There were likely/very likely reductions of small to moderate size in creatine kinase (days 4 and 6) and cortisol (day 6 pre-exercise) in the leucine-enriched protein-carbohydrate condition, also consistent with a delayed effect.
- The increase in superoxide anion production in the leucine-enriched supplement could be due to greater glutamine synthesis as a result of ingested leucine providing the amino group and possibly the ammonia required for glutamine synthesis[6].
- Leucine may enhance the production of superoxide anion via activation of key cytochromes during NADPH-oxidase assembly[7].
- Cellular measurements *in vitro* do not necessarily reflect directly the situation *in vivo* or potential risk of infection.
- These findings agree with the hypothesis that the leucine-enriched protein-carbohydrate recovery supplement attenuated the exercise-induced immunodepressive effects on neutrophil function at the end of a 6-day high-intensity cycling regimen.
- These results suggest that supplementation with a leucine-enriched protein-carbohydrate supplement after exercise may enhance immune function in athletes.

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This study was funded by Nestec (PowerBar), Vevey, Switzerland.

APPENDIX C STUDY 1 INFORMED CONSENT

Effect of LeuPlus protein recovery formulation on subsequent performance and physiological outcomes during one-week of high-intensity cycling

INFORMATION SHEET

The Institute of Food, Nutrition and Human Health

The Institute of Food, Nutrition and Human Health is part of Massey University. It includes a large team of scientists that are interested in human nutrition, exercise science, physiology, and health. The project coordinator is Dr David Rowlands who is Senior Lecturer in Sport and Exercise Science at the Wellington campus. Other researchers working on the project are Mr Andre Nelson (PhD student), Dr Sue Broadbent (immunology), Miss Lara Jackson (Honours student), Mr Jim Clarke (research assistance) and Dr Murray Leikis (medical supervision). In addition, biochemists at the Nestle Research Centre, Lausanne, Switzerland and at McMaster University, Ontario, Canada will analyse some of the blood samples.

Why Are We Doing This Study?

The ability to maintain performance on a daily basis is highly relevant to athletes undertaking intense training and for those in multi-day tournaments. In recent research we observed that a protein-enriched recovery supplement can enhance performance several days after ingestion.. This delayed effect possibly relates to the time-course for muscle repair, enhanced protein synthesis, and possibly to superior glucose homeostasis suggesting glycogen sparing. We found evidence for attenuated muscle damage or faster repair, and reduced general tiredness (implicating a possible link to the brain), but as yet the mechanism for the performance effect remains to be established. From research in rats and isolated muscle cell models, it has been established that the amino acid leucine is the key amino acid responsible for anabolic processes (tissue building) in the cells.

Consequently, we have designed a recovery supplement formulation containing a blend of protein isolates, amino acids, carbohydrates and fats that we think will enhance the rate of recovery from prolonged high-intensity exercise and therefore improve subsequent performance. The purpose of this experiment is to see if it works, and if so it may lead to the development of a new recovery product. This study is funded by Powerbar Performance, a division of Nestec, Switzerland.

In Scientific Terms

Research Question

Does a post-exercise recovery supplement containing protein, leucine, carbohydrate and fat enhance recovery and subsequent high-intensity cycling performance, relative to an isocaloric control containing only carbohydrate and fat?

Participant Recruitment
We have currently tested 7 cyclists out of 10 needed for the study. **We would like to recruit 3 more endurance-trained male cyclists, mountain bikers or triathletes, for trials starting in November.** To participate in the study you should:

- be male and aged 18 to 50 years
- be in regular training – 8 or more hours of cycling per week for the 4-6 months or greater prior to the study, ideally with some racing or exposure to higher-intensity training. The latter is important because of the nature of the performance rides. (NOTE: Participants interested in the study, but training less than but close to the inclusion volume (e.g. 6-8 h per week) but with a demonstrated high-level racing and training history (>2 y) in the sport will be asked to train for around 4-6 weeks above minimum specified volume before starting the study.)
- VO_2max of at least 55 ml·(kg min)$^{-1}$
- Have no food allergies

What is Involved
If you decide to participant in our study, you will make two visits to the laboratory over 1-2 weeks to establish baseline metabolic and fitness parameters, and to practice the performance test (riders who have participated in past studies may omit the latter test provided if racing recently). This will be followed by two 6-day experimental periods interspersed with 1-2 weeks recovery washout. The study design is termed a *double-blind randomized cross-over*. This means that you will be randomly presented with the recovery conditions and neither the researchers or you will not know what recovery supplement condition you are on. The study design is illustrated in Figure 1 and the outline of one experimental block is shown in Figure 2.

Experimental Conditions

During recovery from the lab rides you will ingest servings every 30 min of a milk-like recovery supplement containing either:
Intervention. Whey/casein protein, leucine, carbohydrate, fat.
Control. Carbohydrate and fat only.

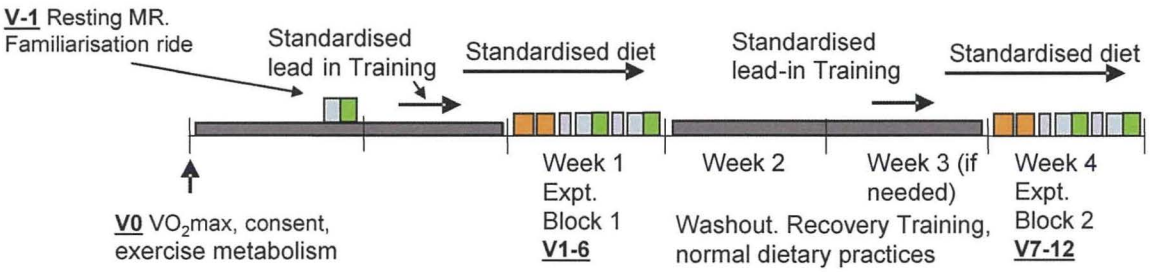

Figure 1. Study plan overview, including the visits (**V**) to the lab.

Details
What will happen prior to and on each day of the two experimental blocks is summarized below.

Baseline (V0) (see Figure 1).

- Report to the lab 1 or more hours after your last meal at an arranged time for screening (health, food allergies, medications, time availability and commitment) and informed consent. If all is in order, you will then cycle for ~15-min to warm up followed by an incremental ride to exhaustion to measure your maximal oxygen uptake (VO_2max) and peak power. On this and all other testing days bring you riding shorts, top, shoes, and your bike or bike set-up measurements. We will set up the lab bikes to replicate your normal position on your bike.

V -1 (familiarization trial)

- Approx. 2-7 days following the baseline visit V0. You will come into the lab in the morning for assessment of your basal metabolic rate (morning fasted). This will then be followed by a light breakfast and a 1 h ride while we collect respiratory gas, and the a full familiarization of the performance test. Following this ride you will be provided with dietary instructions and guidelines for training, and some diet and training diaries.

V1

- Start of experiment. ~7 days following V-1, you will report to lab between 2 and 3 PM. Your last meal will have been standardized and taken at 11 or 12 noon. When you arrive we will ask you to complete two well-being assessments (often used to monitor risks of overtraining) and place cannula into forearm veins for infusion procedures. Blood and urine samples will be collected.
- The ride is 3 h. following a 30 min warm, the consists of 60-min total of intervals at 70-90% of peak cycling power interspaced with recovery periods at 50%; the ride is demanding, so ensure you train lightly and eat sufficiently the day before. A DVD and CD player are available. You will ingest a sports drink during exercise.
- Following exercise, you will begin the recovery supplement ingestion 10-min post exercise, and subsequently at 30-min intervals for 3 h. Additional water will be provided based on body weight loss. During the recovery period we will infuse trace amounts of an amino acid used for determining the effect of the supplement on protein synthesis and breakdown. We will collect breath samples and blood samples from the cannula after exercise and again at 30, 60, 90, 120, and 180 min post to measure metabolic parameters. At 3 h you will ingest a light meal and some fluid and leave to go home. We will collect all urine into labelled containers during recovery and overnight.

V2 (V1 + 1 days)

- Start 6 to 8 AM as arranged. Come into lab fasted (water only - no breakfast, tea or coffee). This is the second standardized exercise day. You will ride for 2.5 during which time you will complete perception and muscle-soreness scales before and during exercise. Blood samples will be collected before and after exercise. The recovery supplement will be ingested immediately post exercise and continue for 3 h, followed by a controlled diet that we will provide (all food provided). Sweat will be collected during exercise for assessment of protein loss. Urine will be collected.

V3 (V1 + 2 days)

- 5 to 6 PM. Recovery day. 60 min @30% of peak power. Recovery supplements ingested immediately post exercise and at 30 min. Controlled diet and urine collection continues.

V4 (V1 + 3 days)

- 6 to 7 AM. Come into lab fasted (water only - no breakfast, tea or coffee). Well being scales; cannula and a blood sample; light breakfast. You will ride for 90 min @50% of peak power (easy-moderate intensity) during which time we will collect breath samples. Following this ride is the first performance test (~80 min). Perception and soreness scales will be taken before and during exercise. Following exercise you will ingest the recovery supplement again for 3 h, but you can leave the lab this time to accommodate work commitments. It is essential that you ingest the supplements at the times specified for the success of the study. Note: we need to conduct morning rides because to measure the effect of the supplement on protein metabolism at rest and exercise, we require overnight fasted metabolism.

V5 (V1 + 4 days)

- 5 PM. Recovery day as for V3. Controlled diet and urine collection continues.

V6 (V1 + 5 days)

- 6 to 7 AM to approx. 10 to 11 AM. A cannula will be placed in a forearm vein of both arms. Baseline blood and breath will be collected then trace amounts of an amino acid and glucose for 90 min at rest and for 90 min during exercise, which will then be followed by the performance test. End of block one of the cross over!

V7-12 (V1 + 14-20 days)

- Repeat block 2 of the cross over on the alternative randomized supplement exactly as specified V1-6.

Standardisation of Training

In preparation for the study we would like you to *standardize your training program* from the week before the first weekly experimental block. What you have to do is simply maintain the same training sessions in terms of time and degree of effort on Days 1-3 and 7 of each proceeding week. A training diary will be provided to help you. Training standardisation is to normalise the physical readiness before starting the experiment. It is important that you don't overtrain in the week leading up to the experiment, and we recommend 1-2 days of light training or complete rest on the day before the first day of the experiment (day V1).

Standardisation and Replication of Diet and the Avoidance of Foods Rich in ^{13}C

During the 2 days preceding and on the morning of day V1 we would like you to consume your normal food and record it for replication preceding the testing in the following experimental block. We will provide sheets for you to do this. The only change that you will need to make to your diet is the omission of certain foods that contain a small amount of the marker that we infuse into your blood on days V1 and V6 to measure protein metabolism. The foods that you may not eat from 1-week before the study are detailed in **Appendix 1**. Like replication of training, a standard diet is important to ensure similar muscle fuel (glycogen and fat) stores are present before exercise testing. During the experiment (days V2-V5) all food will be provided for accurate determination of protein and energy balance.

Are any of the Procedures Harmful or Painful?

Blood Sampling

Cannula are small plastic tubes that are routinely placed into veins of participants in clinical research studies and in hospital patients. There is likely to be mild to moderate discomfort and small risk of bruising associated with the removal of a cannula. The risk of infection is 1 in 20,000. Cannula will be placed 8 times during the study. The researchers are trained in cannula placement and the taking of blood samples via cannula. Between 20 and 100 ml of blood will be collected before and during the exercise procedures. The blood will be stored in a freezer for up to 24 months during which time biochemical analysis will be conducted on it. Approximately 0.5 ml of plasma from each sample will be sent to McMaster University in Ontario, Canada for stable isotope and amino acid analysis. An additional 1.0 ml from each sample will be sent to the Nestle Research Centre in Lausanne, Switzerland for metabolomic analysis.

Infusion of Stable Isotopes to Determine Amino Acid (Leucine) Oxidation and Glucose Utilization

On Day V1 and V6 trace amounts of sterile $NaH^{13}CO_3$, L-[1-^{13}C]leucine and [6,6-^{2}H]glucose will be infused intravenously through a cannula placed into your right forearm. Breath samples will be collected and blood will be drawn from a vein in the opposite arm every half hour to determine leucine oxidation, protein synthesis, breakdown and balance and glucose utilization (rate of appearance and disappearance). This state-of-the-art prime/constant infusion of stable isotopes in humans is a safe, accurate and reliable method of understanding the metabolic processes in vivo. Infusion of L-[1-^{13}C]leucine allows determination of protein synthesis and breakdown. Likewise, the infusion of [6,6-^{2}H]glucose will allow us to measure the rate of appearance and disappearance of glucose, which translate into the rate of release of glucose into the circulation mainly from the liver and the uptake of glucose into the cells mainly by the muscle during exercise, respectively.

Exercise

There is often some physical and psychological discomfort associated with heavy exercise.

Recent evidence has indicated that even among healthy populations of athletes who exercise strenuously and regularly, there is some risk of sudden death due to heart failure. Though rare, such cases can occur in people who may have an undiagnosed condition. If you have any reason to suspect that you may have a cardiovascular problem, we suggest that you see your physician and get an ECG before you agree to participate.

If you have any additional medical concerns associated with this project, please contact your GP, or discuss with the researcher.

Time Commitment

Experiment Component	Time Commitment (h)
Screening, consent, VO_2max test & lab familiarization	1.5 h
Diet and training diaries	0.25 h
Day -1: Familiarisation 2-h ride in lab	2.5 h
Day 1: Loading ride #1	2 x [3h cycling, 3h resting/recovery]
Day 2: Loading ride #2	2 x [2.5h cycling, 1h set up/recovery]
Day 3: Recovery ride #1	2 x [1h cycling]
Day 4: Metabolism and Performance ride #1	2 x [3h cycling, 1h lab]
Day 5: Recovery ride #2	2 x [1h cycling]
Day 6: Metabolism and Performance ride #2	2 x [2h rest, 3h cycling]
Total	~45 h

A reimbursement of $400 in MTA vouchers (or otherwise requested) will be provided on completion of the study to compensate you for the time, travel, and energy. Additional reasonable expenses will also be reimbursed if required (e.g. childcare, overnight accommodation). Parking vouchers will be provided for on-campus parking. All food during the two study blocks is provided for you.

Benefits

You will learn your VO_2max and peak power output. A follow up laboratory test (e.g. VO_2max, lactate threshold) will be provided free of charge if desired, which are normally worth $225. You will partake in some challenging performance tests and likely increase your fitness. You will receive a summary of the results once the final results are available, which will contain a summary on of the effectiveness of the recovery formulation.

What if I Suffer a Personal Injury?

If physical injury results from your participation in this study, you should visit a treatment provider for examination, diagnosis, and treatment and inform the researcher immediately so an indecent form may be completed. The study is conducted principally for the benefit of Nestec to test the performance and physiological responses to the intervention formulation. In the event of injury arising from your participation in the research, an appropriate level of compensation will be awarded, in line with the *New Zealand Researched Medicines Industry Guidelines on Clinical Trials - Compensation for injury resulting from Participation in Industry Sponsored Clinical Trials*. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

Participant's Rights

At any time, you will have the right to:

- decline to participate;
- decline to answer any particular question;
- withdraw from the study;
- ask any questions about the study at any time during participation;
- provide information on the understanding that your name will not be used unless you give permission to the researcher;

- be given access to a summary of the project findings when it is concluded;
- you have the right to have any blood samples returned to you after they have been analysed.

If you have any queries or concerns regarding your rights as a participant in this study you may wish to contact a Health and Disability Advocate, telephone 0800 42 36 38 (4 ADNET).

If you are Interested in Taking Part

CONTACT:

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This project has been reviewed and approved by the Central Regional Ethics Committee, Wellington Application CEN/07/11/077. If you have any concerns about the ethics of this research, please contact Jiska van Bruggen telephone 04 496 2405, email central_ethicscommittee@moh.govt.nz

Guidelines for avoiding maize and sugar in diet in order to lower background ^{13}C enrichment in body-carbohydrate pool

Contamination of the Tracer Signal. We will be measuring the oxidation of the ingested leucine by tracing the appearance of ^{13}C atoms in the expired air. The ^{13}C comes from the trace amounts of infused ^{13}C-labelled leucine on day V6. The tracers used in this study are stable isotopes and completely safe (i.e. non-radioactive). Since some carbohydrate food products (maize and sugar) contain a high natural abundance of ^{13}C in their glucose or starch, you will be asked to avoid eating these products from the week before each experimental block and throughout the experimental blocks (all food during the experimental blocks will be provided). If you fail to do this, our results will be invalid and we will not be able to use your data.

The protocol familiarisation ride 7- days before the exercise test will empty your body of any residual ^{13}C-enriched carbohydrate that might be stored in your muscles after ingesting ^{13}C-enriched carbohydrates, such as corn and sugar. When you follow the low ^{13}C-carbohydrate diet (see below) your body-glycogen stores should be replaced with carbohydrate that contains only the low natural background level of ^{13}C.

What can I eat?
You can eat all bread/pasta/rice/potato products, all vegetables, all meats, some sauces that come in jars (but check for corn flour and cane sugar), milk and dairy products, Barkers non-

sugar jams (check the labels). In fact you'll be amazed how much food you can eat (and you'll eat a healthier diet too!).

What foods do I have to avoid?
Because ^{13}C occurs in high levels in maize (sweet corn) and sugar cane we need to ask you to avoid all products that may include these ingredients. Most processed and many convenience will contain these sugars.

The list includes (more extensive list below)
- Any type of commercial sports drink/sports bar
- Coke and Pepsi, etc (but diet drinks are OK)
- Any soft drink (non-sugar added pure fruit juice – check label - is the only OK drink)
- Cereals made with maize (Cornflakes, sweetened muesli or any other cereals containing corn flour or sugar, ie. Most breakfast cereals. Natural mueslis with no added sugar are OK.)
- Sweetened breads (hot cross buns, teacakes, cakes etc)
- Confectionery (sweets, chocolate etc)
- Cane sugar (honey is OK; or we can provide you with a cane sugar alternative)
- Jelly
- and any refined products that you think may contain cane sugar, corn flour (often used in soups and sauces as a thickener) or maize!
- most processed foods contain maize or sugar, and are therefore unsuitable.
- fructose is usually no good either because most comes from the cheap source – high-fructose corn syrup.

- × Corn Starch
- × Corn Flour
- × Cereals:
  - Cornflakes, weet-bix, instant porridge packets, ricies, mueslis, sweet cereals …etc… they contain sugar.
- × Breakfast shakes/replacements e.g. '*up and go*'
- × Tinned fruit in syrup
- × Jelly, creamed rice, packet/instant desserts, packet yoghurts, raro …etc…
- × Golden syrup
- × Spreads:
  - Marmite
  - '*Kraft*' peanut butter
  - jams, marmalade
  - relishes, chutney, pickle …etc…
- × Drinks
  - Normal coke, sprite and other soft drinks …
  - Ordinary tonic water
  - Soda water with a twist
  - Milo, bournvita, '*Pams*' choca, ovaltine, drinking chocolate
  - Some sports water e.g. '*Mizone*', 'H_2Go'
  - Artificially flavoured drinks e.g. 'e^2', '*G force*'

- Juice concentrates
- Ribena
- Tomato juice

- Herbs and spices
  - '*Masterfoods*' jars of fresh garlic, chilli …etc…
  - '*Pams*' chilli, ginger
  - '*Greggs*' chilli, curries, coriander
  - '*Continental*' curries …etc…
- All mustards
- Tinned beans:
  - Chilli, baked beans, Mexican
- Tinned spaghetti, chick peas, beans …etc…
- Dips (salsa, guacamole, French onion, sundried tomato, sour cream-based …etc…)
- Corn chips (including grain waves!), tortillas, most potato chips
- Tinned/jarred/packet sauces
- All instant, flavoured pasta, rice and noodle snacks/meals
- Tomato puree, sauce, ketchup, chutney
- All tinned and packet (dried or fresh) soups
- All mayonnaise and salad dressings (easy to make your own)
- All stocks (wet or dry) i.e. chicken, beef, vegetable …
- Most soy sauces
- Worcestershire sauce
- Some frozen chips and wedges (corn starch, corn flour in the seasoning)
- Crumbed or battered products
  - Fish cakes, fingers, burgers, nuggets
  - Chicken fingers, burgers, nuggets
  - …etc…
  - lasagne toppas
- Frozen dinners, including pizzas and pies!
- Sweet pastry
- Ice cream (including lite ones)
- Muesli bars, chocolate bars, yoghurt bars
- Crackers:
  - '*Real foods*' corn thins
  - '*Le Snacks*'
  - rice crackers (e.g. '*Pams*', '*Trident*')
  - meal mates
  - '*Huntley and Palmers*' litebread
  - '*Arnotts*' corn or original cruskits
  - '*Weight Watchers*' crispbread
- '*Eta*' roasted peanut items
- Flavoured milks
- Custard
- All yoghurts except unsweetened acidophilus
- Flavoured milks (chocolate, banana …etc…)
- Specialty breads e.g. bagels, sweet breads
- Be wary of what is in grain breads!

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Effect of LeuPlus protein recovery formulation on subsequent performance and physiological outcomes during one-week of high-intensity cycling

PARTICIPANT CONSENT FORM

This consent form will be held for a period of five (5) years

I have read and understand the Information Sheet and have had the details of the study otherwise explained to me.	☐
My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.	☐
I agree to participate in this study under the conditions set out in the Information Sheet.	☐
I understand that my participant is voluntary and that I may withdraw at anytime.	☐
I agree to my blood being taken.	☐
I agree to my blood being stored after collection for later biochemical analysis.	☐
I agree to my blood being flown to McMaster University in Canada and the Nestle Research Centre in Switzerland for analysis. All sample sent overseas will be used in analysis and it will not be possible to send it back to New Zealand.	☐
I agree to the infusion of stable isotope materials.	☐
I agree to the exercise regime/dietary modifications.	☐
I understand that if personal injury results from participation in this experiment, that I may make a claim under Nestec insurance provisions.	☐
I understand that my participation in this study is confidential and that no material which could identify me will be used in any reports on this study.	☐
I know I am to contact the researchers if I have any side effects.	☐

Signature: ..... **Date:** .....

Full Name - printed .....

This project has been reviewed and approved by the Central Regional Ethics Committee, Wellington Application CEN/07/11/077. If you have any concerns about the ethics of this research, please contact Jiska van Bruggen telephone 04 496 2405, email central_ethicscommittee@moh.govt.nz

APPENDIX D STUDY 1 DIET AND TRAINING DIARIES

DIET RECORD

DAY -2	*Name:*

TIME	**FOOD ITEM** and METHOD of PREPARATION	**AMOUNT** eaten

DIET RECORD

DAY -1	*Name:*

TIME	**FOOD ITEM** and METHOD of PREPARATION	**AMOUNT** eaten

DIET RECORD

DAY 1	*Name:*

REMEMBER ONLY LOW PROTEIN FOODS TODAY

TIME	**FOOD ITEM** and METHOD of PREPARATION	**AMOUNT** eaten

INSTRUCTIONS FOR THE DIET RECORD

- List foods soon after they are eaten
- Record only one food item per line
- Be as specific as possible when describing the food item eaten: the way it was cooked (if it was cooked) and the amount that was eaten.
- Record amounts in household measures – *for example: g, ml, tablespoon (tbsp)(= 15ml), teaspoon (tsp) (=5ml), cups (=250ml), slices or units, as in 1 cup non-fat milk, 2 slices of wheat toast, or one raw apple*
- Include brand names whenever possible
- Report only the food portion that was actually eaten
- Include method that was used to prepare food item – *for example: fresh, frozen, stewed, fried, baked, canned, broiled, raw or braised*
- For canned foods include the liquid in which it was canned – *for example: sliced peaches in heavy syrup, fruit cocktail in light syrup, or tuna in water*
- Do not alter your normal diet during the period you keep this diary
- Remember to record the amounts of visible fats (oils, butter, salad dressings, margarine, …) you eat or use in cooking

Diet Instructions for Day 1:
Have your **last meal 4 h before** the testing (i.e. testing at 3pm >> 11am) **Don´t have high/moderate protein-food on Day 1:** **NO** meat, fish, eggs, ham, sausages..., cheese, pulses (beans...), nuts, seeds **LITTLE** milk and milk products, like yoghurt, cream cheese, cream, ...
NOTE: Why?? *Because we are investigating the effect of protein we give during recovery - if you have a lot of protein the day of testing this would affect our results!*

INSTRUCTIONS FOR THE TRAINING DIARY

- Please record your training as detailed as possible
- Record when (date, time), how long at which intensity and which type of exercise it was (cycling, running, weights …)
- If you have races or competitions you have to record them as well

All this is necessary, because you need to repeat the recorded training pattern preceding the second bout of testing!

Remember – it may be fine weather the week before the first block (V1-6) but terrible weather the week before the second block (V7-12), so please avoid doing exercise/rides that cannot be replicated later in the study…

TRAINING INSTRUCTIONS FOR THE LAST 3 DAYS BEFORE TESTING:

- **DAY –3** → 2 – 3 h ride, *light – moderate intensity*
- **DAY –2** → 90 min ride, *light – moderate intensity*
- **DAY –1** → Day off

TRAINING DIARY

Name:

_____

Day	Date	Session	Duration	Exercise		Intensity	
Before V1/7	*dd/mm/yy*	*Number*	*h + min*	*Type*	***Low**/time*	***Mod**/time*	***High**/time*
Comments:							
Day	Date	Session	Duration	Exercise		Intensity	
Before V1/7	*dd/mm/yy*	*Number*	*h + min*	*Type*	***Low**/time*	***Mod**/time*	***High**/time*
Comments:							

APPENDIX E STUDY 1 SUPPLEMENT COMPOSITION

E72027
Stand: 01.04.2007
Version: 6.0

Palmstearin 54

Article number:	72027
Description:	fine powder
Fat content:	100 %
Free fatty acid:	< 0.1 %
Peroxide number:	< 2
Melting point:	51 - 55°C
Water content:	< 0,1 %
Energy:	3.700 KJ 900 Kcal

Fatty acid composition (GC):		
Lauric acid C 12:0:	< 0,5 %	Fatty acid distribution
Myristic acid C 14:0:	1,5 %	iis subject to natural
Palmitic acid C 16:0:	62 %	variations.
Stearic acid C 18:0:	5 %	
Oleic acid C 18:1:	25 %	
Linol acid C 18:2:	5 %	
Others:	1 %	

Trans fatty acid:	< 1,0%
Packaging:	25 kg Paperbag with PE innerliner
Use within:	12 months
Storage conditions:	dry, dark and cool (below 15 °C)
	For commercial processing only

Prepared by	Checked by	Approved by
Date: 01.04.2007	Date: 01.04.2007	Date: 01.04.2007

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DANISCO

First you add knowledge...

CERTIFICATE OF ANALYSIS

Product	**Fructofin C**
Material No	136860
Batch No	0000205352
Customer Order No	4500334275
Invoice No	0090054524
Quantity kg	10200
Manufacturing date	28.10.2007 - 29.10.2007
Best before date	29.10.2008

	Analysis results	Limits MIN	Limits MAX	
Colour	<10		20	ICUMSA
pH 10% w/v	5.5	4.5	7.0	
Moisture	0.03		0.1	%
Specific rotation	-92.7	-93.5	-91.0	°
Fructose	Conforms	99.0	101.0	%
Conductivity ash	Conforms		0.01	%
Arsenic	Conforms		0.5	mg/kg
Heavy metals	Conforms		1	mg/kg
Lead	Conforms		0.1	mg/kg
Chloride	Conforms		40	mg/kg
Sulphate	Conforms		50	mg/kg
Calcium	Conforms		5	mg/kg
Glucose	Conforms		0.1	%
HMF Ph.Eur.	Conforms		0.32	abs.
Mean particle size	0.51	0.35	0.55	mm

Analysis result "Conforms" means that the parameter is subject to In Process testing or Reduced testing based on statistical data.

This is to certify that above lot has been checked by our own Quality Control and found to meet the above values.
This document s generated by the validated computer system and therefore has no signature.

DANISCO SWEETENERS OY
QUALITY CONTROL
SISKO POSTI
05.11.2007

MANUFACTURED BY DANISCO SWEETENERS OY
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ISO/IEC 17025
TESTING
RvA L383

Nestlé Nutrition

ANALYTICAL REPORT

Information provided by requestor:

Sample number PR-400082127
Material PROLACTA 90
Material info WHEY PROTEIN ISOLATE
Material number
Batch code LOT 4
Batch date 20070128
Supplier BBA Lactalis
Order Number
Comments

Requestor: ZWRC

Christophe Schmitt
Nestec Ltd.
Nestlé Research Centre
Vers-chez-les-Blanc
CH-1000 Lausanne
Switzerland
Tel: 00 41217858010
Fax: 00 41217858553
E-Mail To: Christophe.Schmitt@rdls.nestle.com
E-Mail cc:

Report Status: **Final Report**
SampleCondition: **Room Temperature**
Sample Type: **General Sample**
Date of receipt: **02/03/2007**
Report number: **ZWRC702565G1F**
NNQAC Number: **200702565**

Inspection	Report date	Method		WC	UD	Nestlé Norms			Legal Norms		Result	ON	Unit
						Low	High	Expiry	Low	High			
Sulfur Amino acids													
L-Cystine	23/03/2007	PR 95.8660.000		A	28	x	x	x	x	x	2.482		%
L-Methionine	23/03/2007	PR 95.8660.000		A	28	x	x	x	x	x	2.139		%
Standard Amino acids													
L-Aspartic acid	23/03/2007	PR 95.8660.000		A	28	x	x	x	x	x	10.548		%
L-Threonine	23/03/2007	PR 95.8660.000		A	28	x	x	x	x	x	4.672		%
L-Serine	23/03/2007	PR 95.8660.000		A	28	x	x	x	x	x	4.236		%
L-Glutamic acid	23/03/2007	PR 95.8660.000		A	28	x	x	x	x	x	16.501		%
L-Proline	23/03/2007	PR 95.8660.000		A	28	x	x	x	x	x	4.721		%
Glycine	23/03/2007	PR 95.8660.000		A	28	x	x	x	x	x	1.815		%
L-Alanine	23/03/2007	PR 95.8660.000		A	28	x	x	x	x	x	4.685		%
L-Valine	23/03/2007	PR 95.8660.000		A	28	x	x	x	x	x	5.172		%
L-Isoleucine	23/03/2007	PR 95.8660.000		A	28	x	x	x	x	x	5.226		%
L-Leucine	23/03/2007	PR 95.8660.000		A	28	x	x	x	x	x	11.953		%
L-Tyrosine	23/03/2007	PR 95.8660.000		A	28	x	x	x	x	x	3.647		%
L-Phenylalanine	23/03/2007	PR 95.8660.000		A	28	x	x	x	x	x	3.516		%
L-Lysine	23/03/2007	PR 95.8660.000		A	28	x	x	x	x	x	9.445		%
L-Histidine	23/03/2007	PR 95.8660.000		A	28	x	x	x	x	x	1.973		%
L-Arginine	23/03/2007	PR 95.8660.000		A	28	x	x	x	x	x	2.548		%

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General remark:

Deputy Head of Chemical Department: Aad Piekaar

Report date: 23/03/2007 15:00:55

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Intranet: http://intranet.eur.nestle.com/nl/nqac/

ISO/IEC 17025
TESTING
RvA L383

Nestlé Nutrition

ANALYTICAL REPORT

Information provided by requestor:

Sample number PR-400082127
Material PROLACTA 90
Material info WHEY PROTEIN ISOLATE
Material number
Batch code LOT 4
Batch date 20070128
Supplier BBA Lactalis
Order Number
Comments

Requestor: ZWRC

Christophe Schmitt
Nestec Ltd.
Nestlé Research Centre
Vers-chez-les-Blanc
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Fax: 00 41217858553
E-Mail To: Christophe.Schmitt@rdls.nestle.com
E-Mail cc:

Report Status: **Final Report**
SampleCondition: **Room Temperature**
Sample Type: **General Sample**
Date of receipt: **02/03/2007**
Report number: **ZWRC702565G1F**
NNQAC Number: **200702565**

Inspection	Report date	Method	WC	UD	Nestlé Norms			Legal Norms		Result	ON	Unit
					Low	High	Expiry	Low	High			
Standard Amino acids												
Total Nitrogen	23/03/2007	PR 95.8660.000	FL	28	x	x	x	x	x	14.62		%
Other Amino Acids (L-Trypthophan)												
L-Trypthophan	23/03/2007	PR 95.8660.000	A	28	x	x	x	x	x	1.980		%
Minerals analyses												
Calcium	23/03/2007	HI 00.1500.011		28	x	x	x	x	x	295		mg/100g
Magnesium	23/03/2007	HI 00.1500.011		28	x	x	x	x	x	55.5		mg/100g
Sodium	23/03/2007	HI 00.1500.011		28	x	x	x	x	x	100		mg/100g
Potassium	23/03/2007	HI 00.1500.011		28	x	x	x	x	x	408		mg/100g
Phosphorus	23/03/2007	HI 00.1500.011		28	x	x	x	x	x	192		mg/100g
Iron	23/03/2007	HI 00.1500.011		28	x	x	x	x	x	0.86		mg/100g
Copper	23/03/2007	HI 00.1500.011		28	x	x	x	x	x	0.09		mg/100g
Zinc	23/03/2007	HI 00.1500.011		28	x	x	x	x	x	0.71		mg/100g
Manganese	23/03/2007	HI 00.1500.011		28	x	x	x	x	x	6		µg/100g
Halogen analyses and others.												
Chloride	23/03/2007	HI 00.1620.300		28	x	x	x	x	x	16		mg/100g
General outsourced												
Fat content	23/03/2007	PR 95.8660.000	Y	28	x	x	x	x	x	< 0.1		%
Nitrite	23/03/2007	PR 95.8660.000	FL	28	x	x	x	x	x	0.40		mg/kg

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General remark:

Deputy Head of Chemical Department: Aad Piekaar
Pages: Page 2 of 5
Report date: 23/03/2007 15:00:56

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ISO/IEC 17025
TESTING
RvA L383

Nestlé Nutrition

ANALYTICAL REPORT

Information provided by requestor:

Sample number PR-400082127
Material PROLACTA 90
Material info WHEY PROTEIN ISOLATE
Material number
Batch code LOT 4
Batch date 20070128
Supplier BBA Lactalis
Order Number
Comments

Requestor: ZWRC

Christophe Schmitt
Nestec Ltd.
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Report Status: **Final Report**
SampleCondition: **Room Temperature**
Sample Type: **General Sample**
Date of receipt: **02/03/2007**
Report number: **ZWRC702565G1F**
NNQAC Number: **200702565**

Inspection	Report date	Method	WC	UD	Nestlé Norms			Legal Norms		Result	ON	Unit
					Low	High	Expiry	Low	High			
General outsourced												
Nitrate	23/03/2007	PR 95.8660.000	FL	28	x	x	x	x	x	2.5		mg/kg
General												
Ash	23/03/2007	HI 00.0350.000		28	x	x	x	x	x	1.9		%
Proteins	23/03/2007	PR 95.8660.000	FL	28	x	x	x	x	x	91.35		%
Fatty Acids												
C4:0 Butyric	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C6:0 Caproic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C8:0 Caprylic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C10:0 Capric	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C11:0 Triundecanoin	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C12:0 Lauric	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C14:0 Myristic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	0.018		g FA/100g
C14:1 Myristoleic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C15:0 Pentadecanoic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C15:1 Pentadecenoic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C16:0 Palmitic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	0.045		g FA/100g
C16:1 Palmitoleic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C17:0 Margaric	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g

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General remark:

Deputy Head of Chemical Department: Aad Piekaar

Report date: 23/03/2007 15:00:56

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ISO/IEC 17025
TESTING
RvA L383

Nestlé Nutrition

ANALYTICAL REPORT

Information provided by requestor:

Sample number PR-400082127
Material PROLACTA 90
Material info WHEY PROTEIN ISOLATE
Material number
Batch code LOT 4
Batch date 20070128
Supplier BBA Lactalis
Order Number
Comments

Requestor: ZWRC
Christophe Schmitt
Nestec Ltd.
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E-Mail cc:

Report Status: **Final Report**
SampleCondition: **Room Temperature**
Sample Type: **General Sample**
Date of receipt: **02/03/2007**
Report number: **ZWRC702565G1F**
NNQAC Number: **200702565**

Inspection	Report date	Method	WC	UD	Nestlé Norms			Legal Norms		Result	ON	Unit
					Low	High	Expiry	Low	High			
Fatty Acids												
C17:1 Heptadecenoic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C18:0 Stearic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	0.011		g FA/100g
C18:1 Oleic & other cis	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	0.030		g FA/100g
C18:1 Total trans	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C18:2 Linoleic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	0.012		g FA/100g
C18:2 Total trans	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C18:3 alpha-Linolenic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C18:3 gamma-Linolenic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C18:3 Total trans	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C20:0 Arachidic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C20:1 Eicosenoic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C20:2 Eicosadienoic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C20:3 Eicosatrienoic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C20:3 Eicosatrienoic (DHGLA)	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C20:4 Arachidonic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C20:5 Eicosapentanoic (EPA)	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C22:0 Behenic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C22:1 Erucic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g

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General remark:

Deputy Head of Chemical Department: Aad Piekaar

Report date: 23/03/2007 15:00:56

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ISO/IEC 17025
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Nestlé Nutrition

ANALYTICAL REPORT

Information provided by requestor:

Sample number PR-400082127
Material PROLACTA 90
Material info WHEY PROTEIN ISOLATE
Material number
Batch code LOT 4
Batch date 20070128
Supplier BBA Lactalis
Order Number
Comments

Requestor: ZWRC
Christophe Schmitt
Nestec Ltd.
Nestlé Research Centre
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E-Mail To: Christophe.Schmitt@rdls.nestle.com
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Report Status: **Final Report**
SampleCondition: **Room Temperature**
Sample Type: **General Sample**
Date of receipt: **02/03/2007**
Report number: **ZWRC702565G1F**
NNQAC Number: **200702565**

Inspection	Report date	Method	WC	UD	Nestlé Norms			Legal Norms		Result	ON	Unit
					Low	High	Expiry	Low	High			
Fatty Acids												
C22:2 Docosadienoic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C22:6 Docosahexaenoic (DHA)	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C24:0 Lignoceric	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
C24:1 Nervonic	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
Other Fatty Acids (OFA)	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	0.054		g FA/100g
Total trans fatty acids	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	< 0.010		g FA/100g
Total saturated fatty acids	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	0.10		g FA/100g
Total mono unsaturated fatty acid	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	0.03		g FA/100g
Total poly unsaturated fatty acid	23/03/2007	HI 00.0250.000		28	x	x	x	x	x	0.02		g FA/100g
Blocked Lysine												
L-Lysine Blocked	23/03/2007	HI 08.0820.000		28	x	x	x	x	x	1.09		%
L-Lysine Reactive	23/03/2007	HI 08.0820.000		28	x	x	x	x	x	10.70		g/16g N
Lysine as E-DFL	23/03/2007	HI 08.0820.000		28	x	x	x	x	x	0.12		g/16g N

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General remark:

Deputy Head of Chemical Department: Aad Piekaar
Pages: Page 5 of 5
Report date: 23/03/2007 15:00:56

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ANALYTICAL REPORT

Information provided by requestor:

Sample number INTERVENTION TREATMENT 2A
Material CLINICAL TRIAL NUTRITIONAL INTERVENTION
Material info HOMOGENOUS POWDER OF CHO, PRO & FAT
Material number
Batch code
Batch date 20080421
Supplier
Order Number 4520140798
Comments Analyze Leucine only

Requestor: ZWRC

Trent Stellingwerf
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Report Status: **Final Report**
SampleCondition: **Room Temperature**
Sample Type: **General Sample**
Date of receipt: **20/05/2008**
Report number: **ZWRC808914G1F**
NNQAC Number: **200808914**

Inspection	Report date	Method		WC	UD	Nestlé Norms			Legal Norms		Result	ON	Unit
						Low	High	Expiry	Low	High			
Standard Free Amino Acids													
Free L-Leucine	29/05/2008	PR 95.8660.000			7	x	x	x	x	x	5.652		%
General													
Proteins (Total Nitrogen x 6.25)	29/05/2008	PR 95.8660.000		FLN	7	x	x	x	x	x	20.04		%
Blocked Lysine													
Total Nitrogen	29/05/2008	PR 95.8660.000		FLN	7	x	x	x	x	x	3.21		%

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- ON means Result out of Nestlé or Legal norm.
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General remark:

Deputy Head of Chemical Department: Aad Piekaar

Report date: 29/05/2008 15:29:09

Nestlé Nutrition QA Centre Nunspeet

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Intranet: http://intranet.eur.nestle.com/nl/nqac/

ANALYTICAL REPORT

Information provided by requestor:

Sample number Intervention treatment 2B
Material CLINICAL TRIAL NUTRIONAL INTERVENTION SA
Material info HOMOGENOUS POWDER OF CHO, PRO & FAT
Material number
Batch code
Batch date 20080421
Supplier
Order Number 4520140798
Comments Analyze Leucine only

Requestor: ZWRC

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Report Status: **Final Report**
SampleCondition: **Room Temperature**
Sample Type: **General Sample**
Date of receipt: **20/05/2008**
Report number: **ZWRC808915G1F**
NNQAC Number: **200808915**

Inspection	Report date	Method		WC	UD	Nestlé Norms			Legal Norms		Result	O N	Unit
						Low	High	Expiry	Low	High			
Standard Free Amino Acids													
Free L-Leucine	29/05/2008	PR 95.8660.000			7	x	x	x	x	x	5.368		%
General													
Proteins (Total Nitrogen x 6.25)	29/05/2008	PR 95.8660.000		FLN	7	x	x	x	x	x	21.11		%
Blocked Lysine													
Total Nitrogen	29/05/2008	PR 95.8660.000		FLN	7	x	x	x	x	x	5.50		%

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General remark:

Deputy Head of Chemical Department: Aad Piekaar
Pages: Page 1 of 1
Report date: 29/05/2008 15:29:11

Nestlé Nutrition QA Centre Nunspeet

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E.Mail Head Chemical Laboratory: Ton.Noorloos@nl.nestle.com
Intranet: http://intranet.eur.nestle.com/nl/nqac/

ANALYTICAL REPORT

Information provided by requestor:

Sample number Control Treatment 3
Material CLINICAL TRAIL NUTRITIONAL INTERVENTION
Material info HOMOGENOUS POWDER OF CHO, PRO & FAT
Material number
Batch code
Batch date 20080421
Supplier
Order Number 4520140798
Comments Analyze Leucine Only

Requestor: ZWRC

Trent Stellingwerf
Nestec Ltd.
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Report Status: **Final Report**
SampleCondition: **Room Temperature**
Sample Type: **General Sample**
Date of receipt: **20/05/2008**
Report number: **ZWRC808916G1F**
NNQAC Number: **200808916**

Inspection	Report date	Method		WC	UD	Nestlé Norms			Legal Norms		Result	ON	Unit
						Low	High	Expiry	Low	High			
Standard Free Amino Acids													
Free L-Leucine	29/05/2008	PR 95.8660.000			7	x	x	x	x	x	0.687		%
General													
Proteins (Total Nitrogen x 6.25)	29/05/2008	PR 95.8660.000		FLN	7	x	x	x	x	x	0.65		%
Blocked Lysine													
Total Nitrogen	29/05/2008	PR 95.8660.000		FLN	7	x	x	x	x	x	0.10		%

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- UD means Urgency/Days. Priority level
- ON means Result out of Nestlé or Legal norm.
- If x sign is shown in the norm fields, then no norms were provided from the Requestor.

General remark:

Deputy Head of Chemical Department: Aad Piekaar
Pages: Page 1 of 1
Report date: 29/05/2008 15:29:12

APPENDIX F NEUTROPHIL OXIDATIVE BURST ASSAY

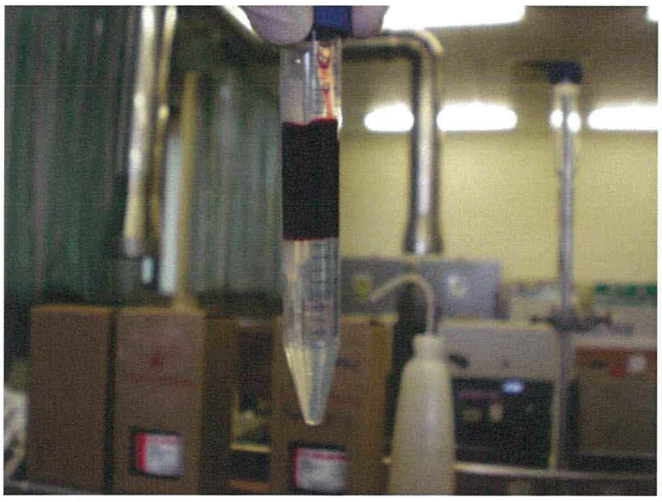
Methods & Materials

1.0 **Blood Collection** – at least 10ml of whole blood per sample is required

- collected in Lithium Heparin 10ml vaccutainers
- all samples tested in duplicate

1.1 **Neutrophil Separation** – place 3ml of Histopaque 1119 in 15ml tube
- carefully layer 3ml of Histopaque 1077 on top
- layer 5ml of whole blood over the 2 density gradients

- density gradients to be at room temperature
- DO NOT MIX the layers
- Histopaque 1119 & 1077 in cold room

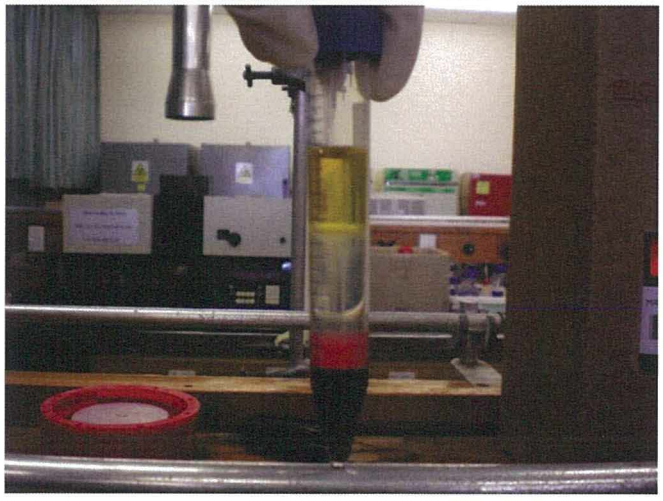


- spin tubes for 30mins at 1000 x g at 18°c with brake turned off.

- adjust centrifuge to 4°c , 300 x g, 10 mins

- carefully aspirate the layer of neutrophils and transfer into another sterile labelled 15ml tube

- Eppendorf Centrifuge 5810R,

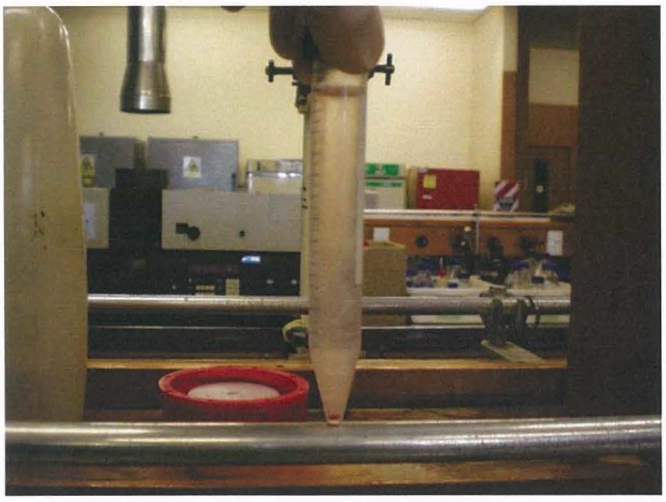


- Neutrophils are just above red blood cells
- avoid aspirating clumps or red blood cells.

- **Hypotonic lysis** – add 5ml of chilled distilled water, mix gently for at least 30 seconds
-add 5ml of Phosphate Buffered Saline (PBS) to restore

isotonicity, mix gently

- Centrifuge at 300 x g for 10mins at 4°c with brake turned off.



- to remove erythrocytes
- distilled water and PBS found in fridge

- Carefully aspirate of supernatant without disturbing pellet.

- will deposit neutrophils on bottom with red cells in suspension
- *adjust centrifuge to 0°c, 300 x g, 5mins*

- Resuspend neutrophils in PBS with glucose (2-3ml) and store on ice

- Gently mix to obtain uniform suspension.
- take at least 100 µL of suspension and run through haematology analyser to obtain percentage neutrophil purity.

- Glucose used to keep neutrophils alive.

- need to obtain neutrophil concentration of ≥95%
- yields 1.0 x 10^6 cells

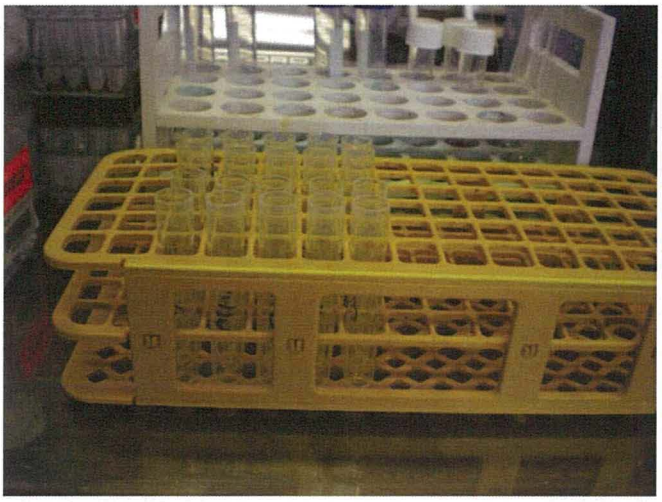
-Superoxide anion, cytochrome-C reduction assay.

- 5 labelled tubes

Control – 2ml Hanks Balance Salt Solution+200µL cytochrome-C
Tube A - Cells + Phorbol Myristate Acetate (PMA) (no Superoxide Dismutase (SOD))
Tube B - Cells + Dimethyl sulphoxide DMSO (no SOD)
Tube C - Cells + PMA + SOD
Tube D - Cells + DMSO +SOD

-use microfuge tubes (2ml)

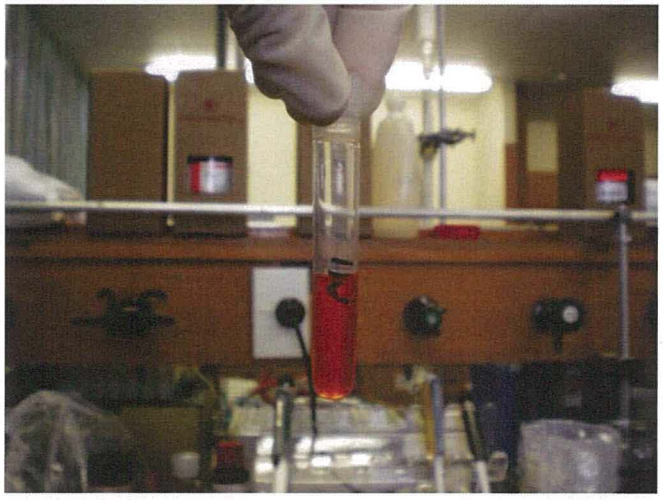
- HBSS in bottom cupboard at room temp
- Cytochrome-c, PMA and SOD in -



20°c freezer

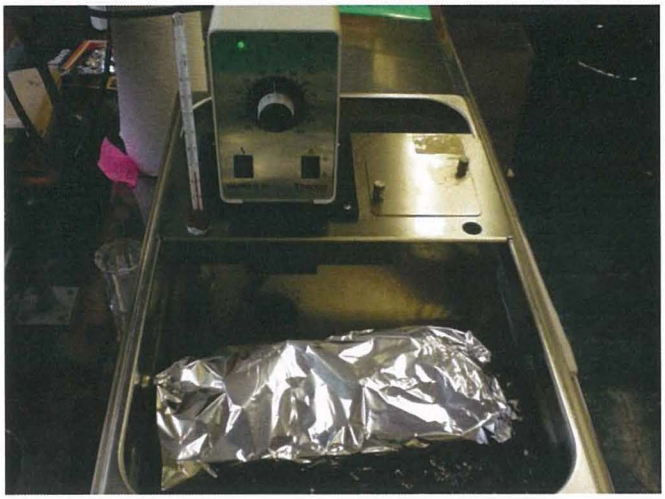
- Turn on spectrophotometer switch at back

- Use 1ml of prepared neutrophil suspension, add 1ml HBSS
- Add 200µL of cytochrome- to all tubes
- Add 200µL of DMSO to tubes B and D
- Pre-incubate neutrophil suspension with HBSS and cytochrome-C for 5mins at 37°c.



- Add 20µL of SOD to tubes C and D and 20 µL of HBSS to A and B and incubate all tubes for 5mins at 37°c.
-Add 200µL of PMA to tubes A and C and incubate for 20mins in gentle warm shaker bath at 37°c (keep covered)

-PMA is light sensitive and needs to be kept covered
-Cover tubes with tin foil while incubating.



- Place all tubes on ice to reduce any further reactions. Keep covered in aluminium foil to prevent any light degradation of PMA

- Spin all tubes at 300 x g in 0°c for 5mins with brake turned off.
-Place all tubes on ice to reduce any further reactions.

- Spectrophotometer
- aspirate at least 1ml of supernatant into quartz cuvet.

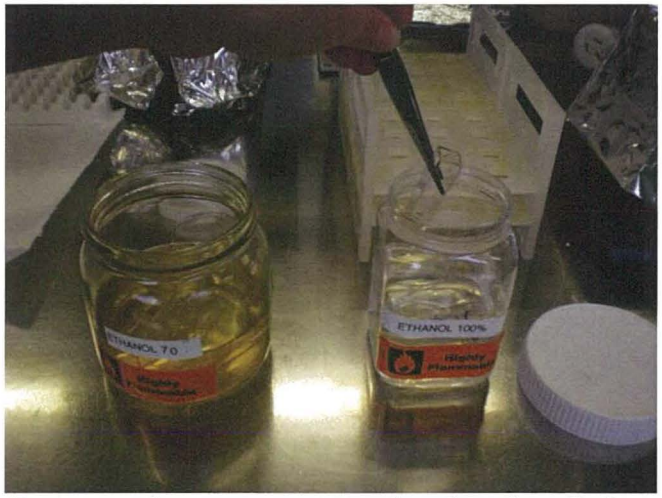
- use distilled water for blank cuvet to 'zero' machine
-allow tubes to stand for 10mins to equilibrate to room temperature (keep covered)

-Read using the control
-Zero using the distilled water and then place in sample A, take reading

-Quantification of superoxide anion determined by difference in absorbance between tubes with and without SOD based on molar extinction coefficient of ferricytochrome-C

-Zero using the distilled water and then place in sample B, take reading
-Zero using the distilled water and then place in sample C, take reading
-Zero using the distilled water and then place in sample D, take reading
-Repeat for all samples to obtain duplicate readings

Cleaning the Cuvets:
-Pour sample into containment jar for sterilisation later
-Place cuvet into lidded jar of 70% ethanol for 10 minutes to sterilise
-Remove and place cuvet into lidded jar of 95% ethanol for 5 minutes to clean



-Do not clean inside the cuvet with paper towel to avoid fibre or dust settling on the inside.

-Remove, place on paper towel on bench to drain and remove excess ethanol from outside of cuvet for 10 minutes

EQUATION TO DETERMINE SUPEROXIDE ANION PRODUCTION PER CELL

$(dE/ Q \times d) \times 10^6 \times 1/L$

Where L = 1×10^6 cells per mL
dE = (absorbance of samples without SOD) - (absorbance of samples with SOD)

Q = coefficient of molar extinction $2.1 \times 10^4\ M^{-1}.\ cm^{-1}$

D = thickness of cuvette == 1 cm

Eg

$$\frac{(A550 \text{ no SOD} - A550 \text{ SOD})}{2.1 \times 10^4\ M^{-1}.cm^{-1} \times 1} \times \frac{10^6 \times 1}{1 \times 10^6 \text{ cells.mL}}$$

DILUTIONS FOR WORKING SOLUTIONS

1) Cytochrome-C: store at -20C: the protein comes as a powder and must be mixed with distilled water. It comes in a 100mg container and should be diluted with 16.1mL of distilled water. Add the water directly to the container of protein powder, agitate till well mixed. This will give 6.2 mg per 1 mL of solution. Aliquot the solution into 5 mL sterile test tubes so that each TT contains 4 mL of working solution. Freeze at -20C.

2) SOD. : working solution of 2 mg/mL HBSS. Is a clear whitish powder. Comes as 20 mg.

- Add 1mL HBSS to powder container and agitate till well mixed. Pipette into eppendorfs. This dilution gives 20 mg in 1 mL. We need 2mg/mL.
- Take 100 µL of the above and add 900 µL HBSS. This gives the working dilution of 2mg/mL; put into 10 eppendorfs and store at -20C.

3) PMA: comes in 1 mg container. 1mg (1000µg) = 1,000,000ng in 1mL of DMSO.

- Add 1mL of DMSO to 1mg powder. This gives 1000 µg in 1000 µL
- Dilute the above to a stock solution by taking 100 µg µL (100µg) and adding 900 µl DMSO in separate eppendorfs. Gives 10 eppendorfs. Stock solution containing 100 µg per mL.
- Working solution: take 10 µL of the above (10 µg) solution and add 990 of DMSO. This actually gives 10,000 **ng PMA** per mL. As we need 50 ng per test tube, we take 200 µL of the working solution to add to each TT.

4) PBS : 1 sachet goes into 1 litre distilled autoclaved water.

To make 5 mM glucose in PBS, add 0.9g glucose to 1 litre of sterile PBS

Note: 180g glucose = 1 Mole glucose. 180g GLU in 1 litre PBS = 1 molar solution.

We need 5mM GLU in PBS therefore divide 180g by 1000 to give 0.18g (1 mMol).
Multiply by 5 to give 0.9g GLU which is put into 1 litre PBS solution.

Materials

- Histopaque 1077 – Lot number – 086K6118, Sigma-Aldrich Co., St Louis, USA
- Histopaque 1119 – Lot number 126K6002, Sigma-Aldrich Co., St Louis, USA
- Phosphate Buffered Saline – Lot number 056K8213, Sigma-Aldrich Co., St Louis, USA
- Hanks Balance Salt Solution – Lot number - 107K2340, Sigma-Aldrich Co., St Louis, USA
- Dimethyl sulphoxide – Lot number – 038K0742, Sigma-Aldrich Co., St Louis, USA
- Superoxide Dismutase, bovine – Lot number – 117K7690, Sigma-Aldrich Co., St Louis, USA
- 4□-Phorbol 12-Myristate 13-Acetate – Lot number – 017K1573, Sigma-Aldrich Co., St Louis, USA
- Cytochrome- C – Lot number – 077K7001, Sigma-Aldrich Co., St Louis, USA
- Centrifuge - Eppendorf Centrifuge 5810R, Global Science, Hamburg, Germany
- Spectrophotometer – Spectronic 2000, Spectronic, New York
- Haematology Analyser - Beckman Coulter Haematology Analyser, Fullerton, USA

Abbreviations

PBS - Phosphate Buffered Saline

HBSS – Hanks Balance Salt Solution

DMSO - Dimethyl Sulphoxide

SOD - Superoxide Dismutase

PMA – 4□-Phorbol 12-Myristate 13-Acetate

APPENDIX G SWEAT COLLECTION METHOD

SWEAT COLLECTION EQUIPMENT

Set up the following aseptically (use gloves so you don't transfer nitrogen and ammonia from the sweat on your finger tips) in Milton cleaned plastic containers.

- Sterile Gauze Pads (7.5cm x 7.5cm) x 2
- Forceps x 2
- Opsite patches (10.0 x 12.0 cm) x 2
- Sterile Parafilm (7.5cm x 7.5cm) x 2
- Sterile Centrifuge Tubes (50mL) x 2
- Disposable razors (for shaving the appropriate collection areas)
- Clippers (if needed)
- Distilled Water x 2
- Disposable surgical gloves
- Alcohol wipes x 2
- Soapy water for shaving
- Waterproof/cryogenic markers to label syringes and freezer tubes (15mL centrifuge tubes)
- Freezer tubes
- Accurate Bathroom Scales
- Accurate analytical Bench Scales

REGIONAL SWEAT COLLECTION METHOD

Prior to subject arrival, tare the following items; gauze, centrifuge container with lid. On arrival at the laboratory ask subjects to urinate. While subjects are changing into exercise clothing, ask them to take nude bodyweight. Using the following anthropometric markers; **Abdominal:** Take measure of the narrowest point between the lower costal (rib) border and iliac crest. Place patch on subject's right side, not overlapping the umbilicus. **Chest at Mesoternale:** Located at the midpoint of the sternum at the level of the centre of the articulation of the 4^{th} rib with the sternum (choldrosternal articulation). Palpate from the top of the right clavicle, feeling the intercostals spaces, count 5 spaces (one for clavicale to first rib, 1^{st} & 2^{nd} ribs, etc). Place bottom of patch at the mesoternale, not overlapping nipple.

Disinfect the marked area with alcohol wipes to avoid possible micro-organism activity, and using soapy water shave the area. Thoroughly rinse with distilled water, and dry with a clean sterile gauze swab. Use gloves and forceps to transfer parafilm to the adhesive side of the Opsite, then gauze, and handle Opsite on the outside surface only (with gloves and forceps) affix to the body. After the ride remove and transfer patches using forceps to the tared centrifuge tubes and weigh (with lid on to prevent evaporation), then an accurately weighed amount of distilled water is added for an approximate 2 x dilution (accurately weighed) in

each of the tubes containing the sweat patches. Meanwhile the subjects are asked to towel dry and take nude body weight.

Refrigerate patches overnight (max 3 days), then centrifuge for 10-20 min at 1700g and 4 °C. Pipette representative samples into spare 50mL centrifuge tube and mix well, then pipette into 15mL centrifuge tubes (leave a 1-2cm gap) in duplicate, and store at -80°C until biochemical analysis can be done.

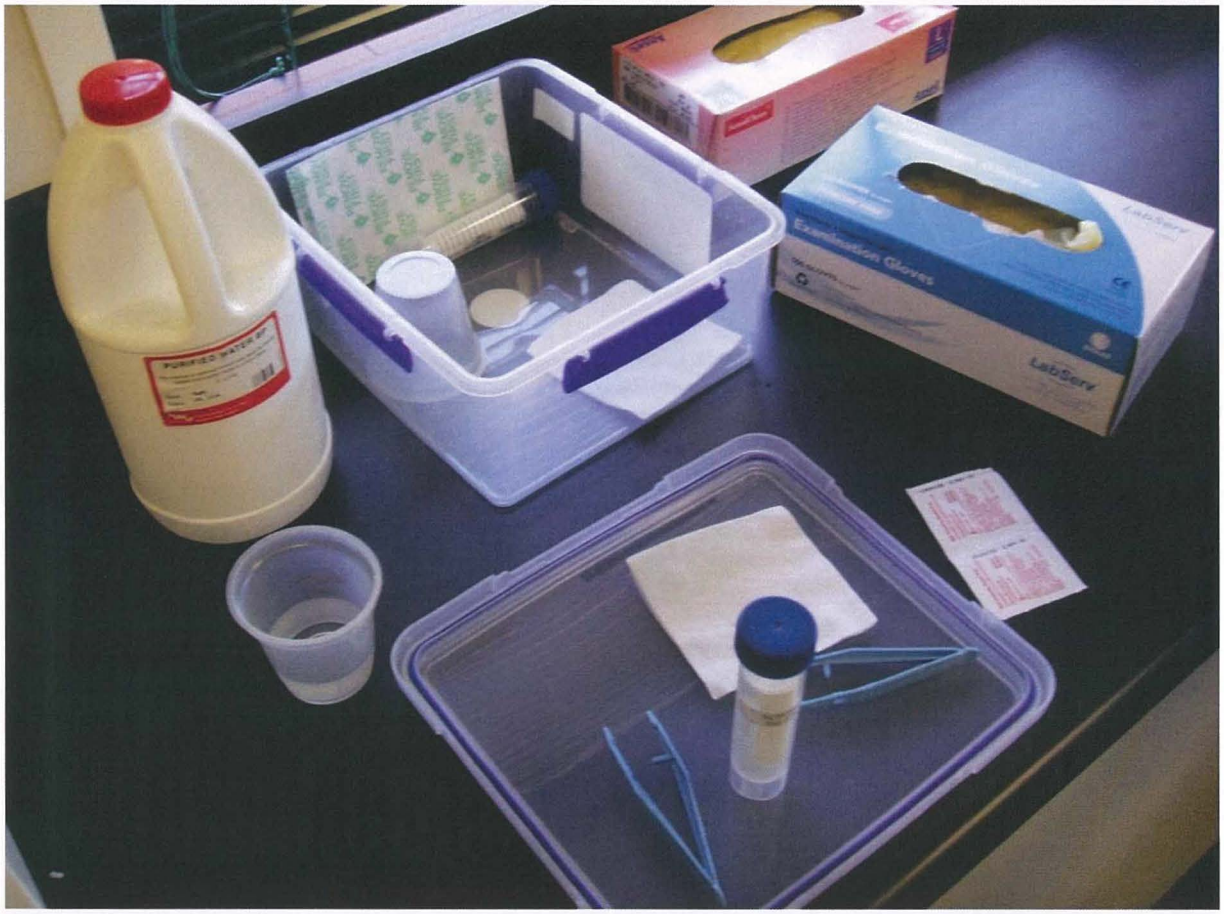
Whole-body sweat loss will be determined by the change in body mass, after correction for fluid intake, and the small changes in mass due to respiratory and metabolic water loss will be ignored.

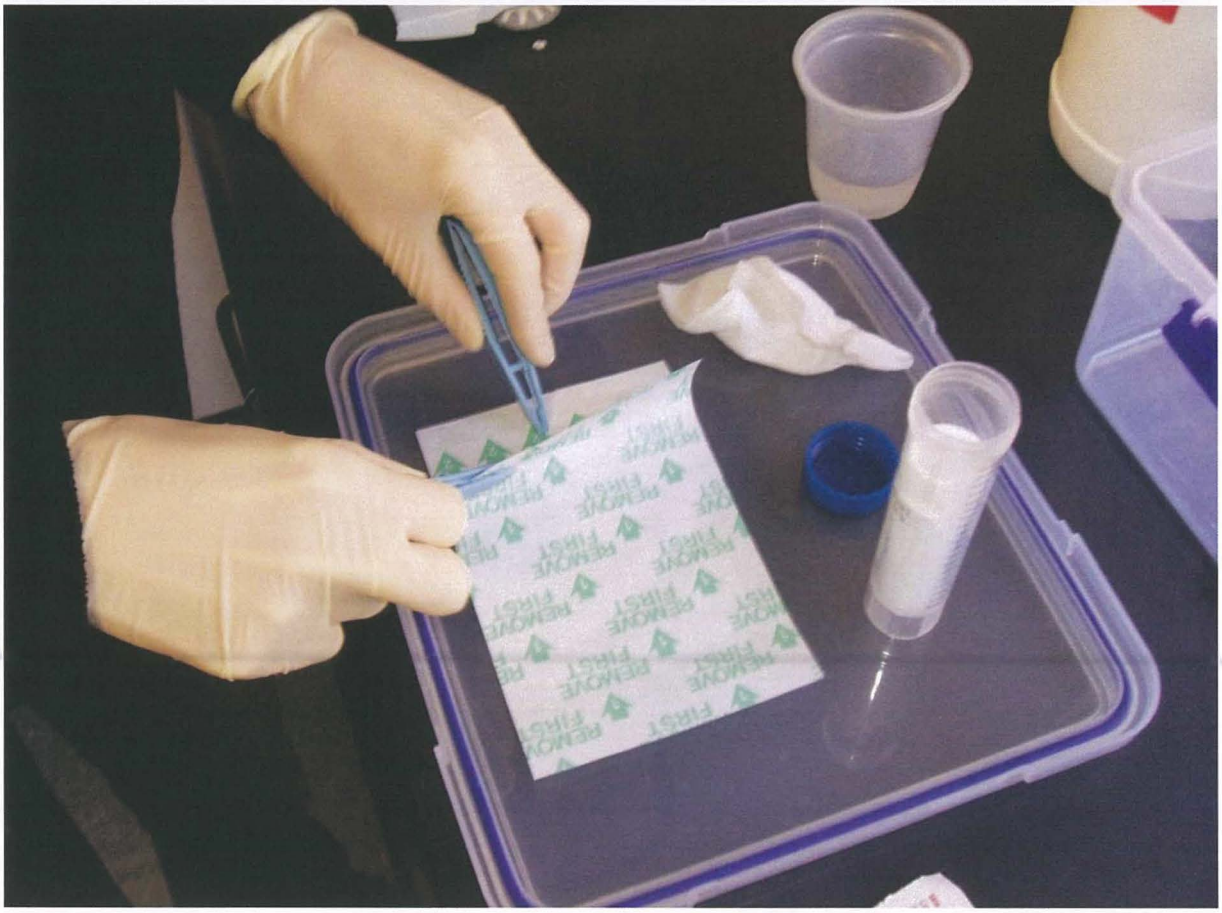
SUBJECT ID _____ DAY _____ DATE _____ BLOCK/BEVERAGE _____

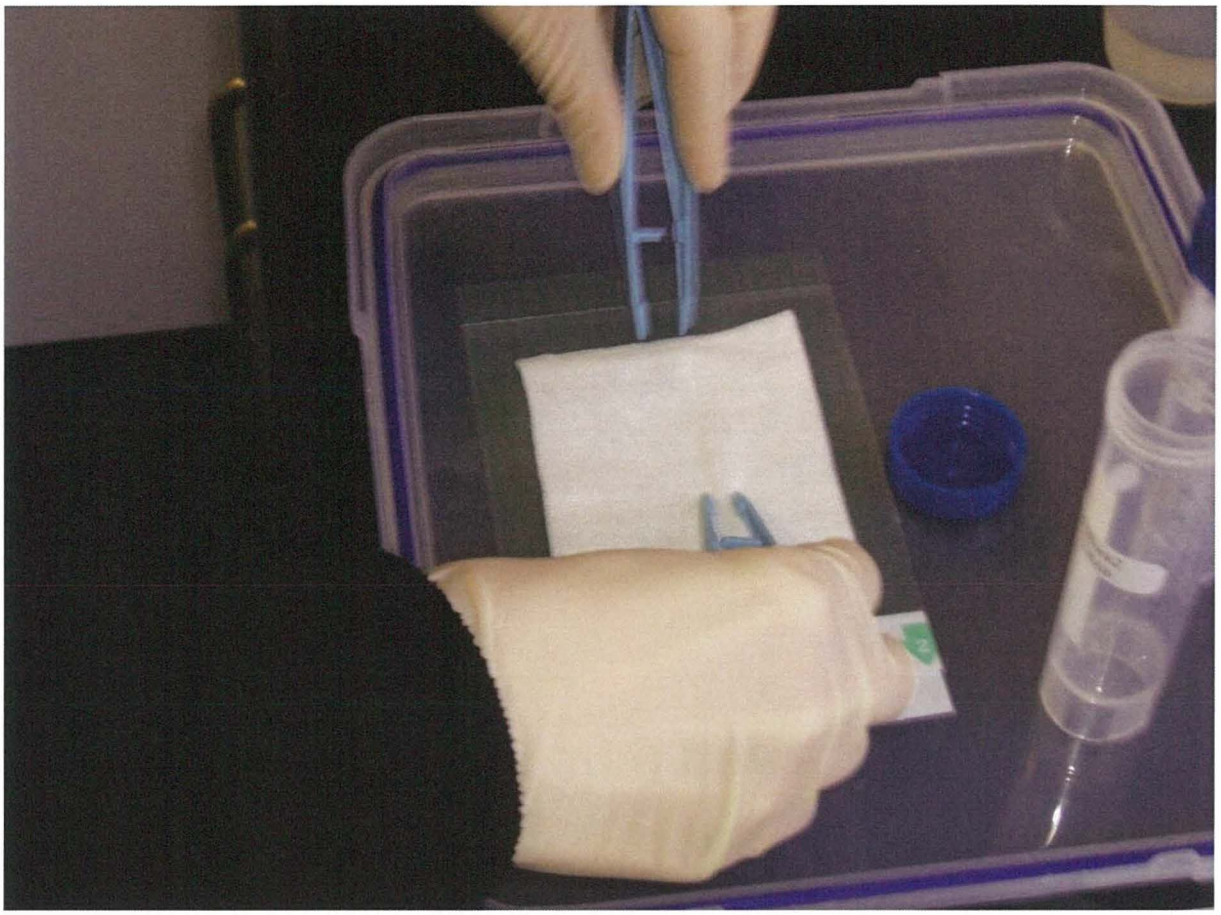
SWEAT COLLECTIONS		Pre Ride	Post Ride	Change	
Time					
Temperature (°C)					
Barometric Pressure (mmHg) *InHg*25.4*					
Humidity (%)					
Nude BW (kg)					
Sports Drink (g)					
Collection Weight (g)	Site	Pre Ride	Post Ride	Sweat Wt (g)	Added Dist H_2O (g)
Centrifuge Tube with lid, Gauze, & Sweat	Abdominal				
	Chest				

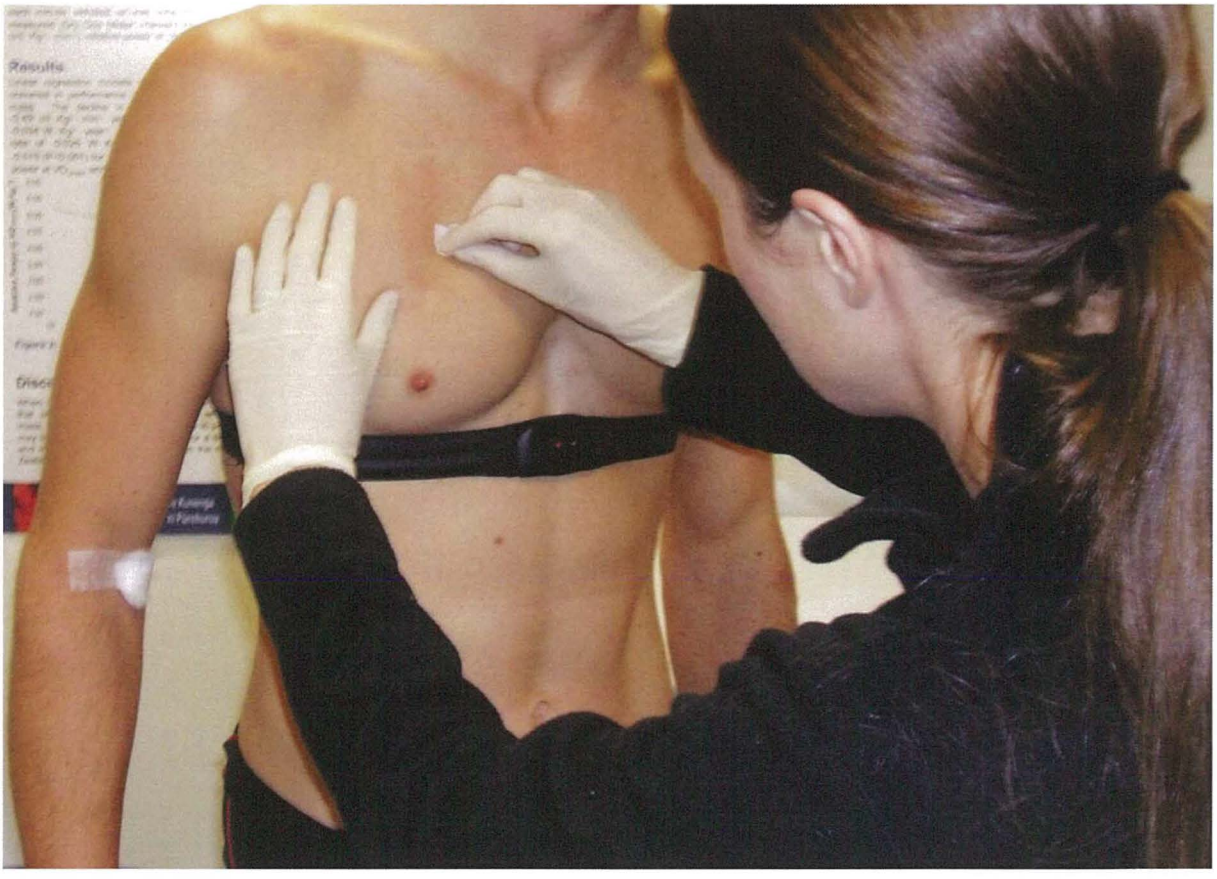
SUBJECT ID _____ DAY _____ DATE _____ BLOCK/BEVERAGE _____

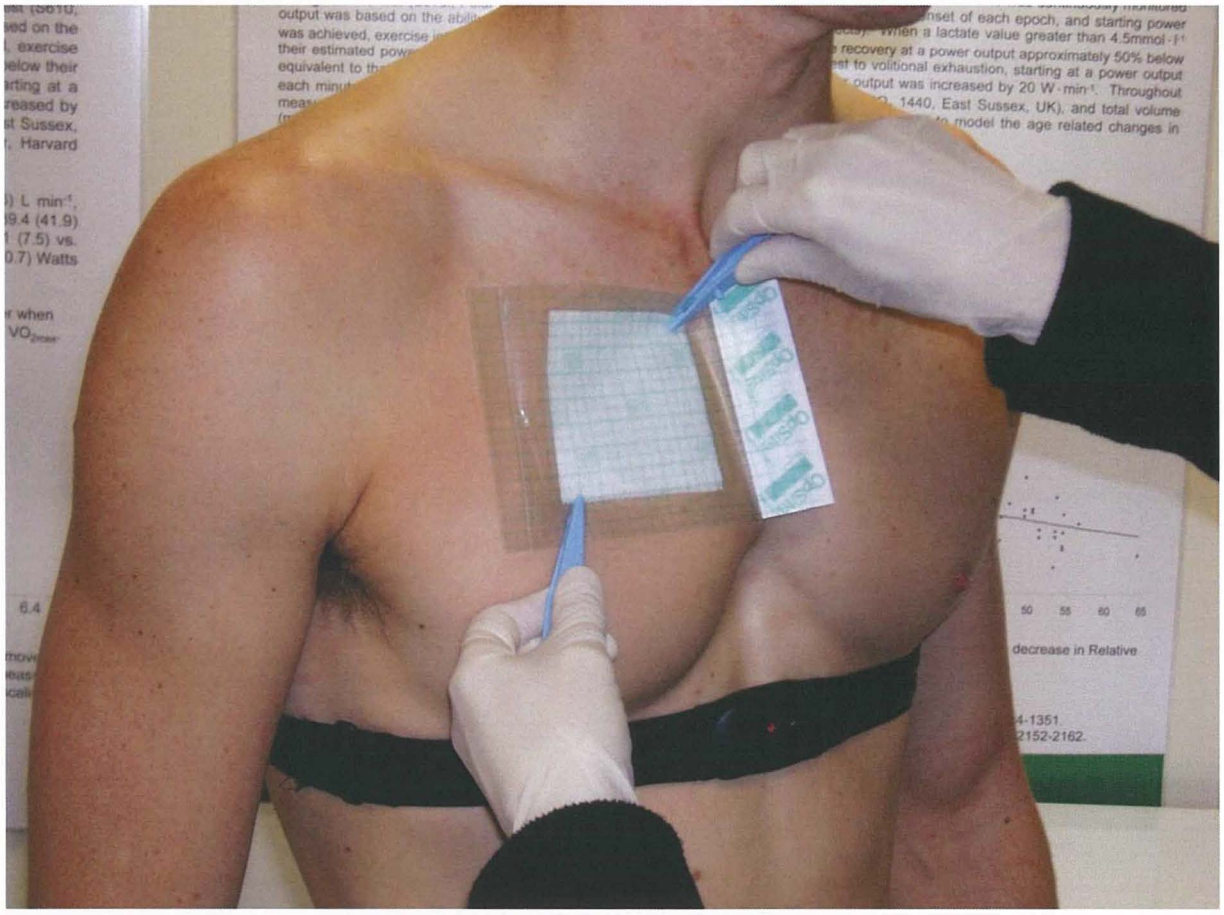
SWEAT COLLECTIONS		Pre Ride	Post Ride	Change	
Time					
Temperature (°C)					
Barometric Pressure (mmHg) *InHg*25.4*					
Humidity (%)					
Nude BW (kg)					
Sports Drink (g)					
Collection Weight (g)	Site	Pre Ride	Post Ride	Sweat Wt (g)	Added Dist H_2O (g)
Centrifuge Tube with lid, Gauze, & Sweat	Abdominal				
	Chest				

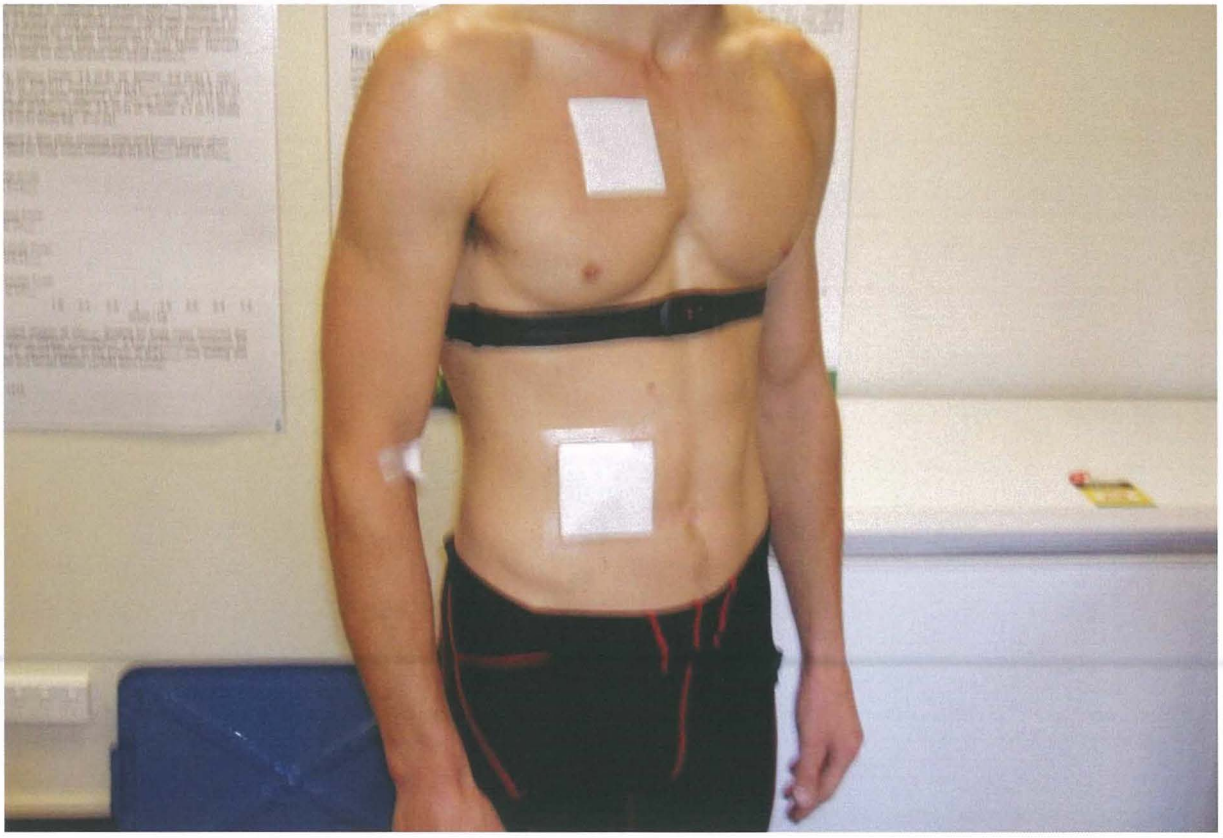

Examination Gloves
LabServ



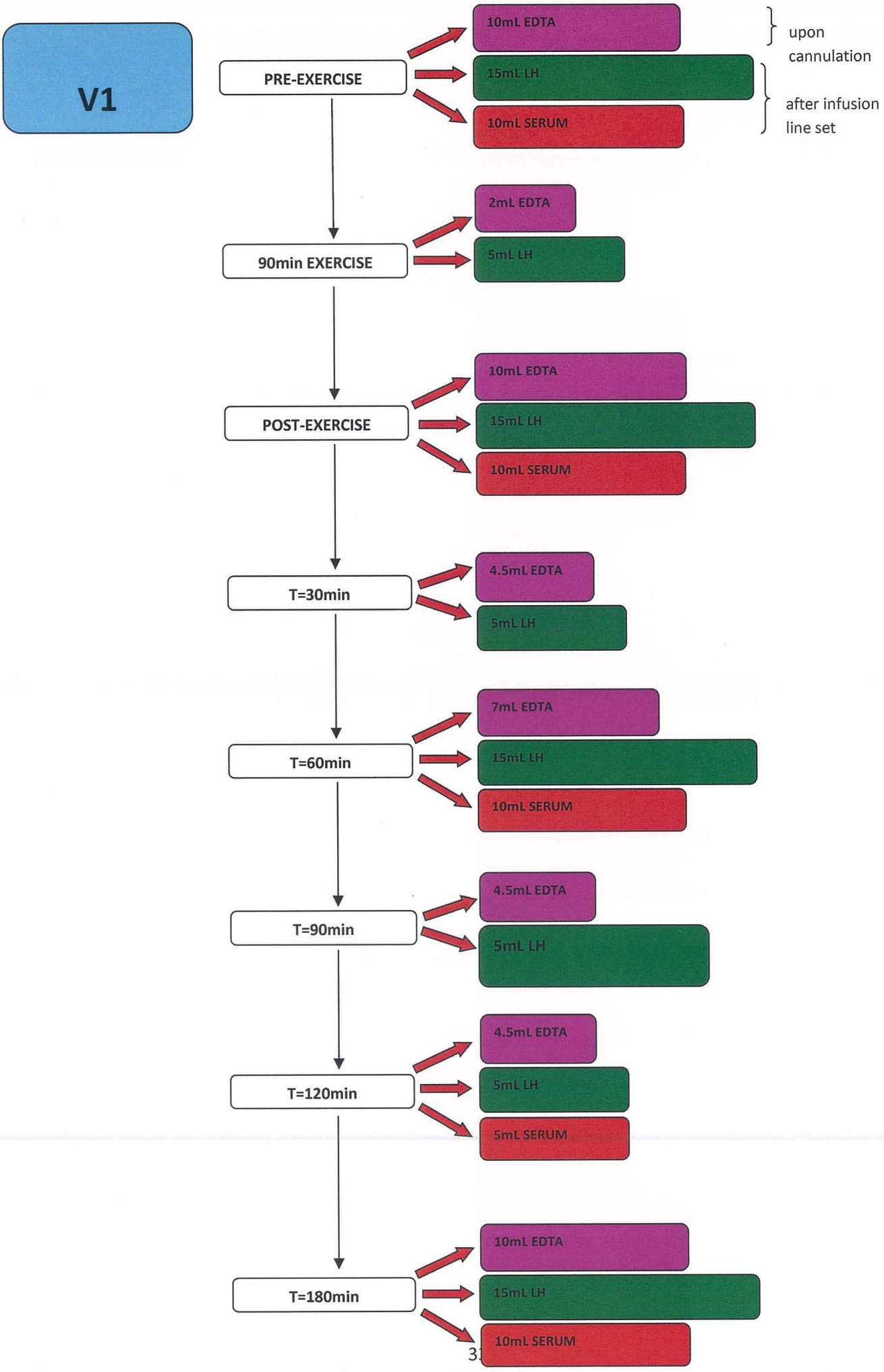


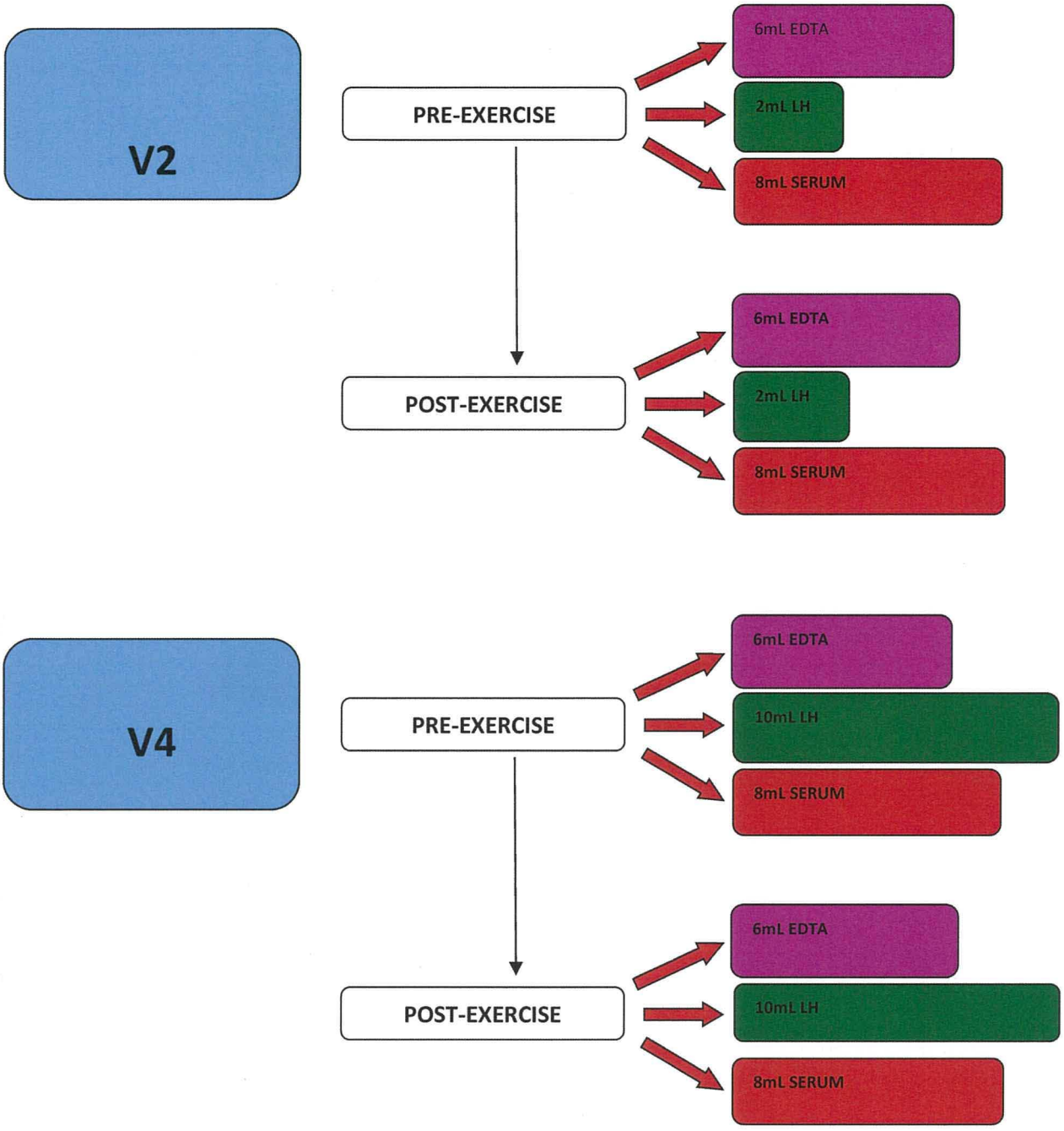

Results





APPENDIX H BLOOD SAMPLING SCHEMA


V1
PRE-EXERCISE
10mL EDTA
15mL LH
10mL SERUM
upon cannulation
after infusion line set
90min EXERCISE
2mL EDTA
5mL LH
POST-EXERCISE
10mL EDTA
15mL LH
10mL SERUM
T=30min
4.5mL EDTA
5mL LH
T=60min
7mL EDTA
15mL LH
10mL SERUM
T=90min
4.5mL EDTA
5mL LH
T=120min
4.5mL EDTA
5mL LH
5mL SERUM
T=180min
10mL EDTA
15mL LH
10mL SERUM


V2
PRE-EXERCISE
6mL EDTA
2mL LH
8mL SERUM
POST-EXERCISE
6mL EDTA
2mL LH
8mL SERUM
V4
PRE-EXERCISE
6mL EDTA
10mL LH
8mL SERUM
POST-EXERCISE
6mL EDTA
10mL LH
8mL SERUM


V6
-90min PRE-EXERCISE
2mL EDTA
5mL LH
PRE-EXERCISE
20mL EDTA
5mL LH
8mL SERUM
60min EXERCISE
2mL EDTA
5mL LH
75min EXERCISE
2mL EDTA
5mL LH
90min EXERCISE
22mL EDTA
5mL LH
POST-EXERCISE
18mL EDTA
2mL SERUM

APPENDIX I STUDY 2 INFORMED CONSENT

How does the dose of protein and leucine ingested with carbohydrate after exercise effect the processes governing adaptation to high-intensity training?

INFORMATION SHEET

The Institute of Food, Nutrition and Human Health

The Institute of Food, Nutrition and Human Health is part of Massey University. It includes a large team of scientists that are interested in human nutrition, exercise science, physiology, and health. The project coordinator is Dr David Rowlands who is Senior Lecturer in Sport and Exercise Science. Other researchers working on the project are Mr Andre Nelson (PhD student), and Mr Andy Hollings, who provides technical support, and Dr Murray Leikis, Dr Mark Fulcher and Mr Sarah Beable who are involved with the muscle biospies. In addition, biochemists at the Nestle Research Centre, Lausanne, Switzerland and at McMaster University, Ontario, Canada will analyse some of the samples.

Why Are We Doing This Study?

The ability to maintain performance on a daily basis is highly relevant to athletes undertaking intense training and for those in multi-day tournaments. In recent research we observed that a protein-enriched recovery supplement can enhance performance several days after ingestion. This delayed effect possibly relates to the time-course for muscle repair, enhanced protein synthesis, and possibly to superior glucose homeostasis suggesting glycogen sparing. We found evidence for attenuated muscle damage or faster repair, and reduced general tiredness (implicating a possible link to the brain), but as yet the mechanism for the performance effect remains to be established. From research in rats and isolated muscle cell models, it has been established that the amino acid leucine is the key amino acid responsible for anabolic processes (tissue building) in the cells. So in addition to faster recovery from heavy exercise, we now suspect that protein and leucine ingested following exercise may accentuate the normal adaptive processes to training.

Consequently, we designed a recovery supplement formulation termed LeuPlus containing a blend of protein isolates, leucine, carbohydrates and fats that we think will enhance the rate of recovery from prolonged high-intensity exercise and therefore improve subsequent performance. Provisional results from the first study, suggests that LeuPlus can enhance immune system function, reduce feelings of tiredness and fatigue, and enhance recovery of subsequent performance. In the muscle, we do not know the mechanisms of action or the effect of dose. The purpose of this experiment is to see determine the cellular mechanism of action and the effect of protein and leucine dose on protein synthesis rates after exercise. This study is funded by Powerbar Performance, a division of Nestec, Switzerland.

In Scientific Terms

Research Question

What effect does the dose of protein and leucine co-ingested with carbohydrate and fat following high-intensity exercise have on fractional protein synthesis and the pattern of gene expression?

Participant Recruitment

We would like to recruit twelve male cyclists or fitness enthusiasts. To participate in the study you should:

- be male and aged 18 to 50 years
- be in regular training – 4 or more hours of cycling per week for the last 4-6 months or greater.
- VO_2max of at least 50 ml·(kg min)$^{-1}$
- Have no food allergies
- Have not participated in a similar trial within the last 2 months prior to starting this trial – discuss with us if you have regarding suitable start dates.

What is Involved

If you decide to participant in our study, you will make one visit to the laboratory several weeks before the experiment to establish baseline metabolic and fitness parameters, to discuss any questions you may have with the researchers, and to complete forms. This will be followed by three experimental periods interspersed with by a 2 week washout period between visits. In the lab, you will conduct a 100 min interval cycle ride, followed by ingestion of the supplement and a 4 h recovery period. The study design is termed a *single-blind randomized cross-over*. This means that you will be randomly presented with the recovery conditions and you will not know what recovery supplement condition you are on.

Experimental Conditions

During recovery from the cycle ride you will ingest 4 servings every 30 min of a milk-like recovery supplement or control containing either:

1) Low protein + free leucine:

- Beverage composition per serve (every 30 min over 90 min): whole protein (5.75 g), leucine (1.875 g), carbohydrate (22.5 g), fat (3.75 g) energy ~637 kJ/154 kcal.

2) High protein + free leucine

- Beverage composition per serve: whole protein (11.5 g), free leucine (3.75 g), carbohydrate (45 g), fat (7.5 g), energy 1260 kJ/302 kcal.

3) Control

- Per serve: carbohydrate (61.9 g), fat (7.5 g); 1260 kJ/302 kcal.

What will happen in the week prior to and on each day of the three experimental blocks is summarised below and in Figures 1 and 2.

STUDY PLAN/FLOW CHART

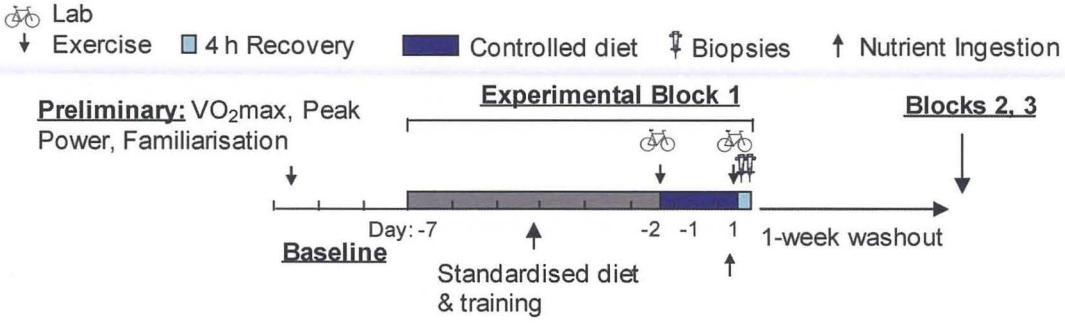


Figure 1. Summary of the experimental design.

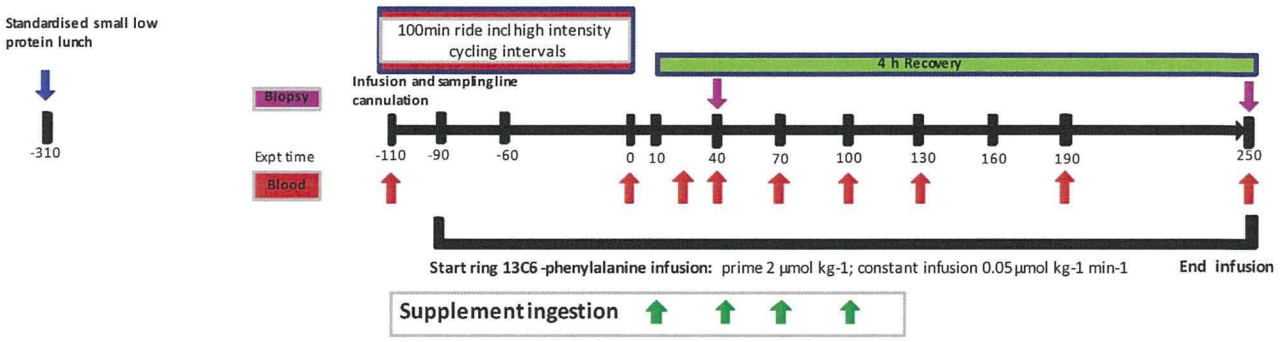


Figure 2. Summary of experimental procedures for the three arms of the triple cross over.

Visit days -7 to -1 (standardised exercise and diet)

- From 6 days prior to each of the three laboratory visits, you will undertake a standardised training regime (record and repeat your own training). In addition, we will provide you with a standardised diet from the day before each of the three experimental days.

Experimental Day

- We will ask you to report to lab at around 3PM. A Teflon cannulae will be placed into both forearm veins and baseline blood samples will be collected.
- Next you will complete 100 min of intermittent-intensity interval cycling. During the ride we will start the infusion of tracer (L-[ring-$^{13}C_6$] phenylalanine) to measure muscle protein synthesis. Upon completion of exercise, we will collect another blood and also a breath sample. We will then ask you to ingest the first serve of recovery supplement, followed by a shower if you desire.
- Blood samples will be collected, and a serving of supplement provided every 30 min until 90 min post exercise.
- We will collect a small sample of muscle from your mid thigh at 30 min and 240 min.
- Shortly after you will be provided with a standardised light meal, then home for sleep.

Standardisation of Training

In preparation for the study we would like you to *standardize your training program* from the week before the first weekly experimental block. What you have to do is simply maintain the same training sessions in terms of time and degree of effort on Days 1-3 and 7 of each proceeding week. We will discuss with you suitable sessions. A training diary will be provided to help you and two sessions will be done in the lab. Training standardisation is to normalise the condition of your muscles before starting the experiment.

Standardisation and Replication of Diet

During the day preceding and on the morning of the first day of the experiment we will provide you with a standardised diet; like the training, this is to standardise the pattern of fuel substrate in your muscles before the experiment. In addition, during the week preceding the first experimental block, we will ask you to record you diet and to replicate the type and amount of food ingested on the same experimental days leading up to the experimental day on blocks 2 and 3. We will provide information and diet record sheets for you to achieve this.

Are any of the Procedures Harmful or Painful?

Blood Sampling

Cannula are small plastic tubes that are routinely placed into veins of participants in clinical research studies and in hospital patients. There is likely to be mild to moderate discomfort and small risk of bruising associated with the removal of a cannula. The risk of infection is 1 in 20,000. There is a rare risk of developing a venous thrombosis (clot), which could lead to a pulmonary embolism or stroke; this risk is elevated in some individuals with hereditary predisposition. Cannula will be placed 3 times during the study. The researchers are trained in cannula placement and the taking of blood samples via cannula. Approximately 100 ml of blood will be collected before and following the exercise procedure. The blood will be stored in a freezer for up to 24 months during which time biochemical analysis will be conducted on it. Approximately 0.5 ml of plasma from each sample will be sent to McMaster University in Ontario, Canada for stable isotope and amino acid analysis.

Infusion of Stable Isotopes to Determine Muscle Fractional Protein Synthetic Rate

On the experimental day, trace amounts of sterile L-[ring-$^{13}C_6$] phenylalanine will be infused intravenously through a cannula placed into a forearm vein. Blood will be drawn from a vein in the opposite arm every half hour for part of the calculations and measurement on insulin and glucose. The incorporation of the labelled phenylalanine into the cytosolic and muscle protein fractions will be determined from the muscle samples at 240 min post exercise, with the 30 min sample providing the baseline point. The state-of-the-art stable isotope procedure is a safe, accurate and reliable method of understanding the metabolic processes. Additionally, the pattern of gene expression in response to the nutrition will be determined from a small part of the muscle; this will be used to determine what molecular processes within the cell are switched on or off in response to the dose of leucine and protein ingested.

Exercise

There is often some physical and psychological discomfort associated with heavy exercise.

Recent evidence has indicated that even among healthy populations of athletes who exercise strenuously and regularly, there is some risk of sudden death due to heart failure. Though rare, such cases can occur in people who may have an undiagnosed condition. If you have any reason to suspect that you may have a cardiovascular problem, we suggest that you see your physician and get an ECG before you agree to participate.

If you have any additional medical concerns associated with this project, please contact your GP, or discuss with the researcher.

Muscle Biopsy

The primary purpose of this study is to determine the nutrient-stimulated mechanisms that facilitate the biological processes involved in adaptation to exercise (e.g. reorganisation and rebuilding of muscle fibres; synthesis of new aerobic energy enzymes). A total of six muscle samples will be collected from your outer quadriceps muscle by a trained individual. It will be performed under local anaesthetic (pain killer). You may have some minor discomfort and bruising when the anaesthetic wears off. There is a risk of fainting. There is also a risk of infection any time a cut is made to the skin. You will be provided with instructions for care to reduce the risk of infection (see additional information provided with this Information Sheet).

If you have any additional medical concerns specifically associated with the muscle biopsies whatsoever, but such as: skin disorders, bleeding or clotting disorder, or vascular malformation, please discuss with your GP and ask for a letter to be prepared and forwarded to the researcher. We will reimburse your medical expenses for this.

Time Commitment

Experiment Component	Time Commitment (h)
Screening, consent, VO_2max test & lab familiarization	1.5 h
Diet and training diaries	0.25 h
Day -7 to -1: Standardised training rides in field and lab (x 2 only per block)	~3 x 6-12 h of cycling (replaces normal training)
Experimental days	3 x 5 h
Total	~45 h (~20 h in the lab)

A reimbursement of $500 in MTA vouchers (or otherwise requested) will be provided on completion of the study to compensate you for the time, travel, and energy. Additional reasonable expenses will also be reimbursed if required (e.g. childcare, overnight accommodation). Parking vouchers will be provided for on-campus parking.

Benefits

You will learn your VO_2max and peak power output. A follow up laboratory test (e.g. VO_2max, lactate threshold) will be provided free of charge if desired, which are normally worth $225. You will partake in some landmark interesting research. You will receive a summary of the results once the final results are available, which will contain a summary of the effectiveness of the recovery formulation.

What if I Suffer a Personal Injury?

If physical injury results from your participation in this study, you should visit a treatment provider for examination, diagnosis, and treatment and inform the researcher immediately so an incident form may be completed. The study is conducted principally for the benefit of Nestec to test the physiological responses to the intervention formulation. In the event of injury arising from your participation in the research, an appropriate level of compensation will be awarded, in line with the *New Zealand Researched Medicines Industry Guidelines on Clinical Trials - Compensation for injury resulting from Participation in Industry Sponsored Clinical Trials*. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

Participant's Rights

At any time, you will have the right to:

- decline to participate;
- decline to answer any particular question;
- withdraw from the study;
- ask any questions about the study at any time during participation;
- provide information on the understanding that your name will not be used unless you give permission to the researcher;
- be given access to a summary of the project findings when it is concluded;
- If requested, we can return the unused and processed tissue samples that are being analysed in New Zealand; due to logistics and cost we are unable to return samples that are being shipped overseas.

If you have any queries or concerns regarding your rights as a participant in this study you may wish to contact a Health and Disability Advocate, telephone 0800 42 36 38 (4 ADNET).

If you are Interested in Taking Part

CONTACT:

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Sport and Exercise Science

Institute of Food, Nutrition, and Human Health

Massey University Wellington Campus

Pvt Bag 756

This project has been reviewed and approved by the Central Regional Ethics Committee, Wellington Application 08/12/065. If you have any concerns about the ethics of this research, please contact Sonia Scott telephone 04 496 2405, email central_ethicscommittee@moh.govt.nz

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How does the dose of protein and leucine ingested with carbohydrate after exercise effect the processes governing adaptation to high-intensity training?

PARTICIPANT CONSENT FORM

This consent form will be held for a period of five (5) years

I have read and understand the Information Sheet and have had the details of the study otherwise explained to me. ☐

My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time. ☐

I agree to participate in this study under the conditions set out in the Information Sheet. ☐

I understand that my participant is voluntary and that I may withdraw at anytime. ☐

I agree to my blood being taken. ☐

I verify that to the best of my knowledge I have no underlying medical issues that could mean that I am medically unsuitable to participate in a study with muscle biopsies. ☐

I agree to the six muscle biopsies. ☐

I agree to my tissue being stored after collection for later biochemical analysis. ☐

I agree to my blood being flown to McMaster University in Canada and the Nestle Research Centre in Switzerland for analysis. All sample sent overseas will be used in analysis and it will not be possible to send it back to New Zealand. ☐

I agree to the infusion of stable isotope materials. ☐

I agree to the exercise regime/dietary modifications. ☐

I understand that if personal injury results from participation in this experiment, that I may make a claim under Nestec insurance provisions. ☐

I understand that my participation in this study is confidential and that no material which could identify me will be used in any reports on this study. ☐

I know I am to contact the researchers if I have any side effects. ☐

Signature: _____ **Date:** _____

Full Name - printed _____

This project has been reviewed and approved by the Central Regional Ethics Committee, Wellington Application 08/12/065. If you have any concerns about the ethics of this research, please contact Sonia Scott telephone 04 496 2405, email central_ethicscommittee@moh.govt.nz

How does the dose of protein and leucine ingested with carbohydrate after exercise effect the processes governing adaptation to high-intensity training?

CONSENT FORM FOR GENETIC ANALYSIS

This consent form will be held for a period of five (5) years

I have read and understand the Information Sheet and have had the details of the genetic analysis explained to me. ☐

I understand that I may ask further questions at any time with respect to the genetic analysis. ☐

I consent to my samples being used in the genetic analysis. ☐

Signature: _____ **Date:** _____

Full Name - printed _____

This project has been reviewed and approved by the Central Regional Ethics Committee, Wellington Application 08/12/065. If you have any concerns about the ethics of this research, please contact Sonia Scott telephone 04 496 2405, email central_ethicscommittee@moh.govt.nz

Wording of Recruitment E-mail to Local Sports Clubs

Massey researchers seek cyclists for recovery nutrition supplement study

Dear Cyclists/Fitness enthusiast [PNP Cycling Club, Wellington Triathlon club members; Wellington MTB Club; vorb cyclists, selected gymnasiums],

Cyclists and fitness enthusiasts interested in taking part in a study to determine the optimal dose of amino acids and protein in a recovery formula are being sought by Sport Scientists at Massey University.

We would like to recruit up to 12 males aged between 18 and 50 years who have been regularly cycling or other activity involving cycling (e.g. triathlon; cardio sessions; spin classes) over the last six months or longer. Participants must be free of illnesses such as diabetes, heart disease, kidney problems, and bleeding or blood-borne disorders.

Participation in the study will involve a preliminary session of fitness testing (VO_2max) in the lab, followed by over the course of six weeks: three standardised 90 min training rides, and three 100 min rides followed by the recovery feeding and physiological measures.

The objective of the study is to test the effectiveness of two levels of protein and the amino acid, leucine, on stimulating the muscle growth and repair process after exercise.

Participants will be reimbursed $500 in vouchers for time and costs and an additional VO_2max test session will be available if requested.

Anyone interested in taking part of or finding out more about the study can contact Mr Andre Nelson or Dr David Rowlands on:

Office: 04 801 5799 ext 6188 (Andre) ext 6940 (David)
Cell: 021 2369914
e-mail: andre.nelson@gmail.com; d.s.rowlands@massey.ac.nz

APPENDIX J STUDY 2 SUPPLEMENT COMPOSITION

Procedure for the Production Milk Drink for Andre Nelson

All drinks were prepared by Mr Garry Radford, Institute of Food Nutrition and Human Health, Massey University, Palmerston North, NZ

Order of addition of the ingredients (if present)

MPC470
WPC894
L-Leucine
Maltrodextrin
Fructose
Canola Oil
Salt
Vanilla essence
Emulsifer
Water

1. Mixed the emulsifier with the fructose.
2. Add the ingredients in the order listed above to hot (about 50°C) water (except the vanilla essence) while stirring with an overhead stirrer
3. Stir for 30 minutes
4. Heat to 75°C in a water bath
5. Homogenise using a 2 stage homogeniser at 200 Bar (1st stage) 50 Bar (2nd stage)
6. Pasteurise at 75°C for 15 seconds. Product temperature leaving pasteuriser was about 10°C
7. Fill 296.4g of milk mixture into bottles containing 3.6g vanilla essence and ensure the final weight of product was 300g
8. Bottles placed in the -18°C freezer until required.

This procedure was used for all the different formulations.

Drinks for a given experimental day were thawed by removing from the freezer and placing in a 6°C refrigerator ~18hr prior to use. Phenylalanine tracer was added to appropriate drinks (High and Low conditions) ~3hr prior to consumption by removing the lid of each drink bottle, placing the lid on a food-only 4dp scale, transferring tracer with a food-only spatula onto the lid and weighing, then transferring from the lid into the bottle to ensure no losses of tracer. The lid was tightened, mixture shaken, and the bottle returned to the fridge until ready to be consumed.

Milk Protein Concentrate 470

PRODUCT BULLETIN

PB.026
Version 8.0307

NZMP Milk Protein Concentrate 470 (also known as ALAPRO™ 4700) from Fonterra is a spray dried, soluble milk protein manufactured by ultra-filtration of fresh skim milk. Low temperature processing maintains native protein structure, ensuring excellent functional, nutritional, and sensory properties.

PRODUCT CHARACTERISTICS

- Excellent flavour profile
- Excellent heat stability
- High nutritional quality
- High level of bound calcium
- High opacity in water
- Good dispersibility

SUGGESTED USES

- Recombined cheeses
- Cultured foods
- Frozen desserts
- Nutritional beverages
- Dry mix meal replacers

PACKAGING

Multi-wall paper which incorporates a moisture barrier and contains the product within an inner polyethylene bag. No staples or metal fasteners are used.

Net Weight	20.0 kg
Gross Weight	20.4 kg

STORAGE AND HANDLING

Milk Protein Concentrates are hygroscopic and can absorb odours. Therefore adequate protection is essential. It is recommended that product is stored at temperatures below 25°C, relative humidity below 65% and in an odour free environment. Stocks should be used in rotation preferably within 24 months of manufacture.

TYPICAL COMPOSITIONAL ANALYSIS

The analysis results listed in this product bulletin are typical as measured on an "as is" basis. Refer to the selling specification for minimum & maximum limits by parameter.

Protein (N x 6.38) (g/100g) as is	70.0
Moisture (g/100g)	4.4
Fat (g/100g)	1.4
Total Carbohydrate (g/100g)	17.0
Ash (g/100g)	7.2
Inhibitory substances (IU/ml)	<0.005

TYPICAL NUTRITIONAL ANALYSIS

Energy (kJ/100g)	1530
Calories (kcal/100g)	365
Energy from fat (kJ/100g)	52
Calories from fat (kcal/100g)	12
Total Sugars (lactose) (g/100g)	17.2
Fibre (g/100g)	0
Cholesterol (mg/100g)	70
Saturated fat (g/100g)	1.0
Trans fat (g/100g)	0.07
Vitamin A (µg/100g)	<6
Vitamin A (IU/100g)	<20
Vitamin C (mg/100g)	<0.9
Iron (mg/100g)	0.4
Sodium (mg/100g)	160
Calcium (mg/100g)	2180

NZMP
New Zealand... making it happen!

Fonterra
Dairy for life

Whey Protein Isolate 894

Instantised

PRODUCT BULLETIN

PB.022
Version 3.1006

NZMP Whey Protein Isolate 894 (also known as ALACEN™ 894) from Fonterra is an instantised whey protein isolate manufactured by cross flow micro-filtration (MF) and ultra-filtration (UF). Micro-filtration naturally isolates undenatured protein, providing a MF WPI product that is low in fat and high in protein. NZMP Whey Protein Isolate 894 is an undenatured, soluble whey protein with excellent dispersibility and solubility combined with excellent nutritive value. NZMP Whey Protein Isolate 894 is ideal for nutritional beverage powders that require spoon stirring.

PRODUCT CHARACTERISTICS

- Excellent nutritional value
- PDCAAS score of 1.00
- Excellent dispersibility and solubility
- Low lactose, low fat and low cholesterol
- Clean flavour
- Soluble over a wide pH range

SUGGESTED USES

- Nutritional powdered beverages

PACKAGING

Multi-wall paper that incorporates a moisture barrier and contains the product within an inner polyethylene bag. No staples or metal fasteners are used.

Net Weight	20.0 kg
Gross Weight	20.4 kg

STORAGE AND HANDLING

Whey Protein Isolates are hygroscopic and can absorb odours. Therefore adequate protection is essential. It is recommended that product is stored at temperatures below 25°C, relative humidity below 65% and in an odour free environment. Stocks should be used in rotation preferably within 24 months of manufacture.

TYPICAL COMPOSITIONAL ANALYSIS

The analysis results listed in this product bulletin are typical as measured on an "as is" basis. Refer to the selling specification for minimum & maximum limits by parameter

Protein (N x 6.38) (g/100g) as is	90.4
Moisture (g/100g)	4.7
Fat (g/100g)	1.0
Total Carbohydrate (g/100g)	0.9
Ash (g/100g)	3.0
Inhibitory substances (IU/ml)	<0.005

TYPICAL NUTRITIONAL ANALYSIS

Energy (kJ/100g)	1,590
Calories (kcal/100g)	380
Energy from fat (kJ/100g)	37
Calories from fat (kcal/100g)	9
Total Sugars (lactose) (g/100g)	0.9
Fibre (g/100g)	0
Cholesterol (mg/100g)	5
Saturated fat (g/100g)	0.6
Trans fat (g/100g)	0.07
Vitamin A (µg/100g)	0
Vitamin A (IU/100g)	0
Vitamin C (mg/100g)	0
Iron (mg/100g)	0.6
Sodium (mg/100g)	130
Calcium (mg/100g)	460

NZMP
New Zealand... making it happen!

Fonterra
Dairy for life

Goodman Fielder

Canola Oil

Description: Oil from the Rapeseed plant with low Erucic acid content refined bleached and deodorised. Designed for general-purpose use where a liquid oil of bland flavour and light colour is required. Canola oil is monounsaturated and is a good source of omega-3 fatty acids. May contain antioxidants.

Allergens: Canola oil is highly purified and free from allergenic substances.

GM Status: The Canola Oil and all ingredients are from non-GM sources.

Nutritional analysis Per 100g

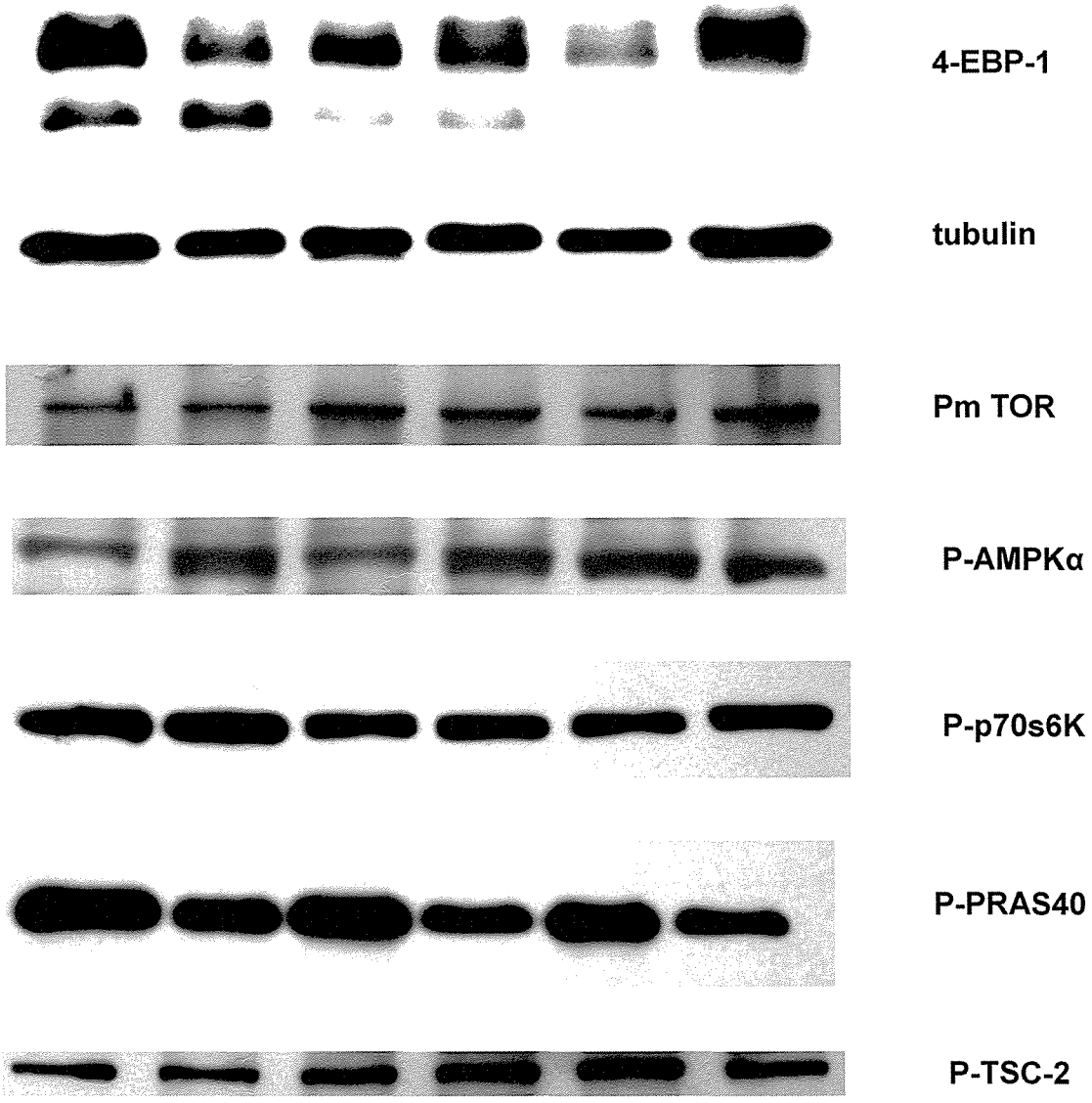
Energy	**3700kJ**
Protein	**0g**
Carbohydrate	**0g**
- sugars	0g
Cholesterol, mg	0mg
Sodium 0mg	
Fat	100g
- Saturates	**7g**
- Trans	**1g**
- Monounsaturates	60g
- Polyunsaturates	32g
- Omega-3	10g

Storage: Ambient temperatures best for long-term storage. Avoid exposure to light, strong-smelling foods or chemicals, cleaning agents and solvents.

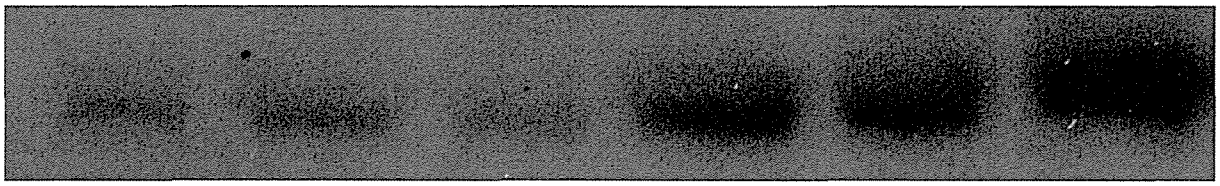
Shelf life: 12 months stored at ambient.

APPENDIX L STUDY 2 WESTERN BLOTS

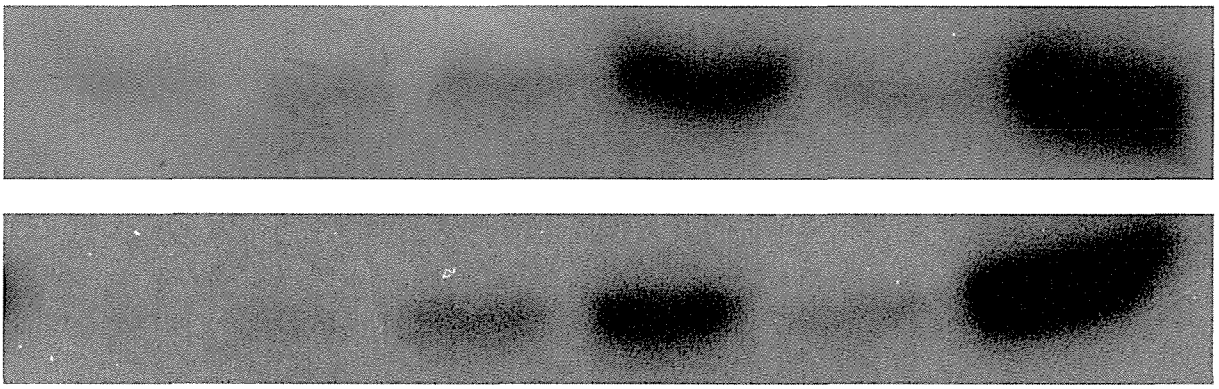
Representative blots from Wakefield Hospital.



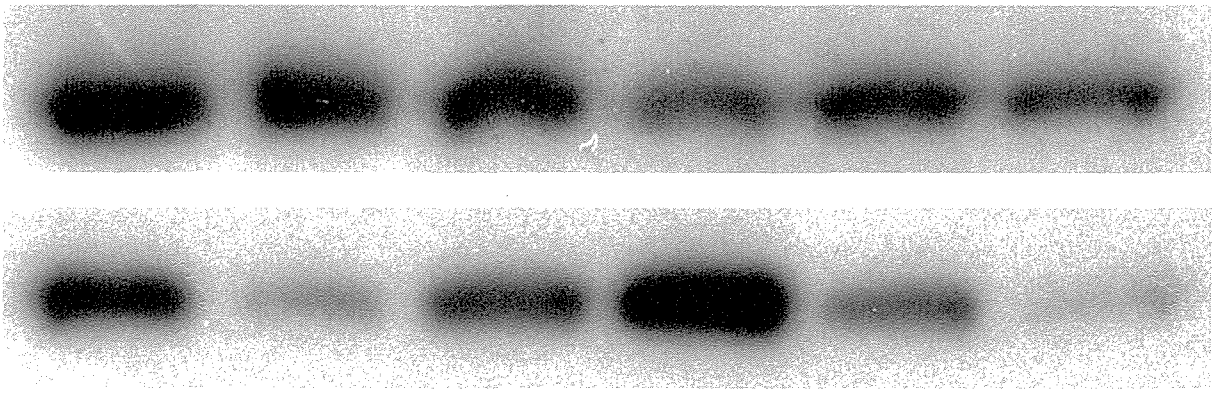
Representative blots from Massey University.



SIRT1



p-rpS6



p-eEF2

APPENDIX M STATEMENT OF CONTRIBUTION

DRC 16

MASSEY UNIVERSITY
GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION TO DOCTORAL THESIS CONTAINING PUBLICATIONS

(To appear at the end of each thesis chapter/section/appendix submitted as an article/paper or collected as an appendix at the end of the thesis)

We, the candidate and the candidate's Principal Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of Candidate: Andre Nelson

Name/Title of Principal Supervisor: Dr David S Rowlands

Name of Published Research Output and full reference: A Protein-Leucine Supplement Increases BCAA and Nitrogen Turnover but not Performance

In which Chapter is the Published Work: Chapter 3

Please indicate either:

- **The percentage of the Published Work that was contributed by the candidate:** _____
  and / or
- **Describe the contribution that the candidate has made to the Published Work:**

The candidate was responsible for set up and complete experiment conduction. First draft manuscript write up and modifications after editing and comments by myself and coauthors. Stats by candidate and myself. Other details noted in Statement of Contribution.

Candidate's Signature — Date: 1/11/2012

David Rowlands — Date: 2/11/12

Principal Supervisor's signature — Date

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