EFFECT OF LEUCINE-PROTEIN HIGH-CARBOHYDRATE POST-EXERCISE NUTRITION ON SUBSEQUENT PERFORMANCE AND THE PROTEIN REGULATED GENOMIC AND SIGNALLING EVENTS GOVERNING ADAPTIVE REMODELLING

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TITLE: Effect of leucine-protein high-carbohydrate post-exercise nutrition on subsequent performance and the protein regulated genomic and signalling events governing adaptive remodelling

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ABSTRACT

Recovery from prolonged endurance exercise requires fuel replenishment and ultrastructure repair to restore cellular homeostasis; and improvement requires adaptive remodelling. Timing nutrient intake to closely follow exercise may be advantageous to recovery and subsequent performance by facilitating the adaptive processes stimulated by exercise. The objective of this research was to firstly determine if leucine-enriched protein feeding after hard training improved subsequent performance, and secondly to explore the candidate means by which protein-rich post-exercise nutrition mediates recovery, primarily transcriptomic and signalling mechanisms.

Study 1 Ten male cyclists ingested leucine-enriched protein-carbohydrate (0.1/0.4/1.2/0.2 $g \cdot kg^{-1} \cdot h^{-1}$ leucine/protein/carbohydrate/fat) or isocaloric high-carbohydrate control (0.06/1.6/0.2 $g \cdot kg^{-1} \cdot h^{-1}$) meals following 2-2.5 h high-intensity interval training on 3 consecutive days. Cyclists performed a repeat-sprint performance test 39 h after training, and markers of physiological recovery and mood state were examined. *Study 2* Eight male cyclists ingested protein (0.4/1.2/0.2 $g \cdot kg^{-1} \cdot h^{-1}$ protein/carbohydrate/fat) or isocaloric high-carbohydrate control (0.03/1.6/0.2 $g \cdot kg^{-1} \cdot h^{-1}$) beverages following a single 1.75 h high-intensity interval cycling bout. Muscle tissue samples were collected from the vastus lateralis before exercise, 3-h and 48-h post-exercise. The transcriptome response was assessed by Illumina microarray, candidate gene expression by real time RT-PCR; and phospho-protein signalling by Western blot.

Leucine-enriched feeding increased mean sprint power by 2.5% (99% confidence limits, ±3.1%; P = 0.013) and reduced overall tiredness during sprints by 13% (90% confidence limits, ±9.2%). Serum creatine kinase was 19% (90% confidence limits, ±18%) lower than control, but difference in lactate dehydrogenase and muscle pain were trivial and unclear. In the second study, protein-carbohydrate feeding led to moderate and very large increases in cell signalling to translation; mTOR, 4E-BP1 and RPS6 phosphorylation by 3-h. Bioinformatics analysis indicates protein ingestion

effects the transcriptome response involved in immune/inflammatory processes, tissue development (extracellular matrix, cytoskeletal, and scarcomere remodelling), and metabolism consistent with increased fatty acid oxidation, compared to control.

Post-exercise protein and carbohydrate coingestion during a period of hard training enhances subsequent high-intensity endurance performance and may reduce membrane disruption in comparison to high-carbohydrate feeding. Furthermore, the mechanism responsible for protein-nutrition mediated adaptation may be through enhancing protein translation and fine-tuning the gene expression profile induced by exercise.

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STATEMENT OF CONTRIBUTION

CHAPTER 3: Leucine-protein supplemented recovery feeding enhances subsequent cycling performance in well-trained men

The principal investigator of this study was Jasmine Thomson. Study conception and design were provided by Jasmine Thomson, Dr. David Rowlands, with contributions from Dr. Ajmol Ali. The ethics proposal was written by Jasmine Thomson. Subjects were recruited and the study was coordinated by Jasmine Thomson. The supplements were formulated and prepared by Jasmine Thomson in the Institute of Food, Nutrition and Human Health Food Technology Laboratories, Massey University, Auckland. The data was collected largely by Jasmine Thomson with help from Dr. Ajmol Ali, Dr. Andrew Foskett, with assistance from Simon Bennett. Blood preparation was done by Jasmine Thomson assisted by Simon Bennett, and analyzed for Creatine Kinase and Lactate Dehydrogenase through LabPlus. Urine and sweat samples were collected and prepared by Jasmine Thomson, Kjeldahl digests and nitrogen determination by distillation and titration performed by Jasmine Thomson. Serum amino acids were analysed by HPLC at research laboratory at McMaster University by Tracey Rerecich. Statistical analyses were performed by Jasmine Thomson with guidance from Dr David Rowlands and Dr Beatrix Jones. The manuscript was written and prepared by Jasmine Thomson with guidance from Dr. David Rowlands, Dr. Ajmol Ali, and Assoc. Prof. Welma Stonehouse.

CHAPTER 4: Increased specificity of immune response, myocyte remodelling, and metabolic gene expression and signalling to translation with protein feeding after prolonged exercise

Study conception and design were provided by Dr. David Rowlands, Dr. Mark Tarnopolsky and Jasmine Thomson. The study was performed within the Departments of Medical Science and Pediatrics and Medicine, McMaster University research laboratories under supervision of Dr. Mark Tarnopolsky. Subjects were recruited by Jasmine Thomson. The supplements were prepared by Stuart Lowther of Life Science Nutritionals Inc. Biological

samples were collected by Dr. Mark Tarnopolsky, Dr. David Rowlands, Dr. Brian Timmons and Jasmine Thomson. Samples were prepared by Holly Robertshaw, Ming-Hua Fu, Changhua Ye, Michaela Devries, David Rowlands and Jasmine Thomson. Insulin assay performed by Suzanne Southward, and glucose by Tracy Rerecich. RNA extraction was done by Holly Robertshaw assisted by Jasmine Thomson. Western blotting was performed by by Elisa Glover, and real-time RT-PCR by Holly Robertshaw, and Changhua Ye. Micro array analysis was done at the Nestle Research Centre, Vers-chez-les-Blanc, Lausanne, Switzerland. Data Analyses were performed by Dr. David Rowlands, Jasmine Thomson and Brian Timmons, apart from the Array data which was performed Frederic Raymond, Robert Mansourian, Sylviane Metairon, Andreas Fuerholz, and Elisa Glover. Bioinformatics was by Andreas Fuerholz, David Rowlands, and Frederic Raymond. The manuscript was written and prepared by Jasmine Thomson with guidance from Dr. David Rowlands, and contributions from Frederic Raymond (Illumina array methods) and Andreas Fuerholz (bioinformatics).

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RESEARCH ETHICS

Ethical approval was obtained from the applicable research ethics bodies for each study (Massey University Palmerston North Ethics Committee; and Hamilton Health Sciences/McMaster University, Hamilton, Ontario). The potential risks and management of risks involved is detailed below;

All participants were screened via a health 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 injury or discomfort during the exercise and performance portions of the research. Some fatigue during exercise and performance trials was to be expected, however this expected to be of a similar level to that normally experienced in participant's own endurance training and competition. Maximal effort was requested of participants in both the VO_{2max} and performance testing and associated discomfort was thought to be normal for this level of athlete, and in fact adaptive and beneficial to health. There may have been some discomfort and minor risk of infection associated with blood catheter insertion and muscle biopsy procedures. Discomfort in each instance was minimised by having subjects lie prone on a hospital bed, and blood and biopsy procedures were performed by trained phlebotomists and medics with experience in the procedures. It was considered that the amount of blood and muscle tissue samples taken to pose no risk of adverse health effects. Risk of infection was minimised by following sterile procedural 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 rights to discontinue or withdraw from the studies at any time. We also ensured there were adequate change and shower facilities, and we minimised the number of observers in the laboratory at any one time while subjects were being examined and tests conducted. Following data

collection and identifying information has been stored securely in locked filing cabinet in locked office with access to only those principally involved in the studies.

We aimed to reduce economic risk to participants by reimbursing them for travel and time where necessary.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS AND ACRONYMS

4EBP1	Eukaryotic initiation factor 4E binding protein 1
AA	Amino acid
AADACL2	Aralkylamine N-acetyltransferase
AANAT	Aralkylamine N-acetyltransferase
ACOX1	Acyl-CoA oxidase 1, palmitoyl
ACSL1	Acyl-CoA synthetase long-chain family member 1
ACTA2	Actin, alpha 2, smooth muscle, aorta
ACTC1	Actin, alpha, cardiac muscle 1
ADCYAP1	Adenylate cyclase activating polypeptide 1 (pituitary)
ADD	Adducin
ADORA2A	Adenosine A2a receptor
ADP	Adenosine diphosphate
AEBSF	Aprotinin, Leupeptin, Bestatin, Pepstatin
AKT	Protein kinase B
AKT1	AKT substrate 1 (proline-rich)
AMP	Adenosine monophosphate
AMPK	Adenosine monophosphate kinase
ANGPT2	Angiopoietin 2
ANOVA	Analysis of variance
AS3MT	Arsenic (+3 oxidation state) methyltransferase
ATP	Adenosine triphosphate
B2M	Beta 2 microglobulin
BCL2	B-cell CLL/lymphoma 2
BCL2L14	BCL2-like 14 (apoptosis facilitator)
BCOADK	Branched-chain 2- oxo-acid dehydrogenase kinase
BDNF	Brain-derived neurotrophic factor
C1QA	Complement component 1, q subcomponent, A chain
C1QB	Complement component 1, q subcomponent, B chain
C1QC	Complement component 1, q subcomponent, C chain
C1S	Complement component 1, s subcomponent
C21orf127	Chromosome 21 open reading frame 127
CABP5	Calcium binding protein 5
CALML5	Calmodulin-like 5
CaMK	Calcium/calmodulin-dependent protein kinase II

CAMKID	Calcium/calmodulin-dependent protein kinase ID
CAMKII	Calcium/calmodulin-dependent protein kinase II
CASP1	Caspase 1, apoptosis-related cysteine peptidase (interleukin 1, beta, convertase)
CASP10	Caspase 10, apoptosis-related cysteine peptidase
CASP3	Caspase 3, apoptosis-related cysteine peptidase
CCL22	Chemokine (C-C motif) ligand 22
CCL5	Chemokine (C-C motif) ligand 5
CD36	Cluster of Differentiation 36
CD86	CD86 molecule
cDNA	Complimentary deoxyribonucleic acid
CEBPB	CCAAT/enhancer binding protein (C/EBP), beta
cFOS	FOS proto-oncogene
CHAT	Choline O-acetyltransferase
CHO	Carbohydrate
CI	Confidence interval
CIB2	Calcium and integrin binding family member 2
cJUN	JUN proto-oncogene
CK	Creatine kinase
CL	Confidence limit
CLCN4	Chloride channel protein 4
COL10A1	Collagen, type X, alpha 1
COL11A2	Collagen, type XI, alpha 2
COL18A1	Collagen, type XVIII, alpha 1
COL1A1	Collagen, type I, alpha 1
COL1A2	Collagen, type I, alpha 2
COL22A1	Collagen, type XXII, alpha 1
COL3A1	Collagen, type III, alpha 1
COL5A1	Collagen, type V, alpha 1
COL5A2	Collagen, type V, alpha 2
COL6A1	Collagen, type VI, alpha 1
COL6A3	Collagen, type VI, alpha 3
COLQ	Collagen-like tail subunit (single strand of homotrimer) of asymmetric acetyl cholinesterase
CON	Control condition

CPS1	Carbamoyl-phosphate synthase 1, mitochondrial
CPT1	Carnitine palmitoyltransferase I
CPT2	Carnitine palmitoyltransferase 2
CREB	cAMP response element binding protein
cRNA	Complementary ribonucleic acid
CROT	Carnitine O-octanoyltransferase
CT	Cycle threshold
CTSC	Cathepsin C
CV	Coefficient of variation
CXCL12	Chemokine (C-X-C motif) ligand 12
CXCL2	Chemokine (C-X-C motif) ligand 2
CXCR4	Chemokine (C-X-C motif) receptor 4
CYCS	Cytochrome c, somatic
CYP2U1	Cytochrome P450, family 2, subfamily U, polypeptide 1
CYP46A1	Cytochrome P450, family 46, subfamily A, polypeptide 1
d	^{15}N dose
DDIT3	DNA-damage-inducible transcript 3 (C/EBP-homologous protein, CHOP, GADD153)
DEPC	Diethylpyrocarbonate
DLAT	Dihydrolipoamide S-acetyltransferase
DMD	Dystrophin
DNA	Deoxyribonucleic acid
DNAJ	40 kDa heat shock protein (HSP40)
Dnase	Deoxyribonuclease
DNMT3	DNA (cytosine-5-)-methyltransferase 3 alpha
DNMT3B	DNA (cytosine-5-)-methyltransferase 3 beta
DSCR1	Regulator of calcineurin 1 (RCAN1, MCIP1)
DTT	Glucose assay buffer
EAA	Essential amino acids
ECH1	Enoyl CoA hydratase 1, peroxisomal
ECM	Extracellular matrix
EDN1	Endothelin 1
EDTA	Ethylenediaminetetraacetic acid
EE	Estimated energy expenditure
EE1A1	Eukaryotic translation elongation factor 1 alpha 1

EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1
EFNB1	Ephrin-B1
EFNB3	EPH receptor B3
EGF	Epidermal growth factor
EGTA	Ethylene glycol tetra acetic acid
EIF1AX	Eukaryotic translation initiation factor 1A, X-linked
eIF2B	Eukaryotic translation initiation factor 2B
eIF4E	Eukaryotic translation initiation factor 4E
eIF4G	Eukaryotic translation initiation factor 4G
EIF5B	Eukaryotic translation initiation factor 5B
ELISA	Enzyme-Linked Immunosorbent Assay, enzyme immunoassay
eNOS	Endothelial nitric oxide synthase 3 (endothelial cell)
EPHA2	EPH receptor A2
ERK 1/2	Mitogen-activated protein kinase
ES	Effect Size
ESR1	Estrogen receptor 1
Ex_{study}	Estimated energy expenditure for study exercise protocol
FABP3	Fatty acid binding protein 3
FABP5	Fatty acid binding protein 5
FAK	Focal adhesion kinase
FBLN1	Fibulin 1
FBLN2	Fibulin 2
FBXL3	F-box and leucine-rich repeat protein 3
FBXO24	F-box protein 24
FBXO32	F-box protein 32
FGF2	Fibroblast growth factor 2
FOXO1a	Forkhead box O1
FOXO3	Forkhead box O3
G6PDH	Glucose-6-phosphate dehydrogenase
GADD45B	Growth arrest and DNA-damage-inducible, beta
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCN5	General control of amino-acid synthesis 5-like 2
GDF1	Growth differentiation factor 1
GDF8	Myostatin
GEA	Global Error Assessment

GLUT4	Glucose transporter type 4
GRS	Graphic rating scale
GSK3	Glycogen synthase
GβL	G-protein beta-subunit like protein
HAT1	Histone acetyltransferase 1
HCHO	High carbohydrate condition
HDAC8	Histone deacetylase 8
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid buffer
HGF	Hepatocyte growth factor
HMGCS2	3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)
HSP70	Heat shock 70 kDa protein 1A (HSPA1A)
HSPA1A	Heat shock 70 kDa protein 1A (HSP70)
hVPS34	Phosphatidylinositol 3-kinase catalytic subunit type 4
ICMT	Isoprenylcysteine carboxyl methyltransferase
IFI6	Interferon, alpha-inducible protein 6
IFITM2	Interferon induced transmembrane protein 2 (1-8D)
IFNA1	Interferon, alpha 1
IGF1	Insulin-like growth factor 1
IKBKB	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
IL	Interleukin
IL17D	Interleukin 17D
IL1F6	Interleukin 1 family, member 6
IL1F8	Interleukin 1 family, member 8
IL22	Interleukin 22
IPA	Ingenuity pathway analysis
IRF8	Interferon regulatory factor 8
IRS1	Insulin receptor substrate 1
ITGAM	Integrin, alpha M (complement component 3 receptor 3 subunit)
ITGB1	Integrin, beta 1
ITGB2	Integrin, beta 2
JNK	c-Jun N-terminal kinases
LAMA4	Laminin, alpha 4
LCHO	Low carbohydrate condition
LDH	Lactate Dehydrogenase

LPIN1	Lipin 1
LPL	Lipoprotein lipase
LRFN5	Leucine rich repeat and fibronectin type III domain containing 5
MAPK	Mitogen-activated protein kinase
MDA	Malonyldialdehyde
MEF2	Myocyte enhancer factor 2
MG	Macro-glycogen
MHC	Myosin heavy chain
MHCII	Major Histocompatibility Complex class II
MLC1SA	Myosin, light chain 6B, alkali, smooth muscle and non-muscle (MYL6B)
MMP13	Matrix metallopeptidase 13 (collagenase 3)
MMP19	Matrix metallopeptidase 19
MMP9	Matrix metallopeptidase 9 (92 kDa gelatinase, type IV collagenase)
MRCL3	Myosin, light chain 12A, regulatory, non-sarcomeric1 (MYL12A)
MRF4	Myogenic factor 6 (also known as Muscle-specific regulatory factor 4)
MRFAP1L1	Morf4 family associated protein 1-like 1
mRNA	Messenger ribonucleic acid
MTMR6	Myotubularin related protein 6
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex 1
MUC4	Mucin 4, cell surface associated
MuRF1	Muscle-specific RING finger protein 1
MYBPH	Myosin binding protein H
MYF5	Myogenic factor 5
MYH1	Myosin, heavy chain 1, skeletal muscle, adult
MYH8	Myosin, heavy chain 8, skeletal muscle, perinatal
MYL1	Myosin, light chain 1, alkali; skeletal, fast
MYL2	Myosin, light chain 2, regulatory, cardiac, slow
MYL3	Myosin, light chain 3, alkali; ventricular, skeletal, slow
MYL4	Myosin, light chain 4, alkali; atrial, embryonic
MYL5	Myosin, light chain 5, regulatory
MYLPF	Myosin light chain, phosphorylatable, fast skeletal muscle

MYOD1	Myogenic differentiation 1
MYOG	Myogenin (myogenic factor 4)
MYST2	MYST histone acetyltransferase 2
n	Sample number
NADP	Nicotinamide adenine dinucleotide phosphate
NCBI GEO	Gene expression omnibus
NDST2	N-deacetylase/N-sulfotransferase (heparan glucosaminyl) 2
N_E	Nitrogen excretion
NES	Mestin
NFAT	Nuclear factor of activated T-cells
NF-kB	Nuclear factor-kB
NFKB1A	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1
N_I	Nitrogen intake
NKIRAS2	Nuclear factor of kappa light polypeptide inhibitor interacting Ras-like 2
NMR	Nuclear magnetic resonance spectroscopy
NPB	Net protein balance
NTN4	Metrin 4
NTNG2	Metrin G2
p	Test statistic probability considered significant at the a-level 0.05
p70S6K	Ribosomal protein S6 kinase, 70 kDa
p90RSK	Ribosomal protein S6 kinase, 90 kDa
PA	Physical Activity
Panther GO	Panther gene ontology mapping
PB	Protein breakdown
PCR	Polymerase chain reaction
PDE	Phosphodiesterase
PDGFB	Platelet-derived growth factor beta polypeptide
PDHA2	Pyruvate dehydrogenase (lipoamide) alpha 2
PDK	Pyruvate dehydrogenase kinase
PDK1	Phosphinositide dependent protein kinase 1
PDZRN3	PDZ domain containing ring finger 3
PECI	Peroxisomal D3,D2-enoyl-CoA isomerase
PFKFB1	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 1

PFKFB3	6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3
PFKM	Phosphofructokinase, muscle
PG	Pro-glycogen
PGC-1α	PPAR gamma coactivator 1 alpha
PH	Plekstrin homology domain containing proteins
PI3K	Phosphinositide 3-kinase
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
PKC	Protein kinase C
PLA_2	Phospholipase A_2
PLIN2	Perilipin 2
PLTP	Phospholipid transfer protein
POMS	Profile of mood states short-form questionnaire
PPAR	Peroxisome proliferator-activated receptor
PPARγ	Peroxisome proliferator-activated receptor gamma
PPARGC1α	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha
PPM2C	Pyruvate dehydrogenase phosphatase catalytic subunit 1 (PDP1)
PRAS40	40 kDa proline-rich AKT substrate
PS	Protein synthesis
PTN, Ptn	Protein condition
Q	Nitrogen flux
qPCR	Real time reverse transcription polymerase chain reaction
r	Pearson's correlation (one-tailed)
Rab 7	GTPase Rab 7
Rag A/B/C/D	Ras-related GTP-binding proteins
RARA	Retinoic acid receptor, alpha
REDD1/2	Deoxyribonucleic acid-damage-inducible transcript 4
RFFL	Ring finger and FYVE-like domain containing 1
RHEB	Ras homolog enriched in brain
RHOA	Ras homolog gene family, member A
RHOC	Ras homolog gene family, member C
RHOJ	Ras homolog gene family, member J
RMR	Resting metabolic rate
RNA	Ribonucleic acid
RNAase	Ribonuclease

ROS	Reactive oxygen species
RPE	Ratings of perceived exertion
RPL27A	Ribosomal protein L27a
RPL7	Ribosomal protein L7
RPLP0	Ribosomal protein, large, P0
RPLP1	Ribosomal protein, large, P1
RPS6	40S ribosomal protein S6
RPS6KA1	Ribosomal protein S6 kinase, 90 kDa, polypeptide 1
rRNA	Ribosomal ribonucleic acid
RSP24	Ribosomal protein S24
RSP3A	Ribosomal protein S3a
RT	Reverse transcription
RT-PCR	Real time reverse transcription polymerase chain reaction
S100A8	S100 calcium binding protein A8
S100A9	S100 calcium binding protein A9
S6K	Ribosomal protein S6 kinase, 70 kDa (formerly known as p70S6K)
SAS	Statistical Analysis Software
SCD	Stearoyl-CoA desaturase (delta-9-desaturase)
SD	Standard deviation
SDC2	Syndecan 2
SDF1	Chemokine (C-X-C motif) ligand 12 (CXCL12)
SDS PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEMA3C	Sema domain, immunoglobulin domain, short basic domain, secreted, (semaphorin) 3C
SEMA3F	Sema domain, immunoglobulin domain, short basic domain, secreted, (semaphorin) 3F
SEMA6A	Sema domain, transmembrane domain, and cytoplasmic domain, (semaphorin) 6A
Ser	Serine rich binding motif
SERCA	Sarco/endoplasmic reticulum calcium transporting ATPase
SIRT5	Sirtuin 5
SIRT6	Sirtuin 6
SLC1A5	Neutral amino acid transporter solute carrier family 1
SLC25A20	Solute carrier family 25 (carnitine/acylcarnitine translocase),

	member 20
SLC2A4	Solute carrier family 2 (facilitated glucose transporter), member 4 (GLUT4)
SLC7A11	Solute carrier family 7, (cationic amino acid transporter, y+ system) member 11
SLC7A5	Solute carrier family 7 (cationic amino acid transporter, y+ system), member 5
SLC7A5/SLC3A2	Bi-directional antiporter
SLC7A8	Solute carrier family 7 (amino acid transporter, L-type), member 8
SLCO1B1	Solute carrier organic anion transporter family, member 1B1
SLCO2A1	Solute carrier organic anion transporter family, member 2A1
SLIT2	Slit homolog 2
SMAD3	SMAD family member 3
SMARCA3	Helicase-like transcription factor (HLTF)
SNP sites	Single nucleotide polymorphism or single nucleotide (A, T, C, or G) difference
SOCS3	Suppressor of cytokine signalling 3
SOD	Superoxide dismutase
SPSS	Statistical Package for the Social Sciences
SR	Sarcoplasmic reticulum
SREBP	Sterol response element binding protein
SULT1A1	Sulfotransferase family, cytosolic, 1A, phenol-preferring, member 1
SYK	Spleen tyrosine kinase
TBK1	TANK-binding kinase 1
TBS-T	Tris buffered saline solution with the detergent Tween-20
TGFB1	Transforming growth factor, beta 1
Thr	Threonine rich binding motif
TIMP1	TIMP metallopeptidase inhibitor 1
TIMP2	TIMP metallopeptidase inhibitor 2
TLR3	Toll-like receptor 3
TNC	Tenascin C
TNF	Tumour necrosis factor
TNFRSF19	Tumour necrosis factor receptor super family, member 19
TNFSF11	Tumour necrosis factor (ligand) super family, member 11

TNN	Tenascin N
TNNT2	Troponin T type 2 (cardiac)
TNXB	Tenascin XB
tr:T	Tracer to tracee ratio
TRIM46	Tripartite motif-containing 46
TRIM5	Tripartite motif-containing 5
TRIM9	Tripartite motif-containing 9
Tris	Tris(hydroxymethyl)aminomethane buffer
TSC1	Tuberous sclerosis protein 1, (hamartin)
TSC1-TSC2	Hamartin-tuberin complex
TSC2	Tuberous sclerosis protein 2 (tuberin)
TUBA1	Tubulin alpha-1A chain
Tween	Polysorbate detergent
Tyr	Tyrosine rich binding motif
UBE2	Ubiquitin-conjugating enzyme E2
UBE2J2	Ubiquitin-conjugating enzyme E2, J2
UCP3	Uncoupling protein 3 (mitochondrial, proton carrier)
VEGF	Vascular endothelial growth factor
VEGFA	Vascular endothelial growth factor A
VIM	Vimentin
VO_{2max}	Maximal volume of oxygen consumed
Vsp15	Vacuole sorting protein 15
W_{max}	Peak power output
WNT5A	Wingless-type MMTV integration site family, member 5A
YY1	YY1 transcription factor
ZNRF2	Zinc and ring finger 2

CHAPTER 1 INTRODUCTION

Recovery from a fatiguing bout of exercise is an integral part of an athlete's training regime, both for maintenance of training intensity and improvement in performance during subsequent bouts of exercise or competition. Without adequate recovery, performance improvement stimulated by training is hampered (Halson, et al. 2002; Jeukendrup, et al. 1992). Recovery following prolonged intense exercise requires replenishment of fuel stores, rehydration, repair of damaged muscle tissue, recovery of cellular homeostasis, and allows for initiation of training-induced adaptations (Ivy 2004; Mahoney, et al. 2005). Whilst duration of recovery is considered the most important factor relating to recovery and adaptation to exercise, the optimum recovery duration in relation to training is poorly defined (Kuipers 1996). Nonetheless, several alternate recovery strategies have been investigated, of which nutritional strategies relating to rehydration and glycogen resynthesis appear to be the most efficacious on subsequent performance (Barnett 2006).

The beneficial effects of consuming a high carbohydrate diet in the recovery period following endurance exercise on subsequent performance is well established (Achten, et al. 2004; Burke, et al. 2004; Halson, et al. 2002). Moreover, it is generally accepted that these benefits to performance result from enhanced muscle glycogen recovery (Burke, et al. 2004; Maughan 2002; Simonsen, et al. 1991). Furthermore, endurance athletes require sufficient protein intakes to not only compensate for the small amount of amino acids oxidized for fuel (Dohm, et al. 1987; Hargreaves & Snow 2001; Kasperek, et al. 1992; Viru 1987) but also to repair damaged muscle proteins and to initiate training-induced adaptations (Tarnopolsky 2004; Tipton & Wolfe 2004). These requirements are easily achieved by most athletes in their habitual diet (Burke 2001; Vogt, et al. 2005; Williams 1998). On the other hand excessively high daily protein intakes (>2.5 $g \cdot kg^{-1} \cdot d^{-1}$) result in increased oxidation of excess protein (Bowtell, et al. 1998; Forslund, et al. 1998; Gaine, et al. 2007) and have also been shown to impair time-to-fatigue performance (Bigard, et al. 1993; Macdermid, et al. 2006). Therefore, at the level of daily intake, athletes need to be cognisant of ensuring high carbohydrate intakes over that of protein as the

more important nutrition strategy to stimulate recovery following endurance exercise.

The timing of nutrient provision in the post-exercise period has received much recent attention. Immediately post-exercise there is increased substrate uptake due to increased blood flow (Biolo, et al. 1995; Biolo, et al. 1997) and substrate transport (Etgen, et al. 1993; Goodyear, et al. 1990), as well as enhanced insulin sensitivity (Garetto, et al. 1984; Richter, et al. 1984); factors which have been suggested to enhance muscle glycogen (Ivy 1998) and protein synthesis (Morrison, et al. 2008) in this 'crucial time window'. Maehlum et al (1977) were the first to highlight the importance of consuming carbohydrate immediately post-exercise and, in a series of studies, Ivy et al (Ivy 1998; Ivy 2004; Ivy, et al. 1988a; Ivy, et al. 1988b) further defined amount of carbohydrate and ingestion frequency for optimal muscle glycogen resynthesis. Comparatively, there is a relative dearth of information regarding timing of protein ingestion in the post-exercise period on replenishment of body protein stores. Levenhagen et al (2001) demonstrated the importance of timing protein-rich feeding immediately following prolonged exercise compared to 3 h delay. Ingesting the protein-carbohydrate supplement early following exercise resulted in increased substrate uptake and 3 times greater protein synthesis in the exercised leg compared to later feeding. However, due to the acknowledged importance of muscle glycogen replenishment, this and other studies in this area have investigated a protein-carbohydrate co-ingestion regimen rather than protein alone (Levenhagen, et al. 2001; Roy, et al. 2002).

The co-ingestion of protein and carbohydrate post-exercise could enhance the recovery process and hence subsequent exercise performance by stimulating greater insulin secretion, which in turn increases glucose uptake and glycogen synthase activity, inducing more rapid glycogen synthesis (Burke, et al. 1995; Ivy 2004; Jeukendrup 2004). This theory was first proposed by Zawadzki et al. (1992), who demonstrated an increase in serum insulin and muscle glycogen with co-ingestion of protein and carbohydrate after exercise compared to carbohydrate alone. Subsequent findings are inconsistent with this theory; studies do not always report enhanced insulin secretion with the addition of protein to carbohydrate (Betts, et al. 2007; van

Loon, et al. 2000a) and, while insulin secretion is positively correlated with glycogen synthesis, elevated plasma insulin does not always result in enhanced muscle glycogen synthesis (Betts, et al. 2008; Howarth, et al. 2009; van Loon, et al. 2000b). Study inconsistencies likely relate not only to the amount of carbohydrate but also the amount and type of amino acids in the protein-carbohydrate supplement, irrespective of caloric balance between supplements. Whilst glucose is the most effective insulin secretagogue (stimulates insulin secretion), certain amino acids have also been found to stimulate insulin secretion via an alternative mechanism (Ahmed, et al. 1999; Newsholme, et al. 2007). The amino acids leucine, phenylalanine and tyrosine act synergistically with carbohydrate to enhance insulin secretion compared to carbohydrate alone (van Loon, et al. 2000a; van Loon, et al. 2000c). The rate of glucose ingestion that maximises insulin secretion is not known (Henquin, et al. 2006), and the addition of protein to high carbohydrate intakes (1-1.2 $g \cdot kg^{-1} \cdot h^{-1}$) has been found to stimulate greater insulin secretion in some studies (Betts, et al. 2007; Betts, et al. 2005; Jentjens, et al. 2001; van Loon, et al. 2000a; van Loon, et al. 2000b). However, unlike insulin secretion, glycogen synthesis appears to be maximised by carbohydrate ingestion at rates of 1-1.2 $g \cdot kg^{-1} \cdot h^{-1}$, fed immediately post-exercise and at frequent intervals (15-30 min) (Ivy 2004; Jentjens & Jeukendrup 2003); whereby the addition of protein fails to enhance glycogen synthesis rate regardless of its influence on insulin secretion (Betts, et al. 2008; van Loon, et al. 2000b). Furthermore, despite an initial enhanced rate of glycogen synthesis, when protein and carbohydrate are combined following exercise, by 24 h the total amount of glycogen storage in muscle is no different to a carbohydrate only recovery diet (Burke, et al. 1995).

Increased muscle glycogen stores are known to lead to enhanced endurance capacity during moderate to high prolonged exercise (Bergstrom, et al. 1967; Bergstrom & Hultman 1967; Hawley, et al. 1997). However, evidence relating post-exercise protein-carbohydrate to subsequent performance benefit relative to carbohydrate-only, is inconsistent. Discrepancies between studies are likely the consequence of different caloric content between post-exercise treatment and control in some studies (Betts, et al. 2007; Williams, et al. 2003), and the use of exercise protocols where muscle glycogen was not limiting for performance in others (Berardi, et al. 2008;

Karp, et al. 2006). Overall, the addition of protein to carbohydrate feeding does not benefit subsequent performance relative to carbohydrate following exercise, unless carbohydrate is inadequate for maximal glycogen synthesis, feeding is infrequent, recovery period between exercise bouts is short, and exercise is at intensity whereby performance is limited by glycogen content. Therefore, as a nutritional recovery strategy, protein and carbohydrate co-ingestion with the intention of enhancing glycogen synthesis has limited application and does not enhance subsequent performance compared with carbohydrate when longer recovery periods are employed.

Cade et al. (1991) proposed post-exercise protein and carbohydrate to benefit muscle repair and recovery from exercise-induced muscle damage. This finding has been largely supported by subsequent studies (Luden, et al. 2007; Millard-Stafford, et al. 2005; Romano-Ely, et al. 2006; Rowlands, et al. 2008). However most studies have used indirect markers of muscle damage, plasma creatine kinase (CK) and lactate dehydrogenase (LDH) activity, and perceived delayed onset muscle soreness to indicate the extent of muscle damage (Luden, et al. 2007; Millard-Stafford, et al. 2005; Romano-Ely, et al. 2006). Creatine kinase and LDH are intracellular enzymes, and their appearance in circulation indicates sarcolemmal permeability and leakage from myocytes, but do not directly infer muscle damage. Delayed onset muscle soreness is theorized to relate to damage to the muscle and connective tissue, and/or subsequent inflammatory responses to the injury site (Cheung, et al. 2003), but also do not directly verify muscle damage. However, plasma CK and LDH activities, and muscle soreness poorly correlate with histological muscle damage (Millard-Stafford, et al. 2005; Stupka, et al. 2000; Van der Meulen, et al. 1991). This relationship is further weakened in females compared to males (Stupka, et al. 2000; Van der Meulen, et al. 1991) and therefore may not be appropriate markers of muscle damage, particularly in mixed gender cohorts such as utilized by Cade, Luden and colleagues (Cade, et al. 1991; Luden, et al. 2007). Additionally, the highly variable nature of plasma CK and LDH release from myocytes and clearance from the circulation (Totsuka, et al. 2002) makes these measures less sensitive for use in between-subject type designs (Cade, et al. 1991; Green, et al. 2008; Millard-Stafford, et al. 2005; Wojcik, et al. 2001). Furthermore, some groups might be criticised for

adding antioxidants to the post-exercise protein-rich supplement, and masking the potential beneficial ingredient (Luden, et al. 2007; Millard-Stafford, et al. 2005; Romano-Ely, et al. 2006). There is some evidence of vitamins E and C attenuating plasma CK and LDH activities (Rokitzki, et al. 1994), as well as muscle soreness following damaging exercise (Kaminski & Boal 1992), thus making it impossible to determine the effect attributable to the protein contained in these supplements.

Not only do CK, LDH, and muscle soreness correlate poorly with muscle damage, they have little relation to functional impairment (Warren, et al. 1999) and hence should be interpreted with caution if no other measures of performance recovery are taken. For example, several studies have reported protein and carbohydrate supplementation following exercise to attenuate plasma CK but fail to show subsequent improvement in performance (Luden, et al. 2007; Millard-Stafford, et al. 2005; Romano-Ely, et al. 2006; Rowlands, et al. 2007). Differences in performance outcomes between studies could result from differences in the degree to which muscle is damaged by initial exercise, imbalanced or uncontrolled daily energy intakes (Millard-Stafford, et al. 2005; Romano-Ely, et al. 2006; Rowlands, et al. 2008), and unmatched caloric content of supplement and control (Luden, et al. 2007). Furthermore, gender may affect recovery of performance (Rowlands & Wadsworth 2010); something that has not been considered in mixed gender studies (Luden, et al. 2007; Millard-Stafford, et al. 2005).

It is well established that feeding protein-rich nutrition during the post-exercise period enhances protein turnover, which is essential for protein synthesis and repair following endurance exercise. Skeletal muscle protein turnover depends on both cellular protein synthesis and degradation (Rennie & Tipton 2000). The key regulator of protein translation, the mammalian target of rapamycin (mTOR), is up-regulated by nutrients after prolonged exercise (Bolster, et al. 2004b). Essential amino acids act to stimulate protein synthesis by not only providing the substrate for translation, but also stimulate translation through mTOR-dependent pathways (Bolster, et al. 2004a; Kimball & Jefferson 2006b). Branched chain amino acids, particularly leucine stimulate translation initiation in a dose-dependent manner (Crozier, et al. 2005). Insulin has only a permissive effect on protein synthesis

through mTOR signalling pathways (Patel, et al. 2001; Prod'homme, et al. 2005); its main contribution is inhibition of protein breakdown through lesser known mechanisms (Greenhaff, et al. 2008). Results from animal (Anthony, et al. 1999; Gautsch, et al. 1998; Morrison, et al. 2008) and human (Kammer, et al. 2009) studies show increased activation of mTOR pathway proteins with post-exercise protein and carbohydrate ingestion compared to carbohydrate only and protein only controls. Correspondingly, studies have also found increased protein synthesis (in the exercised muscle fraction as well as whole-body) with combined protein-carbohydrate intake compared to carbohydrate control. These studies compared protein-rich nutrition following exercise to a control matched to either carbohydrate quantity (Howarth, et al. 2009; Levenhagen, et al. 2002) or total caloric content (Howarth, et al. 2009), thereby isolating the effect of post-exercise protein ingestion from that of energy. The consensus is that enhanced protein synthesis with post-exercise protein provision following endurance exercise is not only related to enhanced repair, but also enhanced synthesis and activity of specific proteins leading to functional adaptation (Tipton 2008). Nevertheless, evidence of the specific protein fraction and correlation with improved performance in this instance is lacking.

In most of the performance studies evaluating attenuation of damage and enhanced repair, focus has been on the 12 to 24 h recovery period from the bout of damaging exercise until performance testing. These protocols might be adequate for the evaluation of serum peak in CK which occurs at 24 h (Koller, et al. 1998; Totsuka, et al. 2002), but may not allow for complete functional recovery which might take up to 2 days after intense endurance exercise (Parra, et al. 2000; Ross, et al. 2010). Hence, studies in which recovery has been examined over a time course of less than 24 h might fail to capture longer term mechanisms driving recovery of endurance exercise performance and facilitated by protein-rich nutrition. A study by Rowlands et al. (2008) supports this notion that a longer recovery period may be needed to benefit performance; they found no clear benefit of protein-carbohydrate feedings on performance at 15 h following initial exercise, but after an additional 45 h there was substantial benefit to performance. The delayed effect on performance might be related to the time required for repair and initiation of adaptation, although evidence of this is lacking. The extended recovery period over which performance was

improved in this study might fit the time course for initiation of adaptation to training reported in previous studies. Repair and adaptation begins immediately following exercise; an increased in gene transcription peaks within hours of exercise (8-12 h) (Yu, et al. 2003), and the appearance of encoded proteins accumulate to a measurable amount within days (Booth & Holloszy 1977; Ren, et al. 1994; Spina, et al. 1996). Adaptive changes resulting in enhanced aerobic performance might take as few as 6 bouts of interval-type training (Burgomaster, et al. 2005; Gibala, et al. 2006).

Nonetheless, Rowlands et al (2008) provided an excessively large amount of protein, 2.9 $g \cdot kg^{-1}$; far in excess of the amount estimated to saturate post-exercise protein synthesis (Moore, et al. 2009). The minimal ingested amino-acid quantity aimed at saturating muscle-protein synthesis rate has been estimated to approximate 0.2 $g \cdot kg^{-1}$ of protein (composed of ~0.1 $g \cdot kg^{-1}$ essential amino acids) following resistance exercise in humans, or alternatively about 0.1 $g \cdot kg^{-1}$ leucine following prolonged exercise in rats (Crozier, et al. 2005). Thus, further research is needed to determine the benefit of a more realistic post-exercise protein supplementation regime on subsequent performance, when adequate time for recovery of performance is provided.

The mechanisms whereby protein-carbohydrate ingestion stimulates protein translation in the initial post-exercise period are becoming characterised, however, there is limited research comparing protein-carbohydrate supplementation to isocaloric control, or utilizing well-trained human sucjects. Furthermore, the protein fraction which is being translated is unknown, and hence the involvement in recovery and adaptation to exercise training is unknown. These gaps in the literature limit understanding of the recovery and adaptation following endurance exercise that is facilitated by nutrition. Within the area of exercise physiology, research is accelerating in areas of cellular regulation (Baar 2006) and gene transcription (Heller 2002), and previously unavailable techniques are now becoming more commonplace. These avenues of research will possibly offer new insights into the mechanisms mediating recovery from prolonged aerobic exercise with protein and carbohydrate nutrition.

1.1.1 PURPOSE OF THESIS

The two studies detailed in this thesis were designed to determine the benefit of post-exercise protein and carbohydrate nutrition on subsequent performance, and mechanisms involved in recovery of muscle cell homeostasis and adaptation to endurance exercise, in comparison to carbohydrate nutrition.

The aims of the first study were to examine possible benefits of protein and carbohydrate feeding following endurance exercise over 3 days on recovery markers and subsequent performance compared to energy balanced carbohydrate control. Unique aspects of this study include; stringent control and balance of daily energy and macronutrient intake; isolation of post-exercise supplement timing by provision of the alternate supplement at the end of the day; inclusion of protein-carbohydrate supplement with ordinary high-carbohydrate food (pasta, cereal bars, juice) to better represent normal dietary practices; provision of saturating quantities of essential amino acids, in particular leucine, for protein synthesis and carbohydrate for glycogen synthesis; and intense training stimulus for adaptation balanced with adequate rest for recovery, over an acute timeframe. It was hypothesised that supplementation of high-carbohydrate post-exercise meals with a leucine-protein beverage would result in enhancement of subsequent performance and markers of recovery including mood state, sarcolemmal damage and perceived muscle soreness.

The aim of the second study was to determine cellular signalling leading to protein translation and profile the global gene expression during early (3 h) and late (48 h) recovery from endurance exercise and facilitated by protein and carbohydrate nutrition. Unique aspects of this study include; the examination of global gene expression during recovery from endurance exercise supported by post-exercise nutrition; the comparison of molecular signalling to translation when isocaloric post-exercise supplements were provided to humans well-trained in endurance exercise; and the isolation of post-exercise supplement timing within a background of balanced energy and macronutrient intake. It was hypothesised that combined protein-carbohydrate ingestion following exercise would differentially regulate signalling to translation, gene expression manifest as enhanced programming for

recovery of homeostasis and adaptation to endurance exercise; and post-translational events.

CHAPTER 2 LITERATURE REVIEW

2.1 EFFECTS OF POST-EXERCISE PROTEIN-CARBOHYDRATE CO-INGESTION ON SUBSEQUENT ENDURANCE EXERCISE PERFORMANCE AND CANDIDATE MECHANISMS

2.1.1 ABSTRACT

Background. Skeletal muscle recovery and subsequent performance are influenced by protein-rich supplements and meals that closely follow endurance exercise. Recently, this has attracted considerable interest, and several mechanisms have been proposed to account for these potential benefits. The purpose of this review is to discuss the conditions under which post-exercise protein and carbohydrate intake benefits subsequent performance compared to carbohydrate intake. *Methods.* Fifty studies met the review criteria; comparison of protein-carbohydrate post-exercise intake with carbohydrate control, taken soon after prolonged exercise (>60 min), over the acute time-frame (<2 weeks), subjects were human and endurance trained. Twenty eight studies reported effects on performance, 25 on insulin concentrations, and 15 on glycogen synthesis. *Results and discussion*. Protein and carbohydrate ingestion after exercise increases serum insulin concentrations and glycogen storage and, in some situations, benefits subsequent performance over the short-term compared to carbohydrate only. Combined ingestion also reduces the appearance in the blood of intracellular enzymes indicating sarcolemmal damage, and lessens perceived muscle soreness, but does not necessarily benefit performance over longer-term compared to carbohydrate alone. Many studies had design limitations such as imbalanced energy provision in treatment and control supplements, lack of control over background diet and training, and limited recovery period. *Conclusions.* Protein-carbohydrate intake after exercise has a greater effect on short-term performance when carbohydrate intake is suboptimal, feeding is infrequent (≥1 h), and the exercise is intense and long enough for muscle glycogen to become limiting. With adequate rest, and high daily energy and carbohydrate intakes, combined post-exercise protein-carbohydrate meals might benefit performance in males several days after intense training. More research is needed to confirm potential benefits for performance when using longer recovery periods after initial fatiguing exercise.

2.1.2 BACKGROUND

Recovery is integral to an athlete's training regime for maintenance of training intensity and improvement in performance. Without adequate recovery, adaptation to the training stimulus will be limited and subsequent performance will deteriorate (Flynn, et al. 1994; Halson, et al. 2002; Jeukendrup, et al. 1992; Simonsen, et al. 1991). Recovery from prolonged intense exercise entails restoration of cellular homeostasis through rehydration, replenishment of fuel stores and repair of muscle damage, as well as initiation of training adaptations leading to enhanced performance (Ivy 2004; Mahoney, et al. 2005). Various nutritional strategies aim to support at least some of these aspects of recovery.

The beneficial effect of a high carbohydrate diet on restoration of glycogen stores during recovery between exercise bouts and endurance performance has been well documented (reviewed by (Burke, et al. 2001a; Burke, et al. 2004; Ivy 1998; Ivy 2004; Tarnopolsky, et al. 2005); however, the daily protein requirement to support endurance training and recovery and enhance performance is still disputed. Chronic high protein intakes (>2.5 $g \cdot kg^{-1} \cdot d^{-1}$) impair endurance performance (Bigard et al. 1993; Jarvis et al. 2002; Macdermid et al. 2006), probably by displacing carbohydrate intake and hampering recovery of muscle glycogen stores. Furthermore, high protein diets do not benefit whole body protein turnover compared to moderate intakes (1.8 $g \cdot kg^{-1} \cdot d^{-1}$) (Forslund, et al. 1999; Gaine, et al. 2007); however, high daily protein may attenuate fasted post-exercise protein breakdown (Bolster, et al. 2005). Thus, research focus has shifted to timing of protein intake within a background of habitual daily protein intakes.

During the hours immediately after prolonged exercise, ingesting nutrients may maximise recovery by exploiting the increased blood flow to the muscle, insulin sensitivity, glycogen synthase activity (Ivy 2004; Levenhagen, et al. 2001) and muscle protein synthesis, thereby enhancing glucose and amino acid uptake and utilization (Carraro, et al. 1990; Sheffield-Moore, et al. 2004; Wilkinson, et al. 2008). Indeed, extensive evidence demonstrates the benefits of timing, frequency, amount and type of post-exercise carbohydrate supplementation for optimizing muscle glycogen recovery (Burke, et al. 2004; Ivy, et al. 1988b; Maughan 2002). In

contrast, research on protein-rich nutrition after prolonged exercise is scarce, although protein intake immediately after exercise seems to stimulate the accretion of new muscle protein (Levenhagen, et al. 2001; Roy, et al. 2002). Carbohydrate and protein together might also reduce muscle-protein breakdown via insulin action and lead to improved net protein balance (Levenhagen, et al. 2002; Miller, et al. 2003). Similarly, protein and carbohydrate might act synergistically to stimulate greater insulin secretion, glucose uptake and glycogen resynthesis (Zawadzki, et al. 1992).

Zawadzki et al. (1992) found serum insulin and muscle glycogen storage both increased when protein and carbohydrate were ingested together after prolonged exercise, compared to carbohydrate alone; however, subsequent results are equivocal. Some studies report enhanced glycogen synthesis (Berardi, et al. 2006; Ivy, et al. 2002; Tarnopolsky, et al. 1997; van Loon, et al. 2000b), whilst others report no difference (Burke, et al. 1995; Carrithers, et al. 2000; Jentjens, et al. 2001; van Hall, et al. 2000). After resistance exercise, the combination of protein and carbohydrate increased muscle protein synthesis compared to amino acids alone and carbohydrate alone (Moore, et al. 2009; Tang, et al. 2007). However, when protein and carbohydrate were taken together after endurance exercise, protein synthesis rates increased compared to equal volume carbohydrate (Howarth, et al. 2009; Levenhagen, et al. 2002) and isocaloric carbohydrate intakes (Howarth, et al. 2009).

While improved recovery suggests performance benefits, several questions have yet to be answered satisfactorily. For instance, does acute protein-carbohydrate co-ingestion benefit subsequent endurance performance? If so, what are the proposed mechanisms? Consequently, this review focuses on the effect of protein and carbohydrate ingested together after exercise on subsequent performance of endurance exercise, compared to ingestion of carbohydrate alone. The review also discusses the conditions under which co-ingestion of protein and carbohydrate is beneficial compared to carbohydrate alone. A second purpose is to summarise current hypotheses about the mechanisms by which co-ingestion of protein and carbohydrate improves subsequent endurance performance.

2.1.3 METHODS

The importance of glycogen for endurance performance and carbohydrate intake on glycogen synthesis is widely acknowledged; consequently, this review does not investigate recovery after protein-only feeding but instead focuses only on studies comparing protein-carbohydrate meals or supplements taken after exercise to carbohydrate only meals or supplements. Within a background of high daily carbohydrate intake, acute post-exercise carbohydrate feeding seems most beneficial to subsequent performance when recovery time is short (<8 h) (Burke, et al. 2004; Parkin, et al. 1997); therefore, studies have been grouped into short-term (<8 h) and longer term recovery (>8 h) categories. The review considers the most important outcome to be the effects of post-exercise nutrition on subsequent performance. Other outcomes that were investigated include insulin concentration and glycogen synthesis over the short-term, and markers of recovery from muscle damage and protein synthesis over longer recovery time course. Studies are discussed in the context of conditions resulting in increased efficacy of post-exercise nutrition, such as the energy, protein, amino acid, and carbohydrate content of treatment and control, intake frequency, gender, and duration of recovery. Finally, the review discusses current hypothesized mechanisms including muscle glycogen enhancement, attenuation of muscle damage and enhanced repair, and the interaction of exercise and nutrients during adaptation to endurance training.

Literature on the effect of post-exercise nutrition on recovery and performance outcome was identified through Internet searches using the databases PubMed and Google Scholar. Keywords used to identify studies included, but were not limited to, post-exercise, endurance, prolonged exercise, protein, recovery, signalling protein synthesis, and adaptation. The reference lists of manuscripts located in this way were further reviewed for relevant studies. Studies were included if they met all the following criteria: they compared protein and carbohydrate co-ingestion to a carbohydrate control, supplementation or feeding was soon after exercise, a prolonged (≥60 min) exercise model was used over an acute time-frame (<2 weeks), and subjects were human, recreationally to well-trained endurance athletes. Some well known studies in this area of research were excluded because treatment and control supplements were provided during as well as after fatiguing exercise or

performance (Saunders, et al. 2004; Saunders, et al. 2009; Watson, et al. 2008) making it difficult to determine the nutrient timing responsible for any effects. The literature search on the proposed mechanisms driving recovery from endurance exercise was classified as short-term (<8 h) or longer-term (>8 h) recovery periods. This search resulted in 14 studies and 25 observations on insulin outcome, and 11 studies and 15 observations on glycogen outcome short-term (Tables 2.1 and 2.2). Biochemical and physiological markers of damage and repair processes were investigated in nine studies longer-term. Finally, the search on performance outcome resulted in eight short-term acute studies and six longer-term acute studies relating to the above mechanisms, respectively. Some studies reported more than one experiment or post-exercise supplement, so each comparison to the control (carbohydrate after exercise) was included, resulting in 12 short-term acute and 11 longer-term acute observations (Tables 2.3 and 2.4).

In the studies reviewed, performance was assessed by running or cycling performance in time to fatigue or time trial test. Where data has been represented as time to exhaustion, it was then converted to the equivalent improvement in mean power as defined by Hopkins et al. (1999) so these studies could be compared with those measuring mean power output. Hopkins et al. (1999) also stated that time to exhaustion tests have larger coefficients of variation (CV) because of the large change in total time resulting from a small change in power output. Outcomes were then represented as percent difference between treatment (protein-carbohydrate) and control (carbohydrate). Effect size (ES) for performance, insulin and glycogen outcomes was calculated using Cohen's effect size, where ES = (mean difference/control *SD*). Effect size was further adjusted for sample size (*n*) using the correction factor 1-3/(4*(*n*-1)-1) multiplied by ES. Performance outcomes were further quantified using Cohen's inferences modified by Hopkins et al. (1999) where the effect is classified as: trivial 0.0–0.2, small 0.2–0.6, moderate 0.6–1.2, large 1.2–2.0, very large 2.0-4.0, enormous >4.0. Correlations in % difference outcomes with treatment due to variations in post-exercise supplementation protocols were expressed as Pearson's correlation, r; where the r is considered to be: trivial 0-0.1, small 0.1-0.3, moderate 0.3-0.7, large 0.7-0.9, nearly perfect 0.9-1.0, and perfect 1.0.

2.1.4 SHORT-TERM RECOVERY STUDIES (<8 h recovery period)

2.1.4.1 Insulin Secretion

The predominant theory explaining enhanced muscle glycogen recovery from exercise with protein-carbohydrate co-ingestion compared to carbohydrate alone was first proposed by Zawadzki et al. (1992); it consists of the ability of protein and carbohydrate to synergistically stimulate greater insulin secretion, thereby causing increased muscle uptake of glucose and enhanced glycogen synthesis. Protein stimulates insulin secretion mainly through certain amino acids acting as insulin secretagogues (agents that stimulate hormone secretion). Certain amino acids, for example those transported with the Na^+-dependent transporter systems, depolarize the pancreas plasma membrane and activate voltage-dependent Ca^{2+} channels, resulting in a Ca^{2+} influx which stimulates insulin secretion (Ahmed, et al. 1999; Newsholme, et al. 2007). Additionally, metabolism of pancreatic β cell amino acids leads to production of ATP, which closes the ATP-sensitive K^+ channels, depolarizes pancreatic cell membranes and activates the Ca^{2+} channel (Newsholme, et al. 2007); however, the physiological significance of this second pathway when glucose is available is unknown (Gao, et al. 2003; Li, et al. 2003).

Studies that investigated the effect of post-exercise protein and carbohydrate intake on insulin are summarised in Table 2.1. Several report an increase in insulin secretion when protein is added to carbohydrate feeding following endurance exercise (Betts, et al. 2007; Betts, et al. 2008; Howarth, et al. 2009; Ivy, et al. 2002; Kammer, et al. 2009; Millard-Stafford, et al. 2005; van Hall, et al. 2000; van Loon, et al. 2000a; Williams, et al. 2003), while others show no added effect on insulin (Carrithers, et al. 2000; Millard-Stafford, et al. 2005; van Loon, et al. 2000a; van Loon, et al. 2000b). van Loon et al. (2000a) were the first to examine the insulin dose-response to protein and the amino acids leucine, and phenylalanine, which are known to be insulin secretagogues. A series of four supplements were compared to 1.2 $g \cdot kg^{-1} \cdot h^{-1}$ carbohydrate control, and the insulin response was greatest when 0.1 $g \cdot kg^{-1} \cdot h^{-1}$ leucine, 0.1 $g \cdot kg^{-1} \cdot h^{-1}$ phenylalanine and 0.2 $g \cdot kg^{-1} \cdot h^{-1}$ wheat protein isolate were added to 1.2 $g \cdot kg^{-1} \cdot h^{-1}$ carbohydrate. However, when van Loon et al. (2000b) compared isocaloric supplements in a second study, they found 0.1 $g \cdot kg^{-1} \cdot h^{-1}$ leucine,

0.1 $g \cdot kg^{-1} \cdot h^{-1}$ phenylalanine, 0.4 $g \cdot kg^{-1} \cdot h^{-1}$ wheat protein isolate and 0.8 $g \cdot kg^{-1} \cdot h^{-1}$ carbohydrate did not significantly differ in insulin response compared to 1.2 $g \cdot kg^{-1} \cdot h^{-1}$ carbohydrate. A further issue is that glucose is known to be the major insulin secretagogue and has previously been shown to produce a dose-dependent response in serum insulin as oral glucose increased from 0.7 to 1.12 $g \cdot kg^{-1} \cdot h^{-1}$ (Wolever & Bentum-Williams 1994); however the rate of glucose ingestion that maximises insulin secretion is not known (Henquin, et al. 2006). Therefore several factors such as energy content, carbohydrate content, as well as type and dose of insulinotropic amino acids are likely candidates for different outcomes in the studies reviewed.

To identify trends from variations in post-exercise supplementation protocols (Table 2.1), the effect of treatment (protein-carbohydrate) on serum insulin concentration relative to control (carbohydrate) was correlated with variations in treatment supplement content. Variations included energy, protein, amino acid, and carbohydrate content, as well timing and frequency of ingestion following exercise.

There was a moderate correlation (r = 0.47; p (one-tailed) = 0.017) between estimated amounts of insulinotropic amino acids (stimulate secretion of insulin, e.g. leucine, phenylalanine and tyrosine) provided by the protein or amino acid source and effect of the protein-carbohydrate supplement on serum insulin concentration relative to carbohydrate. In fact, the synergistic effect on insulin secretion when the post-exercise supplement contains protein and carbohydrate, overshadows the dose response to carbohydrate itself (r = -0.116; p (one-tailed) = 0.290). Increased feeding frequency (15-30 min intervals) had a negative trend (r = -0.31; p (one-tailed) = 0.067) with the effect of protein-carbohydrate on insulin concentration relative to carbohydrate, but not statistically significant. Collectively, these results indicate protein and carbohydrate taken together after exercise can promote insulin secretion when ingested infrequently (≥1 h between feedings), and when amounts of the more potent insulinotropic amino acids are adequate (leucine and phenylalanine content estimated to be > 0.1 $g \cdot kg^{-1} \cdot h^{-1}$).

TABLE 2.1 EFFECT OF COMBINED PROTEIN-CARBOHYDRATE POST-EXERCISE FEEDING ON SERUM INSULIN (< 8 h recovery period)

Authors	*n*	Recovery (h)	Ptn-CHO Supplement		CHO		Feeding Freq ($\cdot h^{-1}$)	Measure	Ptn-CHO		CHO		Outcome		
			Ptn g·kg^{-1}·h^{-1}	Ptn Type	CHO g·kg^{-1}·h^{-1}	CHO g·kg^{-1}·h^{-1}			Mean	*SD*	Mean	*SD*	ES[a]	% difference	Inference[b]
(Carrithers, et al. 2000)	7	4	0.2	Casein WPI MPC	0.7	1	2	Insulin μU·mL^{-1}	4570	2230	5100	1230	-0.37	-10.4	small
	7	4	0.1	AA	0.9	1	2	Insulin μU·mL^{-1}	3220	1670	5100	1230	-1.33	-36.9	large
(van Hall, et al. 2000)	5	1.5	0.5	WPI	1.8	1.8	4	Insulin mU·L^{-1} at 1.5 h	52.0	15.7	48.0	11.2	0.29	8.33	small
	5	4	0.5	WPI	1.8	1.8	4	Insulin mU·L^{-1} from 1.5-4 h	30.0	13.4	36.0	6.70	-0.72	-16.7	moderate
(van Loon, et al. 2000a)	8	3	0.2	Wheat	1.2	1.2	2	Insulin AUC *p*mol·L^{-1}·3 h^{-1} estimated from graph	41.7	11.8	45.8	23.5	-0.16	-8.95	trivial
	8	3	0.4	Wheat	1.2	1.2	2	Insulin AUC *p*mol·L^{-1}·3 h^{-1} estimated from graph	43.8	14.7	45.8	23.5	-0.08	-4.37	trivial
	8	3	0.2 0.05 0.05	Wheat + Leu + Phe	1.2	1.2	2	Insulin AUC *p*mol·L^{-1}·3 h^{-1} estimated from graph	60.4	17.7	45.8	23.5	0.55	31.9	small

Authors	*n*	Recovery (h)	Ptn-CHO Supplement			CHO	Feeding Freq ($\cdot h^{-1}$)	Measure	Ptn-CHO		CHO		Outcome		
			Ptn $g \cdot kg^{-1} \cdot h^{-1}$	Ptn Type	CHO $g \cdot kg^{-1} \cdot h^{-1}$	CHO $g \cdot kg^{-1} \cdot h^{-1}$			Mean	*SD*	Mean	*SD*	ES[a]	% difference	Inference[b]
(van Loon, et al. 2000a)	8	3	0.4 0.1 0.1	Wheat + Leu + Phe	1.2	1.2	2	Insulin AUC $pmol \cdot L^{-1} \cdot 3 h^{-1}$ estimated from graph	81.3	20.6	45.8	23.5	1.34	77.5	large
(van Loon, et al. 2000b)	8	5	0.2 0.1 0.1	Wheat + Leu + Phe	0.8	0.8	2	Insulin AUC at 5 h $U \cdot L^{-1}$	15.9	6.20	8.60	2.50	2.60	84.9	very large
	8	5	0.2 0.1 0.1	Wheat + Leu + Phe	0.8	1.2	2	Insulin AUC at 5 h $U \cdot L^{-1}$	15.9	6.20	12.3	1.80	1.78	29.3	large
(Jentjens, et al. 2001)	8	3	0.4	Wheat Leu Phe	1.2	1.2	2	Insulin AUC $mU \cdot mL^{-1}$ at 180 min	14.7	8.20	7.30	2.50	2.63	101	very large
(Niles, et al. 2001)	10	1.5	0.6	WPI	1.5	2.1	1	Insulin $\mu U \cdot mL^{-1}$ at 90 min peak	60.8	10.0	30.1	3.00	9.37	102	enormous

Authors	*n*	Recovery (h)	Ptn-CHO Supplement			CHO	Feeding Freq (h^{-1})	Measure	Ptn-CHO		CHO		Outcome		
			Ptn $g \cdot kg^{-1} \cdot h^{-1}$	Ptn Type	CHO $g \cdot kg^{-1} \cdot h^{-1}$	CHO $g \cdot kg^{-1} \cdot h^{-1}$			Mean	*SD*	Mean	*SD*	ES[a]	% difference	Inference[b]
(Ivy, et al. 2002)	7	4	0.4	MPC	1.1	1.1	1	Insulin $\mu U \cdot mL^{-1}$ at 180 min peak estimated from graph	27.6	12.7	20.6	16.8	0.36	34.0	small
	7	4	0.4	MPC	1.1	1.5	1	Insulin $\mu U \cdot mL^{-1}$ at 180 min peak estimated from graph	27.6	12.7	20.9	11.3	0.52	32.1	small
(Williams, et al. 2003)	8	0.5	0.1	WPI	0.4	0.2	0.5	Insulin $pmol \cdot L^{-1}$ at 30 min peak estimated from graph	331	173	167	104	1.40	98.2	large
(Betts, et al. 2005)	9	4	0.2	Wheat	1.2	1.2	2	Insulin AUC $mIU \cdot mL^{-1}$ at 4 h	13.5	3.30	11.4	2.70	0.70	18.4	moderate
	9	4	0.1	Wheat	0.8	0.8	2	Insulin AUC $mIU \cdot mL^{-1}$ at 4 h	4.60	1.06	3.10	0.53	2.56	48.4	very large
(Millard-Stafford, et al. 2005)	8	1	0.2	WPI	0.8	0.6	1	Insulin $mU \cdot mL^{-1}$ at 1 h estimated from graph	6.40	0.40	4.20	0.40	4.89	52.4	enormous

Authors	*n*	Recovery (h)	Ptn-CHO Supplement			CHO	Feeding Freq (h^{-1})	Measure	Ptn-CHO		CHO		Outcome		
			Ptn $g \cdot kg^{-1} \cdot h^{-1}$	Ptn Type	CHO $g \cdot kg^{-1} \cdot h^{-1}$	CHO $g \cdot kg^{-1} \cdot h^{-1}$			Mean	*SD*	Mean	*SD*	ES[a]	% difference	Inference[b]
(Millard-Stafford, et al. 2005)	8	1	0.2	WPI	0.8	1	1	Insulin $mU \cdot mL^{-1}$ at 1 h estimated from graph	6.40	0.40	10.60	0.80	-4.67	-39.6	enormous
(Betts, et al. 2007)	6	4	0.3	WPI	0.8	0.8	2	Insulin AUC nmol over 4 h	47.0	18.6	28.9	12.4	1.23	62.6	large
	6	4	0.3	WPI	0.8	1.1	2	Insulin AUC nmol over 4 h	47.0	18.6	43.9	22.9	0.11	7.06	trivial
(Betts, et al. 2008)	6	3.8	0.3	WPI	0.8	0.8	2	Insulin AUC $nmol \cdot L^{-1}$ over 4 h (personal communication)	66.9	8.90	54.6	9.40	1.10	22.5	moderate
(Howarth, et al. 2009)	6	4	0.4	WPI	1.2	1.2	1	Insulin AUC $mIU \cdot mL^{-1} \cdot min^{-1}$	104	18.0	79.0	17.0	1.24	31.6	large
	6	4	0.4	WPI	1.2	1.6	1	Insulin AUC $mIU \cdot mL^{-1} \cdot min^{-1}$	104	18.0	90.0	22.0	0.54	15.6	small

Authors	*n*	Recovery (h)	Ptn-CHO Supplement			CHO		Measure	Ptn-CHO		CHO		Outcome		
			Ptn g·kg^{-1}·h^{-1}	Ptn Type	CHO g·kg^{-1}·h^{-1}	CHO g·kg^{-1}·h^{-1}	Feeding Freq (·h^{-1})		Mean	*SD*	Mean	*SD*	ES[a]	% difference	Inference[b]
(Kammer, et al. 2009)	12	1	0.3	Milk	1.1	1.1	1	Insulin pmol·L^{-1} AUC over 60 min	15500	4320	11900	4190	0.79	30.0	moderate

Abbreviations in order of occurrence: Ptn = protein; CHO = carbohydrate; AA = amino acids; WPI = whey protein isolate; MPC = milk protein concentrate; AUC = area under the curve; Wheat = wheat protein hydrolysate; Leu = leucine; Phe = phenylalanine.

[a]Effect Size adjusted for sample size using the correction factor: 1-3/(4*(n-1)-1) multiplied by [b]Hopkins' modified Cohen's effect size, where ES (mean difference/control SD), and inferences classified as trivial 0.0–0.2, small 0.2–0.6, moderate 0.6–1.2, large 1.2–2.0, very large 2.0-4.0, enormous >4.0

2.1.4.2 Glycogen Synthesis

Several authors suggest the benefit from post-exercise protein and carbohydrate supplementation on muscle glycogen storage is largely related to greater plasma insulin response (Betts, et al. 2005; van Loon, et al. 2000a; Zawadzki, et al. 1992). Studies included in this review have been summarized in Table 2.2. Insulin acts to increase muscle glycogen synthesis in two ways: by potentiating GLUT4 uptake of glucose into muscle and by signalling activation of glycogen synthase. Both processes are key regulators of glycogen synthesis (Cohen 2006) and are represented diagrammatically in Figure 2.1. Insulin activates its receptor on the skeletal muscle plasma membrane; the receptor then transforms to allow it to recruit and dock insulin receptor substrates (IRS), mainly IRS1, which are then activated by phosphoinositide 3-kinases (PI3K). Phosphoinositide 3-kinase signals through phosphoinositide dependent protein kinase 1 (PDK1) and protein kinase B (AKT) (Christ-Roberts, et al. 2003; Nobukuni, et al. 2007). Activated AKT inhibits glycogen synthase kinase 3α/β (GSK 3α/β), allowing the dephosphorylation and activation of glycogen synthase (GS) (Cross, et al. 1995; Hurel, et al. 1996; Yeaman, et al. 2001). Activated AKT also acts to phosphorylate and activate its downstream substrate, termed AKT substrate of 160 kDa (AS160), which mediates translocation of glucose transporter 4 (GLUT4) to the plasma membrane and glucose uptake. The translocation of GLUT4 involves several steps and might also include signalling by the atypical protein kinase Cs (PKC) downstream of IRS-PI3K (Farese 2002; Morifuji, et al. 2009b; Wang, et al. 1999). Physiological hyperinsulinemia can stimulate ribosomal protein S6 kinase (p70S6K), which is mediated by PDK1 (Pullen, et al. 1998); p70S6K has been linked to a feedback loop that deactivates the IRS1-PI3K complex and therefore reduces initiation of the insulin signal (Tremblay & Marette 2001).

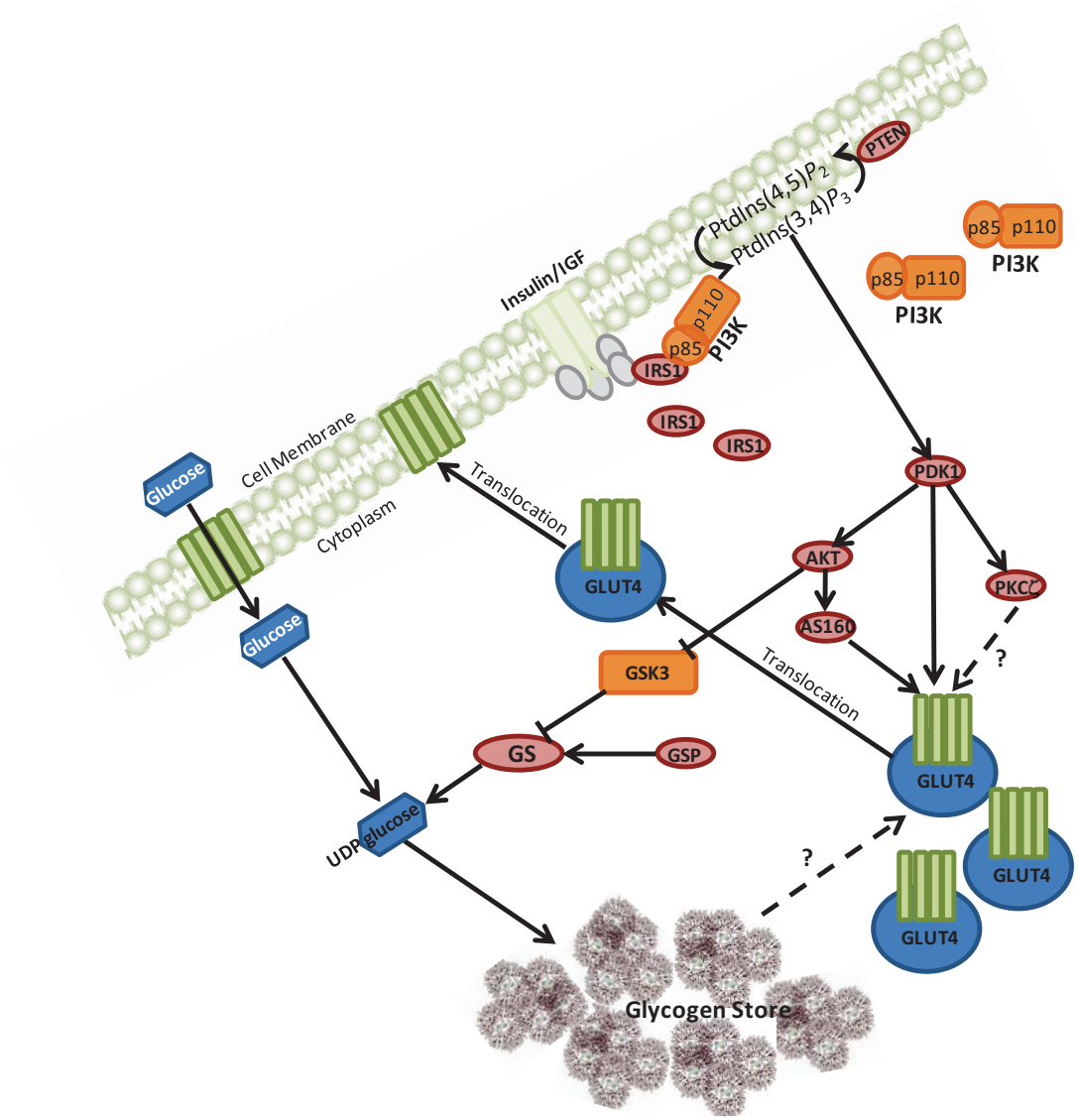

Figure 2.1 Simplified schematic diagram showing insulin regulation of glucose uptake and muscle glycogen synthesis.

Adapted from (Alonso, et al. 1995; Cohen 2006; Zierath & Wallberg-Henriksson 2002).

In addition to the synergistic activation of insulin, co-ingestion of protein and carbohydrate has been hypothesized to enhance signalling to glycogen synthesis (Ivy, et al. 2008). Amino acids and insulin also synergistically up-regulate mammalian target of rapamycin (mTOR) leading to phosphorylation of ribosomal protein S6 kinase (p70S6K) (Anthony, et al. 2007; Kammer, et al. 2009). Ribosomal protein S6 kinase is linked to deactivation of GSK 3α/β and GS activation, up-regulating glycogen synthesis (Armstrong, et al. 2001; Ivy, et al. 2008). However,

others consider this a minor pathway that is not physiologically relevant (Hurel, et al. 1996; Liu, et al. 2004). More importantly, as described above, p70S6K has been linked to a feedback loop that accelerates deactivation of the IRS1-PI3K complex (Baum, et al. 2005; Tremblay, et al. 2005; Tremblay & Marette 2001). High concentrations of amino acids and prolonged hyperinsulinemia are sensed by mTOR and a feedback loop deactivates IRS1-PI3K, effectively capping both glucose uptake in the muscle and the potential for high protein and carbohydrate intakes to increase glycogen synthesis rates (Baum, et al. 2005; Tremblay & Marette 2001).

Seven groups (10 observations) reported increases in both insulin and muscle glycogen concentrations resulting from protein and carbohydrate feeding compared to carbohydrate only (Betts, et al. 2008; Carrithers, et al. 2000; Howarth, et al. 2009; Jentjens, et al. 2004; Jentjens, et al. 2001; Tarnopolsky, et al. 1997; van Loon, et al. 2000a). The change in glycogen concentration was found to be very highly correlated ($r = 0.74$; p (one-tailed) = 0.007) with change in serum insulin concentration after protein-carbohydrate supplementation; however, increased insulin concentration did not consistently lead to enhanced muscle glycogen synthesis (Betts, et al. 2008; Howarth, et al. 2009; van Loon, et al. 2000b). This phenomenon might be explained by a propensity to increase rate of insulin secretion to a far greater extent than rate of glycogen synthesis. Additional insulin is of little benefit to glycogen storage if the rate of glycogen synthesis rapidly reaches its maximum. The rate of synthesis of glycogen in muscle appears to be maximised at carbohydrate intakes of around 1-1.2 $g \cdot kg^{-1} \cdot h^{-1}$ and when post-exercise feeding is frequent (15-30 min) (Jentjens & Jeukendrup 2003). To determine if these trends are shown by more recent literature (Table 2.2), the effects of carbohydrate content and frequency of feeding were each separately correlated with the effect of combined protein-carbohydrate post-exercise feeding on muscle glycogen storage. Carbohydrate content in the control beverage was strongly and negatively correlated with the effect of protein-carbohydrate on muscle glycogen ($r = -0.61$; p (one-tailed) = 0.008); furthermore, increasing frequency of feeding had negative trend with muscle glycogen ($r = -0.26$; p (one-tailed) = 0.177), although not statistically significant. In other words, as carbohydrate is consumed in amounts that support

maximal glycogen synthesis rates, the addition of protein to the post-exercise supplement has no additive effect.

TABLE 2.2 EFFECT OF COMBINED PROTEIN-CARBOHYDRATE POST-EXERCISE FEEDING ON MUSCLE GLYCOGEN (>8 h recovery period)

Authors	*n*	Training	Recovery (h)	Ptn-CHO		CHO	Feeding Freq ($\cdot h^{-1}$)	Measure	Ptn-CHO		CHO Control		Outcome		
				Ptn g·kg^{-1}·h^{-1}	CHO g·kg^{-1}·h^{-1}	CHO g·kg^{-1}·h^{-1}			Mean	*SD*	Mean	*SD*	ES[a]	% difference	Inference[b]
(Zawadzki, et al. 1992)	9	Endurance trained	4	0.3	0.8	0.8	0.5	Glycogen µmol·g^{-1} protein·h^{-1} equiv to mmol·kg^{-1} dry wt·h^{-1}	35.5	9.90	25.6	6.90	1.30	38.7	large
(Tarnopolsky , et al. 1997)	16	Endurance trained	4	0.1	0.8	1	1	Glycogen change at 4 h mmol·kg^{-1} dry wt	103	-	149.4	53.6	-0.83	-31.2	moderate
(Carrithers, et al. 2000)	7	Endurance trained	4	0.2 Ptn	0.7	1	2	Glycogen mmol·kg^{-1} dry wt	118	92.0	107	63.0	0.15	10.3	trivial
	7	Endurance trained	4	0.1 AA	0.9	1	2	Glycogen mmol·kg^{-1} dry wt	87.0	95.0	107	63.0	-0.28	-18.7	small
(van Loon, et al. 2000b)	8	Endurance trained	5	0.4	0.8	0.8	2	mmol glycosol units·g^{-1} dry wt·h^{-1} equiv to mmol·kg^{-1} dry wt·h^{-1}	35.4	14.4	16.6	22.1	0.76	113	moderate
	8	Endurance trained	5	0.4	0.8	1.2	2	mmol glycosol units·g dry wt·h^{-1} (Isocaloric) equiv to mmol·kg^{-1} dry wt·h^{-1}	34.5	14.4	44.8	19.2	-0.48	-23.0	small

Authors	*n*	Training	Recovery (h)	Ptn-CHO		CHO	Feeding Freq ($\cdot h^{-1}$)	Measure	Ptn-CHO		CHO Control		Outcome		
				Ptn $g \cdot kg^{-1} \cdot h^{-1}$	CHO $g \cdot kg^{-1} \cdot h^{-1}$	CHO $g \cdot kg^{-1} \cdot h^{-1}$			Mean	*SD*	Mean	*SD*	ES[a]	% difference	Inference[b]
(Rotman, et al. 2000)	8	Moderately endurance trained	4	0.5	1.2	1.7	0.5	^{13}C magnetic resonance milliIU $\cdot min^{-1}$	2.44	1.36	2.33	0.960	0.10	4.72	trivial
(Jentjens, et al. 2001)	8	Endurance trained	3	0.4	1.2	1.2	2	Glycogen mmol $\cdot kg^{-1}$	252	127	225	62.0	0.39	12.0	small
(Ivy, et al. 2002)	7	Endurance trained	4	0.4	1.1	1.1	0.5	^{13}C-NMR mmol $\cdot L^{-1}$	88.8	11.6	70.0	10.6	1.54	26.9	large
	7	Endurance trained	4	0.4	1.1	1.5	0.5	^{13}C-NMR mmol $\cdot L^{-1}$	88.8	11.6	75.5	7.40	1.56	17.6	large
(Williams, et al. 2003)	8	Endurance trained	4	0.1	0.4	0.2	0.5	Glycogen µmol $\cdot g^{-1}$ dry wt at 4h equiv to mmol $\cdot kg^{-1}$ dry wt $\cdot h^{-1}$	159	50.9	69.0	90.5	0.88	130	moderate
(Berardi, et al. 2006)	6	Endurance trained	5	0.4	0.8	1.2	1	Glycogen resynthesis mmol $\cdot L^{-1}$ over 6 h recovery ^{13}C-NMR	28.6	2.10	22.2	1.19	4.54	28.9	enormous
(Betts, et al. 2008)	6	Endurance trained	4	0.3	0.8	0.8	2	Glycogen mmol glucosyl U $\cdot kg^{-1}$ dry wt	12.1	6.60	12.3	5.40	-0.03	-1.63	trivial

Authors	*n*	Training	Recovery (h)	Ptn-CHO		CHO	Feeding Freq ($\cdot h^{-1}$)	Measure	Ptn-CHO		CHO Control		Outcome		
				Ptn $g \cdot kg^{-1} \cdot h^{-1}$	CHO $g \cdot kg^{-1} \cdot h^{-1}$	CHO $g \cdot kg^{-1} \cdot h^{-1}$			Mean	*SD*	Mean	*SD*	ES[a]	% difference	Inference[b]
(Howarth, et al. 2009)	6	Recreationally active	4	0.4	1.2	1.2	4	Glycogen $mmol \cdot kg^{-1}$ dry $wt \cdot h^{-1}$	25.0	9.80	23.0	7.30	0.23	8.70	small
	6	Recreationally active	4	0.4	1.2	1.6	4	$mmol \cdot kg^{-1}$ dry $wt \cdot h^{-1}$	25.0	9.80	25.0	17.1	0.00	0.00	trivial

Abbreviations in order of occurrence: Ptn = protein; CHO = carbohydrate; wt = weight; AA = amino acids; ^{13}C NMR = nuclear magnetic resonance spectroscopy measurement of ^{13}C; LCHO = low carbohydrate; HCHO = high carbohydrate.

[a]Effect Size adjusted for sample size using the correction factor: 1-3/(4*(n-1)-1) multiplied by [b]Hopkins' modified Cohen's Inferences for outcome, where ES (mean difference/control SD) is classified as trivial 0.0–0.2, small 0.2–0.6, moderate 0.6–1.2, large 1.2–2.0, very large 2.0-4.0, enormous >4.0

2.1.4.3 Effects on Performance

Most studies have not found benefits from post-exercise protein-carbohydrate co-ingestion on subsequent performance compared to carbohydrate alone over the short term (Berardi, et al. 2008; Betts, et al. 2007; Betts, et al. 2005; Karp, et al. 2006; Millard-Stafford, et al. 2005). The characteristics of these studies are included in Table 2.3. While it is generally accepted that increased muscle glycogen leads to enhanced endurance capacity during moderate to high prolonged exercise (Bergstrom, et al. 1967; Bergstrom & Hultman 1967; Hawley, et al. 1997), data on both glycogen concentration and exercise performance (Berardi, et al. 2006; Williams, et al. 2003) are insufficient to draw firm conclusions about the role of protein-carbohydrate post-exercise nutrition. In one study, improved glycogen resynthesis did not lead to performance benefit (Berardi, et al. 2006), but average exercise intensity in this study, which was lower than others reviewed, might have meant that muscle glycogen was not limiting for performance (Berardi, et al. 2008).

Four studies report benefits to performance over the short-term (<8 h) with post-exercise protein-carbohydrate supplementation compared to carbohydrate alone (Berardi, et al. 2008; Betts, et al. 2007; Niles, et al. 2001; Williams, et al. 2003). However, in two of these the benefits appear to be related to increased energy content when protein was added to carbohydrate, rather than a protein effect *per se* (Betts, et al. 2007; Williams, et al. 2003). Williams, et al. (2003) found a moderate increase in glycogen synthesis (130.4%: ES 0.8) and corresponding large benefit to cycling endurance capacity, which equates to a 4.2% increase in mean power (ES 1.7). In this study a high-protein and carbohydrate supplement was compared to a commercially available carbohydrate and electrolyte sports drink. The sports drink was designed for fluid recovery, and contained inadequate amounts of carbohydrate and energy to maximise muscle glycogen synthesis, while the combined protein and carbohydrate beverage contained greater amounts of both. Therefore, the effects of the protein content on glycogen resynthesis and performance are difficult to distinguish from those of increased carbohydrate and energy. Extending this, Betts and co-workers (2007) compared the effects on treadmill-running capacity of combined protein-carbohydrate and carbohydrate only supplements taken after exercise. They found that increasing the energy content of the post-exercise

supplement benefited performance four hours later, regardless of the energy source (protein or carbohydrate). However, not all studies confirm these results: hypercaloric protein-rich nutrition after exercise did not always benefit performance compared to both adequate and sub-optimal carbohydrate for maximal glycogen synthesis (Betts, et al. 2005; Millard-Stafford, et al. 2005).

Two studies found improvements in performance when comparing protein-carbohydrate with isocaloric control containing adequate carbohydrate to maximise glycogen synthesis (Berardi, et al. 2008; Niles, et al. 2001). Niles et al. (2001) found a moderate improvement in performance when both post-exercise supplements were isocaloric and contained adequate amounts of carbohydrate. However, the protein-carbohydrate supplement contained much greater amounts of protein (0.6 $g \cdot kg^{-1} \cdot h^{-1}$) compared to other studies, and recovery times between exercise bouts were extremely short (2 h). Ingestion of protein in this quantity may have slowed absorption and release of glucose, so that the blood glucose profile had a lower peak but remained elevated for longer and benefited performance shortly after. Although peak glucose was apparently lower 30 minutes after the second protein-carbohydrate post-exercise supplement, this was not statistically significant.

Finally, Berardi et al. (2008) compared the effects of combined protein-carbohydrate with carbohydrate control on a 60-min cycling time-trial 6 h after an initial fatiguing time-trial. The authors found the decrease in power output during the second bout relative to the fatiguing bout was attenuated in the protein-carbohydrate condition. The power decrease during the second bout in the protein-carbohydrate condition was 76.6% (ES 0.21) of that experienced by the control condition. During the 6 h recovery period both groups consumed the same calories, which included isocaloric carbohydrate-protein or carbohydrate treatment beverage and an isocaloric macronutrient-balanced meal. In this study, post-exercise carbohydrate provision was considered to be optimal for maximising glycogen synthesis, but supplements were provided infrequently. Although glycogen concentration was not measured in this study, Berardi's group previously found the same post-exercise supplementation protocol enhanced the rate of muscle glycogen synthesis in the protein-carbohydrate condition relative to carbohydrate-control, but failed to benefit performance

(Berardi, et al. 2006). Differences between the performance outcomes of Berardi's two studies are likely due to a lower exercise intensity utilized for the performance test during the first study, and hence glycogen may not have been limiting for performance (Bergstrom & Hultman 1967; Romijn, et al. 1993).

Regardless of whether protein and carbohydrate ingested together enhances glycogen synthesis when carbohydrate content is suboptimal (1-1.2 $g \cdot kg^{-1} \cdot h^{-1}$) and feeding infrequent (≥1 h), the current evidence does not support benefits for subsequent performance. Previous reviews argued that disparate findings regarding the efficacy of protein-carbohydrate intake following exercise on accelerating glycogen synthesis and leading to enhanced subsequent performance may be related to four factors: energy balance between treatments, supplement carbohydrate content, frequency of ingestion, and prior glycogen depletion coupled with short recovery times (Ivy 2004; Millard-Stafford, et al. 2008). While this argument is sound in terms of glycogen resynthesis (as discussed above), recent results suggest a less definite trend in performance outcomes. Contrasting performance findings might additionally relate to the dependence of exercise modality, intensity, and duration on muscle glycogen stores. A further practical aspect is that in all studies reviewed subjects were exercised after fasting, so the influence of carbohydrate feeding before and during exercise on post-exercise supplementation is unknown.

TABLE 2.3 EFFECT OF ACUTE COMBINED PROTEIN-CARBOHYDRATE POST-EXERCISE FEEDING ON SHORT-TERM (<8 h) RECOVERY OF SUBSEQUENT PERFORMANCE

Authors	*n*	Treatment period (d)	Passive Recovery (h)	Ptn-CHO Supplement			CHO Supplement		Performance Protocol[a]	Ptn-CHO Performance		CHO Performance		Outcome		
				E kcal·kg^{-1}·h^{-1}	Ptn g·kg^{-1}·h^{-1}	CHO g·kg^{-1}·h^{-1}	E kcal·kg^{-1}·h^{-1}	CHO g·kg^{-1}·h^{-1}		Mean	*SD*	Mean	*SD*	% difference	ES	Inference
(Niles, et al. 2001)	M=10	1	2	8.4	0.6	1.5	8.4	2.1	Endurance trained; Treadmill Running; Time (s); Time to fatigue; Steady State ~88%VO_{2max}; Lab.	541	91.6	447	97.1	1.5	0.89[e]	moderate
(Williams, et al. 2003)	M=8	1	4	3.8	0.1	0.4	1.0	0.2	Endurance trained; Cycling; Time (min); Time to fatigue; Steady State 85%VO_{2max}; Lab.	31.1	9.10	20.0	5.70	4.2	1.73[e]	large
(Betts, et al. 2005)[h]	M=9	1	4	5.6	0.2	1.2	4.8	1.2	Endurance trained; Treadmill running; Time (min); Time to Fatigue; Steady State 85% VO_{2max}; Lab.	18.0	-	14.5	-	1.8	-	-
	M=9	1	4	3.7	0.1	0.8	3.2	0.8		19.5	-	18.0	-	0.6	-	-
(Millard-Stafford, et al. 2005)	M=3 F=5	1	2	4.0	0.2	0.8	2.4	0.6	Endurance trained; Treadmill running; Time (s); Time to Fatigue; Steady state 90% VO_{2max}; Lab.	31.1[f]	1.67	35.6[f]	3.89	-0.9	-1.03[e]	moderate
	M=3 F=5	1	2	4.0	0.2	0.8	4.0	1.0		31.1[f]	1.67	36.7[f]	2.78	-1.1	-1.79[e]	large

Authors	*n*	Treatment period (d)	Passive Recovery (h)	Ptn-CHO Supplement			CHO Supplement		Performance Protocol[a]	Ptn-CHO Performance		CHO Control Performance		Outcome		
				E kcal·kg^{-1}·h^{-1}	Ptn g·kg^{-1}·h^{-1}	CHO g·kg^{-1}·h^{-1}	E kcal·kg^{-1}·h^{-1}	CHO g·kg^{-1}·h^{-1}		Mean	*SD*	Mean	*SD*	% difference	ES	Inference
(Berardi, et al. 2006)	M=6	1	6	4.8	0.4	0.8	4.8	1.2	Endurance trained; Wind trainer Cycling; Distance (km); time trial 60 min; Lab.	36.3	0.830	36.4	0.800	-0.2	-0.07	trivial
(Karp, et al. 2006)	M=9	1	4	5.2	0.3	1.0	1.5	0.4	Endurance trained; Cycling; Total Power (kJ); Time to Fatigue; Steady state 70% VO_{2max}; Lab.	627	263	591	219	0.6	0.16	trivial
	M=9	1	4	5.2	0.3	1.0	1.5	0.4		399	185	591	219	-3.0	-0.88	moderate
(Betts, et al. 2007)	M=6	1	4	4.3	0.3	0.8	4.3	1.1	Endurance trained; Treadmill running; Time (min); Run to fatigue; Steady state 70%VO_{2max}; Lab.	91.2	15.8	99.9	19.9	-0.8	-0.37[e]	small
	M=6	1	4	4.3	0.3	0.8	3.2	0.8		91.2	15.8	83.7	16.9	0.8	0.37[e]	small
(Berardi, et al. 2008)[g]	M=7T M=8C	1	6	4.8	0.4	0.8	4.8	1.2	Endurance trained; Cycling; Power (W); 60 min time trial; Lab.	3.86	17.1	16.5	56.3	76.6	0.21	small

Abbreviations & acronyms: Exp = experiment; CHO = carbohydrate; Ptn = Protein; Lab = controlled laboratory conditions; Field = field test; T= treatment group C = control group; M = men, F = women, E = energy.

[a]Mode = cycling or running; Output = power, distance, time; Test type = time trial, sprint intervals, time to fatigue; State = steady state, intervals, incremental increase; Test = lab or field.

[b]Metabolic state indicates whether the performance test was performed in fasted or fed state.

[c]Effect Size adjusted for sample size using the following correction factor =1-3/(4*(n-1)-1) multiplied by [d]Hopkins' modified Cohen's effect size, where ES (mean difference/control SD), and inferences classified as trivial 0.0–0.2, small 0.2–0.6, moderate 0.6–1.2, large 1.2–2.0, very large 2.0-4.0, enormous >4.0

[e]Estimated change in mean power from time trials= [(T-C/C)*100] * (6.4/80%VO_{2max}) (Hopkins, et al. 1999).

[f]Mean values taken from graph (Millard-Stafford, et al. 2005).

[g]Performance change from morning baseline trial (Berardi, et al. 2008).

[h]Effect size unable to be calculated for median, non-parametric data without additional information (Leech & Onwuegbuzie 2002).

2.1.5 LONGER-TERM RECOVERY STUDIES (>8 h recovery period)

2.1.5.1 Attenuation of Exercise-induced Damage

Several authors have suggested that adding protein to post-exercise nutrition attenuates exercise-induced muscle damage (Luden, et al. 2007; Millard-Stafford, et al. 2005; Romano-Ely, et al. 2006; Saunders, et al. 2004); however, none of these authors have indicated a mechanism for this. Possible mechanisms might include the direct actions of amino acids such as cysteine and arginine increasing inhibition of the Ca^{2+}-calpain proteolytic pathway (Helman, et al. 2003) and the sulphur containing amino acids (methionine, taurine and cysteine) scavenging reactive oxygen species damage (Atmaca 2004; Metayer, et al. 2008; Moskovitz, et al. 2001) and enhancing antioxidant defences (Grimble & Grimble 1998). Protein may also act indirectly through amino acid metabolites such as beta-hydroxy beta-methylbutyrate (HMB and α-ketoisocaproate (KIC), suppression of the ubiquitin proteasome system (Smith et al 2005).

Several researchers measured serum elevations of the intracellular enzyme creatine kinase (CK); one group (Romano-Ely, et al. 2006), also measured lactate dehydrogenase (LDH). Serum activities of these two enzymes are generally low, but rise after membrane damage and increased membrane permeability. Most researchers found small to enormous reductions in serum CK levels with protein-carbohydrate supplementation after exercise compared to carbohydrate alone (Cade, et al. 1991; Luden, et al. 2007; Millard-Stafford, et al. 2005; Romano-Ely, et al. 2006; Rowlands, et al. 2008; Rowlands, et al. 2007; Saunders, et al. 2004); Romano-Ely et al. (Romano-Ely, et al. 2006) also reported moderate attenuation of LDH (Romano-Ely, et al. 2006). But, in contrast, Rowlands and Wadsworth (2010) reported trivial effects on serum CK in female athletes when supplementing protein-carbohydrate compared to carbohydrate after exercise. This lowered beneficial action could have been due to increased membrane stability provided by oestrogen, limiting CK leakage (Carter et al. 2001) and making any differences from supplementation difficult to determine. Cade et al. (1991), using estimates from graphed results, also found trivial change in plasma CK activities in women compared with small change in male swimmers following the same training and supplementation protocols.

Several groups added antioxidant vitamins to the protein-rich carbohydrate post-exercise supplement, therefore potentially masking the beneficial effects of protein (Luden, et al. 2007; Millard-Stafford, et al. 2005; Romano-Ely, et al. 2006); nonetheless, the data overwhelmingly indicate lower serum levels of intracellular proteins with protein and carbohydrate feeding post-exercise relative to carbohydrate.

On the other hand, evidence from biochemical markers of ROS damage indicates no effect of protein-carbohydrate supplementation after exercise compared to carbohydrate only. For example, Rowlands et al. (2008) found inconclusive effects of protein-rich post-exercise nutrition on malonyldialdehyde (MDA), which is indicative of lipid peroxidation. What is more surprising, Romano-Ely et al. (2006) found protein carbonyl concentrations, which indicate protein oxidation, increased moderately when protein, carbohydrate and antioxidants were supplemented after exercise, compared to carbohydrate only. In this case, the antioxidants provided post-exercise might in fact act as oxidants, thereby increasing ROS-induced damage (Romano-Ely, et al. 2006).

Feeding after exercise might also mitigate damage by providing energy and amino acids as substrates for the inflammatory and immune responses to muscle damage (Flakoll, et al. 2004); for example, branched chain amino acids taken after prolonged exercise have been found to divert lymphocyte immune response towards a Th1 type of response (Bassit, et al. 2002; Negro, et al. 2008). However, data on potential interactions between fuels (glucose, glutamine, and fatty acids) of immune system cells are contradictory (Calder 1995; Wolowczuk, et al. 2008). When combined protein and carbohydrate after exercise was compared to a carbohydrate control, the effects on markers of inflammatory and immune response were ambiguous. Rowlands et al. (2008) found only inconclusive or trivial differences in inflammatory markers Interleukin-6 (IL-6), C-reactive protein (CRP), and tumour necrosis factor-α (TNF-α). But Romano-Ely et al. (2006) found a protein-carbohydrate-antioxidant supplement ingested after exercise caused large increases in IL-6 compared to carbohydrate only. Although, the IL-6 cytokine is thought to have a stronger relationship with glucose homeostasis regulation than inflammation (Helge, et al.

2003). Thus, evidence of post-exercise protein-carbohydrate benefit to immune response compared to carbohydrate only, is indeterminate.

Muscle damage has been inferred from subjective ratings of perceived delayed onset muscle soreness (DOMS), and results show small to very large decreases (37-67%) in perceived soreness with protein-carbohydrate-antioxidant supplements compared to carbohydrate following exercise (Luden, et al. 2007; Millard-Stafford, et al. 2005; Romano-Ely, et al. 2006). Nonetheless, all of the groups reviewed who have measured perceived soreness also added antioxidant vitamins to the protein-rich post-exercise supplement. Since some evidence suggests post-exercise vitamin antioxidants act to decrease the perception of muscle soreness after exercise (Kaminski & Boal 1992) the addition of antioxidant vitamins may have masked the potential beneficial effects of protein.

To summarise, results from most studies show post-exercise ingestion of protein and carbohydrate attenuates the rise in intracellular enzymes (CK, LDH) in circulation. The use of this measure is limited by an inability to distinguish between CK leakage into circulation and reticulo-endothelial system clearance (Hyatt & Clarkson 1998). The mechanism underlying attenuated CK activity is unknown; but likely to result from enhanced repair and protein synthesis. The mechanism is unlikely to result from enhanced antioxidant defence, for two reasons: first, collective results indicate adding antioxidant vitamins to the protein-carbohydrate supplement do not additionally influence peak CK activity; second, protein-carbohydrate supplementation after exercise does not benefit markers of ROS damage (Romano-Ely, et al. 2006; Rowlands, et al. 2008). Furthermore, evidence of post-exercise protein-nutrition benefit to exercise-induced immune and inflammatory response is ambiguous; and the effect on Ca^{2+}-calpain and ubiquitination-proteasome pathways undefined.

2.1.5.2 Effects on Performance

Most studies have found no beneficial effect on performance outcome of combined protein-carbohydrate ingestion after exercise compared to carbohydrate alone (Millard-Stafford, et al. 2005; Romano-Ely, et al. 2006; Rowlands, et al. 2007; Rowlands & Wadsworth 2010); Table 2.4). Several studies have reported protein and

carbohydrate taken together after exercise mitigates exercise-induced damage and/or enhances repair, but this did not improve subsequent performance (Luden, et al. 2007; Millard-Stafford, et al. 2005; Romano-Ely, et al. 2006; Rowlands, et al. 2007; Rowlands & Wadsworth 2010). The inconsistency between the mainly beneficial effects on markers of muscle damage and the equivocal performance improvement could be related to low levels of training-induced functional impairment or inadequate time for repair of damage and recovery of function given the barely adequate energy content of the background diet. More than likely though is the fact that plasma CK activities correlate poorly with impaired muscle function (Warren, et al. 1999).

Luden et al. (2007) suggested low levels of damage to muscle and no impairment of function resulted in limited opportunity for supplement effect. Twenty three cross-country runners were given a protein-carbohydrate-antioxidant beverage (458 kcal, 88 g carbohydrate, 21.1 g protein, 1.8 g fat on average) or a control (370 kcal, 88 g carbohydrate, 1.8 g fat on average) after exercise in a cross-over design. Background diet and training were standardized and replicated but the field-based cross country trials were performed over two different courses, and in different environmental conditions; moreover, to determine performance difference, running times were rated according to average running time of other competitors. This approach has two problems: first, energy and antioxidants added to the protein condition mask the potential beneficial ingredient; second, this system of comparing performance outcomes may introduce errors if race entrants were not identical. Nevertheless, over 6 days of training, protein-carbohydrate-antioxidant supplementation after exercise did not improve performance compared to carbohydrate after exercise. Interestingly, as training mileage increased there was a significant correlation between plasma CK activities and beneficial effect of protein-carbohydrate treatment on subsequent performance. The authors suggest higher mileage runners would have experienced more damage, thereby offering greater opportunity for recovery with protein-rich post-exercise feeding. To determine if this argument is consistent with the literature (Table 2.4), plasma CK activity was correlated with performance improvement in treatment vs. control post-exercise feeding. Although the difference in plasma CK activity between conditions was found to poorly correlate with performance

improvement (r = 0.037; p (one-tailed) = 0.462); the degree of plasma CK activity induced by damaging exercise had a small positive trend, with protein-carbohydrate benefit to subsequent performance (r = 0.25; p (one-tailed) = 0.258), but not statistically significant.

A series of studies by Rowlands' group (Rowlands, et al. 2008; Rowlands, et al. 2007; Rowlands & Wadsworth 2010) investigated the effects of protein-rich supplements after exercise using identical supplemental schema, repeated sprint cycling performance tests, and intense interval cycling bouts to model fatiguing training. In these cross-over studies, subjects with a background diet high in energy and carbohydrate were fed during a 4-h recovery period after exercise with either protein-carbohydrate (1.60 g carbohydrate, 0.80 g protein and 0.29 g fat·kg^{-1} FFM·h^{-1}) or a control (2.35 g carbohydrate and 0.29 g fat·kg^{-1} FFM·h^{-1}; same calorific content). Repeated sprint cycling performance showed no clear benefit by 15 h post-exercise (Rowlands, et al. 2008; Rowlands, et al. 2007) but a substantial benefit with an additional 45 h (60 h from the original damaging exercise) in men (Rowlands, et al. 2008) but not women (Rowlands & Wadsworth 2010). The authors concluded the delayed effect may be related to the time required for repair and recovery; however, this does not explain why only men benefited.

To summarise, several of these studies have shortcomings, including unrealistic hypocaloric dietary designs (Millard-Stafford, et al. 2005), lack of controlled background diet and training (Millard-Stafford, et al. 2005; Romano-Ely, et al. 2006), and imbalanced daily and supplement-delivered energy intakes between supplement and control which might mask the effects of protein taken after exercise (Luden, et al. 2007; Millard-Stafford, et al. 2005). Furthermore, gender may affect the markers of recovery (CK and perception of soreness) and performance; this has not been considered in some mixed-gender studies (Luden, et al. 2007; Millard-Stafford, et al. 2005). However, two interesting trends emerge: performance benefits seem more likely with protein-rich feeding after exercise when training load is high (Luden, et al. 2007), and when passive recovery period is increased to 45 h (45 h from previous performance test and 60 h from the original exhausting exercise) (Rowlands, et al. 2008). However, these theories are currently unsubstantiated and

require further research. Although markers of muscle membrane damage and perceived soreness apparently decrease, protein with carbohydrate fails to deliver better performance than carbohydrate alone, following 15-24 h recovery, and particularly so in the female or mixed-gender population. The conditions under which protein taken with carbohydrate after exercise can improve performance require further study.

TABLE 2.4 EFFECT OF ACUTE COMBINED PROTEIN-CARBOHYDRATE POST-EXERCISE FEEDING ON LONGER-TERM (> 8h) RECOVERY OF SUBSEQUENT PERFORMANCE

Authors	*n*	Treatment period (d)	Passive Recovery (h)	Ptn-CHO Supplement			CHO supplement		Metabolic State[b]	Performance Protocol[a]	Ptn-CHO Performance		CHO Control Performance		Outcome		
				E kcal·kg^{-1}·h^{-1}	Ptn g·kg^{-1}·h^{-1}	CHO g·kg^{-1}·h^{-1}	E kcal·kg^{-1}·h^{-1}	CHO g·kg^{-1}·h^{-1}			Mean	SD	Mean	SD	% difference	ES	Inference
(Millard-Stafford, et al. 2005)	M=3 F=5	1	24	4.0	0.2	0.8	2.4	0.6	Fasted	Endurance trained; Running; Time (s); Time trial 5 km; Field.	1100	36.6	1110	37.3	-0.9	-0.24	small
	M=3 F=5	1	24	4.0	0.2	0.8	4.0	1.0	Fasted		1100	36.6	1120	36.5	-1.6	-0.44	small
	M=3 F=5	1	24	4.0	0.2	0.8	2.4	0.6	Fasted		1070	48.0	1030	37.0	3.7	0.91	unclear
	M=3 F=5	1	24	4.0	0.2	0.8	4.0	1.0	Fasted		1070	48.0	1090	55.0	-1.7	-0.31	small

Authors	*n*	Treatment period (d)	Passive Recovery (h)	Ptn-CHO Supplement			CHO supplement		Metabolic State[b]	Performance Protocol[a]	Ptn-CHO Performance		CHO Control Performance		Outcome		
				E kcal·kg^{-1}·h^{-1}	Ptn g·kg^{-1}·h^{-1}	CHO g·kg^{-1}·h^{-1}	E kcal·kg^{-1}·h^{-1}	CHO g·kg^{-1}·h^{-1}			Mean	*SD*	Mean	*SD*	% difference	ES	Inference
(Romano-Ely, et al. 2006)	M=14	1	22	7.8	0.4	1.5	7.8	1.5	Fed	Endurance trained; Cycling; Time (min); time to fatigue; Steady state at 80%VO_{2max}; Lab.	42.9	21.8	42.3	18.6	0.1	0.03[e]	trivial
(Luden, et al. 2007)	M=11 F=12	6	24	7.6	0.4	1.5	6.2	1.5	Fed	Endurance trained; Cross Country running; Ranked times; Field.	4.80	2.50	4.70	2.50	0.2	0.04	trivial
(Rowlands, et al. 2007)[i]	M=10	1	15	10.8	0.7	1.4	10.8	2.1	Fed	Endurance trained; Cycling; Power (W); Sprint Intervals; Lab.	332	-	328	13.0	1.1	0.25	unclear
(Rowlands, et al. 2008) Day 2[i]	M=10	3	15	10.8	0.7	1.4	10.8	2.1	Fed	Endurance trained; Cycling; Power (W); Sprint Intervals; Lab.	318	17.0	318	16.0	0.0	0.00	trivial
Day 4[i]	M=10	3	45	10.8	0.7	1.4	10.8	2.1	Fed		351	17.0	338	16.0	3.8	0.74	moderate

Authors	*n*	Treatment period (d)	Passive Recovery (h)	Ptn-CHO Supplement			CHO supplement		Metabolic State[b]	Performance Protocol[a]	Ptn-CHO Performance		CHO Control Performance		Outcome		
				E kcal·kg^{-1}·h^{-1}	Ptn g·kg^{-1}·h^{-1}	CHO g·kg^{-1}·h^{-1}	E kcal·kg^{-1}·h^{-1}	CHO g·kg^{-1}·h^{-1}			Mean	*SD*	Mean	*SD*	% difference	ES	Inference
(Rowlands & Wadsworth 2010) Day 2[i]	F=12	4	15	9.7	0.7	1.3	9.7	1.9	Fed	Endurance trained; Cycling; Power (W); Sprint Intervals; Lab.	234	-	237	49.8	-1.3	-0.06	trivial
Day 4[i]	F=12	4	45	9.7	0.7	1.3	9.7	1.9	Fed		250	-	245	49.8	2.0	0.09	trivial

Abbreviations & acronyms: Exp = experiment; CHO = carbohydrate; Ptn = Protein; Lab = controlled laboratory conditions; Field = field test; T= treatment group C = control group; M = men, F = women, E = energy.

[a]Mode = cycling or running; Output = power, distance, time; Test type = time trial, sprint intervals, time to fatigue; State = steady state, intervals, incremental increase; Test = lab. or field.

[b]Metabolic state indicates whether the performance test was performed in fasted or fed state.

[c]Effect Size adjusted for sample size using the following correction factor =1-3/(4*(n-1)-1) multiplied by [d]Hopkins' modified Cohen's effect size, where ES (mean difference/control SD), and inferences classified as trivial 0.0–0.2, small 0.2–0.6, moderate 0.6–1.2, large 1.2–2.0, very large 2.0-4.0, enormous >4.0

[e]Estimated %change in mean power from time trial= [(T-C/C)*100] * (6.4/VO_{2max}) (Hopkins, et al. 1999).

Confidence limits for the within-study effects are not presented because of lack of necessary reported statistical information for most studies

[i]Supplement values converted from fat free mass to body weight so equivalent to other studies.

2.1.5.3 Protein synthesis and repair

The first researchers to describe the importance of immediate rather than delayed protein and carbohydrate supplementation on the rate of protein synthesis after prolonged exercise were Levenhagen et al. (2001). They found greater glucose and amino acid uptake into the leg muscle and increased protein synthesis compared with supplementation 3 h later. Levenhagen et al. (2002) and Howarth et al. (2009) also investigated the effect of protein taken with carbohydrate after prolonged exercise versus traditional post-exercise carbohydrate supplementation. Both groups found increased rates of protein synthesis in muscle when protein was added to carbohydrate (Levenhagen, et al. 2002) or to isocaloric carbohydrate (Howarth, et al. 2009). Later studies found when daily energy and macronutrient intakes are equal, protein-rich supplementation soon after exercise leads to a more positive body protein balance compared to delayed feeding (Roy, et al. 2002) and isocaloric carbohydrate feeding (Rowlands, et al. 2008; Rowlands & Wadsworth 2010).

The mechanisms regulating muscle protein turnover include gene transcription, translation of new and existing mRNA, and mRNA degradation. These mechanisms respond to numerous stimuli including exercise (Fluck & Hoppeler 2003; Hood, et al. 2006; Yu, et al. 2003) and nutrient interrelated signalling cascades (Anthony, et al. 2000; Crozier, et al. 2005; Fujita, et al. 2007; Greenhaff, et al. 2008; Smith, et al. 2008). The mammalian target of rapamycin complex 1 (mTORC1), (comprising raptor, G-protein β-subunit-like protein (GβL) and ras homolog enriched in brain (RHEB)), is the key regulator of translational control and acts as a convergence point for nutrients, hormones and exercise. Translation initiation and elongation are enhanced by p70 ribosomal S6 kinase (p70S6K) and eukaryotic initiation factor 4E binding protein 1 (4E-BP1), downstream of mTOR (Kimball 2006). The key signalling pathways leading to protein translation and described in this review are represented diagrammatically in Figure 2.2.

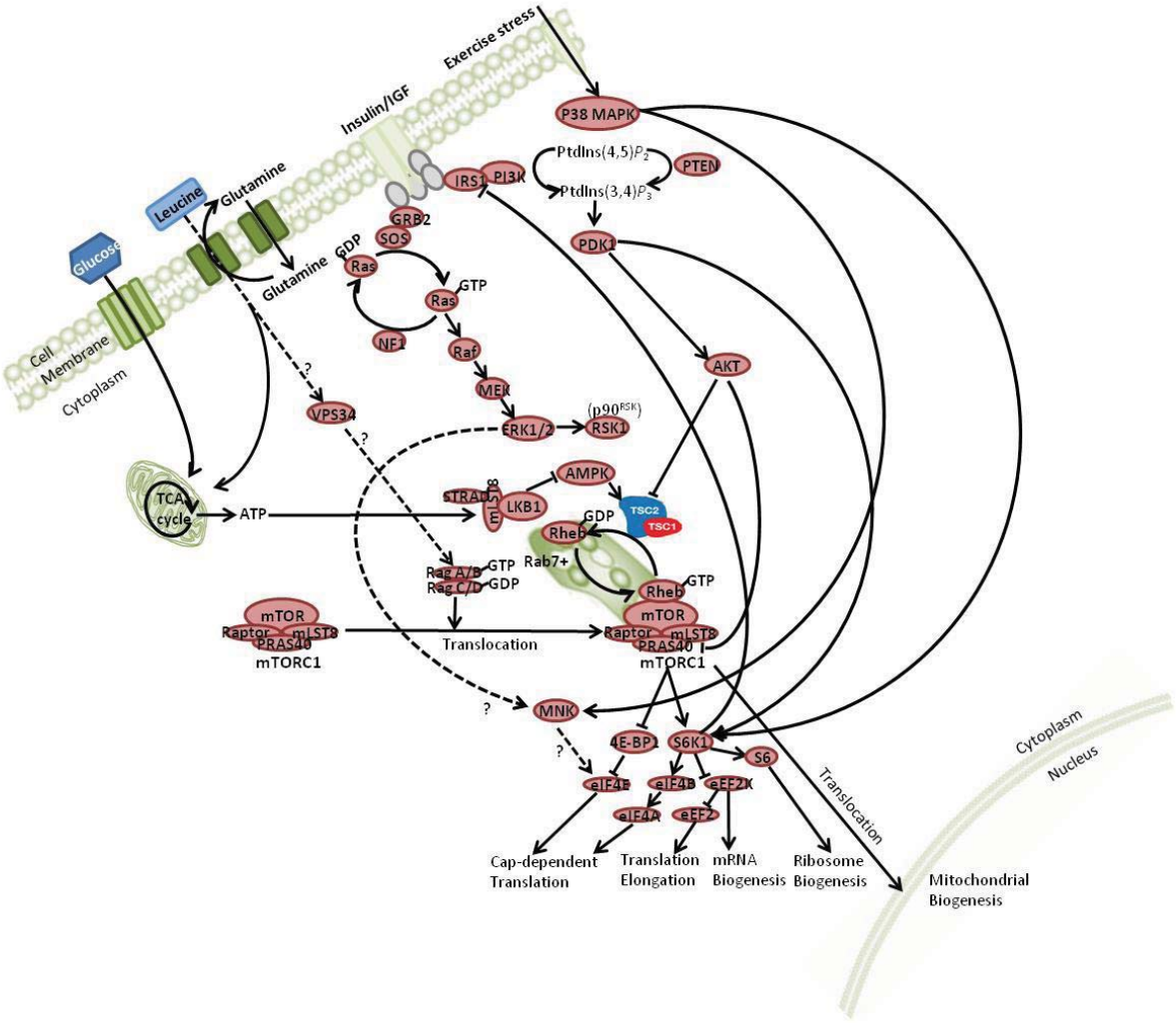

Figure 2.2 Hypothesized regulation of transcript initiaition and elongation induced by post-exercise ingestion of protein and carbohydrate combined.

Adapted from (Kim, et al. 2008; Kimball 2006; Laplante & Sabatini 2009; Roux & Blenis 2004; Ruvinsky & Meyuhas 2006; Sancak, et al. 2008).

Protein synthesis is blunted during prolonged exercise (Deshmukh, et al. 2008; Rose, et al. 2009a; Rose, et al. 2009b) and remains depressed initially following exercise (Gautsch, et al. 1998). During fasting, protein synthesis increases slightly (Carraro, et al. 1990; Sheffield-Moore, et al. 2004); this is induced by the MAPK-ERK1/2-p90^{RSK}-RPS6 pathway (Hawley, et al. 2006; Ivy, et al. 2008; Mascher, et al. 2007), or is related

to glucose, produced by gluconeogenesis, stimulating insulin-mTOR signalling (Morrison, et al. 2008). Until adequate energy and protein are provided, protein turnover remains negative (Norton & Layman 2006).

The main agents inducing protein synthesis after feeding are amino acids and insulin. Insulin acts through IRS1-PI3K-AKT signalling to activate mTOR and consequently stimulate signalling to translation initiation and elongation. AKT inactivates the tumour suppressor complex TSC1-TSC2, thereby releasing its inhibition of RHEB, and allowing mTOR activity (Harrington, et al. 2004). AKT also phosphorylates and inactivates the proline-rich AKT substrate 40 kDa (PRAS40), releasing its inhibition of mTOR activity (Haar, et al. 2007; Sancak, et al. 2007). Insulin also stimulates translation initiation independently from mTOR; IRS1-PI3K inhibits GSK3, which reduces its inhibition of eukaryotic initiation factor 2B (eIF2B) (Greenhaff, et al. 2008; Hawley, et al. 2006; Miyazaki & Esser 2009). Amino acids act on mTOR by TSC1-TSC2 dependent and independent pathways (Bolster, et al. 2004b; Gao, et al. 2002; Meyuhas 2000). Essential amino acids stimulate mTOR activity through RagA/B-RagC/D complex binding to raptor and by mediating mTORC1 relocalisation to endomembrane system rab7+ vesicles, therefore allowing activation by Rheb (Avruch, et al. 2009; Gwinn, et al. 2008; Kim, et al. 2008; Sancak, et al. 2008). Amino acids might also act on the human vacuolar protein sorting 34 (hVPS34) and its partners, VPS15 and Beclin, which contribute to rab 7+ vesicle trafficking (Gwinn, et al. 2008). Lastly, when nutrient availability increases, ATP consequently increases, and AMPK activation decreases, subsequently permitting mTOR activity (Deshmukh, et al. 2008; Gwinn, et al. 2008; Yu, et al. 2003). Thus there are several potential mechanisms whereby consumption of combined protein-carbohydrate following prolonged exercise act to increase protein translation converging at mTOR.

Comparisons of protein-carbohydrate vs. carbohydrate ingestion after exercise in animals (Anthony, et al. 1999; Gautsch, et al. 1998; Morifuji, et al. 2009a; Morrison, et al. 2008) and humans (Kammer, et al. 2009) indicate increased phosphorylation and activation of several of the proteins involved in mTOR signalling cascades. In

endurance-trained humans, Ivy et al. (2008) found supplementation with protein-carbohydrate after 45 min of intense cycling increased AKT, mTOR, and RPS6 phosphorylation compared to a non-caloric placebo. Another study by this group (Kammer, et al. 2009) compared consumption of cereal and milk to a carbohydrate beverage after prolonged, moderate intensity cycling by fasted subjects. Phosphorylation of AKT and mTOR increased; however, protein-carbohydrate feeding provided greater energy than the carbohydrate control or fasting, and this may have exaggerated the role of energy rather than protein on signalling pathways leading to protein synthesis.

Clearly, activation of proteins involved in signalling to protein synthesis increases when protein-carbohydrate ingestion follows endurance exercise, at least compared to fasting and hypocaloric carbohydrate nutrition. However, these results do not clarify the influence of post-exercise energy imbalance on ATP:ADP-AMPK-mTOR signalling (Deshmukh, et al. 2008; Gwinn, et al. 2008; Yu, et al. 2003), nor do they clarify the effects of dissimilar exercise protocols on regulation of protein synthesis by MAPK-ERK1/2-p90^{RSK}-RPS6 (Hawley, et al. 2006; Ivy, et al. 2008; Mascher, et al. 2007; Widegren, et al. 2000). More importantly, these data do not show which muscle protein fraction is synthesised, or how the increase in protein synthesis relates to enhanced functional recovery.

2.1.5.4 Adaptive Processes

A single bout of prolonged exercise produces chemical and mechanical stresses in skeletal muscle; these stresses act as potent stimuli which are sensed and transmitted through signalling cascades like Calcinerurin-CaMK-PKC, MAPKs and AMPK; these in turn regulate gene expression through downstream nuclear and mitochondrial transcription components (Hawley, et al. 2006; Koulmann & Bigard 2006; Wackerhage & Woods 2002; Yu, et al. 2003). Most transcription is inhibited during exercise but expression increases greatly during recovery (Coffey, et al. 2007; Mahoney, et al. 2005; Pilegaard, et al. 2005; Yang, et al. 2005). The generally accepted model of exercise-induced adaptation involves peaks in transcript expression following each bout of exercise and subsequent translation of their encoded proteins; repeated bouts lead to

accumulation of proteins, resulting in a change in phenotype. This theory of adaptation to endurance training is represented in Figure 2.3.

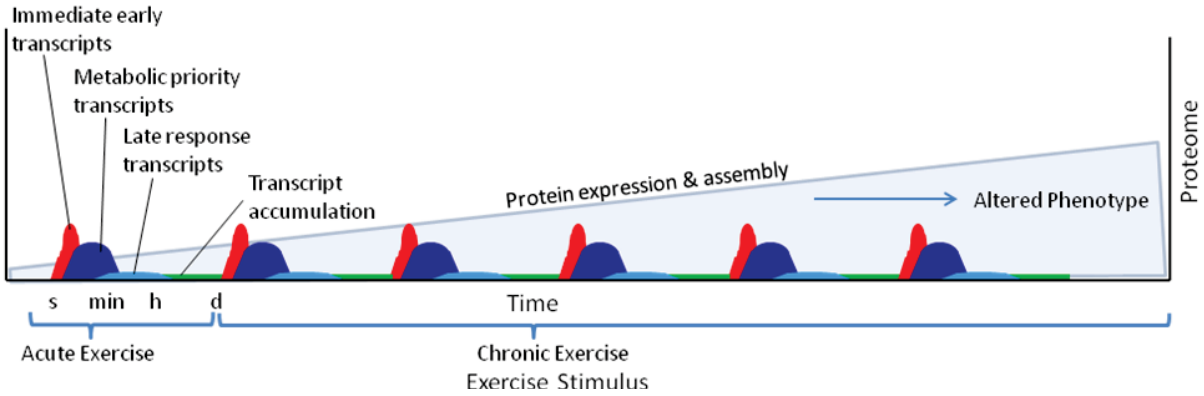

Figure 2.3 Hypothesized transcript and protein accumulation resulting in phenotype change with successive bouts of endurance exercise

Adapted from (Booth & Neufer 2005; Fluck 2006; Hawley, et al. 2006; Mahoney, et al. 2005).

Booth and Neufer (2005) further classify exercise-induced transcripts into three groups, depending on the duration of enhanced expression and their encoded protein products. Immediate early transcripts are transient and return to baseline levels within minutes; these transcripts are thought to be involved in regulation of transcriptional and stress responses to the exercise stimulus (Hoppeler & Fluck 2002). Metabolic priority genes are expressed at high levels, peak within a few hours after exercise and return to pre-exercise levels within 24 h. Genes whose protein products have mitochondrial and other metabolic roles have lower mRNA peaks but persist longer and are therefore thought to play a key role in muscle adaptation (Booth & Neufer 2005). Peaks in transcripts maintained over prolonged periods not only accumulate with successive exercise bouts, but translation and accumulation of their protein products also increase towards higher steady-state concentrations (Booth & Neufer 2005; Fluck 2006; Hawley, et al. 2006; Mahoney, et al. 2005).

Numerous studies show the effect of dietary macronutrients (carbohydrates, fatty acids and sterols) and micronutrients (minerals and vitamins) on transcription regulation induced by prolonged exercise (Cluberton, et al. 2005; Towle 1995). Pilegaard et al.

(2005) compared the effect of high and low carbohydrate availability after prolonged cycling and found high carbohydrate intakes retarded the exercise-induced increase in gene expression involved in glucose and lipid uptake and metabolism. However, none of these studies investigated the addition of protein to carbohydrate after exercise. Therefore the prolonged exercise and protein nutrition interaction on gene expression is largely unknown.

2.1.5.5 Potential Performance Effect

No studies directly investigated the effect of protein and carbohydrate-rich nutrition after exercise on exercise-induced adaptive processes and later performance. However, the protocol timelines in one study (Rowlands, et al. 2008) might suggest a relationship between post-exercise nutrition and transcriptional and translational response, leading to an improved functional phenotype. For example, Rowlands et al. (2008) found protein-carbohydrate co-ingestion after exercise had no effect on performance relative to carbohydrate alone at 15 h, but benefited performance 45 h later (by 60 h). This delay is suggestive of the recovery time required for re-establishment of homeostasis, as well as initiation of adaptative mechanisms. This might fit with the time course for adaptation found in previous studies utilizing aerobic intervals and prolonged exercise training. For example, adaptation begins immediately after exercise, and the accumulation of new proteins can be measured within a few days of training (Booth & Holloszy 1977; Ren, et al. 1994; Spina, et al. 1996). Further, adaptive changes result in enhanced aerobic performance after as few as 6 bouts of interval training (Burgomaster, et al. 2005; Gibala, et al. 2006).

2.1.6 CONCLUSION AND DIRECTIONS FOR FURTHER RESEARCH

The greatest potential for performance improvements lies not in superior glycogen availability, or mitigation of damage, but in processes that enhance repair and that are facilitated by nutrition. Supplements rich in protein and carbohydrate taken after exercise may act as substrates and signals to initiate the processes of synthesis and repair, as well as signals mediating exercise-induced gene expression.

Most studies have focused on acute short-term timelines: namely, whether ingesting protein and carbohydrate after exercise increases muscle glycogen stores and therefore benefits same-day performance. When carbohydrate intake is low, and feeding infrequent, glycogen resynthesis benefits from protein and carbohydrate taken together after exercise. However, many of these studies were designed to favour the likelihood of performance benefit, for example subjects performed exercise in a glycogen depleted or fasted state, whereas this is not reflective of current practices of endurance athletes and results might lack ecological validity. Therefore further research set in a background of adequate daily energy, protein and carbohydrate intakes, is warranted.

Protein and carbohydrate taken together after exercise have consistently been shown to attenuate the release of intracellular enzymes (CK, LDH) and reduce muscle soreness. This probably results from faster repair processes; however, over most experimental time frames subsequent performance does not improve. Therefore, further research should focus on integrating longer recovery times and/or including passive rest days to allow optimum recovery.

Furthermore, this review has identified a large gap in the literature regarding the effects of protein-rich nutrition after exercise on adaptive mechanisms. The importance of these nutrients on protein synthesis after endurance exercise is becoming clear, and the signalling pathways stimulated by a combination of endurance exercise, amino acids and insulin are rapidly being characterised, although mostly in animals. Finally, the exercise-induced transcriptome facilitated by nutrition and involved in functional adaptation to exercise training remain largely unknown.

CHAPTER 3 STUDY 1

ACCEPTED: Applied Physiology, Nutrition, and Metabolism

3.1 LEUCINE-PROTEIN SUPPLEMENTED RECOVERY FEEDING ENHANCES SUBSEQUENT CYCLING PERFORMANCE IN WELL-TRAINED MEN

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Running Head: Leucine-protein enriched recovery feeding and performance

Key Words: muscle damage, mood state, nitrogen balance, creatine kinase

3.1.1 ABSTRACT

The purpose of this study was to determine whether a practical leucine-protein high-carbohydrate post-exercise feeding regimen could improve recovery, as measured by subsequent cycling performance and mechanistic markers, relative to control feeding. In a crossover, 10 male cyclists performed 2-2.5 h interval training bouts on 3 consecutive evenings ingesting either leucine-protein high-carbohydrate (0.1/0.4/1.2/0.2 $g \cdot kg^{-1} \cdot h^{-1}$ leucine/protein/carbohydrate/fat) or isocaloric control (0.06/1.6/0.2 $g \cdot kg^{-1} \cdot h^{-1}$ protein/carbohydrate/fat) nutrition for 1.5 h post-exercise. Throughout the experimental period diet was controlled, energy and macronutrient intake balanced, and protein intake clamped at 1.6 $g \cdot kg^{-1} \cdot d^{-1}$. The alternate supplement was provided the next morning thereby isolating the post-exercise nutrition effect. Following 39 h recovery, cyclists performed a repeat-sprint performance test. Post-exercise leucine-protein ingestion improved mean sprint power by 2.5% (99% confidence limits, ±2.6%; $p = 0.013$) and reduced perceived overall tiredness during the sprints by 13% (90% confidence limits, ±9.2%), but perceptions of leg tiredness and soreness were unaffected. Recovery pre-exercise serum creatine-kinase concentration was lowered 19% (90% confidence limits, ±18%), but lactate dehydrogenase and pressure-pain threshold were unaltered. There was a small reduction in anger (25% ±18%), but other moods were unchanged. Plasma leucine (3 fold) and essential amino-acid (47%) concentrations were elevated post-exercise. Net nitrogen balance trended mildly negative in both conditions (mean ± *SD*: leucine-protein -20 ± 46 $mg \cdot kg^{-1} \cdot 24\ h^{-1}$, control -25 ± 36 $mg \cdot kg^{-1} \cdot 24\ h^{-1}$). The ingestion of a leucine-protein supplement along with other high-carbohydrate food following intense training on consecutive days enhances subsequent high-intensity endurance performance and may attenuate muscle membrane disruption in well-trained male cyclists.

3.1.2 INTRODUCTION

There is considerable interest on the potential role of protein ingestion following endurance exercise in improving recovery and promoting training adaptations within the skeletal muscle (Hawley, et al. 2007; Rodriguez 2009). Intense endurance exercise damages muscle necessitating repair (Koller, et al. 1998), stimulates adaptive remodelling (Hawley, et al. 2007) and can increase protein oxidation (Koopman, et al. 2004), which act to increase the dietary protein requirement relative to the sedentary condition (Tarnopolsky, et al. 1988). These effects are compounded in competitive endurance athletes who often train or compete intensely on a daily basis and sometimes over multiple daily sessions generating a constant state of tissue catabolism and recovery (Rodriguez 2009). Intake of essential amino acids is necessary to elevate protein synthesis (Borsheim, et al. 2002; Moore, et al. 2009), which is an important process in tissue remodelling. It is now established from animal studies that leucine is the key amino acid stimulating protein synthesis (Crozier, et al. 2005), and there is some evidence for a moderate enhancement in protein synthesis in young men after resistance exercise with the addition of leucine to protein and carbohydrate (Koopman, et al. 2005). In addition to regenerating glycogen (Ivy 2004), carbohydrate 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 co-ingestion 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 (Ivy 2004; Levenhagen, et al. 2002; Miller, et al. 2003).

In terms of performance subsequent to an initial strenuous bout of exercise, protein (typically 1.5-2% whey protein) co-ingested within a carbohydrate sports drink after exercise has been shown to enhance acute (2-15 h) later performance over carbohydrate only solutions (Berardi, et al. 2008; Niles, et al. 2001; Saunders, et al. 2004). This effect has been attributed to enhanced glycogen resynthesis (Berardi, et al. 2008; Zawadzki, et al. 1992). However, some authors report no clear effect (Betts, et al. 2005) and the

practical relevance of providing only a protein-carbohydrate drink to athletes for overnight recovery is questionable (e.g. due to hunger, negative energy and nitrogen balance). While the glycogen paradigm is interesting, it was hypothesized that the role for protein in training adaptation and performance is more likely to be realised not through superior glycogen availability but via enhanced adaptive tissue remodelling associated with up-regulation of endurance-exercise specific gene expression (Hawley, et al. 2007; Yang, et al. 2005) and amino-acid stimulated protein synthesis (Howarth, et al. 2009; Morrison, et al. 2008). The mRNA response to endurance exercise peaks 8-12 h post exercise (Yang, et al. 2005) and adaptive protein synthesis is a cumulative process augmented by recovery periods between bouts (Hood, et al. 2006), so a period of more than 2-24 h may be required for the putative effects of post-exercise protein ingestion on cellular repair and adaptation to exert any measurable effect on the performance phenotype.

With repair and remodelling in mind, the effect of a high protein-carbohydrate recovery diet was compared versus isocaloric control (0.7/1.4/0.26 versus 0.1/2.1/0.26 $g \cdot kg^{-1} \cdot h^{-1}$ protein/carbohydrate/lipid) ingested for 4 h following 2.5 h high-intensity cycling (energy expenditure 10.5 MJ) on 15 and 60 h subsequent performance in well-trained cyclists (Rowlands, et al. 2008). Importantly, in this study adequate energy and carbohydrate were supplied to saturate glycogen synthesis to objectively remove glycogen as a covariate, which was a probable performance mechanism in earlier studies (Berardi, et al. 2008; Niles, et al. 2001). Under these conditions, the high-protein diet had no impact on 15 h, but substantially enhanced 60 h subsequent performance suggesting time of the performance measure is important and that a protein-dose sensitive adaptive response could account for the accrued benefit of recovery protein feeding. However, the quantity ingested (36-53 $g \cdot h^{-1}$) was possibly excessive given chronic exposure to elevated dietary protein content increases protein turnover (Bowtell, et al. 1998) and decreases protein synthesis rates (Bolster, et al. 2005). A further consideration was that while the acquisition of 4 h recovery nutrition entirely from

hypertonic beverages affords control in the laboratory and was useful in blinding in the proof-of-principle study, it is unrepresentative of practice.

Therefore, the aim of the present study was to determine if subsequent performance and indicators of recovery could be improved following the ingestion of a concentrated leucine-protein carbohydrate-lipid supplement taken with ordinary high-carbohydrate food (pasta, cereal bars, juice) during the first 1.5 h after interval cycling over 3 days. Total nitrogen intake was reduced to 35% of that used in a previous performance study (Rowlands, et al. 2008) during the recovery period, but it was anticipated that signalling potency would remain high via the addition of a saturating quantity of free leucine estimated from earlier animal research (Crozier, et al. 2005; Koopman, et al. 2005). Within treatment block, the specific effect of the post-exercise supplement (evening) was isolated and total daily protein and leucine intake balanced by providing the alternate supplement at the opposite end of the day (morning). Under the stringent condition of daily energy balance, clamped protein and high dietary carbohydrate intake, it was demonstrated for the first time that supplementation of high-carbohydrate post-exercise meals with a leucine-protein drink results in a meaningful enhancement of subsequent performance and other recovery measures.

3.1.3 MATERIALS AND METHODS

3.1.3.1 Subjects

Ten well-trained male cyclists and triathletes completed the study. Average maximal oxygen uptake (VO_{2max}) and peak power outputs were (mean ± *SD*) 68.2 ± 8.3 $mL \cdot kg^{-1} \cdot min^{-1}$ and 343 ± 27 W, respectively. Training experience was 3.3 ± 2.6 y and, during the previous 6 months, subjects performed 13.2 ± 4.4 $h \cdot wk^{-1}$ and 8.1 ± 2.5 $h \cdot wk^{-1}$ of aerobic training and cycling specific training, respectively. Subjects were aged 33 ± 9 y, with body mass of 76 ± 5 kg. All subjects received verbal and written descriptions from the researchers detailing the purpose of the study, rights, requirements, and associated risks; subjects were screened for precluding health conditions and gave written consent

to participate. The study was approved by the Massey University Human Ethics Committee.

3.1.3.2 Experimental Design

The design was a randomized double-blind, placebo-controlled cross-over comprising two 9-day blocks of exercise training followed by a performance test (Figure 3.1). Each block started with 3 days of reproduced standardized lead-in training followed by a rest day: training on days -8 and -7 was conducted in the field on a standardized course with intensity maintained by the subject using heart rate; day -6 was a 2 h controlled ride in the laboratory at 50% of peak power. During the 4-day Lead-in period, training and diet was recorded using a diary, which was checked by researchers and returned to the subject as the schedule for replication over the same period during the second arm of the cross over Then, the cyclists completed 3 consecutive days of controlled high-intensity interval training (test days -4, -3, -2) in the laboratory, a rest day (day -1), followed by the performance test (day 0). There was a 2-week washout between blocks. Beginning 2 weeks prior to the first experimental block, preliminary tests were conducted over 3 consecutive days.

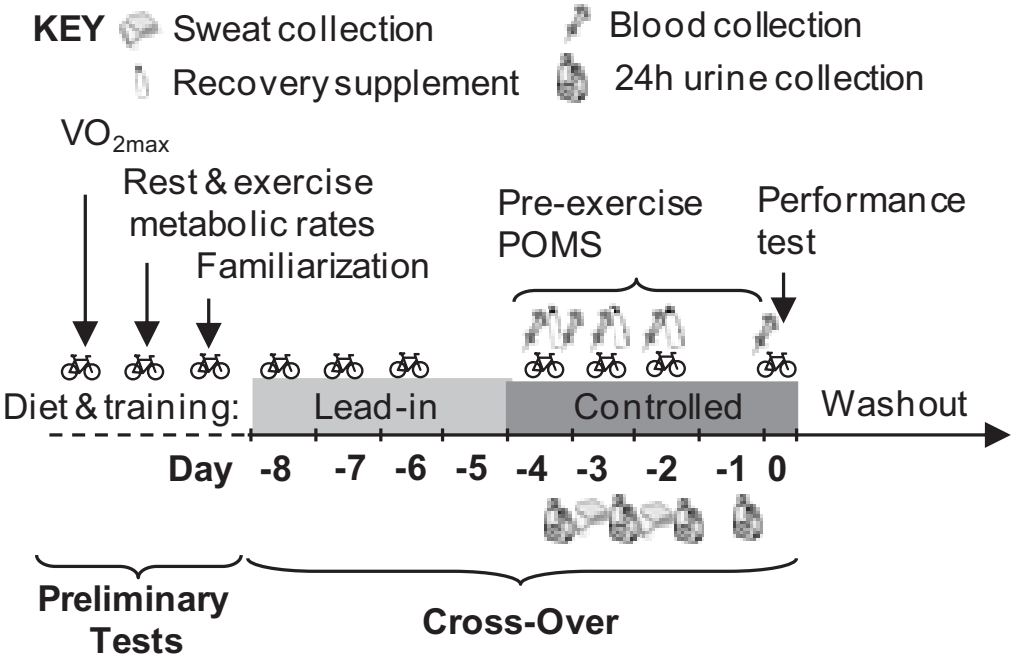

Figure 3.1 Experimental design

Following preliminary testing, each cyclist completed the two blocks of the cross over which comprised a 4-day standardized lead-in period of training and diet and the controlled experimental period comprising three loading rides, a rest day, and the repeated-sprint test to measure the effect of the recovery supplement on subsequent performance.

3.1.3.3 Preliminary Testing

Maximum aerobic power. Maximum power output (W_{max}) and VO_{2max} were estimated using a 2.5-min stage, 25 W incremental test on an electromagnetically-braked cycle ergometer (Velotron, Version 1.5 Software, Racermate Inc., Seattle, U.S.A) as described previously (Rowlands, et al. 2007). Expired gas fractions were collected by Douglas bag and analyzed using a gas analyzer (Servomex 1440, Applied Instruments Auckland, New Zealand) calibrated with blank (99.9% N_2), high calibration gas (26% O_2 and 4% CO_2) and ambient air prior to each test. Ambient bag volume was measured using a dry gas meter (Harvard, Biolab, New Zealand). All cycle ergometer testing was performed

under standardized conditions of 20 ± 0.5°C, 53 ± 6% humidity and subjects were cooled with fans to minimize thermal distress.

Metabolic rate. As a component of measures taken to balance energy expenditure with intake during the experimental blocks, the fasted resting and sub-maximal metabolic rates for each subject were determined. Following a 12-14 h overnight fast and abstinence from exercise subjects drove or were driven to the research laboratory where they lay supine in a darkened, temperature controlled (21-24°C) room for 30 min, followed by the collection of expired air for 6-10 min. Immediately following, subjects rode three consecutive 10-min stages at fixed workloads equivalent to intensities of 40, 50, and 60% of VO_{2max}. Metabolic rate ($kJ \cdot min^{-1}$) was estimated from the VO_2 calculated from expired air collected during the final 2 min of each workload. The VO_2 was adjusted for the energy equivalent of oxygen according to the measured non-protein respiratory-exchange ratio (Peronnet and Massicotte 1993).

Familiarization. Prior to each study block, subjects performed a full familiarization trial of the repeat-sprint performance test (see below for details) which included exposure to all outcome measures used to determine the performance response to treatment: ratings of perceived exertion (RPE) and graphic rating scales (GRS), psychometric questionnaire, reported muscle soreness, and pressure algometer pain threshold measurement.

3.1.3.4 Procedures

High-intensity interval training. The training sessions on days -4, -3, and -2 were adapted from the loading protocols used previously (Rowlands, et al. 2008) and were designed to replicate very hard, moderate, and hard effort interval-training sessions, respectively. Cyclists reported to the laboratory between 16:00-18:00 h, and arrival times were replicated during the subsequent experimental block to within 60 min. Each interval training protocol was pre-programmed into the Velotron software and exercise intensities based on W_{max}. All 3 interval sessions were preceded by a warm up comprising 15 min at 30% W_{max}, 10 min at 40% W_{max}, and 5 min at 50% W_{max}, and

completed with a 10-min cool down at 30% $W_{max.}$ The respective interval protocols comprised: day -4, five blocks of 5 x 2-min intervals (1 x 90%, 2 x 80%, and 2 x 70% of W_{max}) interspersed with 2-min recovery intervals at 50%, and each block separated by 5 min recovery at 50% W_{max} (total 150 min cycling); day -3, 8 x 5-min intervals at 70% W_{max} interspersed with 5-min recovery intervals at 50% W_{max} (total 120 min); day -2, 5 blocks of 5 x 1-min intervals (2 x 90%, 2 x 80%, and 1 x 70% W_{max}) interspersed with 2-min recovery intervals at 40%, and each block separated by 5-min recovery at 40% W_{max} (total 130 min).

Psychometric questionnaire and muscle soreness. To evaluate the effect of treatment on mood state, a short form of the profile of mood states questionnaire (POMS) was presented to subjects prior to each training session and prior to the performance test (Wadsworth, et al. 2010). The effect of treatment on the soreness threshold of cycling-specific muscle groups were quantified prior to the performance test using a pressure algometer (FDK 60, Wagner Instruments, Greenwich, CT) (Wadsworth, et al. 2010).

Physiological measures. Timed 24 h urine samples were collected following first void immediately post-exercise on day -4 until immediately prior to performance test on day 0 for analysis of nitrogen excretion. Regional sweat collection using gauze pads at the chest and abdomen during training rides on days -3 and -2 for quantification of exercise sweat nitrogen content were undertaken according to the method of Colombani et al. (1997). On day -4, blood was collected via Teflon catheter (Becton Dickinson, Sandy, UT) placed in an antecubital vein for analysis of muscle damage markers creatine kinase (CK) and lactate dehydrogenase (LDH), and also immediately following and 30, 60, 90, and 120 min after exercise for the representative plasma amino-acid concentrations. Additional venepunctures were taken prior to exercise on days -3, -2, and 0 for analysis of CK and LDH. Blood was dispensed into ice-chilled EDTA vacutainers for amino-acid analysis or into silicone-coated serum vacutainers for CK and LDH. Serum tubes were allowed to clot at room temperature then underwent centrifugation at 20°C for serum and 4°C for EDTA tubes for 5-10 min at 3500 rpm, and serum/plasma aspirated into Eppendorf microtubes and stored at -80°C until analysis.

Repeat-sprint performance test. Subjects reported to the laboratory at 08:00 h following an overnight fast on the morning of day 0. Psychometric data, soreness, and blood were collected as described above. A standardized pre-exercise meal as provided 1-h prior to exercise consisting of 2 slices of toasted white bread, 10 g margarine, 15 g plum jam and 160 mL of sports drink to increase liver-glycogen content. In addition, a cereal bar (LCM, Kelloggs, Charmhaven NSW, Australia) and water were ingested 15 min preceding cycling. The nutrition provided approximately 1 $g \cdot kg^{-1}$ of carbohydrate and 300 mL of fluid within 1 h of exercise. During all exercise sessions and performance tests sports drinks were ingested providing: 2.2 $mL \cdot W_{max}^{-1} \cdot h^{-1}$ fluid, 0.13 $g \cdot W_{max}^{-1} \cdot h^{-1}$ carbohydrate and 1.4 $mg \cdot W_{max}^{-1} \cdot h^{-1}$ sodium.

Performance was evaluated via analysis of sprint mean power in a repeat-sprint protocol comprising 10 maximal sprints (typical range 1.5-3 min) interspaced with standardized recovery periods (5.43 min) at 40% W_{max}, as described previously (Rowlands, et al. 2007). Briefly, internal work to be done (kilocalories) during the sprint and recoveries was determined by individual W_{max} (kilocalories=0.125 x W_{max}). Fixed linear workloads approximately equivalent to the load created by riding a 28, 39, or 48 front chain ring and a10-sprocket, 21- to 11-tooth rear cluster were selected from the Velotron software. An up-or-down gear switch was positioned on the end of the right handlebar brake hood to provide convenient changing of the gearing. Cyclists self-selected cadence and gearing but were instructed to sprint as fast as possible until the required kilocalories were achieved. No verbal encouragement was provided to the participants; the only information provided during the sprints was elapsed work completed (kilocalories) shown on a computer screen. Participants were given a verbal countdown in preparation for the start of each sprint and at 20, 10, 5, and 2 kilocalories to go in preparation for the end of each sprint. Testing was performed under standardized environmental conditions as described above. Perceptual responses during the performance test were evaluated using RPE and GRS scales representing tiredness, soreness, and sprint power, previously described (Rowlands, et al. 2008; Rowlands, et al. 2007).

3.1.3.5 Nutritional Procedures

Controlled diet. A key objective of the experimental design was to isolate the effect of the experimental nutritional intervention. To facilitate, subjects were provided with menu plans and pre-weighed, pre-packaged items for the entire duration of the Controlled period of the experimental block (Figure 3.1). During this period, total daily energy and macronutrient intakes were balanced by providing the alternate beverage supplement to subjects with the morning meal (i.e. when the leucine-protein supplement (see below) was provided post exercise, the control supplement was provided in the morning and vice versa). Total daily energy requirement was estimated from the resting and sub-maximal exercise metabolic rates, and predicted daily incidental activity:

$$EE = ((PA \times RMR) \times PA_{duration}) + Ex_{Study}$$

Where PA is incidental physical activity in $kJ \cdot min^{-1}$ derived from daily activity records over 3 d, RMR is resting metabolic rate in $kJ \cdot min^{-1}$, Ex_{Study} is the study cycling training in $kJ \cdot min^{-1}$. Ex_{Study} energy expenditure was estimated based on plotting measured energy expenditures versus fixed workloads (40, 50, and 60% of VO_{2max}) to derive individual regression equations predicting energy expenditure and accounting for work efficiency during cycling exercise.

Mean total daily energy requirement during the 3-d controlled training block was calculated to be 0.24 $MJ \cdot kg^{-1} \cdot 24\ h^{-1}$. Total prescribed dietary protein intake was 1.6 $g \cdot kg^{-1} \cdot 24\ h^{-1}$ in line with estimated daily requirement for male endurance athletes (Tarnopolsky, et al. 1988); actual intake was $1.62 \pm 0.07\ g \cdot kg^{-1} \cdot 24\ h^{-1}$. Prescribed dietary carbohydrate intake was 9.3 $g \cdot kg^{-1} \cdot 24\ h^{-1}$ to maintain high intramuscular glycogen concentration (Ivy 2004). The remainder of estimated energy requirement was provided by fat intake and approximated 1.5 $g \cdot kg^{-1} \cdot 24\ h^{-1}$.

Intervention. The nutritional intervention was ingested over the first 90 min of recovery from exercise on days -4 to -2. The nutrition treatments were delivered to the subjects within normal post-exercise recovery food items: cereal bars (LCM) and fruit juice, and pasta with sauce, supplemented with two identically flavoured milk-like intervention

beverages. Details of the post-exercise feeding schedule are provided in Table 3.1. Three primary biological factors were utilized in the design of the leucine-rich high-protein supplemented-feeding protocol. Firstly, the aim was to confirm the efficacy of the high protein-carbohydrate post-exercise feeding intervention on performance observed previously (Rowlands, et al. 2008) but with a reduced feeding duration from 3.5 h to 1.5 h (which is closer to practice), lower nitrogen loading, and with retention of physiological signalling potency achieved the addition of free leucine (Crozier, et al. 2005). Retention of effect with reduced nitrogen input is equivalent to increased supplemental nitrogen efficiency, and this was achieved through the ingestion of 0.92 $g \cdot kg^{-1}$ of whole protein providing 0.6 $g \cdot kg^{-1}$ EAA, and 0.29 $g \cdot kg^{-1}$ of leucine, where the protein intake was derived from a mid-range estimate of EAA requirement to saturate muscle protein synthesis (0.45 $g \cdot kg^{-1} \cdot h^{-1}$) (Biolo, et al. 1999; Borsheim, et al. 2002; Koopman, et al. 2005; Miller, et al. 2003). Secondly, sufficient carbohydrate was provided to saturate the glycogen synthesis rate (Ivy 2004). Thirdly, a moderate quantity of lipid was retained to successfully aid in blinding the cyclists to the protein component in the intervention beverages (subjects reported not being able to distinguish between beverages), and reduce beverage volume to model a commercial product. The supplement beverages were prepared in the food technology laboratory within the department, 1-4 d previously and kept refrigerated until consumption. Subjects reported no gastrointestinal disturbances.

TABLE 3.1 COMPOSITION OF POST-EXERCISE SUPPLEMENT BEVERAGE AND ACCOMPANYING RECOVERY FOOD

INGREDIENT	Leucine-protein		Control	
	$g \cdot kg^{-1} \cdot h^{-1}$[a]	Total[b] (g)	$g \cdot kg^{-1} \cdot h^{-1}$	Total (g)
Supplement Beverage				
L-Leucine (free)	0.10	17.9 (8.0)	-	-
Milk Protein Concentrate	0.24	37.1 (2.1)	-	-
Whey Protein Isolate	0.15	22.8 (1.3)	-	-

INGREDIENT	Leucine-protein		Control	
	$g \cdot kg^{-1} \cdot h^{-1}$[a]	Total[b] (g)	$g \cdot kg^{-1} \cdot h^{-1}$	Total (g)
Maltodextrin	0.28	42.6 (2.5)	0.79	120.8 (7.0)
Sucrose	0.37	57.1 (3.3)	0.37	57.0 (3.3)
Canola Oil	0.15	23.3 (1.4)	0.15	22.6 (1.3)
Emulsifier	0.01	2.0 (0.1)	0.02	2.3 (0.1)
Vanilla flavouring	0.05	8.2 (0.5)	0.05	8.2 (0.5)
Splenda	0.07	10.2 (0.6)	0.01	2.0 (0.1)
Water	2.96	452 (26)	3.23	493 (29)
Additional Food Items				
Cereal Bar	0.29	22.4 (1.3)	0.29	22.4 (1.3)
Orange and Mango Juice	2.68	204.5 (42.9)	2.68	204.5 (42.9)
Pasta (dry weight)	1.07	81.5 (4.7)	1.07	81.5 (4.7)
Bolognese sauce	2.37	181.2 (9.9)	2.37	181.2 (9.9)
Total Nutrient Profile[a]				
Energy (kJ)[c]	36.6	5600 (330)	36.7	5600 (330)
Protein	0.46	70.1 (4.2)	0.06	9.6 (0.93)
Essential amino acids	0.30	46.2 (2.8)	0.02	3.7 (0.22)
Total leucine	0.14	22.0 (1.3)	0.005	0.90 (0.06)
Carbohydrate	1.20	180 (11)	1.57	239 (14)
Fat	0.21	31.8 (2.0)	0.20	31.1 (2.0)

[a] The per hour rate as an equivalent rate representing the total ingested over the 90-min supplementation period (4 servings) divided by two, as the first serving is at recovery time=0.

[b]Mean (*SD*) for the 90-min supplementation period.

[c]Energy represented as $kJ \cdot kg^{-1} \cdot h^{-1}$ and Total kJ.

3.1.3.6 Biochemical Analyses

Plasma amino-acid concentrations were measured from the perchloric acid extract by HPLC as described previously (Moore et al. 2005). Serum CK and LDH activities were measured with commercially available *liquid assay* kits (*Roche Diagnostics* NZ Ltd., Auckland, New Zealand). Net nitrogen balance (subtraction of nitrogen excretion from dietary nitrogen intake) was determined over the training block following the first training ride up to the final urine collection before the performance test. Nitrogen intake was estimated from dietary analysis of the pre-weighed, pre-packaged controlled diet using software and nitrogen factors from the Australia New Zealand Food Standards NUTTLAB database (http://www.foodstandards.gov.au/consumerinformation/nuttab2006/). Dietary compliance were accounted for by daily dietary check sheets and return of uneaten portions reweighed in the laboratory. To determine nitrogen excretion, 24-h urine, and representative regional sweat collections during exercise were analyzed by micro-kjeldahl technique for total nitrogen percent. Total sweat loss during exercise was calculated following the methods of Maughan et al. (2007). Additional nitrogen losses were estimated from faeces and resting sweat losses (modified for body surface area) observed previously in endurance-trained males on similar protein intakes (Tarnopolsky, et al. 1988).

3.1.3.7 Statistical Analysis

Sample size. Sample size was generated based on sufficient power to detect a likely substantial benefit to overall sprint mean power within the performance test. Consistent with the general method, sample size was estimated by magnitude-based inference (Hopkins, et al. 2009). The smallest worthwhile effect of treatment on performance is between 0.3-0.7 times the estimate for coefficient of variation (CV) (Hopkins, et al. 1999). The relationship between performance in the repeat-sprint performance test (CV=3.1%; (Rowlands, et al. 2008)) and performance during competition has not been established; but the CV is within the range of other estimates for repeated-sprint tests and cycling competitions (Paton & Hopkins 2005). Therefore, the statistical model was

powered to detect 0.5 x CV (0.5 x 3.1% = 1.55%); with the anticipated effect magnitude of 4.1% (Rowlands, et al. 2008) the resulting sample size yielded n=10.

Analysis. The effects of treatment on dependent outcome variables were estimated with mixed linear modelling (Statistical Package for the Social Sciences, Version 16.0, SPSS Inc., Chicago, IL). Statistical models followed the prescriptions of Rowlands et al. (2008). Dependent variables were analyzed after log transformation to reduce effects of heteroscedasticity, except nitrogen balance data which had negative values that were undefined with log transformation. Outcomes from the log transformed analyses were expressed as percent or fold differences with appropriate geometric measures of variation or error. A fixed constant of 1.0 was added to perceptual GRS data so that the minimum value was anchored at 1.0. Fixed effects were treatment and order (to account for any physical or psychological learning effects, and carryover in the physiological measures between trials). For the appropriate repeated measures analysis day or sample time was also added following transformation by grand mean centering, and for analysis of sprint mean power sprint number was fitted as a numeric (scale) effect with polynomial contrast following transformation by grand mean centering; the polynomial contrast was chosen as it best represented the data and resulted in the tightest model fit. Reported outcomes for the analysis of sprint mean power, are the overall average effect (derived from the within-subject position coefficient) and the linear decline or fatigue effect (derived from the slope coefficient). When appropriate, baseline measures were standardized relative to the sample variation and defined as numeric covariates. Temperature and humidity were added as numeric covariates but found to have no appreciable effect and were excluded from the model. Subject identity was the random effect.

Precision of estimation and statistical inference. In keeping with recent trends in inferential statistics the estimate precision is presented as 99% confidence limits for performance outcomes and 90% confidence limits for other outcomes, and statistical inference was by magnitude-based inference (Hopkins, et al. 2009). For performance, effect magnitude was qualified as the product of the coefficient of variation for the

performance measure and the following thresholds: trivial 0.0-0.3, small 0.3-0.9, moderate 0.9-1.6, large 1.6-2.5 (Hopkins, et al. 2009). The CV for the performance measure is 3.1% (Rowlands, et al. 2008), which concurs with the range and uncertainty of published estimates of error for high-intensity cycling competition (Paton & Hopkins 2005). Following standardization, inferences about the true population value for mechanism outcomes were qualified using the standardized difference (mean difference/appropriate *SD*) classification (trivial 0.0–0.2, small 0.2–0.6, moderate 0.6–1.2, large 1.2–2.0, very large 2.0-4.0), where the threshold for the smallest meaningful or *substantial* effect is 0.2. An analysis of the likelihood of a substantial increase, decrease, or negligible effect from the t-distribution was performed (Rowlands, et al. 2008), with likelihoods ordered into cut-offs and inferred as: <1%, almost certainly not; 1–5%, very unlikely; 5–25%, unlikely; 25–75%, possible; 75–95%, likely; 95–99%, very likely; >99%, almost certain. In the case where the majority (>50%) of the uncertainty lies between the threshold for a substantial increase and decrease, the likelihood of the effect being *trivial* (negligible, unaffected) is qualified. Effects were described as *unclear* or *inconclusive* if the confidence interval overlapped into both positive and negative values.

3.1.4 RESULTS

3.1.4.1 Plasma Amino Acids

Plasma leucine, essential amino acids, and total amino-acid concentrations during the 2 h recovery from exercise is shown in Figure 3.2. Overall, the leucine-rich supplemented meal lead to large increases in total (24% 90% confidence interval ±20%) and essential amino-acid concentrations (47% ±39%) and very large increases in leucine concentration (3.0 fold ±2.8 fold), relative to the control.

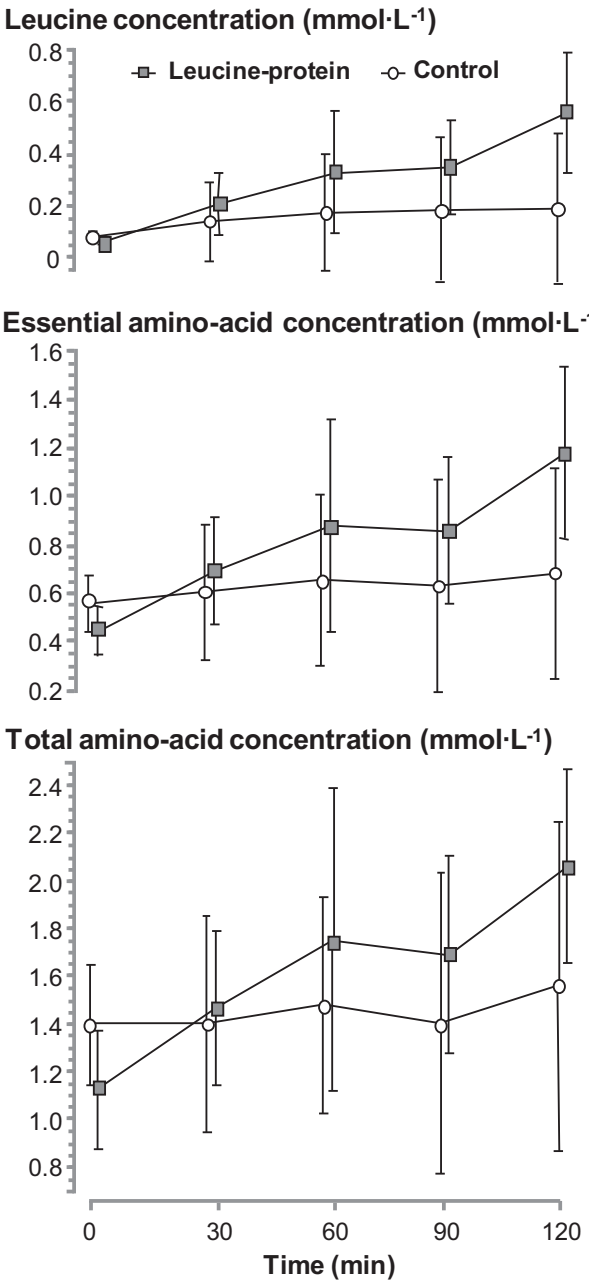

Figure 3.2 Plasma leucine, essential and total amino-acid concentrations following exercise and for 120-min after ingestion of the leucine-protein and control supplements.

Data are raw mean ± *SD*.

3.1.4.2 Performance

Overall mean sprint power (CV) in the leucine-protein and control conditions was 325 W (12%) and 320 W (12%), respectively, which represents a small increase in sprint mean power of 2.5% (99% confidence limits (CL) ±2.6%; likelihood of substantial harm/trivial/benefit 0.0%/6.7%/93.3%; p = 0.013) with respect to the magnitude-based inferential threshold for small. of 0.93%. There was a mean trivial effect of test order on overall mean sprint power with performance in the second experimental block -0.58% slower (±2.59%) that in the first. The effects of treatment on linear slope (fatigue) (-1.3% ±5.3) and curvature (-6.6% ±17.9%) were unclear. The leucine-protein supplement reduced perceived overall tiredness during the performance test by 13% (90% confidence limits (CL) ±9.2%), but effects on ratings of perceived exertion, leg tiredness, muscle soreness, and leg soreness were trivial.

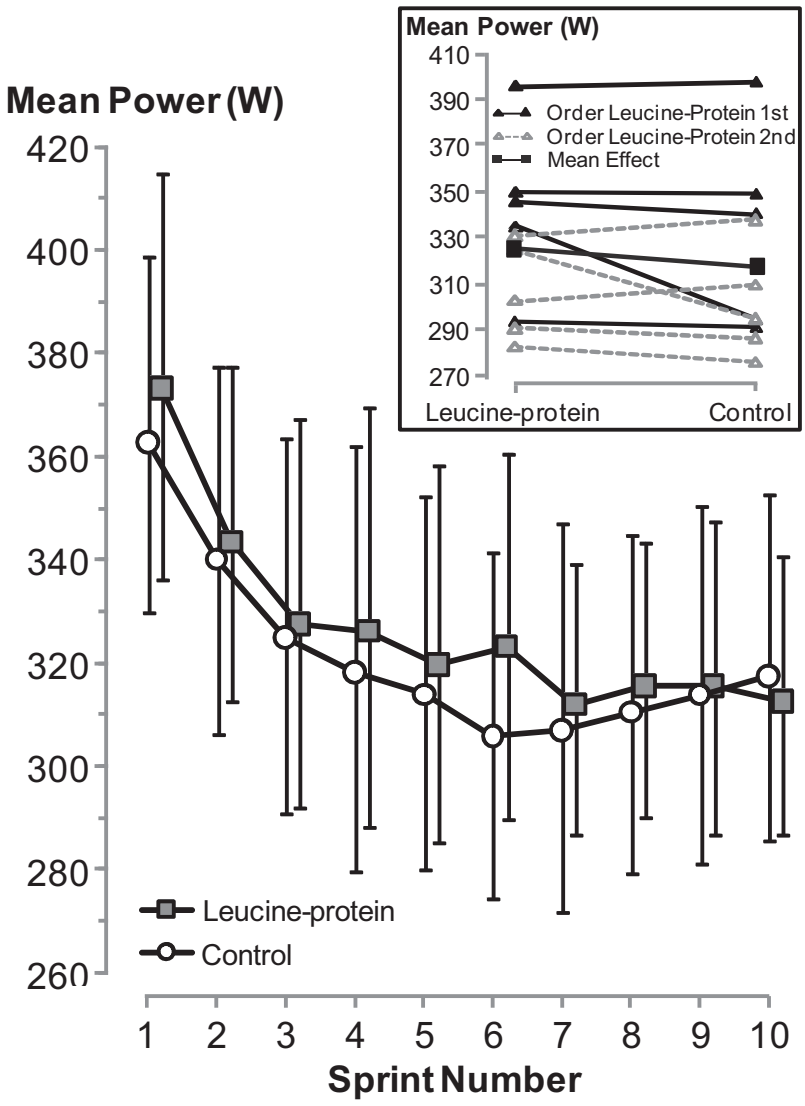

Figure 3.3 Effect of the leucine-protein supplement on sprint mean power during the performance test

Data are back-transformed means. Bars are the pooled sample variation derived from the analysis. The threshold change for a small effect was 0.93%. Inset: within-subject overall mean power response derived from the analysis.

3.1.4.3 Muscle membrane disruption and soreness

Analysis of markers of muscle disruption taken before exercise, on days -3 and -2, and the day of the performance test revealed leucine-protein supplement caused an overall

small reduction in serum CK activity, but had a trivial effect on LDH activity (Table 3.2). There was a likely trivial increase in muscle soreness threshold (pressure algometer) of 0.2% (90% CL ±0.3%) measured immediately prior to the performance test.

TABLE 3.2 EFFECT OF POST-EXERCISE SUPPLEMENTATION CONDITION ON ENZYME MARKERS OF MUSCLE MEMBRANE DISRUPTION DURING THE 4 d BLOCK

SAMPLE	Control[a] $U \cdot L^{-1}$	Leucine-protein[a] $U \cdot L^{-1}$	Outcome		
			Effect (%) ±90% CL[c]	Effect size ±90% CL	Inference
Creatine Kinase Activity					
Baseline[b]	136 (30)	176 (125)	4.8 ±28	0.08 ±0.45	Unclear
Day -3	270 (256)	236 (149)	-16 ±23	-0.25 ±0.36	Small decrease possible
Day -2	211 (165)	187 (113)	-18 ±23	-0.28 ±0.37	Small decrease possible
Day 0	136 (55)	134 (52)	-9.4 ±24	-0.15 ±0.39	Unclear
Average[d]	188 (162)	184 (117)	-19 ±18	-0.30 ±0.29	Small decrease likely
Lactate Dehydrogenase Activity					
Baseline[b]	166 (26)	172 (29)	1.0 ±5.2	0.06 ±0.30	Unclear
Day -3	166 (24)	169 (31)	-0.8 ±5.1	-0.05 ±0.30	Unclear
Day -2	159 (20)	162 (20)	-1.1 ±5.1	-0.07 ±0.30	Unclear
Day 0	157 (21)	162 (20)	1.1 ±5.2	0.07 ±0.30	Unclear
Average[d]	162 (22)	166 (25)	-1.2 ±4.2	-0.08 ±0.26	Likely trivial

[a] Data are raw mean with standard deviation (*SD*) for the control condition as reference and leucine enriched as treatment.

[b] Prior to training on Day -4.

[c] Mean effect of leucine-enriched versus control after adjustment for baseline bias.

[d] Average is the mean response to treatment over days -4 to 0 derived from the analysis.

3.1.4.4 Nitrogen balance

Nitrogen intake and output data are presented in Table 3.3. Nitrogen balance was found to be negative (effect size -0.46 to -0.51) for each sample period in both experimental conditions, except on day -2 in the leucine-protein condition where the increase was trivial (0.18) and on day -3 in the control condition where the reduction was trivial (-0.01). In terms of 24 h differences, balance was less negative in the leucine-protein condition on day -4, (20 mgN·kg^{-1} 90%CL: ±29 mg N·kg^{-1}; effect size 0.45 ±0.65), but more negative on day -3 (-18 ±20 mgN·kg^{-1}; -0.40 ±0.45), and there were trivial differences on days -2 and -1 (26 ±31 mgN·kg^{-1}; 0.59 ±0.69 and -5.2 ±45.0 mgN·kg^{-1}; -0.12 ±0.51).

TABLE 3.3 NITROGEN BALANCE SUMMARY OVER THE 4 d BLOCK

DAY	N Intake[a]	N Excretion *mg·kg^{-1}·d^{-1}*					Balance[e]
	mg·kg^{-1}·d^{-1}	Urine	Exercise sweat[b]	Resting sweat[c]	Misc.[d]	Total	*mg·kg^{-1}·d^{-1}*
				Leucine-protein rich			
Day -4	283 (4)	215 (60)	6.5 (1.3)	11.2 (0.1)	42 (2)	277 (62)	-14 (61)
Day -3	262 (9)	221 (28)	5.5 (1.7)	11.6 (0.2)	42 (2)	282 (29)	-20 (32)
Day -2	262 (5)	193 (39)	5.8 (1.4)	11.5 (0.1)	42 (2)	254 (39)	7.9 (38.2)
Day -1	261 (19)	254 (42)	-	14.0 (0.0)	42 (2)	311 (44)	-50 (42)
Overall[f]	-	-	-	-	43 (2)	-	-19 (48)

DAY	N Intake[a]	N Excretion $mg \cdot kg^{-1} \cdot d^{-1}$					Balance[e]
	$mg \cdot kg^{-1} \cdot d^{-1}$	Urine	Exercise sweat[b]	Resting sweat[c]	Misc.[d]	Total	$mg \cdot kg^{-1} \cdot d^{-1}$
Control							
Day -4	263 (4)	233 (37)	6.9 (2.3)	11.2 (0.1)	42 (2)	295 (39)	-32 (36)
Day -3	262 (9)	202 (32)	5.2 (2.6)	11.6 (0.1)	42 (2)	262 (32)	-0.5 (37.8)
Day -2	262 (5)	216 (26)	6.7 (1.9)	11.5 (0.1)	42 (2)	278 (27)	-16 (26)
Day -1	261 (19)	249 (31)	-	14.0 (0.0)	42 (2)	306 (32)	-45 (46)
Overall[f]	-	-	-	-	-	-	-23 (39)

[a] Data are mean with standard deviation (*SD*) adjusted to daily 24 h equivalent. Beginning on Day -4, sampling periods were timed from the end of each training ride through to the shortly before beginning the performance test on day 0.

[b] Exercise sweat estimated from regional sweat collection and accounts for body mass change, respiratory water loss, substrate mass loss and metabolic water in accordance with the methods of methods of Maughan et al 2007.

[c] Resting sweat nitrogen excretion was estimated over 24 h minus duration of exercise, and was based on values derived from Tarnopolsky (1988) altered to reflect body surface area

[d] Misc. is defined as miscellaneous nitrogen losses which are based on Calloway et al (1971) and include estimated faecal nitrogen losses based on Tarnopolsky (1988).

[e]Nitrogen balance is the difference between dietary nitrogen intake and total nitrogen excretion.

[f]Mean daily-equivalent total intake minus excretion adjusted for body mass.

3.1.4.5 Mood State

There was no clear effect of the leucine-protein supplement on POMS global mood state scores over the training period (Figure 3.4); however, the mood subscale anger showed an overall small decrease (-25% 90%CL ±18%) compared to control. On the day of the performance test (day 0) there was a likely small decrease in anger (-40.0% ±30.3%) and possible small decreases on days -3 and -2 of the training program (-28% ±37%, -34% ±34%). On day -3 there was a possible small decrease in fatigue (-26% ±17%), and possible small increase in esteem on day -2 (28% ±51%). Differences in vigour, tension, confusion, and depression subscales were trivial or unclear.

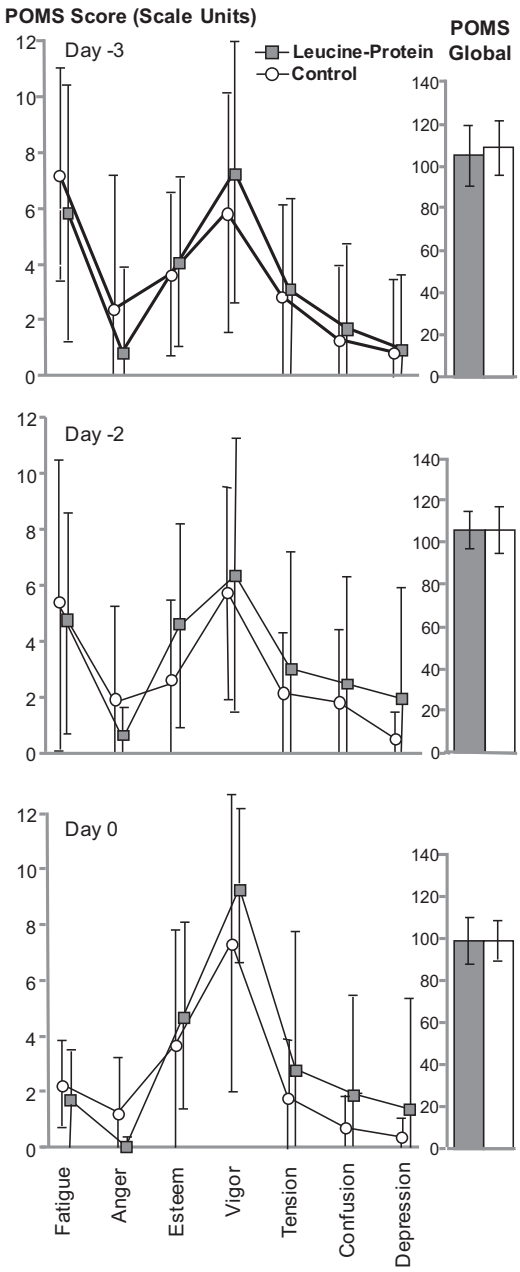

Figure 3.4 The effect of treatment on mood state.

Data are mean ± *SD*.

3.1.5 DISCUSSION

The primary outcome of the current study was that a leucine-protein enriched supplement combined with high-carbohydrate meals ingested following high-intensity

interval training for 3 days lead to a small enhancement of subsequent high-intensity cycling performance. Associated was a small reduction in CK activity suggestive of reduced disruption to skeletal muscle integrity or faster repair over the 3 d intense training block. These findings are particularly novel in the area of research on the role of protein nutrition post endurance exercise and relevant to real-life practice because the supplement was provided along with normal food items, ingested following exercise in a realistic time frame and provided with an appropriate quantity of carbohydrate to optimize glycogen repletion. Stringent control of exercise, protein intake clamped at estimated daily requirement, and a 24 h balanced diet that included ingestion of the alternative supplement at the other end of the day, provide firm evidence that performance and recovery outcomes were a direct result of the post-exercise intervention.

In the present study, particular care was taken to control extraneous dietary bias that has clouded inference as to any supplement effect in previous longer-duration recovery studies. The finding of a meaningful effect on performance due to the immediate post-exercise supplementation follows on directly from the primary objective of a previous protein-feeding recovery study (Rowlands, et al. 2008), which was to secure or refute the principle that protein quantity in the recovery diet of an endurance athlete is an important determinant of recovery and adaptation to intense exercise stress. In an earlier study (Rowlands, et al. 2008), cyclists were fed a comparably high quantity of dairy protein (~55 $g \cdot h^{-1}$ of casein and whey isolate) and carbohydrate to ensure saturation of protein synthesis (23 g $EAA \cdot h^{-1}$) and glycogen repletion against an isocaloric low-protein high-carbohydrate control at 15 and 60 h following an initial intense training session. Performance was enhanced by a moderate 4.1% at 60 h, but there was no clear effect at 15 h. These data suggested that a delayed mechanism of action rather than superior short-term energy substrate repletion is responsible for better subsequent performance induced by protein in the 3-5 day high-intensity training model. The lower CK is consistent with other reports in trained cyclists (Rowlands, et al. 2008) indicating lower relative disruption to cellular structural integrity. The mechanism might be linked

to reduced proteolysis of cytoskeletal or membrane-extracellular matrix components as evidenced by general down-regulation of proteolytic and apoptotic process gene expression after exercise following protein-carbohydrate feeding (Rowlands et al. 2008). In the current study, total nitrogen provided by the recovery feeding intervention was reduced to 35% of that used previously (Rowlands, et al. 2008) on the rationale that 0.7 g protein·kg^{-1}·h^{-1} might be excessive and beneficial effects might be obtained with lower total protein intake via the addition of free leucine. Interestingly, the magnitude of the performance outcome in the current study with the leucine-protein supplement was nearly half that of previous high-protein feeding intervention. Possibly differences in study design (such as 2 days of high-intensity intervals and post-exercise feeding in the previous study versus 3 days in the current study), the total post-exercise protein intake, feeding duration, nitrogen status or simply sampling variation, might account for different magnitudes of the mean effects.

The rationale for adding leucine was to try to retain the signalling potency of the protein-based supplement in the face of the 2.9-fold lower protein intake. In hindsight and if funding and human resources had permitted, it would have been ideal to have compared the leucine-protein supplement to an isocaloric protein-only supplement plus high-carbohydrate meals to obtain the leucine differential (if any). In young men ingesting leucine-protein-carbohydrate following resistance exercise, whole-body net protein balance was improved and there was a moderate increase in mixed-muscle fractional synthetic rate (0.136 ± 0.020 %·h^{-1} vs. 0.117 ± 0.040 %·h^{-1}; effect size 0.61) vs. protein-carbohydrate ingestion and vs. carbohydrate only (0.097 ± 0.037 %·h^{-1} (Koopman, et al. 2005). Following endurance exercise, Howarth et al. (2009) reported a 50% enhancement in mixed-muscle protein fractional synthesis rate with similar quantities of whole protein plus carbohydrate vs. carbohydrate only following intense endurance exercise. While probably correlated, it is unknown if changes in mixed muscle protein fractional synthetic rate transpire to substantial changes in endurance performance. This absent comparison, nevertheless, does not detract from the possible practical implications of the leucine-protein supplement, and follow-up work is required

to clarify the relative independent contributions of leucine and protein to the processes governing recovery, and on the question of whether the independent effects of leucine and whole protein are sufficient to lead to a substantial enhancement of subsequent performance.

There are four other published studies that have also investigated the effect of post-exercise high protein-carbohydrate supplement interventions on subsequent performance with the assay time >15 h post the initial loading exercise; however, none of the authors report clear performance effects (Luden, et al. 2007; Millard-Stafford, et al. 2005; Romano-Ely, et al. 2006; Rowlands & Wadsworth 2010). Loading, supplementation and duration before performance is measured, gender effects - blunted response in females (Millard-Stafford, et al. 2005; Rowlands & Wadsworth 2010), uncontrolled intervening exercise during the experimental period (2007), and uncertainty surrounding the addition of antioxidants to the protein feeding (Romano-Ely, et al. 2006) might have contributed to the absence of an observed protein-nutrition mediated effect on subsequent performance. The Luden study is by far the most comparable to the present study and earlier work by Rowlands et al. (2008), but uncontrolled background diet (including energy status and protein intake) and training in the runners, exercise type (running vs. repeated-sprint cycling), or nature and quantity of the supplement might have contribute to the non-significant effects on run performance in this study.

With respect to nitrogen balance, data suggest whole-body protein turnover might be important in determining the efficacy of post-exercise protein-based nutritional interventions on subsequent performance outcomes. A noteworthy strength of the current study was tight control over exercise, incidental activity, and diet, in an effort to balance energy turnover (Todd 1984) and daily protein intake as the mechanisms influencing post-exercise protein metabolism. In clamping 24-h protein intake at 1.6 $g \cdot kg^{-1} \cdot d^{-1}$ the daily requirement estimated from nitrogen equilibration research (Tarnopolsky, et al. 1988), it was observed that the cyclists were in a state of mild protein deficiency equivalent to -0.12 and -0.14 g $protein \cdot kg^{-1} \cdot d^{-1}$ in the leucine-protein and control conditions, respectively (Figure 3.4). In a previous performance study

(Rowlands, et al. 2008) total daily dietary protein was not balanced between conditions (average daily protein intake equated to 2.97 $g \cdot kg^{-1} \cdot 24\ h^{-1}$ in the high-protein condition and 0.92 $g \cdot kg^{-1} \cdot 24\ h^{-1}$ in the low-protein condition), 24 h net nitrogen balance was negative in the low-protein (-0.28 g protein$\cdot kg^{-1} \cdot 24\ h^{-1}$) and positive in the high-protein condition (0.49 g protein$\cdot kg^{-1} \cdot 24\ h^{-1}$), and non-adapted nitrogen requirement was estimated subsequently (but after initiation of the present study) at 1.98 $g \cdot kg^{-1} \cdot 24\ h^{-1}$ (Rowlands & Wadsworth 2010)). Work by Rowlands et al. (2008) was the first demonstration of a relationship between dietary protein intake, nitrogen balance and endurance performance in trained men, but without clamping daily protein intake in this current study, it could not be determined whether the improvement in subsequent performance was due to the nitrogen-balance differential or specifically to the post-exercise feeding. Ingalls et al. (1998) demonstrated that negative nitrogen balance following eccentric exercise limited functional recovery, so it is possible that the performance benefit in Rowlands et al. (2008) study is simply a result of impaired recovery, secondary to inadequate protein intake in the low-protein condition; however, this explanation cannot apply to the present data because protein intake was balanced and similarly negative in both conditions. Therefore, the present outcome suggests that the post-exercise leucine-protein supplement is beneficial for recovery from consecutive days of high-intensity exercise when protein intake is clamped at a daily intake estimated to be marginally below the actual requirement. The observed state of mild negative balance might be transitory until new protein equilibrium is established with a 10 d adaptation period possibly required (Tarnopolsky, et al. 1988); this supposition is supported by the pre-study dietary diaries of the cohort suggesting the cyclists were ingesting an average daily protein intake of 2.0 $g \cdot kg^{-1} \cdot d^{-1}$. Therefore, selection of 1.6 $g \cdot kg^{-1} \cdot 24\ h^{-1}$, based on a robust and fair literature estimate, might have been insufficient to meet the demands of consecutive-day high-intensity training. Nitrogen balance studies are inherently methodologically limited. Although urinary nitrogen loss was measured and total sweat loss extimated from regional patch collection, and corrections for faecal and other losses used, loss may have been greater because the nitrogen balance method tends to underestimate nitrogen excretion either by lack of complete

sample collection or through unmeasured losses (breath ammonia, skin desquamation, hair, nails, mucous, semen). Urine and sweat loss accounts for ~85% of total (Tarnopolsky, et al. 1988), so 15% of the loss estimate was subject to unknown error. With respect to sweat collection, regional collection was previously found to overestimate (Lemon, et al. 1986) as well as underestimate (Colombani, et al. 1997) urea nitrogen excretion compared with whole-body wash-down, potentially adding variation to the present measures; however, Lemon et al. (1986) compared only urea nitrogen between methods. In a latter and more comprehensive comparison of methods, Colombani et al. (1997) compared the sum of urea plus ammonia nitrogen and reported no significant difference between patch and whole-body wash-down, except for one site (upper back). In the present study, total nitrogen, and selected upper body sites (excluding the upper back) were measured because it was not practical to use thigh patches due to the loss of patch adhesion under cycling shorts and the effect of covering clothing on the rate of sweat evaporation and corresponding sweat nitrogen concentration. Therefore, the present method is likely to provide a good approximation to whole-body wash-down, and error would be within subject and trivial comprising only a fraction of the exercising sweat loss component of the 24 h nitrogen balance of 2% (Table 3.3). To close, there were no other studies in men to determine the relationship between post-exercise protein nutrition, nitrogen balance and performance in the literature at the time of publication, so it is recommended that future supplementation studies determine the impact of nitrogen status on supplement efficacy, and these might include isotope methods to more accurately measure nitrogen status and to capture protein turnover.

Mood state profile monitoring is used as a method for quantifying training-induced stress and indirectly used to infer the extent of recovery processes. The leucine-protein supplementation led to a small reduction in anger throughout the training period, and small reduction in fatigue following the hardest training session. This result is interesting, but similarly minor in line with Berardi et al. (2008) who found reductions only in the fatigue score with protein-carbohydrate feeding. The study protocol utilized

by Berardi and colleagues was designed to maximize glycogen resynthesis during short-term recovery, and results are similar with the pattern observed by Achten et al. (2004) in response to the ingestion of a carbohydrate-enriched diet during intensified training (higher fatigue score with normal carbohydrate diet). Saturating quantities of carbohydrate were provided during the post-exercise period and total daily diet leading to the conclusion that the leucine-protein component of the recovery nutrition is more likely responsible for the effects on mood state.

In summary, supplementing post-exercise high-carbohydrate meals with a leucine-protein rich beverage during a 3 d block of intense cycling in the laboratory enhanced subsequent repeated-sprint performance. The improved recovery might be associated with lower disruption to skeletal muscle integrity or to minor change in mood state. Twenty-four hour energy, leucine, and macronutrient intake was balanced suggesting the supplement is effective when ingested post-exercise. More research is required to determine if a nitrogen status influences the efficacy of the supplement on subsequent performance. To conclude, combining a high-carbohydrate post-exercise diet with a leucine-protein rich supplement may be beneficial during periods of intense endurance training or competition when dietary protein intake is mildly below the dietary requirement.

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3.1.7 GRANTS

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CHAPTER 4 STUDY 2

ACCEPTED: Physiological Genomics

Author Contributions: Experiment conception and design; DR MT JT. Experiment implementation; JT DR MT BT. Data Analysis; DR JT BT FR RM SM AF EG M-CZ. Reagents/materials/analysis tools contribution; MT TS FR RM SM AF MK. Chapter composition; JT with assistance from DR.

4.1 TRANSCRIPTOME AND TRANSLATIONAL SIGNALING FOLLOWING ENDURANCE EXERCISE IN TRAINED SKELETAL MUSCLE: IMPACT OF DIETARY PROTEIN

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Running Head: Protein-Exercise induced Muscle Transcriptome

Key Words: dietary protein, skeletal muscle, gene expression, adaptation, mTOR, nutrigenomics.

4.1.1 ABSTRACT

Physical endurance activity and subsequent protein-rich diet were fundamental to early human survival and fitness evolution. However, only recently the timing of post-exercise protein-rich feeding was connected to functional adaptation in human skeletal muscle. Therefore the impact of adding protein to high-carbohydrate feeding following intense endurance exercise on genomic output, signalling regulating translation and whole body protein turnover was examined, to better understand the role of nutrition in adaptive remodelling. In a crossover, 8 well-trained men were biopsied at rest, 3 h and 48 h following 105-min cycling. Beverages containing 0.4/1.2/0.2 (PTN) or 0.0/1.6/0.2 (CON) $g \cdot kg^{-1}$ protein/carbohydrate/fat were ingested immediately and 1 h post-exercise. Ontology analysis of microarray results revealed over-represented developmental processes, signal transduction, ion transport, and muscle contraction (at both time points), immunity and defence, sensory perception and neuronal activities (3 h), glycolysis, lipid, fatty acid and steroid metabolism (48 h) with PTN; whereas, nucleoside, nucleotide and nucleic acid metabolism, protein transport, metabolism and modification were under-represented. Top differentially affected canonical pathways were; axonal guidance, actin cytoskeleton, calcium signalling, apoptosis, ephrin signalling, fatty acid metabolism, glycolysis/gluconeogenesis, dendritic cell maturation, Ca^{2+} binding proteins and PPAR signalling, signifying protein-nutrient facilitation of known exercise-regulated mitogenic, myofibril, and metabolic responses. Early post-exercise phosphorylation of $mTOR^{Ser2448}$, $RPS6^{Ser240/244}$, and 4E-BP1-γ increased, and AMPKα^{Thr172} decreased, indicative of enhanced translation initiation and elongation. Surprisingly, AMPK phosphorylation had increased by 48 h. Despite anabolic signalling, PTN had a trivial impact on whole-body protein turnover. These findings indicate protein-carbohydrate nutrition following endurance exercise refines and modulates the global gene expression response to endurance exercise. Adding protein to high-carbohydrate feeding may enhance recovery and adaptation via early attenuation of immune/inflammatory activity, coupled with regulation of myocyte extracellular matrix, cytoskeletal, and sarcomere remodelling, metabolic and mitochondrial function. Protein phosphorylation observations suggest an early increase in translation initiation and elongation, but also a later role for AMPK in the protein-

nutrition mediated metabolic transcriptome. In summary, protein-rich nutrition mediated fine-tuning of the broad adaptive response to endurance exercise.

4.1.2 INTRODUCTION

Human physical endurance and the intake of dietary protein were interconnected and fundamental environmental factors determining survival and human evolutionary fitness (Bortz 1985). The daily energy expended in physical activity of a Palaeolithic hunter-gather 91 $kJ \cdot kg^{-1}$ (Eaton 2003) was within the range of modern endurance athletes 75-230 $kJ \cdot kg^{-1}$ (van Erp-Baart, et al. 1989), and largely involved in procurement of a diet likely high in protein consumed within the hours following exercise (Bortz 1985; Eaton 2003; Eaton, et al. 1997). Considering that the musculoskeletal homeostatic systems and physical endurance capacity likely evolved during the last 2 million years (Bramble & Lieberman 2004), and that trained skeletal muscle is the probably the natural expression condition from an evolutionary perspective (Booth, et al. 2000), the endurance-exercise induced disturbance to skeletal muscle homeostasis, coupled with a post-exercise protein-rich diet might be considered normal environmental cues for adaptive remodelling. However, only recently has the connection between post-exercise protein feeding and improved functional adaptation in endurance-trained human skeletal muscle been demonstrated empirically (Rowlands, et al. 2008; Thomson, et al. 2011).

The endurance-exercise imposed myocellular stress includes generation of reactive oxygen species, inflammation, and disruption of cellular energy homeostasis and cell integrity (Morton, et al. 2009). These stimuli are rapidly sensed, transduced, and integrated through signalling pathways into a coordinated transcriptional response and eventual synthesis of proteins needed to ameliorate subsequent threats to cellular homeostasis (Fluck & Hoppeler 2003; Hood, et al. 2006; Yu, et al. 2003), and with chronic exposure, to eventually direct the accumulation of specific proteins leading to changes in the muscle phenotype contributing to enhanced endurance capacity (Choi, et al. 2005; Fluck 2006; Hawley, et al. 2006; Wittwer, et al. 2004). Recently, gene microarray analysis has provided an unbiased interrogation of the transcriptome response to endurance exercise and has uncovered novel pathways for discovery. Even after a single bout of endurance exercise, there is a coordinated up-regulation

of many of the mRNA transcripts encoding for components of the extracellular matrix and mitochondria in human skeletal muscle (Mahoney, et al., 2005). In endurance-trained skeletal muscle, over expressed of gene clusters involved in energy metabolism, mitochondrial biogenesis (Stepto, et al. 2009; Yoshioka, et al. 2003), inflammation and stress response (Wittwer, et al. 2004; Yoshioka, et al. 2003), membrane transport, (Stepto, et al. 2009), cellular signalling (Wittwer, et al. 2004), extracellular matrix (Timmons, et al. 2005a; Yoshioka, et al. 2003), angiogenesis, contractile (Stepto, et al. 2009) and cytoskeletal genes (Timmons, et al. 2005a) have been reported. Combined, the microarray data suggest gene expression reprogramming is a major mechanism driving phenotypic adaptation to exercise.

Recently, dietary intervention trials and the outcomes from several cell culture and animal studies suggest a considerable potential for nutritional modulation of the metabolic and myogenic gene expression profile following endurance exercise (Arkinstall, et al. 2004; Pilegaard, et al. 2005). For example, carbohydrate restriction following endurance exercise augmented metabolic gene expression and mitogen-activated protein kinase p38 (p38-MAPK) signalling (Cochran, et al. 2010; Pilegaard, et al. 2005), while peroxisome proliferator-activated receptor-α coactivator-1 alpha (PGC-1α), forkhead box protein O1 (FOXO1) and PDK4 expression were enhanced with high fat-protein feeding following endurance exercise (Pilegaard, et al. 2005). In addition to gene expression, it has been known for some time that protein feeding, in particular the branched chain amino acids, can up-regulate the protein synthetic machinery in human skeletal muscle after acute exercise (Rasmussen, et al. 2000; Volpi, et al. 2003). Carbohydrate ingestion through insulin signalling has a permissive effect on protein synthesis via protein kinase B (AKT) and glycogen synthase kinase 3 (GSK3) (Greenhaff, et al. 2008; Hawley, et al. 2006; Miyazaki & Esser 2009), although the main role of insulin in protein balance is attenuation of protein degradation (Greenhaff, et al. 2008).

Together, amino acids, insulin, and exercise act through both separate and common pathways to control protein synthesis, with many converging on the mammalian target of rapamycin complex I (mTORC1). mTORC1 is a key regulator of translation initiation through the downstream targets ribosomal S6 kinase 1 (p70S6K) and 4E

binding protein 1 (4E-BP1) (Kimball & Jefferson 2006b), and stimulates ribosomal biogenesis boosting capacity for protein synthesis (Welle, et al. 2009; Yang, et al. 2008). Moreover, a role has also been established for mTORC1 in activation of mitochondrial biogenesis, through an interaction with the transcriptional factor, yin-yang 1 (YY1) and PGC-1α (Cunningham, et al. 2007). More recently, it was shown that long-term feeding of branched chain amino acids to mice led to greater mitochondrial capacity, improved endurance exercise capacity, lower oxidative stress and higher anti-oxidant enzymes, and enhanced survival; acutely, the amino acids were shown to function in part through an (eNOS)/mTORC1 pathway (D'Antona, et al. 2010). These findings raise the strong possibility that the consumption of protein in the early post-endurance exercise period could potentiate and refine the exercise-induced signalling pathways. However, to the best of the researcher's knowledge the broad molecular program specifically induced in response to protein nutrition following exercise has not yet been explored, and this rationale forms the first principle objective of the current study based on microarray analysis of the global muscle transcriptome.

To complement the transcriptome profiling, the specific protein-nutritional effect on up- and down-stream components of the mTORC1 signalling pathway was investigated, to isolate the effect of protein co-ingestion on signalling activation of translation initiation. Furthermore, because the gene-expression response changes over time (Mahoney, et al. 2005; Yang, et al. 2005), the expression and signalling over both acute (3 h) and longer (48 h) post-exercise time points (Mahoney, et al. 2005) was profiled. In line with the established classical myofiber and metabolic-biochemical responses to endurance exercise (Holloszy & Booth 1976; Holloszy & Coyle 1984), it was hypothesised that post-exercise protein nutrition would direct a molecular program favouring slow fibre type transformation, angiogenesis, mitochondrial biogenesis, and increased fatty acid oxidation. This study provides novel insights into the role of dietary protein in nutrient-stimulated signalling and processes governing regeneration and adaptation from an acute bout of endurance exercise in endurance-trained skeletal muscle.

4.1.3 MATERIALS AND METHODS

4.1.3.1 Subjects

Eight healthy endurance-trained male cyclists and triathletes with mean ± *SD* age of 32.8 ± 6.4 y, height of 178.8 ± 3.6 cm, and weight 76.7 ± 5.2 kg completed the study. Maximal oxygen uptake (VO_{2max}) was 4.6 ± 0.6 $L \cdot min^{-1}$ and corresponding peak power output (W_{max}) 349 ± 34 W. The cyclists had been training for 11.7 ± 6.3 y with a reported weekly average training duration of 9.3 ± 4.5 h over the 6 months prior to the study that included high-intensity training or competition. All subjects provided informed written consent to participate in the study, prior to any measurement or testing. The study was approved by the McMaster University Hamilton Health Sciences Human Research Ethics Board.

4.1.3.2 Experimental Design

The design was a single blind, randomized, crossover involving the ingestion of a protein-enriched and control supplement following high-intensity interval cycling exercise. Detail of the design and an experimental block is illustrated in Figure 4.1 and described below. The washout period between blocks was one week.

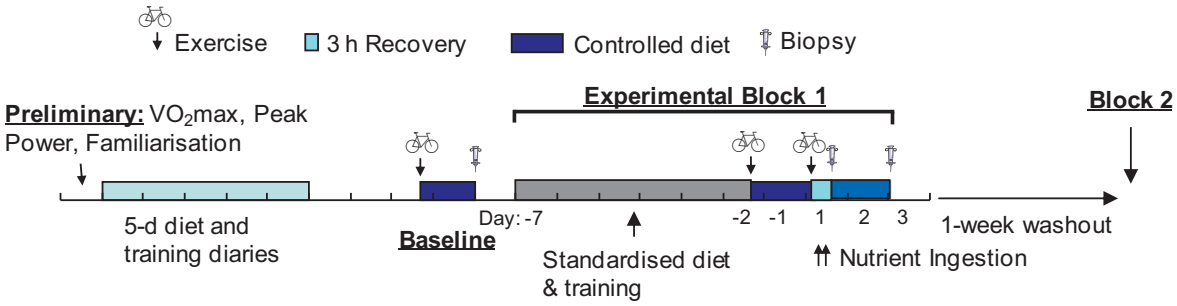


Figure 4.1 Experimental design

Preliminary Testing

One to 2 weeks prior to the start of the first experimental block, subjects reported to the laboratory for measurement of body mass, maximal oxygen uptake (VO_{2max}), and peak power. VO_{2max} and peak power were ascertained via cycle ergometry (Excalibur Sport V2, Lode BV Groningen, The Netherlands) and on-line assessment of external respiration (Moxus Modulator VO_2 system; AEI Technologies,

Pittsburgh, PA) using a continuous graded exercise test to exhaustion as described previously (Thorburn, et al. 2006). Following the VO_{2max} test, each cyclist completed the first two blocks of intervals of the loading ride (see below) for familiarization and to verify fitness status. For the following 5 days, cyclists compiled a 5-day diet and training diary that included one weekend day for calculation of average energy intake during normal activities and training. Diet records were analyzed using appropriate software (Nutritionist Pro, version 2.2; First DataBank, San Bruno, CA). No testing was conducted on or around major holidays, shortly before or after any major competitions, or during times when subjects' diets or training deviated significantly from normal.

Baseline

Seven days prior to the first experimental block, a baseline muscle biopsy (see below) was collected at rest at 0900 h following an overnight fast. The prior effects of physical activity and diet were controlled: at 1700 h two days prior (minus 40 h) participants cycled for 90 min, then consumed packaged meals to a prescribed schedule provided by the researchers and abstained from alcohol for the remaining evening and following day. The 90-min exercise consisted of a 30-min graded warm up to 50% of peak power, followed by 5 min intervals of 70% and 50% peak power respectively for 45 min, and a cool down at 30% peak power for 15 min. Cyclists ingested water *ad libitum* during exercise. The following day did not involve any exercise training.

Experimental Block

From 7 days prior to each experimental block subjects recorded their diet and training for 5 days (Figure 4.1; day -7 to day -2) for replication during the second arm of the study. The controlled exercise and dietary regime experienced prior to the baseline muscle biopsy were then repeated in preparation for the interval cycling and recovery nutrition intervention. On day 1, cyclists reported to the laboratory fasted between 0800-0900 h (start time standardized for each cyclist). The inserted catheter was kept patent with isotonic saline. To simulate real-life normal practice, and to replenish liver glycogen and reduce hunger, participants received a light pre-exercise

meal consisting of 1 granola bar (Quaker® Crunchy Granola Bar, The Quaker Oats Co., Chicago, IL) and 250 mL water.

Fifteen minutes later, cyclists started 105 min of high-intensity interval cycling. Briefly (Figure 4.1), following 3 x 10-min blocks of 30-50% peak power, cyclists completed 4 blocks of high intensity intervals interspaced with 6 min recovery at 50% peak power. The first two blocks consisted of 4 x 2-min intervals at 90% alternating with 2-min at 50% peak power. The following two blocks consisted of 4 x 2-min intervals at 80% alternating with 2-min at 50% peak power. The ride was modelled on the near exhaustive loading ride used previously in a performance study of national-level cyclists and triathletes (Rowlands, et al. 2008), but moderated to ensure completion by all riders of the present sample. During the high-intensity interval cycling protocol, cyclists received 10.7 $mL \cdot kg^{-1} \cdot h^{-1}$ artificially sweetened electrolyte solution (18 $mmol \cdot L^{-1}$ Na^{+}, 3 $mmol \cdot L^{-1}$ K^{+}) to maintain hydration but allow for substantial diminishment of glycogen stores. During all exercise procedures, the cyclists were cooled with fans in order to minimize thermal distress.

Following exercise, participants voided urine and then recovered in the laboratory for 3 h. The nutritional intervention was consumed immediately after exercise and again at 1 h post exercise. Muscle biopsies were collected at 3 and 48 h post exercise, with the latter sample being taken following an overnight fast. Baseline, 3 h and 48 h sampling times were chosen to examine both the rapid (3 h) and delayed/sustained (48 h) response of protein-nutrition to post-exercise recovery muscle gene and protein responses. The expression of metabolic and myogenic genes have been shown to typically peak at 3-8 h post exercise before beginning a subsequent decline toward baseline (Pilegaard, et al. 2000; Yang, et al. 2005).

4.1.3.3 Skeletal Muscle Tissue Collection

Needle biopsy samples were taken from the vastus lateralis approximately 15-20 cm proximal to the lateral knee joint under local anaesthesia (1% lindocaine, Astra Zeneca) using a 5 mm Bergström needle with manual suction. All biopsies were taken from separate incisions on alternating legs and 5 cm proximal from previous incisions, to minimize the potential effect of the initial biopsy injury on gene expression in subsequent biopsy samples (Aronson, et al. 1998; Lundby, et al. 2005;

Malm, et al. 2000). Approximately 150 mg of muscle was taken from each biopsy, immediately dissected from any visible fat and connective tissue, and then sectioned. Three sections of approximately 25 to 50 mg were placed in RNAse-free polyethylene tubes, snap-frozen in liquid nitrogen, and stored at -80^{o}C until analysis. A fourth section was added to Gluteraldehyde solution (2% Gluteraldehyde in Sodium Cacogylate) and stored at 2-4^{o}C until staining and examination by light microscopy.

4.1.3.4 Blood and Urine Collection

Blood was transferred into EDTA vacutainers before exercise, and at 0 and 180 min, and 48 h post exercise. Blood was stored on ice, then centrifuged at 3000 rpm for 10-15 min (Centrifuge, Haraeus Sepatech Medifuge, Germany), followed by aspiration of plasma and storage at -80ºC for later analysis. Total urine output was collected from the end of exercise until the morning of day 3 into 4 L containers containing 15 mL of 6 M hydrochloric acid to preserve ammonia and urea. Following collection, volume was measured and an aliquot stored at -80^{o}C.

4.1.3.5 Nutritional Intervention and Dietary Control

Immediately and at 1 h following exercise, subjects ingested 1.9 g·kg^{-1} (dry weight) of experimental nutritional powder made up to 500 mL with water. Each of the two serving units were constructed to provide 1.2 g·kg^{-1} carbohydrate, 0.4 g·kg^{-1} protein, and 0.2 g·kg^{-1} fat (abbreviated PTN or protein-rich nutrition depending on the grammatical context), or 1.6 g·kg^{-1} carbohydrate, and 0.2 g·kg^{-1} fat (CON or control nutrition). The PTN formulation provided carbohydrate shown to induce hyperinsulinemia and maximize the glycogen synthesis rate after exercise (Ivy 2004) and protein calculated to provide excess amino-acid signal and/or substrate to maximize post-exercise protein synthesis rates for ≥6 h (Wagenmakers 1999). For each representative 500 mL serving the PTN beverage contained 44.5 g maltodextrin, 40 g fructose, 21.3 g freeze dried canola oil, 19.8 g milk protein concentrate and 13.3 g whey isolate, vanilla flavouring, and 0.22 g sodium chloride; the control condition contained the same ingredients with the omission of the milk and whey protein and the caloric equivalent replaced with maltodextrin.

To standardize diet, subjects ingested a controlled diet during day -1, and for the entire period beginning 5 h post exercise on day 1 until bed on day 2. The controlled diet was designed to provide energy equivalent to the previously determined average daily intake of each subject, with macronutrient composition of 55% carbohydrate, 16% protein, and remaining energy provided by dietary fat (controlled diet: 3459 ± 662 kcal·d^{-1}, 506 ± 91 g·d^{-1} carbohydrate, 132 ± 25 g·d^{-1} protein, 104 ± 23 g·d^{-1} fat; values are means ± standard deviation). Dietary intake on day 1 included both the PTN and CON nutrition powders in order to balance total daily nutrient intake and hence by design, isolate the specific effects of post-exercise protein nutrition. In both experimental conditions, the alternate nutritional powder was ingested at 5 and 9 h post exercise, along with a salad roll and evening meal, respectively. The control diet consisted of a prescribed schedule that included milk drinks, eggs, cheese, supermarket-packaged salads with dressing, nuts, dried fruit, granola bars, bananas, commercially prepared standard vegetarian salad rolls.

4.1.3.6 Biochemical and Molecular Analysis

4.1.3.6.1 Illumina Microarray

Total RNA extraction, labelled-cRNA synthesis, and hybridization

Muscle tissue for gene microarray analysis (approximately 10 mg wet weight) were disrupted and homogenized in lysis buffer using a FastPrep instrument and lysing tubes containing ceramic beads (MP Biomedicals, Irvine, CA, USA). Total RNA was then extracted and purified with the RNAdvance tissue kit (Agencourt, Beverly, MA, USA) through an automated procedure. After the extraction, RNA samples were quantified with the RiboGreen RNA Quantification Kit (Molecular Probes, Eugene Oregon), and then monitored with the Agilent 2100 Bioanalyser, to check RNA quality (RNA integrity numbers ≥ 8 for high quality).

All cRNA targets were synthesized, labelled and purified according to the Illumina TotalPrep RNA amplification protocol (Applied Biosystems/Ambion, Austin, TX, USA). These operations were done on an automated system originally developed for the preparation of samples for Affymetrix arrays (Raymond, et al. 2006), which was then adapted for the Illumina procedure.

Briefly, 100 ng of total RNA were used to produce double-stranded cDNA, followed by *in vitro* transcription, and cRNA labelling with biotin. This method is based on the Eberwine T7 procedure (Van Gelder, et al. 1990). Prior to the hybridization on the gene microarrays, 750 ng of biotin-labelled cRNAs were added to the hybridization mix, which contained control oligonucleotides (such as negative and hybridization controls), hybridization buffer, and water. Then, 15 µl of each hybridization mix were dispensed on the arrays. After an overnight hybridization (16 hours, 58°C), the arrays were washed to remove non-hybridized material and stained with Streptavidin-Cy3, which covalently binds to biotin.

Microarray Selection

All samples were analyzed with HumanRef-8 v2.0 Expression BeadChips (Illumina, San Diego, CA, USA), which comprise probes to interrogate 22 184 transcripts. The content of the BeadChip is based on the curated content from the NCBI Reference Sequence (RefSeq) database, release 17 and includes probes for genes with unique locations in the human genome. Probes were specifically designed to avoid querying pseudo-genes and SNP sites.

To quantify mRNA levels for each sample, 900,000 beads are available on the BeadChip, with an average redundancy of 40 beads per bead type with one bead type per transcript. Each bead contains multiple copies of a gene-specific 50mer probe. Probe length and design, as well as bead redundancy ensure measurement sensitivity, selectivity, and precision. Concatenated to the probe is a short *address sequence*, which is used during the array manufacturing process to identify the beads (Gunderson, et al. 2004).

Processing and statistical analysis of microarray

Scanning was performed using the BeadArray Reader (Illumina), which provides intensity values for all transcripts by measuring the signal emitted by the Biotin-Streptavidin-Cy3 conjugates responding to a laser excitation. Genome Studio software (Illumina) was used to extract and summarize signal intensity, which was expressed in absolute units, to which a background correction was applied and estimated for each array from the average signal of the negative control probes. 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 (Pearson correlation coefficient = 0). A mixed model analysis of variance for repeated measures was constructed within software (Partek, St Louis, MO) to determine the differential effects of treatment for balanced crossover design. The model parameters included as fixed effects sequence, treatment, time and the interactions treatment*sequence, treatment*subject, treatment*time (PTN effect), sequence*time (carryover), sequence*treatment*time (period), and subject*time (biological variability); subject was nested within sequence (subject (sequence)). A moderated F test was then applied using the Global Error Assessment (Mansourian, et al. 2004) to take into account sample size leading to a more robust analysis. The following temporal and treatment (PTN) comparisons were taken from the analysis of variance for informatics evaluation: the reference environment – expression relative to baseline in the control condition at 3 h and 48 h following exercise (CON-Pre at 3 h, CON-Pre at 48 h, PTN-Pre at 3 h, PTN-Pre at 48 h), and the differential expression profile in response to the addition of protein to the post-exercise nutritional milieu (PTN-CON 3 h, PTN-CON 48 h). For the array analysis, it is acknowledged that developments in statistical analysis are on-going and that global effect-size or biological-variance standardized likelihood-based gene selection criteria are probably inferentially superior; however, in the absence of a satisfactory validated approach a null-hypothesis based gene selection criteria ($p<0.05$) was utilized. In addition to the Log2 difference and fold change, effect size magnitude was qualified from the by-gene differential/composite standard deviation obtained from the Robust GEA error. As discussed in the statistics selection below, this, like other null-hypothesis testing approaches generates inflated-effect bias within the dataset, as illustrated by effect sizes of only moderate or greater magnitude selected for consideration within the informatics analysis.

Bioinformatics

Several bioinformatics tools were used to predict biological response in skeletal muscle to protein feeding following strenuous endurance exercise. To provide a

global unfiltered predictive primary classification analysis of the transcriptomal response based on evolutionary relationships the Panther (Protein ANalysis THrough Evolutionary Relationships) Classification System (http://www.pantherdb.org) release 6.1 (Thomas, et al. 2003) was used. The gene Expression Analysis tool within Panther, was used to classified the differentially expressed genes by molecular function and biological processes (Booth & Thomason 1991). Gene selections for each of the six primary differential comparisons were uploaded and used for the skeletal muscle tissue reference list all Illumina transcripts significantly (16357 Transcripts, $P<0.001$) expressed at least once in the analysis (including the Pre sample). Within Panther, molecular function is the function that a protein encoded by the expressed gene performs on its direct molecular targets. Biological process covers the biological systems to which a protein contributes. Panther utilizes the Gene Ontology™ (GO, http://www.geneontology.org) database, which provided reference and cross matching for construction of a Family-based gene ontology. Evaluation of ontology families delivered a road map of the principle evolutionary-based linkages differentially affected by treatment and time, which combined with targeted network and canonical pathway analysis using Ingenuity Pathway Analysis (IPA, http://www.ingenuity.com) provided the evidence base for evaluation of the *a priori* hypotheses and novel discovery analysis. A combination of manual extraction from the Panther, followed by sorting and data manipulation processing in spreadsheets (Excel, Microsoft, Seattle, WA) and the Statistical Analysis System (SAS Vers 9.1, SAS Institute, Cary, NC) were used to build the ontology list. This ontology-family based analysis approach circumvented the potential inferential errors from biologically spurious networks and canonical pathways, and the prospect of a difficult and unwieldy analysis that resulted when the complete differential gene selections were uploaded directly into IPA. Within IPA, the analysis criteria comprised of Ingenuity Expert Information (IPA knowledge base) + GO databases. Gene data was filtered by selecting only species=human and tissue=skeletal muscle with relaxed filter (analyze molecules). In exploratory analysis of the biological process ontology family immune and defence, an approximately two-fold increase in the number of genes included by the IPA selection was found when immune cells were included with skeletal muscle tissue within the filter selection. Leucocytes are

present in skeletal muscle tissue, and are known to play an important role in function including cellular communication, inflammation, and phagocytotic clean up following stress (Pimorady-Esfahani, et al. 1997). Consequently, for the ontology family immune and defence, the IPA analysis was run with the additional inclusion of immune cells and novel and noteworthy findings reported. Within each primary biological process family ontology, IPA network, function, and canonical pathway analysis outcomes were examined and the top two processes assigned a focus score of 3.0 or higher were selected for presentation and further evaluation. An IPA score of 3.0 is based on the $-\log_{10}$ (*p*-value, where the *p*-value is generated from the IPA-generated Fisher exact test) and both contains the volume and complexity of the present dataset and presents a conservative 0.05% chance that the pathway or process is not differentially regulated based on the number of pathways or process associated with the filtered gene selection. Biologically non-relevant networks, functions, and canonical pathways were filtered out. From the Panther classification analysis, it was evident that the primary PTN differential expression was directing expression of genes broadly involved in four primary clusters: tissue developmental processes, muscle contraction, metabolism, and cellular immunity and defence (responding to stress). Consequently, the nodal network analysis on the top IPA functions derived from input of the respective ontology clusters were the focus. Finally, skeletal muscle is primarily concerned with torque generation to facilitate limb movement, but the protein nutrition intervention was also predicted to alter amino acid and other substrate metabolism. It is noteworthy that while the selected database software substantially extend knowledge of the pathways and molecular processes differentially activated by exercise and the protein nutrition intervention, the databases are finite and reflect the current knowledge, criteria, and limitations of the respective software. The Illumina array output dataset will be deposited in NCBI's Gene Expression Omnibus upon submission for publication.

4.1.3.7 Analysis of Candidate Genes by Quantitative PCR

The expression of candidate gene responders to the post-exercise protein nutrition was quantified by reverse-transcriptase polymerase chain reaction (qPCR). The qPCR was later used to confirm microarray expression data. Primer and probe sets for the analysed transcripts were designed using Primer Express Software (Applied

Biosystems) (Supplementary Table T1). Total RNA was extracted from muscle tissue using a commercially available Trizol®Reagent (Invitrogen, Burlington, ON, Canada) following manufacturer's instructions. Briefly, 25 to 40 mg of muscle were immersed in 2 mL of Trizol reagent. Muscle samples were then homogenized using a glass homogenizer immersed in ice, and aqueous and organic phases were separated with 400 μL of chloroform. Total RNA was precipitated using 500 μL of isopropyl alcohol, washed with 75% ethanol, re-suspended in 14 μL of 0.1% diethylpyrocarbonate (DEPC) treated H_2O, and stored at -80 ^{o}C. The quantity of isolated RNA was determined using the GeneQuant Pro Spectrophotometer (GE Healthcare, Piscataway, NJ). Samples were treated with DNase inhibitors (Ambion, Austin, TX). A Biorad iCycler thermal cycler system and TaqMan reagents (Applied Biosystems, Streetsville, ON, Canada) were used to run the qPCR and fluorescence for mRNA amplification and quantification. The cycling parameters were as follows; reverse transcription at 48ºC for 30 min, AmpliTaq activation at 95ºC for 10 min to inactivate the reverse transcriptase enzyme, polymerase chain reaction denaturation at 95ºC for 15 s followed by polymerase chain reaction annealing/extension at 60ºC for 1 min, repeated for approximately 40 cycles to propagate the amplification phase of the reaction.All samples were run simultaneously in duplicate along with negative controls that lacked RNA. The housekeeping gene was Beta 2 microglobulin (Mahoney, et al. 2004).

4.1.3.8 Analysis of Protein Phosphorylation by Gel Electrophoresis and Western Blot

The control of global protein synthesis through the mTORC1 pathway was determined through densitometric (Western blot) quantification of the phosphorylation status of mTORC1, and of several up-stream affecters and down-stream targets of mTORC1. In short, 25-40 mg of wet muscle tissue was homogenized in sucrose-mannitol buffer (70 mM sucrose, 220 mM mannitol, 5 mM HEPES, 1 mM EGTA, pH 7.5), protease inhibitor cocktail (AEBSF, Aprotinin, Leupeptin, Bestatin, Pepstatin A, E-64). Protein concentration was determined by the method of Lowry et al. (Lowry, et al. 1951) and quantified using spectrophotometry (Cary 300 Bio UV-Visible spectrophotometer, Varian, Palo Alto, CA). Twenty μg of protein samples were boiled in Laemmli buffer and resolved and separated by SDS-

polyacrylamide gel electrophoresis (Mini-PROTEAN II Electrophoresis Cell Biorad, Mississauga, ON, Canada). Proteins were then transferred onto polyvinyldifluoride membrane gels (Millipore, Bedford, MA). Primary antibodies were: phospho-AKT (Ser473, 1:1000, #9271, Cell Signaling, Danvers, MA), total-AKT (1:1000, #9272, Cell Signaling), phospho-AMPKα (Thr172, 1:1000) (#4188, Cell Signaling), total-AMPKα (1:1000, #07-363, Millipore, Billerica, MA), phospho-p38-MAPK (Thr180/Tyr182, 1:1000, #9212, Cell Signaling), total-p38-MAPK (1:1000, #9215, Cell Signaling), phospho-ERK1/2 (Thr202/Tyr204; 1:1000, #9101, Cell Signaling), total-ERK (1:1000, #9102, Cell Signaling), phospho-mTOR (Ser2448, 1:1000, #2971, Cell Signaling), total-mTOR (1:1000, #2972, Cell Signaling), phospho-S6 ribosomal protein (Ser240/244, 1:1000, #4838, Cell Signaling), total-S6 ribosomal protein (1:2000, #2217, Cell Signaling), total-4E-BP1 (1:1000, #9452, Cell Signaling), phospho-p70S6K (Thr389, 1:400, #11759, Santa Cruz Biotechnology), total p70S6K (1:400, #230, Santa Cruz Biotechnology). Loading standards were anti-actin (1:10000) (#612657, BD Biosciences, Mississauga, ON, Canada) and anti-tubulin (1:1000) (#2125 Cell Signaling). Membranes were probed with a horseradish peroxidase-conjugated secondary antibody (Amersham, Piscataway, NJ), visualized with chemiluminescence (ECL Plus, Amersham Piscataway, NJ) and quantified with densitometry (*ImageJ* v1.34s software, *rsbweb.nih.gov/ij/*). Within-subject control was achieved by running the two levels of treatment and the phosphorylated and total protein on the same blot, with the exception of AMPK and p70S6K which were run as two separate gels due to problems with the phospho blots after stripping, and were compared against the actin standard. For the 4E-BP1 blot analysis, the band representing the 4E-BP1 gamma-isoform was compared with the total protein in the blot (alpha + beta + gamma bands), and represented as the fraction of gamma-isoform.

4.1.3.9 Muscle Glycogen, Plasma Insulin and Glucose

Pro-glycogen (PG) and macro-glycogen fractions (MG) were extracted based on the method of Adamo and Graham (1998) and the resulting glucose concentration determined fluoremetrically according to the method of Passoneau and Lowry (Passoneau & Lowry 1993). Plasma insulin and glucose concentrations were determined by enzyme-linked immunoabsorbant assay (IS130D Insulin ELISA kit,

Calbiotech, Spring Valley, CA) and enzymatic method (Passoneau & Lowry 1993), respectively.

4.1.3.10 Histological Assessment of Myofibrils

Whether the protein-rich nutrition had a detectable effect on muscle damage was evaluated via histological quantification of Z-disk streaming and the number of necrotic fibbers. Toluidine blue stained semi-sections (~1μm think) were examined at 1000x magnification (100x oil with 10x ocular) using light microscope. The degree of muscle damage was determined via assessment of the amount of Z-disk streaming (ultra structural skeletal muscle pathology). Z-disk streaming was quantified as the number of damaged Z-disks per fibre per mm^2 multiplied by severity, where foci were assigned the ordinals 0 = no Z-disk streaming, 1 = focal, 2 = moderate streaming 3 = extreme focal. The number of fibres per unit area assessed ranged from 18 to 95 with a median of 42. Necrotic or dying fibres were characterized by internalized nuclei, oedema, and loss of Z-disk, disorganization, and pallor.

4.1.3.11 Whole Body Protein Turnover

Oral ^{15}N-glycine ingestion was used to assess whole-body protein turnover during the recovery period according to previously described methods (Waterlow, et al. 1978). Briefly, a single 2 $mg \cdot kg^{-1}$ dose of ^{15}N-glycine (98% enriched, Cambridge Isotope Laboratories, Andover, Mass.) was added to the first serving of the recovery beverage. Nitrogen flux was calculated from the accumulation of ^{15}N-ammonia and nitrogenous compounds in the 22-h urine collection on day 1 of recovery. The enrichment (ratio of tracer–tracee, tr:T) of ^{15}N-ammonia were determined in duplicate using isotope ratio mass spectrometry (Metabolic Solutions, Nashua, NH). The tr:T for the accumulative sample was corrected for background ^{15}N-ammonia enrichment established from the analysis of a urine sample collected before exercise on day 1. Whole body nitrogen flux (Q), protein synthesis (PS), protein breakdown (PB), and net protein balance (NPB = PS – PB) were calculated as follows: Q = d/corrected tr:T, PS = $(Q - N_E) \times 6.25$, and PB = $(Q - N_I) \times 6.25$, where d is the oral ^{15}N dose (g glycine × 0.1972), N_E is the total 22 h nitrogen excretion in grams, N_I is 22 h total dietary nitrogen intake over the sample period, and 6.25 is the amino acid

nitrogen constant. In presentation, PS and PB are normalized by dividing through body mass. Total ingested dietary nitrogen intake on day 1 and 2 of recovery was 22.3 ± 0.36 and 20.5 ± 0.44 $g \cdot d^{-1}$, respectively. Urinary urea concentration was measured by spectrophotometry (Benchmark Plus Microplate reader, Bio-Rad, Hercules, California) and creatinine by high-performance liquid chromatography (Agilent-Hewlett Packard, 1100 Series, Waldbronn, Germany). Additional (~21%) nitrogen loss from urinary creatine, and total nitrogen losses from sweat, faeces, and miscellaneous at rest was added to measured urinary urea and creatinine output based on ratios established previously in endurance athletes (Tarnopolsky, et al. 1988).

4.1.3.12 Statistical Analysis

Sample size. The sample size required for magnitude-based mechanistic inference (Hopkins, et al. 2009) was estimated to detect the smallest mRNA expression by qPCR and protein phosphorylation detection by Western blot of 1.25-fold change, assuming within-subject reliability of 25%. The resulting sample size was n=8. The Illumina microarray was exploratory and not subject to *a priori* power analysis. *Post-hoc* analysis revealed sufficient power to detect moderate effect size (0.9-1.1).

General method. The effect of PTN on all dependent outcome variables except the Illumina microarray (described above) was estimated from mixed models in SAS. To accommodate heteroscedastic variance all data were log transformed prior to analysis, except the ordinal light microscope data. Consequent outcomes were presented as the adjusted or geometric mean and the appropriate error and uncertainty as factors of the mean. Estimate precision was by 95% confidence interval. Sole reliance on null-hypothesis testing has been criticized (Cohen 1994; Rozeboom 1960; Sterne & Smith 2001); and whilst *p*-values were provided for reference to the null hypothesis, interpretation via magnitude-based inference with a modified Cohen's effect size classification to describe magnitude (Hopkins, et al. 2009; Rowlands, et al. 2008) was also utilized. Confirmation of the magnitude of the log2 differential expression estimated by qPCR vs. Illumina was provided by Pearson correlation.

4.1.4 RESULTS

4.1.4.1 Candidate gene expression analysis provides limited insight to protein-nutrition mediated metabolic, stress, and growth related responses

In the initial investigation of the transcriptional response, real time qPCR was utilized. Data from the qPCR was used to address whether the addition of protein to the post-exercise nutrition could regulate genes associated with cell energy homeostasis, growth and differentiation, inflammation, cholesterol biosynthesis, membrane stress, calcium signalling, and branched-chain amino-acid metabolism. The response and statistical summary is illustrated in Supplementary data sheet S1 (All supplementary sheets are on a CD attached to the back cover of this thesis and a summary of the material can be found at the end of the chapter). In response to exercise relative to baseline (comparisons include CON-Pre at 3 h, CON-Pre at 48 h, PTN-Pre at 3 h, and PTN-Pre at 48 h), likely moderate to extremely large increases in *DDIT3*, *PPARGC1α*, *FOXO1A*, *NFkB*, *PDK4*, *DSCR,* were observed, but only small increases in *BCKDK* and *SREBF2* in PTN at 3 h. In the control condition SIRT3 experssion was decreased at both time points (CON at 3 and 48 h), and *PDK4* down-regulated at 48 h. With post-exercise protein nutrition a moderate increases was observed in genes associated with mitochondrial and carbohydrate metabolism *PPARGC1α* (encoding PGC-1α) and *PDK4*, and endoplasmic reticulum stress and growth *DDIT3* and *FOXO1A* (Supplementary data sheet S1) at 3 h; and likely moderate up-regulation of *PPARGC1α* and *PDK4* expression at 48 h in comparision to control. Expression of *IGF-1* was unresponsive to either exercise or protein.

4.1.4.2 High correlation between differential log2 qPCR and robust array gene expression

A high correlation was found between quantitative change in expression of candidate genes measured by qPCR and change in the same transcripts in the Illumina microarray, following background correction and normalization (Supplemental Figure F1 Relationship between qPCR and microarray).

4.1.4.3 Illumina microarray analysis shows a large number of genes differentially expressed in response to exercise and high protein-carbohydrate nutrition

Following the exciting initial evaluation of the qPCR outcomes, it was decided that transcriptomics would be used to explore the impact of adding protein to post-exercise carbohydrate-lipid nutrition on global gene expression. Furthermore, the microarray permitted the exploration and identification of previously undescribed nutrient-responsive biological processes, molecular functions, and candidate epigenomic regulatory events. The complete statistical analysis is provided in Supplemental data sheet S9. Table 4.1 presents the total number of differentially expressed genes derived from the robust GEA analysis and the subsequent Panther mapped gene ontology. Refer to Supplementary data sheet S10 for full names of unlabelled abbreviated genes.

TABLE 4.1 SUMMARY OF GENE SELECTION

Analysis	Comparison					
	CON-Pre 3 h	CON-Pre 48 h	PTN-Pre 3 h	PTN-Pre 48 h	PTN-CON 3 h	PTN-CON 48 h
GEA ANOVA	3389	1811	3610	2037	1219	1399
Panther Classification	3293	1744	3511	1973	1172	1352

Data are number of genes selected following the respective filtering criteria: global error assessment (GEA) analysis of variance, $p<0.05$ and Panther Classification mapped genes.

4.1.4.4 Ontology analysis identified a temporally affected molecular program regulating gene expression, tissue development and remodelling, and metabolic adaptation in response to protein nutrition following intense endurance exercise

The Panther classification analysis provided the ontology of over- and under-represented biological processes and molecular functions differentially affected in the skeletal muscle tissue by exercise and protein nutrition (summarized in Figure 4.2 and see Supplemental data sheets S4-6). At 3 h post-exercise relative to the resting state, the ontological family groupings for biological processes that were

over-represented in both PTN and CON conditions; were developmental processes, carbohydrate metabolism, lipid, fatty acid and steroid metabolism, immunity and defence (including under-represented stress response, and natural killer cell mediated immunity), muscle contraction, protein metabolism and modification (over-represented in PTN-Pre 3 h, and under-represented in CON-Pre 3 h), signal transduction (varied functions were over/under-represented), and transport processes. Biological processes under-represented at 3 h in both PTN and CON relative to baseline were nucleoside, nucleotide and nucleic acid metabolism, neuronal activities, and sensory perception. Noteworthy, genes involved in apoptosis induction were also over-represented but apoptosis inhibition under-represented in PTN; and did not reach the selection threshold for differential in CON at 3 h. Genes involved in cell cycle control and cell structure and motility, coenzyme and prosthetic group metabolism, and porphyrin metabolism were over-represented in CON-Pre only. Adding protein to the post-exercise recovery environment differentially affected most of the biological processes that were over- or under-represented 3 h following exercise relative to baseline. Nuclear processes and regulatory events around transcription (nucleoside, nucleotide and nucleic acid metabolism) and post-transcriptional (protein metabolism and modification, targeting and localization; median expression down-regulated) were under-represented. In contrast to 3 h, greater variation in the global expression profile was observed at 48 h between the PTN and CON comparisons, but protein nutrition drove over-representation of developmental processes, muscle contraction, signal transduction, glycolysis, fatty acid and sulfur metabolism, and anion transport. As at 3 h, the expression of genes involved in nucleoside, nucleotide and nucleic acid metabolism were under-represented. Intracellular and nuclear protein trafficking were also under-represented (Supplemental data sheet S5).

Notable ontological family groupings of molecular functions relative to resting baseline (Supplemental data sheet S5) which were over-represented included genes for heat shock protein chaperones in CON at 3 h, while the defence and immunity classification was over-represented at 3 h with PTN, and both PTN and CON at 48 h. Extracellular matrix protein expression was over-represented in CON at 3 h and both CON and PTN at 48 h; median expression was up-regulated in CON at 3 h, however

mostly down-regulated in PTN at 3 and 48 h relative to baseline. Genes coding for cytoskeletal proteins and related functions were some of the most over-represented in response to both exercise and protein nutrition, and provide strong evidence for an impact of post-exercise protein nutrition on cell structure. At 48 h however, only the molecular function actin binding motor protein was over-represented with all 8 actin-binding motor protein transcripts up-regulated with PTN (Supplemental data sheet S6). Select calcium binding proteins were over-represented in PTN at 3 h and did not reach selection criteria in CON, but over-represented in both by 48 h. Signaling molecules were over-represented and under-represented in PTN at 3 and 48 h respectively, whereas CON exhibited the opposite pattern. Related to excitation-contraction coupling, genes for several voltage-gated (calcium, sodium, potassium) ion channels were over expressed in response to PTN compared with CON at 3 h, but there were no genes which reached selection threshold for differential in CON versus baseline comparisons. Of note, nuclear processes surrounding regulatory events around transcription (nucleic acid binding) were mixed over/under-represented at 3 h and under-represented by 48 h. Within the transcription factor ontology family the majority were over-represented throughout. Molecular functions under-represented at 3 h included genes for protease, protease and metalloprotease inhibitors, and hydrolase, in CON. Biological processes and molecular functions that were differentially over- and under-represented are presented in Figure 4.2. Next, the key relationships, genes, and functions derived from the ontology selections, canonical pathway and network analysis are described.

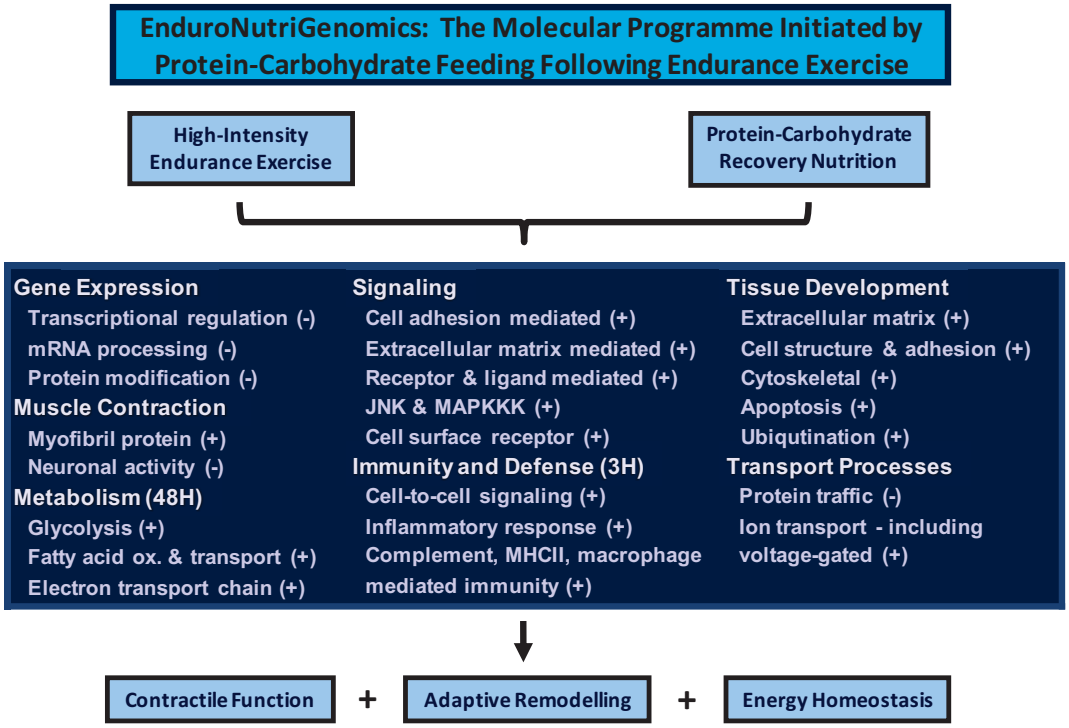


Figure 4.2 Summary of the molecular programme and affected biological processes initiated by the addition of protein to high carbohydrate-lipid nutrition following high-intensity endurance exercise

4.1.4.5 Protein nutrition directs a temporally-responsive molecular program governing adaptive tissue development and remodelling

Developmental processes and muscle contraction (Figure 4.3) represent two biological processes of high functional interest that were differentially affected by protein nutrition following exercise. The top IPA canonical pathway identified in the analysis of the developmental processes was axonal guidance signalling (Supplemental data sheet S8), which was also top canonical pathway in the analysis of the signal transduction ontology family. In the context of skeletal muscle, axonal guidance signalling is associated with assembly and organization, attraction and adhesion, and signalling through the extracellular matrix and cell surface receptors (Dickson 2002). Network analysis (Supplemental data sheet S7 Developmental Processes) and further investigation (Supplemental data sheet S6) revealed ephrin B (*EFNB1*, *EFNB3*) expression was mixed up and down with PTN at 3 h. Ephrin receptor transcript A2 (*EPHA2*) was up-regulated at 3 h with PTN, while A7 and A10 were down-regulated at 48 h. Semaphorin (*SEMA3F*, *SEMA6A*, *SEMA6D*)

expression was down-regulated with exercise, but *SEMA3C*, *SEMA3F* and the semaphorin ligand-binding subunit neurophilin (*NRP1*), were up-regulated with PTN at 48 h. Netrin (*NTN4*, *NTNG2*) expression was up/down at 3/48-h with exercise, but up-regulated with PTN at 48 h. The Slits/Robo mediated pathway (*SLIT2*, *RHOA*, *RHOC*, *RHOJ*, *CXCL12*, and *CXCR4*) genes were variably expressed, but without PTN affect. Ephrin and Semaphorin signalling is also involved in angiogenesis (Serini & Bussolino 2004). In the present study, PTN attenuated the exercise-induced increase in the gene encoding angiopoietin-related protein (*ANGPT2*) expression at 3 h, and up-regulated platelet-derived growth factor beta polypeptide (*PDGFB*) and fibroblast growth factor 2 (*FGF2*) gene expression at 3 h and 48 h, consistent with vascular remodelling. Vascular endothelial growth factor (*VEGF*) was up-regulated in both conditions, but with no PTN differential.

The molecular function ontology family extracellular matrix (ECM) was over-represented in CON at 3 and 48 h and PTN at 48 h, but down-regulated in PTN at both time points compared to CON (Supplementary data sheet S5). Expression of genes classed as ECM structural proteins were up-regulated with exercise but down-regulated with PTN at 3 h (*COL5A1, COL6A3, COL10A1, COL22A1, COL3A1, COL5A2, COL11A2, COL1A1, COL1A2, COL18A1*) and 48 h (*COLQ, COL3A1, COL22A1, COL6A1, COL1A1*). Glycoprotein gene expression was down-regulated with PTN at 3 h and 48 h (*FBLN2, FBLN1, EFEMP1, SDC2, LRFN5*), except for except for laminin 4 (*LAMA4*) and the tenacins (*TNC, TNN, TNXB*) which were up at 3 h and down at 48 h. Extracellular matrix communication, cell-cell and cell-matrix gene expression were up-regulated with exercise, and differentially mixed up and down-regulated with PTN at 3 h (*TGFBI, CSPG2, TNFAIP6, MUC4, TNXB,* and *LAMA4)*. Additionally, the integrins *ITGAM, ITGB2, ITGB1* were up-regulated with exercise, but only *ITGB1* was differentially up-regulated with PTN at 3 h. The exact role of integrin in the present analysis is not entirely clear, but the integrins are key receptors involved in cell and ECM interactions and could function in the convergence of growth factor signalling with extracellular matrix, cytoskeletal reorganization and cell development via modulation of FAK-JNK signalling events (Legate, et al. 2009). Another novel observation was that transmembrane transport activity, specifically, 18 voltage-gated ion (Na^+, Ca^{2+}, K^+) transporter protein

subunits, were differentially expressed with protein nutrition at 3 h. There was no differential expression in ion transporter genes relative to rest; but gene expression was up-regulated with protein nutrition in comparison to carbohydrate control.

Examination of developmental processes ontology revealed a protein nutrition differential in expression of several well characterized myogenic control factors (Supplemental data sheet S6). Myogenin (*MYOG*, myogenic factor 4) was up-regulated with both exercise and PTN at 3 h. The growth differentiation factors, *GDF1*, *GDF7*, and *GDF8* were varied up and down-regulated with exercise. PTN influenced expression of growth differentiation factor *GDF1* via down/up-regulation at 3/48-h; while *GDF7* was up-regulated at 3 h. Myostatin (*GDF8*) was down-regulated with exercise at 3 h with the reduction non-significantly 1.5 fold greater with PTN. Myogenic factor 5 (*MYF5*) was up-regulated at 48 h with PTN, while myocyte enhancer factor 2C (*MEF2C*) was down-regulated at 3 h. Myogenic differentiation 1 (*MYOD1*) was up-regulated with exercise at 3 h and with protein nutrition at 48 h. The expression of *WNT5A* was down with exercise at 3 h, but attenuated with PTN. The WNT5A protein is involved in myogenic gene induction (*MYF5*, *MYOD*) and muscle development and slow-fibre specific transcription via the noncanonical pathway involving CAMKII (calcium calmodulin mediated kinase II), calcineurin and NFAT (Yang 2003).

Ontology analysis revealed PTN under-represented but up-regulated expression of apoptotic inhibition genes (*BCL2, BCL2L14,* and *IFNA1*) at 3 h. Further investigation of protease molecular function and extracellular matrix biological process (Supplemental data sheet S6), uncovered that PTN differentially regulated the expression of several apoptotic genes as well as those involved in major protein breakdown mechanisms. The pro-apoptotic caspases (*CASP3, CASP10, CASP1*) were up-regulated at 3 h (no differential by 48 h), while several cathepsin genes, involved in the lysosomal degradation pathways, were down-regulated with PTN at 3 h (*CTSC, H, L, K, O, Z*). Metalloproteinases, *MMP9, 13, 19* were up-regulated, while the metalloproteinase inhibitors *TIMP1, TIMP2* were down-regulated with PTN at 3 h. Ubiquitin-protein ligases E2 and E3, involved in substrate specific targeting of proteins for ubiquitination and degradation (Murton, et al. 2008), were differently

down/up-regulated by exercise and protein nutrition at 3/48-h, respectively. Genes encoding several E2 and E3 proteins (*FBXO32, TRIM 5, UBE2J2, PDZRN3, TRIM 46, RFFL*) were down-regulated with PTN at 3 h and genes encoding several E3 proteins (*FBXL3, FBXO24, SMURF1, UBE2, TRIM9, ZNRF2*) comparatively up-regulated by 48 h.

Within the ontology muscle contraction, the IPA interrogation resolved 7 canonical pathways with IPA score over 3 at 3 h, but only 1 pathway at 48 h. The top 3 focus genes (*MYLPF*, *MYL4*, and *MYL1*) were common to all pathways at 3 h and encode for myosin light chain, which are involved in actinomyosin assembly. The top pathway at 3 h, actin cytoskeletal signalling, and the pathway common to both 3 h and 48 h; calcium signalling are presented (Supplemental data sheet S8). At 3 h myosin light chain, myosin complexes, and actin filament exhibited mixed up and down transcript expression in response to exercise. With exercise, alpha actin skeletal and cardiac isoforms (*ACTA2, ACTC*) were up-regulated at both time points but with no protein nutrition differential. Varied up/down-regulation of fast twitch (*MYH1, MYL1, MYL5, MYLPF, MYBPH, MYL3, MLC1SA*) and slow twitch myosin isoforms occurred at both time points in response to exercise; with PTN, mainly relative down-regulation at 3 h, but very large to extremely large (1.2-3.6 fold change) differential by 48 h were observed. The temporal PTN differential pattern was manifest especially in the embryonic and perinatal myosin heavy and light chain isoforms (*MYH3, MYH8, MYL4*) and cardiac troponin T type 2 (*TNNT2*), with down/up-regulation at 3/48-h. (Supplemental data sheets S3, S6). Evidence from canonical pathway and networks analysis suggests myosin expression may be regulated up-stream through integrin or *RHOA* associated signalling. Analysis of genes encoding other myofibril protein complexes within the calcium signalling pathway (troponin T and I, and tropomyosin), supports evidence that protein ingestion has minor generally down-regulatory impact on myofibril protein expression at 3 h, but up at 48 h. With respect to the cytoskeletal transcriptome, within the ontology families cell structure and motility (biological process), and cytoskeletal proteins (molecular function), PTN facilitated median down-regulation of tubulin, cytoskeletal proteins, mixed up and down intermediate filament expression by 3 h, and up-regulation of actin binding proteins by 48 h. Notably, PTN

attenuated the exercise-induced increase in expression of α/β tubulin subunit transcripts, caused mixed up and down expression of keratin and keratin-associated genes, down-regulation of nestin (*NES*), dystrophin (*DMD*), and vimentin (*VIM*), but had no impact on desmin (Supplementary data sheet S6). Interrogation of the signal transduction ontology in IPA confirmed cytoskeletal and extracellular matrix associated signalling processes were differentially affected by PTN at 3 h. For instance the two top canonical pathways, axonal guidance signalling and ephrin receptor signalling, were previously reported under developmental processes ontology. Moreover, signalling mediating structural modification was retained at 48 h in PTN compared to CON. In addition to the two top canonical pathways resolved at 3 h, canonical pathways included relaxin, cAMP-mediated, ERK/MAPK, PKA, IGF-1, and G-protein coupled receptor signalling (Supplementary data sheet S8 worksheet Signal Transduction). Noteworthy, genes within the adenylyl cyclase (*ADCYAP1*) and $Ca2^{+}$/calmodulin related proteins (e.g. *CALML5*, *MYLPF*, *MYL3*, *MYL2*, *MYL1*, *FBLN2*, *FBLN1*, *MRCL3*, *S100A8*, *S100A9*, *CABP5*, *CIB2*, *CAMK1D*, *MLC1SA*, *ADD*) were up-regulated with PTN at 3 h, but not significantly affected at 48 h. G-protein coupled receptor genes were mixed up/down-regulated with PTN at both time points, and ribosomal protein S6 kinase, 90 kDa, polypeptide 1 (*RPS6KA1*, *p90RSK* and cAMP-response element binding protein (*CREB1*) genes were unaffected at 3 h, but up-regulated with PTN at 48 h. Phosphodiesterase (*PDE*) was differentially up-regulated with PTN at both 3 h and 48 h. Temporal and nutrition induced differences in *ERK1/2* expression suggests some increased activity within the ERK1/2-MAPK signalling pathway. Finally, expression of transcription factor gene *cFOS* (which forms AP-1 dimer with cJUN and regulates cell proliferation, differentiation, and apoptosis) was up-regulated in response to exercise at both 3 h and 48 h, but not differentially affected by PTN.

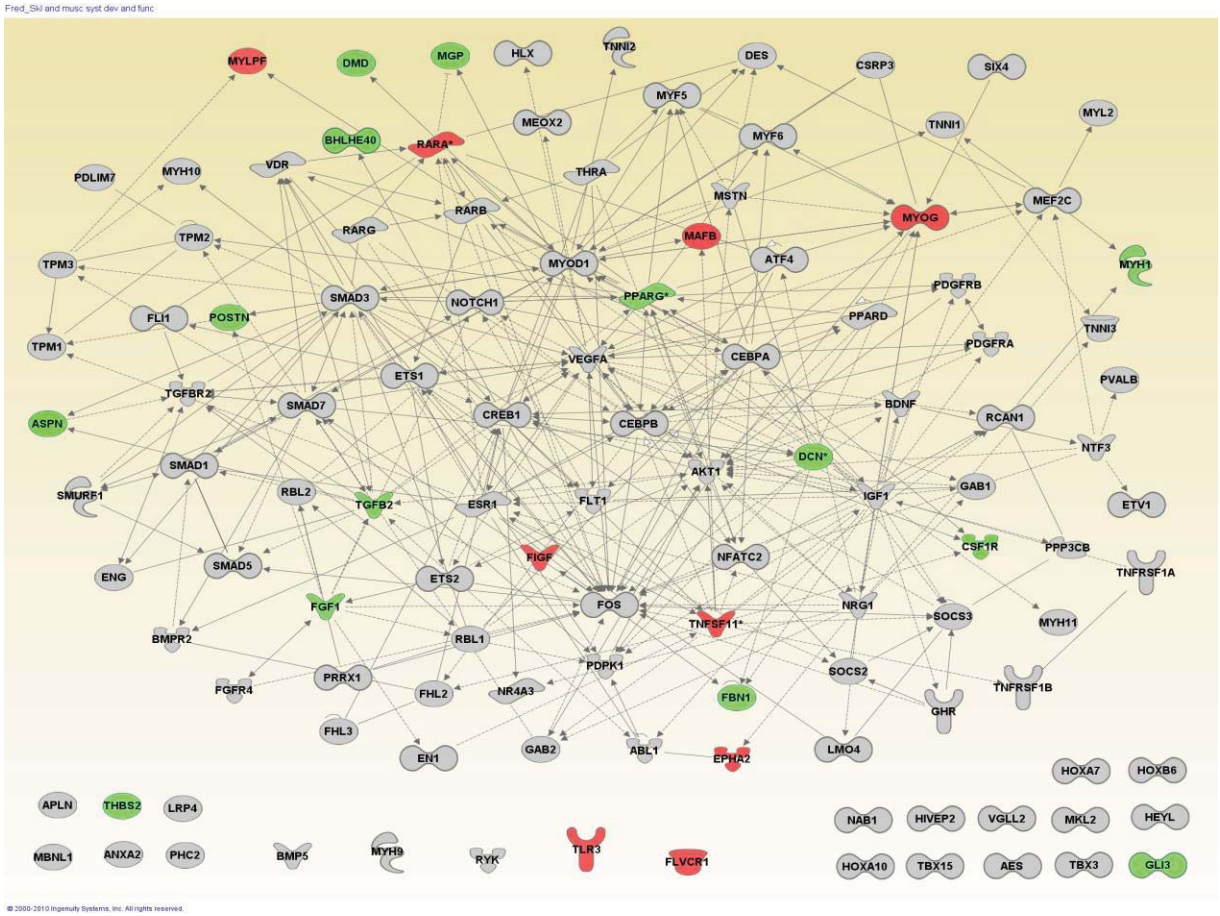
4.1.4.6 Protein nutrition attenuates the exercise-induced stress, immunity and defence response

Dendritic cell maturation was the top canonical pathway differentially affected by PTN at 3 h, with the majority of inclusive genes down-regulated (Supplementary data sheet S8 worksheet Immunity and Defence). Cell-to-cell signalling and interaction and the inflammatory response were the two top functions resulting from

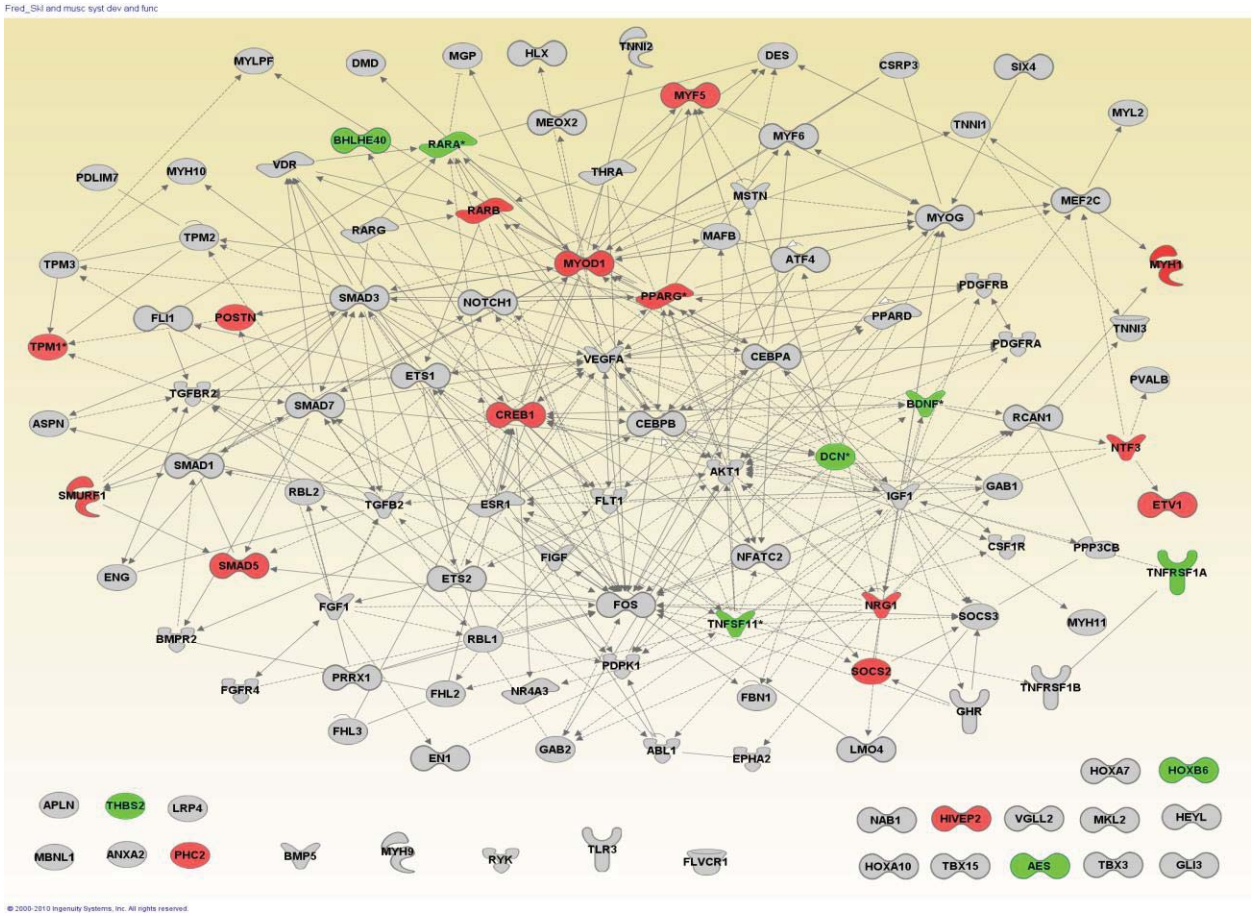
the IPA interrogation of the immunity and defence ontology family; with significant PTN differential expression identified at 3 h only. In the network analysis of cell-to-cell signalling and interaction (Figure 4.3; Supplementary data sheet S7 worksheet Immunity and DefenseSKM+Immune), the most interconnected genes in were associated with activation, recruitment, adhesion, and development of leukocytes. The nuclear factor of kappa B (*NFκB*), a key regulator of the inflammatory response, although up-regulated with exercise at 3 h was not differentially affected by PTN (Supplementary data sheet S1). Although several NFκB regulatory genes (*IKBKB*, *NFKBIA*, *TBK1*) were down-regulated, whereas (*NKIRAS2, TLR3*) were up-regulated with PTN at 3 h. Differentially down-regulated genes included *IKBKB* which mediates NFκB activation, and *GADD45B* which regulates the NFκB signalling pathway in response to stress; indicating a role for PTN in reduction of NFκB activity. Inflammatory cytokines the interferons, along with tumour necrosis factor are involved in dendritic cell maturation and a number of cytokines and chemokines are expressed by dendritic cells themselves (Olex, et al. 2010; Pimorady-Esfahani, et al. 1997). With PTN, gene expression of the interferons (*IFNA1*, *IFITM2*, *IFI6*, and *IRF8*) was mixed up and down, and tumour necrosis factor (TNFSF11) and interleukins (*IL1F6*, *IL1F8* transcript variant 2, *IL17D*, *IL22*) were up-regulated at 3 h. Eighteen other cytokines were expressed in response to exercise, but only 6 differentially affected by PTN. At 48 h the tumour necrosis factor family were mixed up and down with PTN. Further inflammatory response expression included genes involved in macrophage associated-processes which were differentially down-regulated with PTN, such as*ADORA2A* (adenosine receptor A2a) and the chemokine ligands (*CXCL2*, *CCL22*, *TNFRSF19*). Major histocompatibility complex (MHC) class II transcripts were down-regulated with PTN, indicating decreased antigen presentation function. Complement component 1 (*C1QA*, *C1QB*, *C1QC*, *C1S*) were down-regulated with PTN, while the complement cascade inactivator CFI was up-regulated. Furthermore, PTN attenuated the exercise-induced up-regulation of *CD86* (CD86 antigen) involved in T-cell activation. Together expression within the immunity and defence ontology indicates diminished immune response. Alternatively, the heat shock proteins (*HSPA1A, DNAJ*) were over-expressed and up-regulated with exercise at 3 h, and the *HSP70* (heat shock protein

70) gene family significantly up-regulated with PTN. Common to several other cell homeostasis-linked responses within the gene array, *HSP70* demonstrated an up-regulation at 3 h and down-regulation at 48 h temporal response.

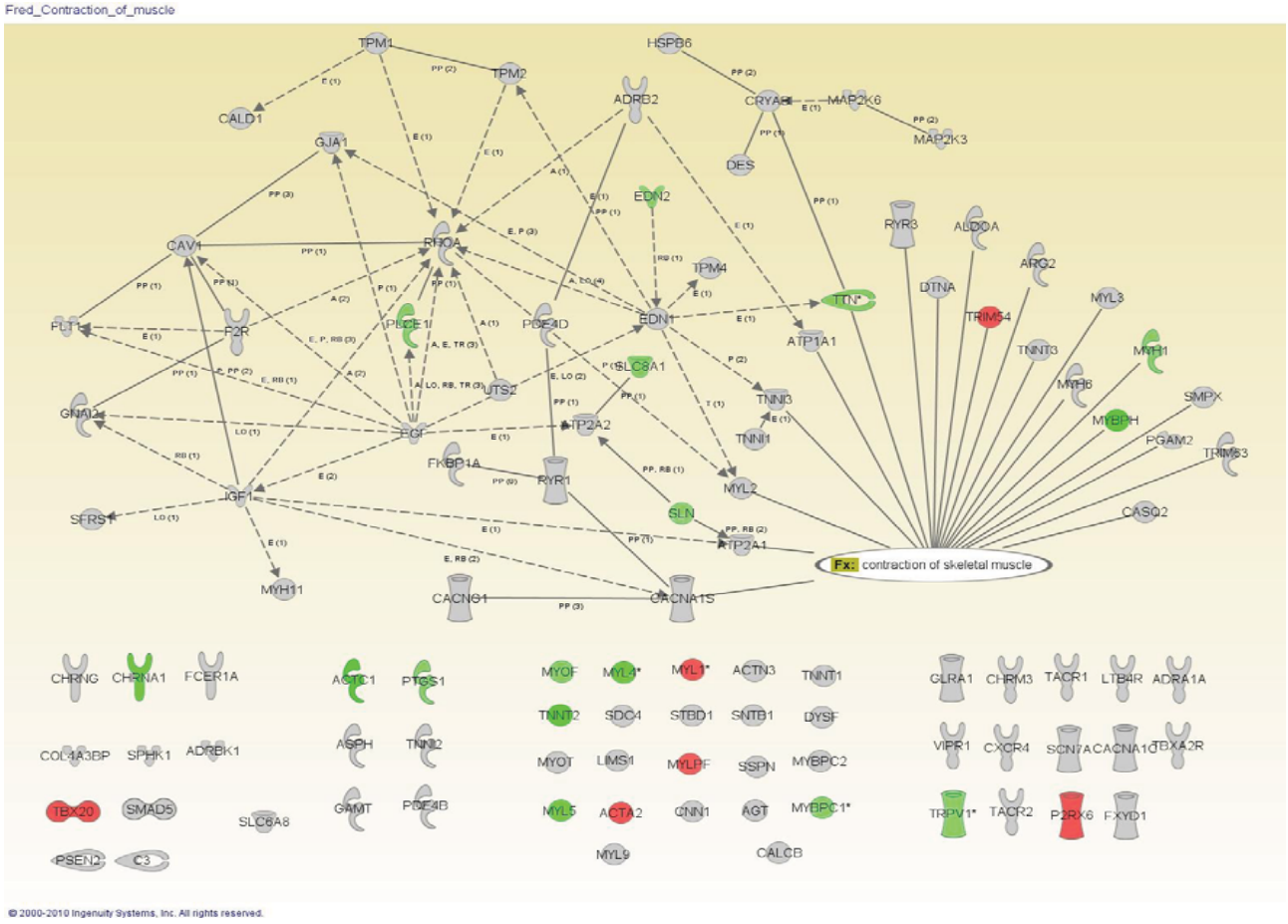
A. Skeleton and Muscular System Development and Function 3 h



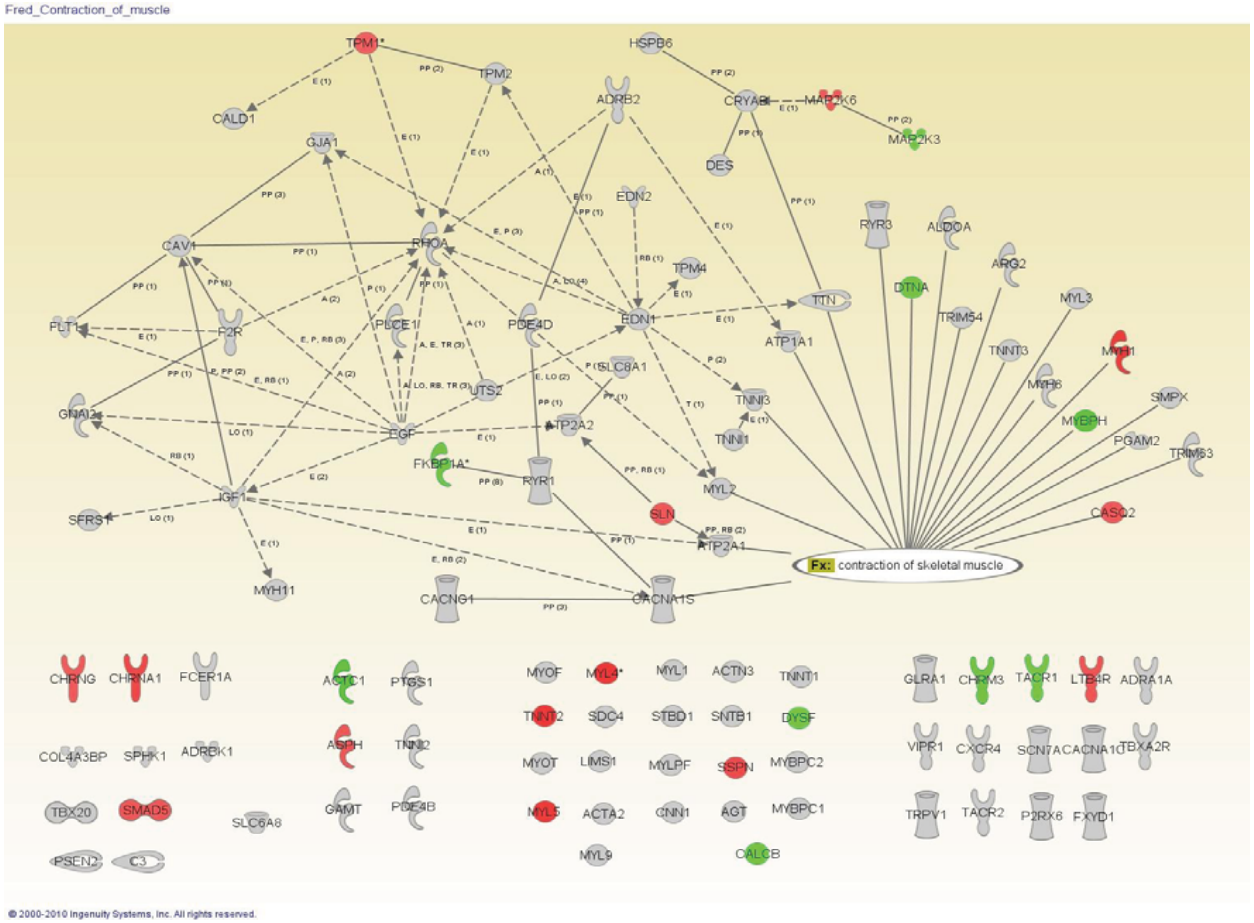
A. Skeleton and Muscular System Development and Function 48 h



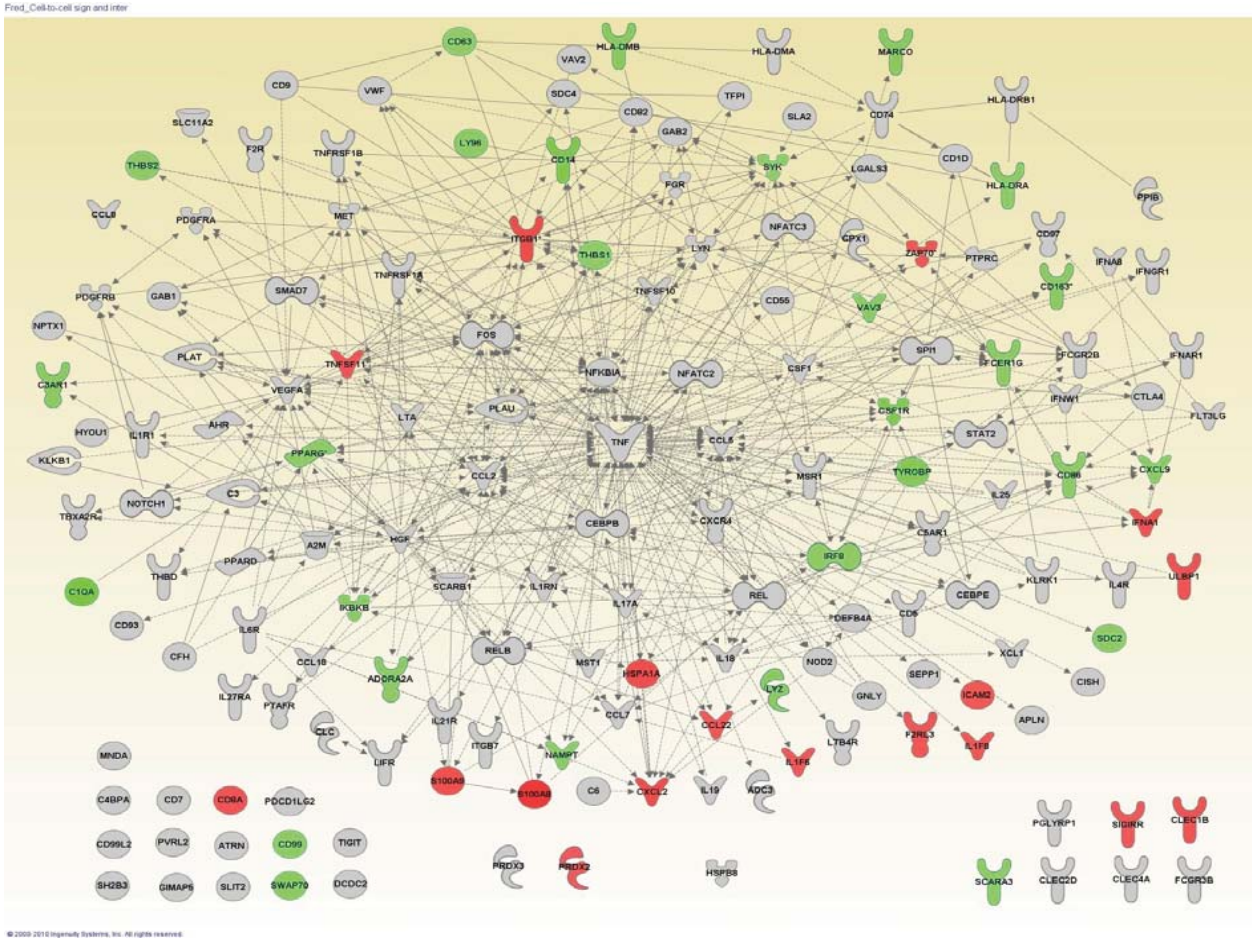
B. Muscle Contraction 3 h



B. Muscle Contraction 48 h



C. Cell-To-Cell Signaling and Interaction 3 h



C. Cell-To-Cell Signaling and Interaction 3 h

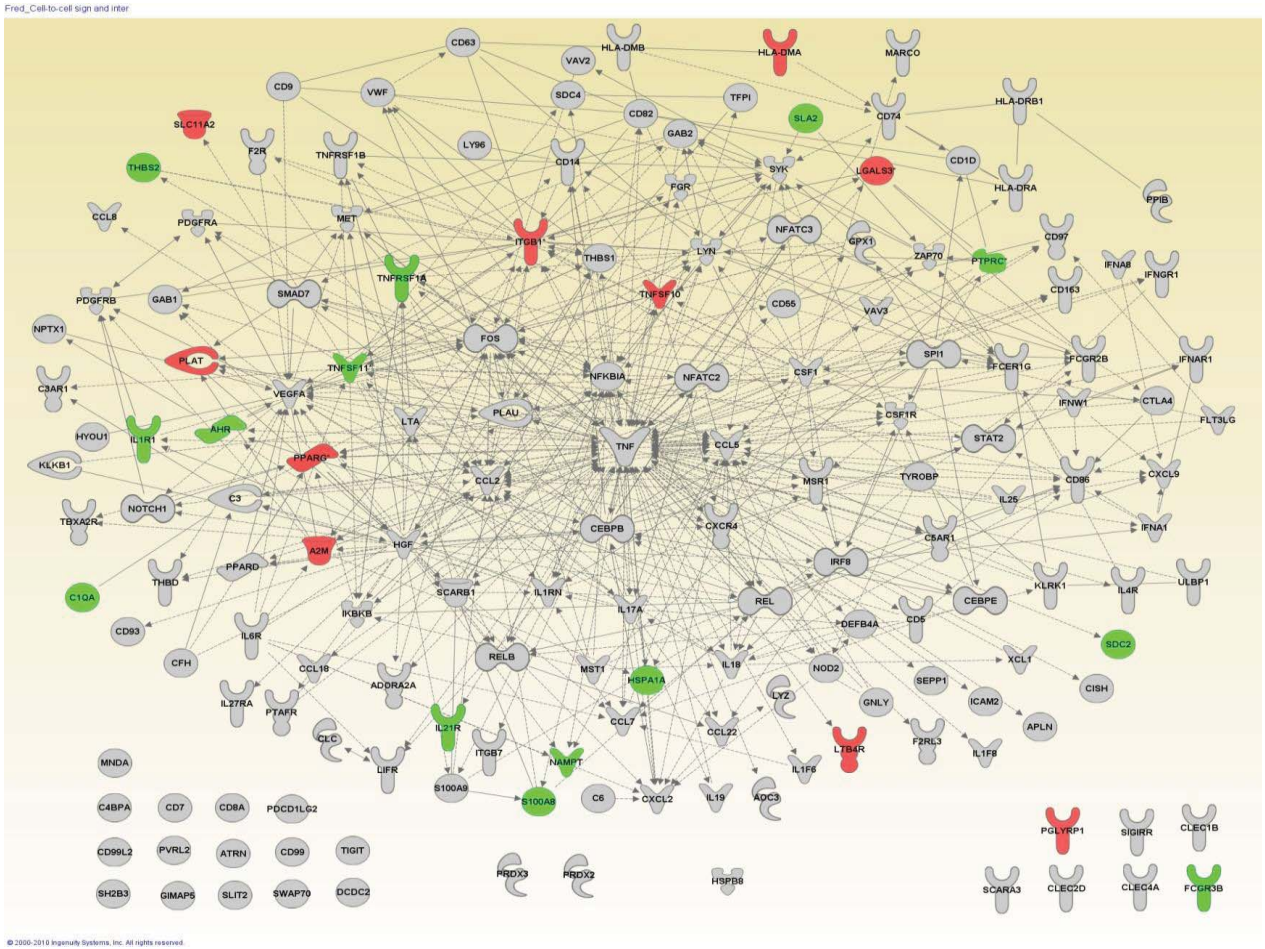


Figure 4.3 Top IPA networks for ontology families developmental processes, muscle contraction, and immunity and defence responding to the differential effect of PTN at 3 h and 48 h

A. Developmental processes IPA function: skeleton and muscular system development and function, **B. Muscle contraction**, **C. Immunity and defence** cell-to-cell signalling and interaction. Networks are generated for the respective ontologies from the gene selections inclusive of all six differential comparisons. Gene names shown in red are up-regulated and green down-regulated, whereas genes in grey are differentially expressed at one or more of the other five time points considered in the analysis. The intensity of red or green colour increases or decreases with expression magnitude (fold change relative to the reference condition). The protein-nutrition differential networks should be viewed in conjunction with the networks overlaid with the differential response for the four comparisons relative to rest; these networks are deposited in Supplementary data sheet S7. Top nodes are: skeleton and muscular system development and function PPARG (PPARγ), MYOG, MYOD1, VEGFA, CEBPB, RARA, AKT1, CREB1, FOS, SMAD3, ESR1, IGF1, SOCS3, TNFSF11, BDNF; muscle contraction RHOA, EGF, EDN1; cell-to-cell signalling and interaction TNF, VEGFA, FOS, NFKBIA, ITGB1, CEBPB, COL5, PPARG (PPARγ), CD86, SYK, CSF1, HGF, CXCL, CCL2, CCL5, PLAU.

4.1.4.7 A temporally affected metabolic control response governed by protein nutrition directs metabolism away from glucose and towards fatty-acid transport and oxidation

Analysis of the ontologies carbohydrate metabolism, and lipid and steroid metabolism suggest that post-exercise protein consumption modifies metabolic and mitochondrial respiratory gene expression. Gene expression of hexokinase and glucokinase, which phosphorylate and regulate entry of glucose into glycolysis were up-regulated in response to exercise; with no significant PTN differential at 3 h but relative down-regulation at 48 h (see Supplemental data sheets S8 worksheet Carbohydrate Metabolism and Supplemental data sheet S6). Genes for the glycolytic flux regulating enzymes *PFKFB* and *PFKM* (6-phosphofructo-2-kinase/fructose-2,6-biphosphatase and 6-phosphofructokinase, respectively) were down-regulated after exercise; but not differentially affected by PTN; although two exceptions were *PFKFB1* which was up-regulated with exercise at 3 h and *PFKFB3* up-regulated with PTN at 48 h. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was down-regulated with PTN relative to rest at 3 h and 48 h but had no significant differential. Further evidence was provided by genes involved in control of pyruvate entry into the mitochondria. With exercise *PDHA2* (pyruvate dehydrogenase alpha 2) was down-regulated, but no PTN differential was evident. Pyruvate dehydrogenase kinase isozyme 4 gene was up-regulated after exercise at 3 h without a PTN differential, but was up-regulated with PTN at 48 h (see Supplemental data sheet S8 worksheets Carbohydrate Metabolism and PPAR Signaling). The *DLAT* gene (dihydrolipoamide S-acetyltransferase) which encodes the E2 component of pyruvate dehydrogenase complex was also up-regulated with PTN at 48 h. The pyruvate dehydrogenase phosphatase catalytic subunit 1 (*PPM2C*) was down-regulated with exercise at 3 h, but up-regulated with PTN at 48 h. No other citric acid cycle genes were affected by exercise or protein nutrition.

The established endurance-exercise regulation of metabolic and mitochondrial gene expression by peroxisome proliferator-activated receptor-gamma and peroxisome proliferator-activated receptor-gamma coactivator 1 alpha were differentially

affected by PTN. Peroxisome proliferator-activated receptor-gamma (*PPARγ*) was up-regulated at 3 h and 48 h with exercise although relatively down-regulated with PTN at 3 h. Peroxisome proliferator-activated receptor-gamma coactivator 1 alpha (*PPARGC1α*) was extremely up-regulated by 2.8 fold at 3 h with exercise but not significantly affected by PTN. Conversely, by 48 h *PPARCC1α* had no exercise differential, but was moderately up-regulated by PTN (see Supplemental data sheet S8 worksheet PPAR Signaling; Supplemental data sheet S3). It is noteworthy that although the protein versus control differential comparisons were not significantly different, further transcriptional regulation of genes involved in pyruvate and fatty-acid oxidation may have been attained by up-regulation of *PPARδ* at 3 h (PTN-Pre comparison) and by down-regulation at 48 h (PTN-Pre comparison) (Supplemental data sheet S8 worksheet PPAR Signaling). In the network analysis of genes involved in the IPA defined functions Fatty Acid Metabolism and Transport of Fatty Acids; the most interconnected gene was *PPARγ* (Figure 4.3; Supplemental data sheet S7 worksheet Developmental Processes). The majority of genes involved in lipid metabolism were up-regulated by PTN at 48 h (Supplemental data sheet S6); and included those involved in the functions lipid transport (*ACSL1*, *CD36*, *CPT2*, *SLC25A20*, *CROT*, *CYP46A1*, *FABP5*, *LPL*, *PLIN2*, *PLTP*, *PPARγ*), oxidation of fatty acids (*ACOX1*, *ACSL1*, *CPT2*, *LPIN1*, *LPL*, *PECI*, *PPARγ*, *PPARGC1α*), and modification of lipids (*ACOX1*, *ACSL1*, *CPT2*, *CYP2U1*, *CYP46A1*, *LPIN1*, *LPL*, *MTMR6*, *PECI*, *PIK3CA*, *PPARγ*, *PPARGC1α*, *SCD*, *SULT1A1*). Interestingly, exercise up-regulated expression of HMGCS2 (3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2, mitochondrial) involved in fatty acid biosynthesis and fatty acid elongation, was attenuated with PTN. Furthermore, two genes encoding prostaglandin transporters (*SLC02A1*, *SLC01B1*) were down-regulated by PTN at 48 h, which taken together indicate that PTN might down-regulate prostaglandin synthesis and export in skeletal muscle.

Mitochondrial/metabolic components of considerable interest were the electron transport chain proteins. Expression at 3 h was mixed up/down with exercise and protein nutrition had little impact compared with baseline (Supplemental data sheet S7 worksheet Mitochondrial Electron Transport), with some exceptions. Transporter

gene expression was up-regulated by 48 h in response to exercise, with a further increase in expression of transcripts encoding mitochondrial electron transport chain components cytochrome c oxidase, NADH2 dehydrogenase (ubiquinone), and ubiquinol-cytochrome-c reductase with PTN. Whilst, uncoupling protein 3 (*UCP3*) was down-regulated at 48 h by exercise and protein.

4.1.4.8 Transcriptional regulation, post-translational processing, and epigenetic associated responses

Seventy-six and 63 transcripts classified under mRNA transcription regulation and mRNA transcription, respectively, differentially expressed at 3 h in response to PTN were selected (Supplementary Tables S5, S6). Several possible epigenetic factors were differentially affected by PTN. With regards to histone methylation, *DNMT3B* was down-regulated by PTN at 48 h. The DNA (cytosine-5-)-methyltransferase 3 beta has been shown to down-regulate *PPARGC1α* (encoding PGC-1α) and mitochondrial gene expression in muscle cell culture (Barrès, et al. 2009). N-6 adenine-specific DNA methyltransferase 1 (*c21orf127*) and isoprenylcysteine carboxyl methyl transferase (*ICMT*) were down-regulated at 3 h and both time points, respectively. Alternatively, arsenic (3+ oxidation state) methyl transferase (*AS3MT*) was up-regulated with PTN at 48 h. Other enzymes involved in histone remodelling, including the histone acetyltransferases and deacetylases; histone acetylatransfease 2 (*MYST2*) and heparin glucosaminyl 2 (*NDST2*) were down-regulated at 48 h and dihydrolipoamide S-acetyltransferase (*DLAT*) up-regulated at 48 h by PTN. The function of most of these enzymes remains largely unknown. Sirtuin 6 (*SIRT6*) and 5 (*SIRT5*) were up-regulated with PTN at 3 h and 48 h, respectively. Sirtuin 6 is involved in DNA repair and links histone H3 lysine 9 deacetylation to NF-kappaB-dependent gene expression (Kawahara, et al. 2009), while SIRT5 is located within the mitochondrial matrix and may have a role in mitochondrial protein deacetylation and amino acid metabolism, modulated by nutritional status (Gertz & Steegborn 2010). Interestingly, the SIRT5 substrate mitochondrial carbamoyl-phosphate synthetase 1 (*CPS1*) (Nakagawa, et al. 2009) was also up-regulated at 48 h by PTN. The transcription factor, SMARCA3 (SWI/SNF related, matrix associated, actin dependent regulator of chromatin, subfamily A, member 3) was also down-regulated with PTN at 48 h. The expression

of several key transcription factors and regulatory elements were recognized to regulate tissue development and metabolism; together with under-represented mRNA transcription regulation ontology, suggests tighter control of the transcriptome at 3 h. Furthermore, 136 genes classified as protein metabolism and modification and 48 classified as protein modification were differentially regulated at 3 h, suggesting PTN additionally influences the recovery phenotype via post-translational processing.

4.1.4.9 Protein nutrition induces acute activation of mTORC1, 4E-BP1 and RPS6 without impact on AKT or ERK1/2-p38-MAPK signalling, but has temporal impact on AMPKα phosphorylation

The effect of PTN on the phosphorylation status of signalling proteins regulating mTORC1 and translation initiation are shown in Figure 4.4, while the statistical summary is provided in Supplementary data sheet S2. At 3 h, large and very large (effect size) respective increases in the phosphorylation of the translation initiation regulators RPS6 and 4E-BP1 were observed, which was associated up-stream with a possibly trivial increase in p70S6K but moderate increase in mTORC1. AMPKα phosphorylation was not clearly different from baseline, but moderately increased control (Figure 4.4). The effect of PTN on the up-stream mTORC1 effectors AKT was trivial, and on p38-MAPK and ERK1/2-p42 unclear. By 48 h, the effect of PTN on RPS6 phosphorylation was negligible, however, 4E-BP1 and AMPKα phosphorylation were reduced and increased, respectively; responses in the other proteins were unremarkable.

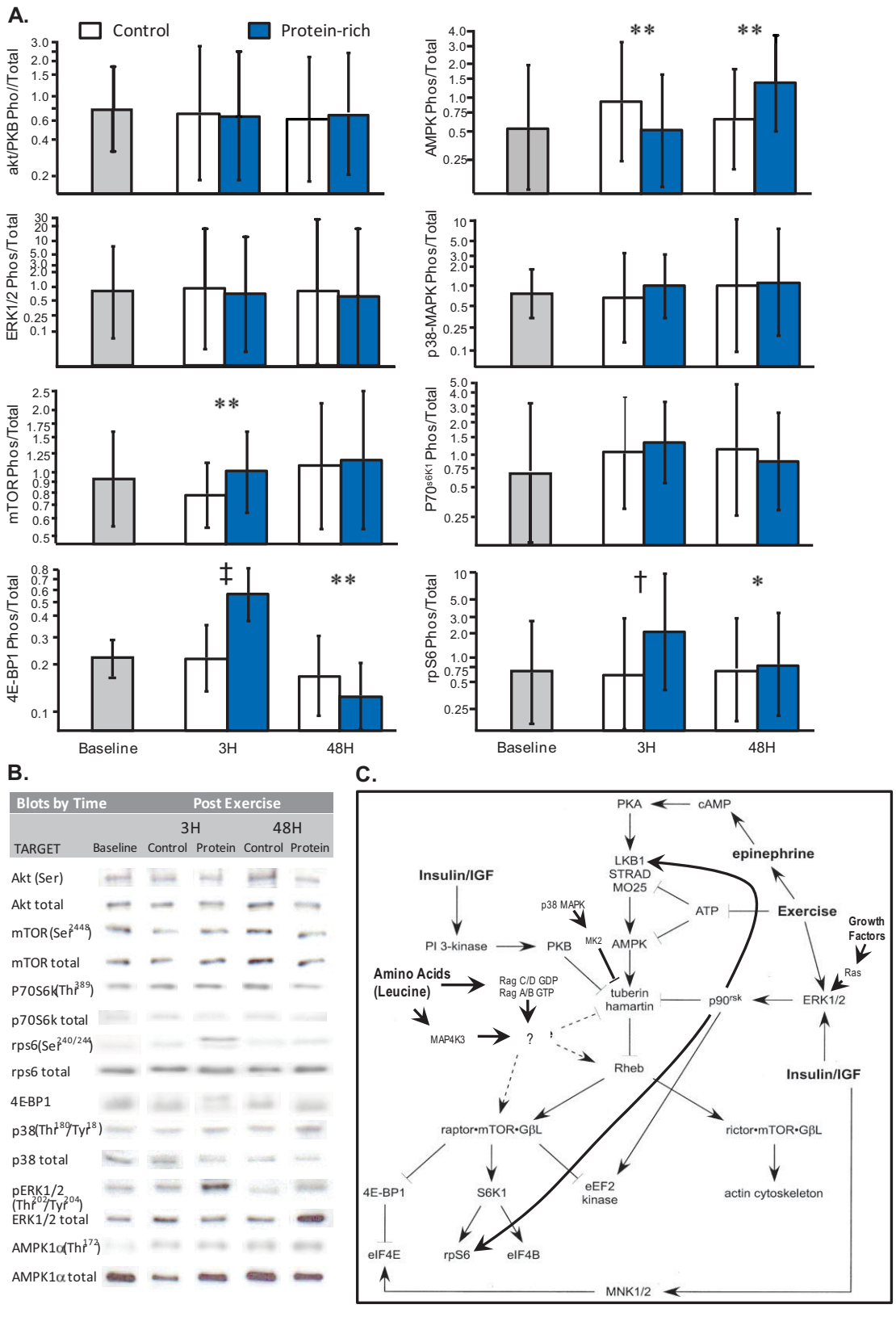


Figure 4.4 Effect of the addition of protein to high carbohydrate-lipid nutrition following high-intensity endurance exercise on the phosphorylation of proteins regulating mTORC1 and downstream translation initiation

A. Phosphorylation of proteins regulating mTORC1 activity and downstream factors regulating translation initiation. Where the symbols; * indicates small, ** moderate, † large, and ‡ very large effect sizes. **B.** Representative gels. **C.** Schematic summary of regulation of exercise and nutrient signalling through mTORC1; adapted from (Kim, et al. 2008; Kimball & Jefferson 2004; Kimball & Jefferson 2006a; Sancak, et al. 2008). Abbreviations with phosphorylation site and activation status in parentheses: AMP-activated protein kinase, AMPK (Thr^{172}); extracellular signal-regulated kinases 1 and 2, ERK1/2 (Thr^{202}/Tyr^{204}); eIF4E binding protein 1, 4E-BP1 (gamma-isoform/alpha + beta + gamma-isoforms); protein kinase B, AKT (Ser^{473}); ribosomal protein S6, RPS6 ($Ser^{240/244}$); p70 RPS6 kinase, p70S6K1 (Thr^{389}); mammalian target of rapamycin, mTOR (Ser^{2448}).

4.1.4.10 Whole-Body Protein Turnover

The addition of protein to the post-exercise nutrition had no clear effect on 22-h subsequent whole-body protein turnover (Table 4.2).

TABLE 4.2 WHOLE-BODY PROTEIN TURNOVER OVER THE RECOVERY PERIOD 22 h FOLLOWING EXERCISE

METHOD	Condition[a]		Outcome			
	Protein-Rich	Control	Protein Fold Effect ×/÷ 95% CL	Effect Size	*p*-value	Inference[b]
Nitrogen flux ($g \cdot d^{-1}$)	0.47 (15)	0.47 (28)	1.1; ×/÷1.49	0.04	0.94	unclear
Protein synthesis ($g \cdot kg^{-1} \cdot day^{-1}$)	1.9 (33)	1.9 (31)	1.0; ×/÷1.68	0.00	0.99	unclear
Protein breakdown ($g \cdot kg^{-1} \cdot day^{-1}$)	1.0 (97)	1.1 (71)	0.95; ×/÷2.38	-0.06	0.89	unclear
Net protein balance ($g \cdot kg^{-1} \cdot day^{-1}$)	0.72 (59)	0.69 (61)	1.04; ×/÷1.68	0.06	0.84	unclear

[a] Data mean with coefficient of variation (%) in parenthesis.

[b]Inference refers to the likelihood of an effect size >0.2 as defined by the effect-size descriptors: trivial <0.2, small (Sm.) 0.2–0.6, moderate (Mod.) 0.6–1.2, large (Lg.) 1.2–2.0, very large (V. lg.) 2.0-4.0, extremely large (Extr. Lg.) >4.0. An effect is unclear if its confidence interval includes both substantial increases and decreases.

4.1.4.11 Plasma Insulin and Glucose, and Muscle Glycogen Resynthesis

The PTN condition had trivial impact on circulating insulin (mean effect ±95%CL: 2.0% ±15% and glucose (4.9% ±7.0%) over three 3 h post exercise. The quantity of glycogen in dried muscle was not clearly affect by PTN at 3 h (-3.0% ±18.0%) and 48 h (2.2% ±18.0%) (Figure 4.5 A-C).

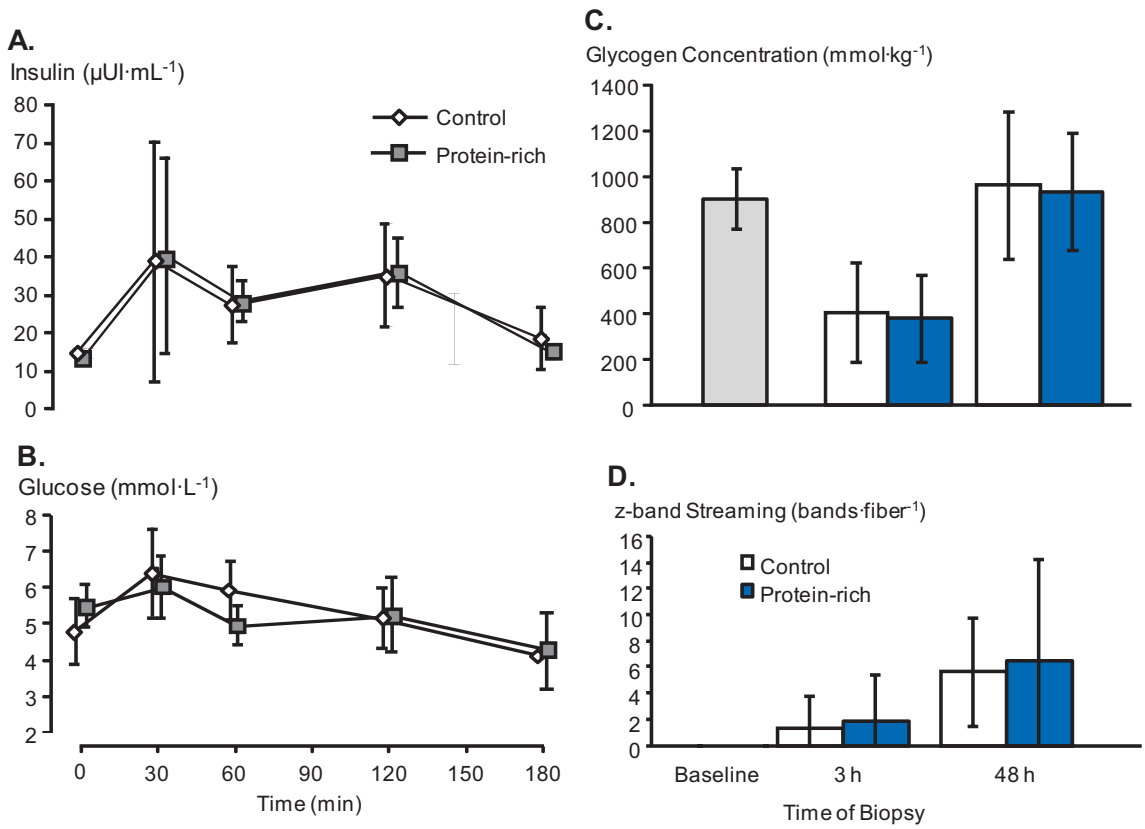


Figure 4.5 Effect of the addition of protein to high carbohydrate-lipid nutrition following high-intensity endurance exercise on plasma insulin and glucose, muscle glycogen, and Z-disk streaming

A. Plasma insulin and **B**. glucose concentrations during the first 3 h of recovery, **C**. total muscle-glycogen concentration and **D.** Z-disk steaming at 3 h and 48 h.

4.1.4.12 Muscle Damage (Histology)

There was no Z-disk streaming in baseline muscle (Figure 4.5 D). A small increase in streaming was observed at 3 h but there was no clear effect of treatment (PTN minus CON: 0.6 ±3.5 units). There was a large increase in streaming at 48 h from the value at 3 h (ES: Control 1.7 ±1.4; PTN 1.3 ±1.0), but also no clear effect of PTN

(ES: 0.09 ±1.9). Four of the 48 h samples comprising two in each condition contained necrotic fibres (percent necrotic fibres: 3, 28, 28, 33); however, there were no necrotic fibres identified at baseline or at 3 h.

4.1.5 DISCUSSION

The analysis strategy allowed us to infer associations between the observed molecular responses and the emerging phenotype for reduced muscle soreness and damage (Rowlands, et al. 2008; Saunders, et al. 2004; Thomson, et al. 2011), up-regulated signalling to translation initiation (Morrison, et al. 2008), and protein synthesis (Howarth, et al. 2009), and enhanced subsequent physical work capacity (Rowlands, et al. 2008; Thomson, et al. 2011) with high protein-carbohydrate feeding following endurance exercise. Bioinformatics analysis revealed that adding protein to the post-exercise diet induced a unique temporally biphasic molecular program supporting tissue development and metabolic adaptation to endurance exercise. The early (3 h) protein-nutrition mediated transcriptomal program was characterized by over-represented gene ontology families directing developmental processes including extracellular matrix and cytoskeletal structure, muscle contraction and synaptic transmission, cell adhesion and signalling, voltage-gated ion channels, and calcium-binding proteins. Supporting tissue remodelling were the under-represented apoptosis and over-represented proteolytic processes differentially affected with protein nutrition. Supporting restoration of tissue homeostasis was over-represented immunity and defence responses. Conversely, protein metabolism and modification was under-represented with protein nutrition at 3 h. Phospho-protein analysis demonstrated a protein nutrition induced up-regulation of mTORC1, RPS6, and 4E-BP1 suggesting increased protein synthesis, while AMPK phosphorylation was negated and unchanged from rest. The later (48 h) transcriptional program facilitated by protein nutrition maintained an over-representation of developmental processes, extracellular matrix, muscle contraction, voltage-gated ion channels, and immunity and defence gene expression, supporting tissue remodelling. In addition there was significant impact on metabolism, mostly through down-regulation of glycolytic processes and substantial over-representation of fatty acid oxidation and transport genes, and mitochondrial respiratory protein expression. The mitochondrial processes were linked to increased *PPARγ/δ* and

PPARGC1α expression and moderately increased AMPK phosphorylation at 48 h. Equally noteworthy, nuclear and post-transcriptional mRNA regulatory processes were differentially under-represented in response to protein nutrition both early and later (48 h) after exercise, with modifications including candidate epigenetic responses (methyltransferases, acetyltransferases, and deacetylases). While transcription factors were under-represented at 3 h and over-represented at 48 h with protein. Finally, the absence of a clear increase in whole-body protein turnover and muscle glycogen concentration due to the post-exercise protein feeding, suggest these mechanisms are unlikely to account for previously reported improved subsequent endurance performance phenotype (Rowlands, et al. 2008).

4.1.5.1 Post-exercise protein nutrition directs a molecular program regulating developmental remodelling and contractile function in skeletal muscle

Skeletal muscle contractile protein (O'Neill, et al. 1999), cytoskeletal (McGivney, et al. 2009), and extracellular matrix remodelling are central to phenotype adaptation to endurance exercise (Timmons, et al. 2005a; Timmons, et al. 2005b). Genes coding for cytoskeletal and contractile proteins were some of the most over-represented ontologies in response to both exercise and protein nutrition. Myogenic regulatory factors (*MYOD1, MYF5,* and *MYOG)* were up-regulated with protein nutrition at 3 h. With protein nutrition, a temporal differential in expression was observed, with mainly down-regulation at 3 h, but very large to extremely large differential (up- and down-regulation) by 48 h. The protein nutrition differential was especially evident in the developmental isoforms; embryonic and perinatal myosin heavy and light chain, and cardiac troponin were up-regulated. The biological function of these transient isoforms is believed to be in muscle fibre transition or formation of new fibres during muscle regeneration and development in adult skeletal muscle (Kadi & Thornell 1999). Although fast and slow twitch myosin isoforms had varied up- and down-regulation at both time points; differential expression within the calcium signalling canonical pathway (Supplementary Table S8 Worksheet Muscle Contraction) supports a role for protein nutrition directing slow myofibrillar development. Calcium-calmodulin signalling and activation of calcineurin may affect gene expression through transcription factors CREB, NFAT and MEF2

leading to increased expression of slow-fibre type MHC, MYL, troponin, and SERCA isoforms with endurance training (Grondard, et al. 2008; Pette 1998), a pattern consistent with the present protein-nutrition mediated differential expression response. Signaling activity was not examined, but *NFATc* and *CREB* expression was up-regulated with protein nutrition at 48 h, and evidence for other calcium signalling activity at 3 h and 48 h was provided by calcium related canonical pathways (Supplementary Table S8, worksheets Muscle Contraction, Select Ca2+ Binding Proteins). Furthermore, at 3 h exercise up-regulated *DSCR1* (modulatory calcineurin-interacting protein 1 (Grondard, et al. 2008)) whose protein products are inhibitory to NFATc. Exercise up-regulated *HDAC* expression at 3 h and protein nutrition differentially up-regulated expression at 48 h; which regulate myogenesis and slow fibre muscle gene expression through association with MEF2 (Haberland, et al. 2009; Schiaffino & Serrano 2002). Collectively, the post-exercise protein mediated myogenic response coupled to calcium-activated transcriptional regulation supports a role for protein nutrition in slow fibre type development and contractile function remodelling in response following endurance exercise.

With respect to the cytoskeletal transcriptome, protein nutrition facilitated median down-regulation of tubulin microtubules and mixed up and down expression of the actin microfilaments and intermediate filaments at 3 h. Protein nutrition also facilitated up-regulation of actin binding protein transcript expression at 48 h (myosin complex), discussed previously within the contractile transcriptome. Of particular interest is early regulation of actin microfilaments and intermediate filament expression with protein nutrition. The actin microfilaments of the cytoskeleton are composed of a minor population of actin isoforms, the beta and gamma actins, and have important roles in maintaining cell shape and structure (Sonnemann et al 2006), cell motility (Seigel et al 2009), vesicle and organelle trafficking (Tsakiridis et al 1994), and membrane channel activity (Johnson et al 2005). Actin cytoskeletal signalling was the top canonical pathway at 3 h, and expression was mainly up-regulated with protein nutrition. Transcripts encoding actin beta, and actin filament capping protein were up-regulated, actin gamma and actinin alpha isoforms were up and down, although dystrophin was down-regulated with protein nutrition following exercise compared to carbohydrate control.

The intermediate filaments of the skeletal muscle cytoskeleton have roles in sarcomere integrity, and provide mechano-chemical links between the sarcolemma, nuclei and mitochondria (Capetanaki, et al. 2007). The role of intermediate filaments in sarcomere integrity has lead to speculation that adaptation of intermediate filaments comprised of desmin and the cyto-keratins offer increased protection against sarcolemma disruption (Stone, et al. 2007). There was a substantial up-regulation of kertatin-19 gene expression; however protein nutrition had no impact on the exercise-induced down-regulation of desmin at 3 h. On the other hand, the developmental intermediate filament transcripts, vimentin, and nestin, which have been described as markers of skeletal muscle injury (Michalczyk K & M. 2005; Vaittinen, et al. 2001), were down-regulated with protein nutrition at 3 h. Additionally the intermediate filament titin (Gregorio, et al. 1999) transcript expression was down-regulated in the protein condition; indicating reduced sarcomerogenesis (addition of sarcomeres in series). The regulation of sarcomerogenesis has been reported to depend on the extent of muscle damage (Proske & Morgan 2001) and is associated with eccentric contractions, while loss of sarcomeres in series is associated with concentric muscle contractions (Butterfield, et al 2005). Although an increase in Z-disk streaming was observed; no protein nutrition differential was found. Nonetheless, these data implicate protein nutrition in regulation of cytoskeletal actin microfilament and intermediate filament remodelling and function, and this response was most prevalent at 3 h post exercise, while regulation of actin binding proteins transcription was delayed and up-regulated by 48 h. Cytoskeleton gene expression has previously been reported to increase in response to 6-weeks of endurance exercise training in men (Keller, et al. 2007), and following a single bout of intense incremental aerobic exercise in untrained horses (McGivney, et al. 2009). Observations from the current study, therefore, are the first to illustrate a substantial cytoskeletal molecular program in response to a single bout of intense prolonged exercise in chronically well-trained humans, and the first to show a protein-nutrition mediated differential expression.

This is the first research to show an impact of post-exercise nutrition on two of the key adaptations within the muscle extracellular matrix phenotype with endurance training. Protein nutrition following endurance exercise differentially affected

vascular and neuronal development relating to increased tissue capillarization (Andersen & Henriksson 1977; Timmons, et al. 2005a) and motor neuron diameter and terminal branching, respectively (Gardiner, et al. 2006). Remodelling of these processes not only share common guidance cues through the extracellular matrix (Serini & Bussolino 2004), but also have a strong interconnection with that of cytoskeletal remodelling through cell adhesion molecules such as the integrins (Chen et al 2007; Dickson 2002). To date a number of studies provide evidence of up-regulated gene expression in axon guidance and angiogenesis ontologies in untrained (Mahoney, et al. 2005), short-term training interventions (Chen, et al. 2007; Timmons, et al. 2005a; Timmons, et al. 2005b) and with chronic endurance training (Stepto, et al. 2009); however this is the first research to show an impact of post-exercise protein on these processes. However in general, compared to the broad exercise stress and adaptive program in the control condition, with protein nutrition there was a delayed and refined genomic profile mediating adaptive remodelling within the muscle extracellular matrix. Indeed, protein nutrition was observed to differentially up-regulate expression profiles including the integrins, ephrins, and semaphorins which are involved in extracellular matrix signalling, development, adhesion, attractive and repulsive cues, as well as several angiogenic factors and matrix metalloproteinases. Along with mainly down-regulation of extracellular matrix structural protein (e.g. type IV collagens) and glycoprotein genes at both 3 h and 48 h; suggestive of attenuation and refinement of the exercise induced extracellular matrix transcriptome by protein nutrition. The mechanism might be lower relative stress on cell and tissue structural integrity with the addition of protein to the post-exercise nutrition environment, which may also partly explain the frequent attenuation of circulating damage markers observed in previous studies (Rowlands, et al. 2008; Thomson, et al. 2011).

Several biological process and molecular function gene ontologies associated with protein breakdown were differentially regulated with protein nutrition, with most expression activity occurring at 3 h. Lysosomal proteases (cathepsins) were mostly down-regulated with exercise and protein nutrition, consistent with decreased indiscriminate lysosomal protein destruction and autophagy, and protein turnover (Bechet, et al. 2005). Differential expression of pro- and anti-apoptosis genes,

including several caspases, suggests tight regulation of apoptosis with protein nutrition. Protein nutrition regulated the expression of a large number of genes encoding ubiquitination targeting proteins (E2, E3), suggesting protein nutrition mediated tightening of this proteolytic process. Non-lysosomal and Ca^{2+}-independent proteolysis (ubiquitination) are primarily responsible for myofibrilar protein breakdown and may therefore play a major role in skeletal muscle remodelling (Taillandier, et al. 2003; Taillandier, et al. 2004). Elevated proteolytic gene expression in the 1-4 h following high-intensity endurance exercise has been previously reported following acute exercise independent of training status (Coffey, et al. 2006; Louis, et al. 2007; Mahoney, et al. 2005). The mechanism reducing protein degradation following high-intensity exercise with protein nutrition could be associated with up-regulated mTORC1 signalling (Nicklin, et al. 2009) leading to increased translation of specific mRNA (Kimball & Jefferson 2006a) and possibly an anti-autophagy response (Zhao, et al. 2007). Alternatively, the mechanism could be down-regulation of AMPK and the Forkhead box O3 (FOXO3) class of transcription factors, although there was no significant exercise or nutrition differential expression of *FOXO3* in the current study. Nonetheless, the reduction in the degradative transcriptome, combined with evidence for increased mTORC1 signalling activity at 3 h, supports a model of muscle remodelling proposed by Tipton (Tipton 2008), where increased muscle protein synthesis and targeted muscle protein breakdown with protein-carbohydrate nutrition following strenuous endurance exercise represent important components of adaptive remodelling.

4.1.5.2 Stress, immune, and inflammatory response

Within the immune and defence ontology in the immediate hours post exercise, protein nutrition down-regulated the exercise-induced inflammatory response while also offering acute protection from stress through increased HSP70 chaperone expression. Macrophages play a key role in modulation of immune response by producing chemokines and cytokines as well as phagocytic clean up of damaged tissue following strenuous endurance exercise (Fehr, et al. 1989; Pimorady-Esfahani, et al. 1997); the over-represented expression at 3 h suggests a role for protein nutrition in macrophage function. Dendritic cells are the key antigen presenting cells, which stimulate B and T cells and thereby the initial immune response (Banchereau

& Steinman 1998). Reduced dendritic cell maturation and MHC II antigen processing and presentation are in line with reduced post-exercise inflammatory response mediated by protein nutrition at 3 h. The complement cascade is also part of the acute phase inflammatory response and attracts leukocytes to the site of injury (Frenette, et al. 2000) as well as mediating clean up of cellular debris by cooperation with phagocytes (Engel & Biesecker 1982). Therefore, the effects of protein nutrition in reducing complement and macrophage-mediated gene expression suggests reduced requirement for clean up of cellular debris, which is consistent with the moderated proteolytic transcriptome. Relative down-regulation of immune and inflammatory response and an increased protection from stress with protein nutrition is suggestive of more rapid restoration of cellular homeostasis and initiation of the adaptive program.

The HSP70 family function primarily as molecular chaperones, cell signalling, mRNA stabilization and degradation, protein degradation, and as regulators of cell death (Morton, et al. 2009), and offer protection against subsequent stressors (Maglara, et al. 2003). Up-regulation of *HSP70* suggests protein nutrition might adapt to better support post-translational processing during a subsequent exercise bout. An expression response signifying down-regulated NFκB activity at 3 h was found, whereas the majority of evidence suggests that high-intensity endurance exercise increases NFκB activity (Kramer & Goodyear 2007). Putative functions of NFκB are countering oxidative stress, inducing a proinflammatory response, and stimulating cytokine and metabolic responses following exercise (Kramer & Goodyear 2007). However, inflammatory-response genes were mostly down-regulated, and there was limited evidence for an antioxidant response (*GPX3* down-regulated with protein-rich nutrition at 3 and 48 h) and metabolic signal at 3 h.

4.1.5.3 A temporally affected metabolic response governed by protein nutrition directs metabolism away from glucose and towards fatty-acid transport and oxidation

Extensive transcriptomal and some signalling evidence for a protein-nutrition mediated metabolic/mitochondrial program following intense endurance exercise was found. The present data comprise the first report of an association between

dietary protein and endurance exercise transcriptomal response which is broadly in line with the metabolic and mitochondrial adaptive responses to endurance training (Holloszy & Coyle 1984). The expression of lipid metabolic genes, electron chain components, along with mitochondrial genes were differentially up-regulated with protein nutrition at 48 h. Evidence of up-regulation of key metabolic regulators *PPARGC1α*, *PPARγ*, *CREB* combined with increased AMPKα phosphorylation at 48 h, provide a candidate signalling mechanism for post-exercise protein to facilitate the exercise-induced metabolic/mitochondrial transcriptomal program. Once phosphorylated and activated, AMPK has been found to increase expression of genes involved in skeletal muscle oxidative capacity and mitochondrial biogenesis via direct phosphorylation of the PGC-1α protein (encoded by *PPARGC1α*) and increased *PPARGC1α* expression (Jäger, et al. 2007). In turn PGC-1α induces transcription of various factors, including PPARγ and genes associated with lipid metabolism (de Lange, et al. 2007). Indeed, this was confirmed in the network analysis; protein-nutrition mediated up-regulation of *PPARγ* and genes involved in lipid metabolism at 48 h. The protein nutrition mediated increased in AMPK-PGC-1α associated signalling at 48 h may also have been associated with regulation of genes involved in the mitochondrial electron-transport chain. In rodent skeletal muscle cells, Jager et al. (Jäger, et al. 2007) reported that posttranslational modifications of PGC-1α by AMPK induced PGC-1α, GLUT4, and cytochrome c gene expression in a PGC-1α-dependent way, and via an additional pathway AMPK-mediated induction of UCP-3 and PDK4. In the current gene array, neither exercise or protein nutrition affected GLUT4 gene expression (*SLC2A4*); *UCP-3* was down-regulated with protein nutrition at 3 h and up-regulated in both conditions at 48 h, while *PDK4* was up-regulated in the protein condition at 48 h. A further candidate mechanism for protein facilitation of metabolic/mitochondrial transcriptomal program is via eNOS-mTORC1-PGC1α signalling, which is not only known to play roles in mitochondrial biogenesis (Cunningham, et al. 2007; D'Antona, et al. 2010), but has recently been shown to be elevated with branched chain amino acid supplementation (D'Antona, et al. 2010). In a series of studies D'Antona et al (D'Antona, et al. 2010) found that branched chain amino acids up-regulated eNOS activity, mTORC1 activation and PGC1α mRNA, all of which associate with the

transcription factor yin-yang 1 (YY1) (Cunningham, et al. 2007; Karantzoulis-Fegaras, et al. 1999) to enhance mitochondrial biogenesis. In the current study protein nutrition mediated increased mTORC1 activity; however no evidence of eNOS or YY1 gene expression with protein nutrition was found. Nonetheless these data suggest a transcriptional program favouring down-regulation of glucose metabolism with protein nutrition, facilitating later (48 h) adaptive response to endurance exercise.

Other signalling pathways might have integrated the protein-nutrition signal with the metabolic/mitochondrial transcriptome. Candidate signalling mechanisms include cAMP (Miura, et al. 2007) and Ca^{2+}-calmodulin-dependent kinases (Wu, et al. 2002). Evidence for this was provided by expression profiles within the canonical pathways for muscle contraction and signalling (Supplementary data sheet S8). In addition, both p38-MAPK and insulin-stimulated AKT signalling through CREB and FOXO1A have been associated with increased PGC-1α transcript expression in hepatic cell lines (Knutti, et al. 2001; Li, et al. 2007). While in the present study *CREB* expression was affected; there was no evidence for a protein-nutrition mediated effect on these signalling pathways. Activated calcineurin has also been associated with a strong coordinated increase in the expression of lipid metabolic genes and the protein content of mitochondrial electron chain components (Long, et al. 2007). There was some evidence for involvement of calcineurin signalling; firstly, the slow muscle fibre gene program described above, and secondly the observed increase in *NFATc* expression at 3 h could be linked to calcineurin activation (Matsakas & Patel 2009). Finally, we present the novel and interesting possibility that dietary protein ingested after exercise might influence the metabolic/mitochondrial gene expression profile through an epigenetic mechanism, and therefore impact on principle adaptations to endurance exercise training. The possibility that a reduced suppression of *PPARGC1*α (encoding PGC-1α) expression by DNA methyltransferase DNMT3B (Barrès, et al. 2009) could have contributed to the observed effects requires follow up investigation. Other acetylation and methylation events stimulated by exercise or protein nutrition may have also impact on PGC-1α. For example, the acetyltransferase GCN5 acetylates and represses PGC-

1α (Rodgers, et al. 2008), and in the current study was down-regulated at 3 h but without protein nutrition differential.

The reason for the increase in AMPK phosphorylation at 48 h with the current intervention is unknown. Previously the ingestion of a leucine-enriched essential amino acid–carbohydrate mixture increased skeletal-muscle protein synthesis and AKT, mTORC1, p70S6K and 4E-BP1 phosphorylation, while causing a small decrease in AMPKα phosphorylation 1 h post-prandial at rest (Fujita, et al. 2007). Phosphorylation of AMPK is frequently associated with cell energy stress and acts to inhibit mTORC1 activity through TSC2, thereby maintaining cellular amino acid concentration by lowering the suppression of autophagic amino acid provision, and reducing translation initiation and cell growth (Deldicque, et al. 2005; Kimball 2006). Therefore, the present attenuation of exercise induced AMPKα phosphorylation at 3 h, is suggestive that post-exercise protein feeding improves initial cellular energy homeostasis, which complements the moderated transcriptional regulatory response and improved stress response which was also found. However, by 48 h AMPK phosphorylation was moderately increased with protein-nutrition, and hence could be the mechanism for up-regulated metabolic/mitochondrial transcriptome and down-regulated translation initiation signalling reported. Further support for lowered translational activity in the protein condition at 48 h comes from up-regulation of *DDIT4* (DNA damage-inducible transcript 4; synonym REDD1) transcripts, which inhibit cell growth by negatively regulating the mTORC1 pathway (Sofer, et al. 2005).

The phospho-protein findings at 3 h are suggestive of increased translation initiation and are generally consistent with post-exercise protein feeding studies in animals (Anthony, et al. 2007; Gautsch, et al. 1998; Morrison, et al. 2008). The atypical kinase mTORC1 integrates nutritional and mitogenic signals to control translation, autophagy and cell growth via phosphorylation of downstream effectors. The phosphorylation of two key effectors 4E-BP1 and RPS6 (Avruch, et al. 2009) were substantially increased with protein nutrition at 3 h. These and other effectors regulate protein synthesis rate primarily at mRNA translation initiation (Kimball & Jefferson 2006b). The present data are the first to identify that the addition of protein

to high-carbohydrate nutrition following endurance is responsible for increased mTORC1 activity in well-trained men. In the only other investigation in humans, Ivy et al. (Ivy, et al. 2008) also reported increased AKT, mTORC1, and RPS6 Ser240/244 phosphorylation with protein-carbohydrate feeding after endurance exercise, but the control was a water-placebo. The authors also reported no significant change in p70S6K phosphorylation. Therefore, unlike after resistance exercise, where increased p70S6K phosphorylation is regularly observed with amino acid or protein feeding (Dreyer, et al. 2008; Karlsson, et al. 2004); the trivial p70S6K signal following endurance exercise appears insufficient to account for the large increase in RPS6 phosphorylation. Nevertheless, RPS6 phosphorylation has previously been observed to remain elevated for longer duration than p70S6K once phosphorylated (Anthony, et al. 2002). Whatever the mechanism, phosphorylation of RPS6, 4E-BP1 and eIF4B has been found to enhance translation of a subset of mRNAs that encode components of the translational apparatus (Kimball & Jefferson 2006a; Meyuhas 2000). However, 7 cytoplasmic ribosomal protein transcripts (*RPL7*, *RPL27A*, *RPLP1*, *RSP24*, *RSP3A*, *MRFAP1L1*, *RPLP0*) within the ribosomal protein molecular function classification, were down-regulated with protein at both 3 h and 48 h (Supplementary data sheet S6). Along with the eukaryotic translation elongation (*EEF1A1*) and initiation (*EIF1AX*, *EIF5B*) factors also down-regulated with protein-rich nutrition at 3 h. The reported ribosomal transcripts represent only 8% of the total ribosomal protein transcripts; and the long half-life of ribosomes means that a transient reduction in ribosome biogenesis may not have a large impact on global translation (Reiter, et al. 2008). However, further research targeting translational protein and ribosomal activity would be required to confirm the direction of protein nutrition influence on translation regulation.

The trivial impact of post-exercise protein nutrition on serum insulin concentration and AKT phosphorylation suggests that factors downstream of AKT are unlikely to have been responsible for the increase mTORC1 phosphorylation at 3 h. A large increase in the expression of p90RSK transcript at 48 h was observed with protein-rich nutrition, but unclear p38-MAPK and ERK1/2 phosphorylation outcomes (Figure 4.4, Supplementary data sheet S2). However, a possible limitation in the current study was that the 3 h post-exercise muscle biopsy may have missed an

exercise-protein feeding induced elevation in p38-MAPK or ERK1/2 activation; these kinases have been previously shown to rapidly peak then return to baseline within 30-60 min following cycling exercise (Richter, et al. 2004; Widegren, et al. 1998). These data, therefore, leaves the impact of post endurance-exercise protein feeding on MAPK-stimulated mTORC1 signalling unresolved. Recently, an amino-acid transporter system was shown to be necessary for regulation of mTORC1 signalling and autophagy in vitro (Nicklin, et al. 2009). Cellular uptake of L-glutamine and its subsequent rapid efflux in the presence of essential amino acids was the rate limiting step which activated mTORC1. L-glutamine uptake was regulated by solute carrier member SLC1A5 and by bidirectional transport of extracellular leucine and intracellular glutamine through SLC7A5/SLC3A2 (Nicklin, et al. 2009). The present ontology analysis provides some suggestion for an impact of protein nutrition on amino acid transporter expression (Supplementary data sheet S5), however on further examination, moderate to extremely large increases in *SLC3A2* transcript variant 1, *SLC7A5*, *SLC7A11*, *SLC7A8* transcript variant 1 expression with exercise at 3 h but no nutrition differential was found (Supplementary data sheet S9). In the present study, the pattern of amino acid transporter expression was not coupled to increased mTORC1 phosphorylation with protein nutrition at 3 h. Therefore, the *in vivo* transcriptomal data suggests that endurance exercise up-regulates the amino acid transporter system proposed by Nicklin et al. (2009), but protein nutrition is required to increase mTORC1 Ser2448 phosphorylation, suggesting other amino-acid linked signalling mechanisms are involved.

Despite anabolic signalling at the cellular level, no clear differences in whole body protein turnover rate was found between conditions over 24-h. Previous studies (Howarth, et al. 2009; Levenhagen, et al. 2002) have shown acute fractional skeletal muscle protein synthesis and whole body nitrogen balance (Howarth, et al. 2009) were improved with combined protein-carbohydrate ingestion following prolonged exercise. On the other hand, Greenhaff et al (2008) found anabolic signalling to be dissociated from change in muscle protein synthesis. The degree of phosphorylation of signalling proteins temporal relationship between anabolic signalling with measurable protein synthesis output is at present unknown. Furthermore provision of

the alternate nutrition bolus at the opposite end of the day in the current study, may have stimulated whole body protein turnover to the same degree as the post-exercise meal over 24 h, although might not have affected the fractions of muscle protein, stimulated by exercise performed within close time proximity.

4.1.5.4 Design considerations

A limitation of the present study includes the impracticality of obtaining numerous samples, and therefore biopsies were limited to baseline, as well as 3 h and 48 h post exercise for both conditions. This timeline was chosen in order to focus on time points previously shown to represent early (3 h) and later (48 h) transcriptome response to a single bout of endurance exercise (Mahoney, et al. 2005). However, it is acknowledged that these time points may have resulted in failure to observe transient peak in mRNA for some immediate early genes such as the transcription factors c-FOS, c-JUN as well as rapid transient activation of signalling proteins p38-MAPK and ERK1/2 (Richter, et al. 2004; Widegren, et al. 1998). A further limitation is the unknown variability in fibre and other cell types included in the vastus lateralis tissue samples. For instance, muscle fibre types, and the content of other cell types such as resident endothelial cells, macrophages, or stem cell populations of the tissue samples were not determined. The microarray technology itself provides inherent limitations related to sensitivity (around half of the transcriptome may be below microarray sensitivity threshold), accuracy (above the sensitivity threshold, microarrays accurately indicate expression change and direction of 70-90% of genes; however absolute magnitude is beyond microarray capability), specificity (some degree of cross-hybridization is expected to occur despite controls), and precision between platforms (correlation between microarray platforms is about 0.7) (Draghici, et al. 2006). A final limitation is in the interpretation of expression data; gene expression rates do not always correlate with protein expression rates, and do not give any indication of the activity of the encoded protein once synthesized (Booth & Baldwin 1996).

4.1.6 Summary

In this first analysis, the effect of adding protein to post-exercise carbohydrate-lipid nutrition on the endurance exercise transcriptome in trained muscle is reported.

Protein nutrition was found to lead to refinement and increased specificity of the broad global expression response to endurance exercise exemplified within the control condition, and is somewhat similar to the fine-tuning typified by endurance training in comparison to untrained skeletal muscle (Egan 2008; Mahoney & Tarnopolsky 2005). Specifically, protein nutrition induced a molecular programme supporting early attenuation of immunity and defence response and later tissue development and metabolic-mitochondrial adaptation to endurance exercise. The immune and inflammatory ontology was characterized by relative down-regulation of innate immune response expression while enhanced stress response and defence expression corresponded to increased heat shock protein chaperone mRNA. Developmental processes were directed at cytoskeletal, extracellular matrix, and sarcomere signalling and remodelling, regulation of proteolytic process and targeted protein breakdown. Metabolic gene expression favoured increased fatty acid metabolism and mitochondrial electron transport, some of the primary metabolic adaptations to endurance training. Furthermore, evidence of altered gene expression of proteins involved in acetylation and methylation of the nucleosome is provided, thereby contributing the first evidence for possible nutri-epigenomic regulation of adaptation to endurance training. Finally, the phospho-protein observations suggest an early increase in translation initiation consistent with previous investigations, but also a later role for AMPK in the protein-nutrition mediated metabolic transcriptome. To conclude, the protein component of post-exercise nutrition facilitates targeted adaptive responses to high-intensity exercise stress.

4.1.7 Supplementary Information

Supplementary Spreadsheets, Figures, and Tables can be found in the CD on the back cover of this thesis.

F1. Relationship between qPCR and microarray.

T1. Probe and primer set for qPCR analysis.

S1. Statistical summary of qPCR outcomes. Histograms of raw means and *SD*s for each gene by post-exercise nutrition condition and biopsy sample time.

S2. Statistical summary of western blot outcomes.

S3. Listings of all selected genes differentially expressed in response to exercise and protein coingestion at 3 h and 48 h.

S4. Complete Panther classification analysis for the differential gene expression in response to exercise and post-exercise nutrition condition.

S5. Panther ontology analysis for the differential gene expression in response to exercise and protein nutrition. Biological processes and molecular functions meeting the arbitrary selection criteria of $p<0.05$ (summarized in Supplementary data sheet S4) are organized by Panther Family ontology. The number and percentage of genes up- and down-regulated are listed and shown graphically. Descriptive statistics are provided by Panther classification. Over- (red) and under-represented (green) classifications are shaded.

S6. Full listing of gene selection grouped and organized by Panther gene ontology classification system.

S7. Top networks constructed by the researchers in IPA following IPA interrogation of the respective Panther biological process ontology families: developmental processes, muscle contraction, lipid and steroid metabolism, and immunity and defence. Networks comprised all genes significantly selected in one or more of the six differential comparisons. The respective top and corresponding IPA functions from which the IPA networks were derived were: skeletal and muscular system development and function, muscle contraction, fatty acid metabolism and transport of fatty acids, cell-to-cell signalling and interaction and inflammatory response. Additionally, differentially-affected mitochondrial respiratory capacity with protein nutrition was investigated via an analysis of the expression profile of all transcripts listed under the IPA function mitochondrial electron transport. Gene names shown in red are up-regulated and green down-regulated, whereas genes in grey are differentially expressed at one or more of the other five time points considered in the network analysis. The intensity of red or green colour increases or decreases with expression magnitude (fold change relative to the reference condition). Uncoloured genes (white) were those that had not reached the selection criteria.

S8. Top canonical pathways resulting from the IPA interrogation of the respective Panther biological process ontology families: developmental processes, muscle contraction, apoptosis, signal transduction, lipid and steroid metabolism, carbohydrate metabolism, immunity and defence. Additionally, the molecular function ontology select Ca^{2+} binding proteins and the manual analysis PPAR signalling supported the primary top IPA-selected pathways. Gene names shown in red are up-regulated and green down-regulated, whereas genes in grey are differentially expressed at one or more of the other five time points considered in the analysis. The intensity of red or green colour increases or decreases with expression magnitude (fold change relative to the reference condition). Uncoloured genes (white) were those that had not reached the selection criteria.

S9. Complete differential statistical analysis.

S10. Abbreviations and acronyms.

External. Microarray raw data will be deposited in NCBI's Gene Expression Omnibus at the time of submission of the primary manuscript to journal (2011).

CHAPTER 5 GENERAL DISCUSSION

5.1.1 INTRODUCTION

Over the previous 2 decades there has been substantial interest in the effects of post-exercise protein-carbohydrate ingestion on protein turnover and synthesis. More recently, interest has focused on the effects of post-exercise protein-carbohydrate coingestion on recovery from damage and substrate depletion induced by prolonged exercise and subsequent benefit to performance compared to post-exercise carbohydrate. However, results of rate of glycogen replenishment and benefit to subsequent performance are inconsistent. In the conclusions to the literature review (Chapter 2) is was suggested that this is likely due to different caloric content of treatment and control (Betts, et al. 2007; Williams, et al. 2003) and inappropriate testing protocols for determining the effect of differences in pre-exercise glycogen concentration on performance (Berardi, et al. 2008; Karp, et al. 2006). In several studies it has been reported that combined protein and carbohydrate ingestion following exercise has mitigated exercise induced damage and/or enhanced repair however no improvement in subsequent performance was found (Luden, et al. 2007; Millard-Stafford, et al. 2005; Romano-Ely, et al. 2006; Rowlands, et al. 2007; Rowlands & Wadsworth 2010). Overall, evidence of a post-exercise protein-carbohydrate mediated benefit to performance relative to carbohydrate, is lacking. Furthermore, almost all research in this area has focused on acute mechanisms, which would occur within 24 h after initial exercise stimulus. In fact, most research has concentrated on the rate of glycogen synthesis, and therefore has utilized short recovery periods of 1-5 h (Berardi, et al. 2006; Betts, et al. 2007; Betts, et al. 2005; Betts, et al. 2008; Carrithers, et al. 2000; Howarth, et al. 2009; Ivy, et al. 2002; Jentjens, et al. 2001; Kammer, et al. 2009; Millard-Stafford, et al. 2005; Niles, et al. 2001; Tarnopolsky, et al. 1997; van Hall, et al. 2000; van Loon, et al. 2000a; van Loon, et al. 2000b; Williams, et al. 2003; Zawadzki, et al. 1992). Others have investigated the attenuation of muscle damage over 12-24 h recovery periods (Cade, et al. 1991; Luden, et al. 2007; Millard-Stafford, et al. 2005; Romano-Ely, et al. 2006; Rowlands, et al. 2007; Saunders, et al. 2004). However, longer recovery periods may be needed for complete functional recovery after intense endurance exercise (Parra, et al. 2000; Ross, et al. 2010).

A single bout of intense prolonged exercise produces strong chemical and mechanical signals which are transmitted to the nuclei and mitochondrion, and then directly and indirectly co-ordinate gene transcription (Fluck & Hoppeler 2003; Hoppeler & Fluck 2002). Dietary nutrients are also potent stimuli, sensed by various response elements and signals transmitted to transcription factors, cofactors and other components involved in regulation of metabolic gene expression (Feige & Auwerx 2007). However, few investigations have evaluated the effect of post-exercise protein and carbohydrate consumption on signalling to protein translation and subsequent protein synthesis (Anthony, et al. 2007; Gautsch, et al. 1998; Ivy, et al. 2008; Kammer, et al. 2009; Morrison, et al. 2008). To the best of the researcher's knowledge no other study has investigated global skeletal muscle transcription induced by prolonged exercise and facilitated by protein-rich nutrition.

Therefore, in the studies outlined in this thesis; firstly, the effect of post-exercise leucine-rich protein-carbohydrate was compared to carbohydrate-control on subsequent performance, markers of muscle damage, and psychometric recovery. Exercise stimulus entailed a short (4.5 d) endurance training program, which included 3 bouts of high-intensity interval training and 36 h rest prior to performance testing. The supplementation protocol consisted of supplementing 4 ordinary high-carbohydrate recovery meals with leucine-rich protein and carbohydrate in the 1.5 h following exercise. In the second study, the effect of post-exersie protein-carbohydrate co-ingestion was compared to carbohydrate-control on global gene expression, activity of signalling cascades known to be regulated by contractile activity and the principle proteins mediating translation, and visual evidence of muscle damage. Specifically, the effect of post-exercise protein-carbohydrate co-ingestion on early (3 h) and later (48 h) time points was studied in order to capture the mecahanisms driving recovery and adaptive responses, facilitated by post-exercise protein-rich nutrition.

Both studies were unique, given that isolation of post-exercise nutrient timing was achieved by providing the alternate supplement at the opposite end of the day within stringently controlled and balanced daily energy and macronutrient diets. Combined, the main aims in conducting this research were to investigate the effect of immediate

post-exercise protein-carbohydrate ingestion following prolonged intense exercise on subsequent performance and the mechanisms involved.

5.1.2 PERFORMANCE OUTCOME

5.1.2.1 Leucine-Rich Protein and Carbohydrate Post-Exercise Feeding Benefits Subsequent Performance

One of the main outcomes from this research is evidence of a likely small benefit to performance with leucine-rich protein and carbohydrate post-exercise feeding compared to carbohydrate control. These results have relevance since they not only follow on from previous work by Rowlands et al. (2008) confirming that immediate timing of protein in the recovery diet is beneficial to subsequent performance; but also demonstrate that the performance benefit is achievable with a more practical amount of protein when its potency is increased with leucine. Moreover, Rowlands et al (2008) found that the performance benefit was not realised at an earlier time point of 15 h, but by 60 h after the initial exercise stimulus there was a moderate-sized performance enhancement. Combined with the current performance study, in which performance was tested 39 h after the final training session; results are suggestive of a delayed mechanism facilitated by post-exercise protein-rich feeding.

It appears a period for consolidation of recovery, repair and adaptation processes following exercise bout or training might be important for the realization of functional improvement. However results from the current performance study are inconsistent with many other post-exercise protein-carbohydrate interventions in which recovery was >15 h (Luden, et al. 2007; Millard-Stafford, et al. 2005; Nelson, et al. 2010; Romano-Ely, et al. 2006; Rowlands & Wadsworth 2010; Skillen, et al. 2008). Dissimilarity of results likely relates to differences in exercise loading; provision of sufficient rest duration for recovery and adaptation; supplement contents; and possibly gender effects. In relation to the time required for adaptation; increased content and activity of mitochondrial enzymes and resultant improved performance was reported to occur after as little as 6-7 bouts of high-intensity sprint and endurance exercise training (Burgomaster, et al. 2005; Gibala, et al. 2009); however few studies on post-exercise protein-carbohydrate nutrition have utilised more than a single bout of prolonged exercise (Nelson, et al. 2010; Rowlands &

Wadsworth 2010; Skillen, et al. 2008). Nonetheless, performance improvements were of the magnitude trivial (Nelson, et al. 2010; Rowlands & Wadsworth 2010; Skillen, et al. 2008) to moderate (Rowlands, et al. 2008) in said studies. In addition to repeated exercise stimulus, it appears a rest period for recovery might also be important for realization of performance benefits. For instance, consecutive days of intense prolonged exercise have been found to result in reduced Ca^{2+} cycling properties of the sarcoplasmic reticulum (Duhamel, et al. 2007) which was detrimental to subsequent performance when adequate rest was not provided (Parra, et al. 2000). Differences in composition of the post-exercise supplements between studies might have had a role in disparate outcomes. For example, Skillen et al (2008) provided a meagre 0.07 $g \cdot kg^{-1}$ of protein during the recovery period following exercise, compared to the much larger protein content provided in the current performance study (0.92 $g \cdot kg^{-1}$). Although the researchers did provide an additional 0.14 $g \cdot kg^{-1}$ prior to and during the exercise protocol the amount remaining for protein synthesis and repair is unknown. Furthermore, Nelson et al (2010) reported instances of gastric distress, potentially detrimental to performance; however it was not known which of the supplement ingredients was the contributing factor. Finally, two studies (Millard-Stafford, et al. 2005; Rowlands & Wadsworth 2010) suggest a plausible blunted response in females compared to males. Male subjects were studied in the present performance study.

An alternative possible explanation for inconsistent findings between studies is an enhanced efficacy of protein-rich post-exercise supplementation within a background of inadequate daily protein intake. There is some indication that protein-rich post exercise supplementation is more efficacious during situations that would otherwise result in negative protein balance. In Rowlands et al (2008), subjects following the control condition were in negative protein balance, while the protein-rich condition lead to more positive daily protein balance, likely due to differences in daily protein intakes. The authors (Rowlands, et al. 2008) reported a moderate benefit to performance in the protein-rich post-exercise feeding condition relative to carbohydrate feeding. In the current performance study, subjects were found to be in mildly negative nitrogen balance in both conditions, and a small performance improvement in the protein-rich condition was demonstrated. Whereas, Nelson and

colleagues (2010) found no clear benefit to performance when both conditions remained in positive balance. Taken together, these data suggest the post-exercise protein-carbohydrate condition may improve subsequent performance to a greater degree when larger or more potent amounts of protein is provided during periods of negative protein balance; however this remains to be conclusively determined.

Finally, a simple explanation for inconsistent findings between studies may be due to uncertainty surrounding the performance effects measured between various studies. However, the uncertainty (confidence interval) of the performance effect of many of the studies reviewed was not able to be determined due to lack of statistical information, specifically exact *p*-values.

5.1.3 MECHANISMS FOR DELAYED PERFORMANCE BENEFIT

5.1.3.1 Protein-Rich Nutrition Following Exercise Modulates and Refines Exercise-Induced Gene Expression

Another major outcome from this research is the evidence of differentially expressed exercise-induced gene networks resulting from post-exercise protein-carbohydrate nutrition compared with isocaloric carbohydrate. The early transcriptomal programme was characterized by over-represented developmental processes, muscle contraction, cytoskeletal proteins, extracellular matrix, cell adhesion, cell structure and motility, immunity and defence, signal transduction, ion channels, calcium binding proteins, and proteases. Under-represented processes included apoptosis, nucleic acid processing and metabolism, transcription factors, and protein metabolism and modification. The later transcriptomal programme was characterized by over-represented developmental processes, muscle contraction, immunity and defence, signal transduction, ion channels, lipid, fatty acid and steroid metabolism, and transcription factors. Whereas, nucleic acid processing and metabolism, remained underrepresented with protein-rich nutrition following exercise. Where over-representation is the probability that observed expression is greater than that resulting from random chance, and under-representation is the probability that observed expression is lower than that resulting from random chance. Protein-rich nutrition differentially regulated gene expression in three transcriptional programmes

that were especially exciting and include those involved in governing tissue development and remodelling, immunity and defence, and metabolism.

5.1.3.1.1 Post-exercise protein-rich nutrition directs a molecular program of contractile, cytoskeletal and extracellular matrix development and remodelling

Protein-carbohydrate nutrition following prolonged exercise directed tissue development and remodelling at contractile, cytoskeletal protein, and extracellular matrix. In support, gene expression regulating proteolytic processes and targeted protein breakdown was refined. Skeletal muscle contractile (O'Neill, et al. 1999), cytoskeletal (McGivney, et al. 2009), and extracellular matrix remodelling are central adaptations to endurance exercise (Timmons, et al. 2005a; Timmons, et al. 2005b). Furthermore, this is the first research to show an impact of post-exercise nutrition on two of the key adaptations within the muscle extracellular matrix, vascular and neuronal development, to endurance training. Genes encoding contractile and cytoskeletal proteins were also some of the most over represented gene ontologies in response to both exercise and protein nutrition.

A temporal difference was observed in gene expression profiles in the biological process muscle contraction in the post-exercise protein-carbohydrate condition relative to carbohydrate control. Gene expression mediated by the protein-rich condition was mainly down-regulated for myosin light and heavy chains, troponin and other genes encoding contractile proteins at 3 h, and then up-regulated myosin light chain, troponin, and myosin complex expression by 48 h relative to carbohydrate nutrition. The temporal differential was especially evident in the developmental isoforms; embryonic and perinatal myosin heavy and light chain, and cardiac troponin all of which were unaffected by protein nutrition at 3 h and up-regulated at 48 h compared to carbohydrate-control. The biological function of these isoforms is believed to be in muscle fibre transition or formation of new transient fibres during muscle regeneration in adult skeletal muscle (Kadi & Thornell 1999). Interestingly, the opposite trend was evident in the expression of contractile alpha-actin, which was differentially up-regulated at 3 h and had no protein differential by 48 h. The developmental isoform of alpha-actin (cardiac actin alpha) was

differentially reduced at both time points mediated by protein-rich nutrition. Gene expression regulating fast and slow twitch myosin isoforms was varied up and down at both time points; however expression activity within the calcium signalling canonical pathway indicated a trend for slow twitch expression. Calcium-calmodulin and calcineurin activities modulate gene expression through transcription factors CREB, NFAT and MEF2 leading to increased expression of slow-fibre type myosin heavy chain, myosin light chain, troponin, and SERCA (sarco/endoplasmic reticulum Ca^{2+}-ATPase) isoforms with endurance training (Grondard, et al. 2008; Pette 1998). Genes encoding the transcription factors *NFATc* and *CREB* were up-regulated with protein-rich nutrition relative to carbohydrate control at 48 h. Furthermore, *HDAC,* the product of which regulates myogenesis and slow fibre muscle gene expression through association with MEF2 (Haberland, et al. 2009; Schiaffino & Serrano 2002), was differentially up-regulated with protein-rich nutrition at 48 h. In other words, the expression profile suggests that remodelling of the contractile apparatus, mediated by protein nutrition, was down-regulated at 3 h, mRNA peaked at 48 h, and was directed towards a slow twitch phenotype.

With respect to the cytoskeletal transcriptome, protein nutrition facilitated a difference in expression of the three components of the cytoskeleton. Median down-regulation of tubulin microtubules, and mixed up and down expression of actin microfilaments and intermediate filaments were found at 3 h. Protein-rich nutrition also promoted up-regulation of actin binding protein transcript expression at 48 h (myosin complex), discussed previously within the contractile transcriptome. Of particular interest; was the early regulation of actin microfilament and intermediate filament gene expression observed with post-exercise protein-rich nutrition. Actin microfilament transcripts encoding beta-actin, and actin filament capping protein were up-regulated, gamma-actin and alpha-actinin isoforms were up and down, although dystrophin was down-regulated with protein nutrition following exercise at 3 h, compared to carbohydrate control. Within the intermediate filament expression profile, protein-rich nutrition mediated an expression profile indicative of increased sarcomere protection and reduced disruption. The intermediate filament proteins desmin and the cyto-keratins (keratin-8 and keratin-19) have known roles in sarcomere integrity (Stone, et al. 2007). Protein-rich nutrition mediated up-regulation

of keratin-19 transcript; however there was no protein differential effect on desmin at 3 h. On the other hand, the developmental intermediate filament transcripts, vimentin, and nestin, which have been described as markers of skeletal muscle injury (Michalczyk K & M. 2005; Vaittinen, et al. 2001), were down-regulated with protein-rich nutrition at 3 h, relative to carbohydrate control. Consistent with reduced muscle injury, the intermediate filament titin and the actin filament capping protein transcripts, were down-regulated, suggestive of protein mediated reduction in sarcomerogenesis (Gregorio, et al. 1999). The addition of sarcomeres in series, known as sarcomerogenesis, has been reported to depend on the extent of muscle damage caused by exercise (Butterfield, et al. 2005; Proske & Morgan 2001). Conversely, no histological evidence of a reduction in damage with protein nutrition was found; there was an equal increase in Z-disk streaming in both conditions. Nonetheless, these data implicate protein nutrition in regulation of actin microfilament and intermediate filament remodelling within the cytoskeleton, and delayed expression of actin binding protein transcripts at 48 h. Cytoskeleton gene expression has previously been reported to increase in response to 6-weeks endurance training in men (Keller, et al. 2007), and following a single bout of intense incremental aerobic exercise in untrained horses (McGivney, et al. 2009). However, the current observations are the first to illustrate a substantial cytoskeletal molecular program in response to a single bout of prolonged exercise in chronically well-trained athletes, and the first to show a protein-rich nutrition mediated expression.

Protein-rich nutrition following endurance exercise mediated mainly down-regulation of gene expression within the extracellular matrix compared to carbohydrate-control. However, a protein nutrition differential in angiogenesis and axonal guidance expression, which are related to two key adaptations to endurance exercise; tissue capillarization (Andersen & Henriksson 1977; Timmons, et al. 2005a) and motor neuron size and terminal branching (Dickson 2002; Gardiner, et al. 2006). Both vascular and axon guidance processes share common guidance cues through the extracellular matrix (Serini & Bussolino 2004) and are interconnected through cell adhesion molecules, the integrins (Chen et al 2007; Dickson 2002). Provision of post-exercise protein nutrition differentially up-regulated expression

profiles including the integrins and ephrins at 3 h, and semaphorins and netrin at 48 h; which are involved in extracellular matrix signalling, development, adhesion, attractive and repulsive cues. Additionally several angiogenic factors such as platelet derived growth factor (*PGDFB*) and fibroblast growth factor (*FGF2*) expression were differentially up-regulated, and angiopoietin-related protein (*ANGPT2*) expression attenuated at 3 h and 48 h. To date a number of studies provide evidence of up-regulated gene expression in axon guidance and angiogenesis following acute exercise in untrained (Mahoney, et al. 2005), short-term endurance trained (Chen, et al. 2007; Timmons, et al. 2005a; Timmons, et al. 2005b) and with chronically endurance trained men (Stepto, et al. 2009); however this is the first research to show an impact of post-exercise protein-rich nutrition on these processes. Interestingly, protein nutrition differentially down-regulated the majority of expression within the extracellular matrix compared with carbohydrate control, including the structural protein (e.g. type IV collagens) and glycoprotein genes at both 3 h and 48 h. One might speculate that attenuation of damage mediated by protein-rich nutrition reduced the signal for extracellular matrix remodelling and refined the exercise induced transcriptome. Consistent with a reduction in damage is the differential down-regulation of tenascin C transcript expression; tenascin C is associated with extracellular matrix remodelling following muscle injury and damage to the sarcolemma (Järvinen, et al. 2000). Together, these data indicate that protein nutrition mediated reduced expression relating to recovery of extracellular matrix homeostasis, but also increased expression promoting adaptation to endurance exercise.

In support of tissue remodelling gene expression associated with protein breakdown differed between conditions at 3 h. Protein-rich nutrition mediated down-regulation of lysosomal proteases (cathepsins) transcripts, consistent with decreased indiscriminate protein destruction and autophagy (Bechet, et al. 2005). Protein-rich nutrition also regulated the expression of a large number of genes encoding ubiquitination targeting proteins (E2, E3), suggestive of tight control of the ubiquitin-proteasome proteolytic pathway. Ubiquitination is primarily responsible for myofibrilar protein breakdown, and may therefore play a major role in skeletal muscle remodelling (Taillandier, et al. 2003; Taillandier, et al. 2004). The reduction

in the degradative transcriptome combined with increased mTORC1 signalling to protein translation observed, supports the model of adaptation proposed by Tipton (Tipton 2008), whereby protein-carbohydrate nutrition following strenuous endurance exercise promotes adaptive remodelling by increasing muscle protein synthesis and targeted muscle protein breakdown.

5.1.3.1.2 Post-exercise protein-rich nutrition mediates down-regulation of stress, immune, and inflammatory response

In the immediate hours post-exercise protein-rich nutrition mediated down-regulation of exercise-induced inflammatory gene expression and up-regulated defence through increased chaperone transcript expression relative to carbohydrate control. The NF-kB transcription factor is a master regulator of genes involved in immune and inflammatory responses, and the majority of evidence suggests that high-intensity endurance exercise increases NFκB activity (Kramer & Goodyear 2007). During the early immune response to strenuous endurance exercise, macrophages play a key modulatory role by producing chemokines and cytokines, as well as providing phagocytic clean up of damaged tissue (Fehr, et al. 1989; Pimorady-Esfahani, et al. 1997). Skeletal muscle-antigen processing is primarily performed by dendritic cells (DCs) (Banchereau & Steinman 1998), and macrophages (Fehr, et al. 1989; Pimorady-Esfahani, et al. 1997), via association with the major histocompatibility complexes I and II, which present peptide fragments for recognition and stimulation of the B and T cell-mediated response (Banchereau & Steinman 1998). The complement cascade is also part of the acute phase inflammatory response, and attracts leukocytes to the site of injury (Frenette, et al. 2000) as well as mediating clean up of cellular debris by cooperation with phagocytes (Engel & Biesecker 1982). Therefore, the down-regulation of macrophage, complement, and major histocompatibility complex II gene expression at 3 h, with post-exercise protein-rich nutrition, indicates reduced requirement for clean up of cellular debris compared with carbohydrate control. These data are consistent with the moderated proteolytic transcriptome described previously; specifically down-regulated cathepsin expression, which are known regulatory molecules for major histocompatibility complex II function (Riese & Chapman 2000). In addition, a gene expression profile suggestive of down-regulated NFκB

activity was observed; which indicates attenuation of the exercise-induced inflammatory response. In contrast to the immune and inflammatory response, post-exercise protein-rich nutrition mediated up-regulation of the defence related molecular chaperones, the 70 kDa heat shock protein family, to a greater extent than carbohydrate control. The 70 kDa heat shock proteins have well documented roles in cell protection arising from their role in maintaining correct protein folding, correcting misfolded proteins, preventing protein aggregation, and chaperoning molecules (Morton, et al. 2009). Previous studies have shown the HSP70 family of chaperones to be up-regulated with prolonged cycling exercise (Febbraio & Koukoulas 2000; Khassaf, et al. 2001), and it was postulated that the HSP70 family protects skeletal muscle from exercise-induced damage and facilitates remodelling. However, this is the first study to show up-regulation mediated by post-exercise protein-rich nutrition. Overall, this data supports a role for protein-carbohydrate nutrition following prolonged exercise in facilitating increased defence and decreased inflammatory responses.

5.1.3.1.3 Protein-rich nutrition directs a temporal metabolic response away from glucose and towards fatty-acid transport and oxidation

Both transcriptomal and signalling evidence of a protein-nutrition mediated metabolic/mitochondrial program following intense endurance exercise was demonstrated. The expression of lipid metabolic genes, electron chain components, along with mitochondrial genes was differentially up-regulated with protein nutrition at 48 h. This response is consistent with well known metabolic and mitochondrial adaptive responses to endurance training; however this data is novel because this is the first evidence of a differential effect mediated by protein-rich post-exercise nutrition. Expression of genes encoding the key metabolic regulators, *PPARGC1α*, *PPARγ*, and *CREB* were upregulated, combined with increased phosphorylation of the signalling protein AMPKα at 48 h. Hence, AMPK signalling might be a candidate mechanism by which post-exercise protein-rich nutrition facilitates the exercise-induced metabolic/mitochondrial transcriptomal program. Phosphorylated AMPK has been found to increase the expression of genes involved in skeletal muscle oxidative capacity and mitochondrial biogenesis via direct phosphorylation of the peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-

1α) protein and increased *PPARGC1α* (PGC-1α gene) expression (Jäger, et al. 2007). In turn PGC-1α induces transcription of various factors, including peroxisome proliferator-activated receptor (PPARγ) and genes associated with lipid metabolism (de Lange, et al. 2007). A protein nutrition mediated increase in AMPK-PGC-1α signalling at 48 h may have been associated with up-regulation of genes involved in the mitochondrial electron-transport chain. For instance, in a series of *in vitro* and *in vivo* experiments Jäger et al, (2007) reported that posttranslational modifications of PGC-1α by AMPK induced up-regulation of PGC-1α itself, as well as cytochrome c gene expression, in a PGC-1α-dependent way. Via an alternate pathway, AMPK also mediated induction of the mitochondrial uncoupling protein UCP-3 and the mitochondrial enzyme pyruvate dehydrogenase kinase (PDK4) (Jäger, et al. 2007). The *UCP-3* transcript was down-regulated at 3 h in the protein-carbohydrate condition and up-regulated by 48 h but no nutrition differential, while *PDK4* and *PPARGC1α* were both up-regulated at 48 h with protein-rich post-exercise nutrition. A further candidate mechanism for ingested protein facilitation of the metabolic/mitochondrial transcriptomal program is via eNOS-mTORC1-PGC1α signalling. Not only does this signalling pathway have a role in mitochondrial biogenesis (Cunningham, et al. 2007; D'Antona, et al. 2010), but was recently shown to be elevated with branched chain amino acid supplementation (D'Antona, et al. 2010). In a series of studies D'Antona et al (2010) found branched chain amino acids up-regulated eNOS activity, mTORC1 activation and PGC1α mRNA, all of which associate with the transcription factor yin-yang 1 (YY1) (Cunningham, et al. 2007), and consequently lead to enhanced mitochondrial biogenesis. Protein-rich nutrition following prolonged exercise mediated increased mTORC1 activity at 3 h relative to carbohydrate control; however no evidence of eNOS or YY1 gene expression was evident. A final candidate mechanism is the novel possibility that protein-rich nutrition might influence metabolic/mitochondrial gene programming through an epigenetic mechanism at the level of transcription factor acetylation. Differentially down-regulated DNA methyltransferase *DNMT3B* at 48 h was observed with protein-rich nutrition, indicating the possibility of reduced PGC-1α suppression (Barrès, et al. 2009). Activity of the PGC-1α transcription factor is also repressed by acetyltransferase GCN5 (Rodgers, et al. 2008), which was found had down-regulated

transcript expression at 3 h, although with no protein-nutrition differential. Nonetheless, overall the data suggests a transcriptional program supporting down-regulation of glucose metabolism and enhanced lipid metabolism and mitochondrial activity with protein-rich nutrition response following endurance exercise.

Previous studies of the global gene expression profile induced by endurance exercise have investigated untrained individuals (Mahoney, et al. 2005; Schmutz, et al. 2006), following acute training (Schmutz, et al. 2006; Timmons, et al. 2005a; Timmons, et al. 2005b), and chronic endurance training (Stepto, et al. 2009; Wittwer, et al. 2004); but there has been little work on post-exercise nutritional manipulation. In comparison to untrained individuals, the exercise-induced gene expression in well-trained athletes after a single bout of exercise is more conservative (Coffey, et al. 2006; Schmutz, et al. 2006; Wittwer, et al. 2004). It is interesting and novel, therefore that differences in transcription response to post-exercise nutrition following a single bout of exercise were found in well-trained men. This research presents for the first time, a comparison of global gene expression resulting from prolonged exercise combined with protein-carbohydrate or isocaloric carbohydrate post-exercise nutrition in well-trained endurance athletes. The likely mechanism of action of post-exercise protein-rich nutrition on recovery and adaptation leading to benefit to subsequent performance is via facilitation of contractile, cytoskeletal and extracellular matrix remodelling, improved defence, and enhanced lipid metabolism and mitochondrial responses.

5.1.3.2 Enhanced Signalling to Protein Translation

In addition to gene transcription, the effect of protein-carbohydrate supplementation following high intensity cycling on signalling to protein translation was compared to isocaloric carbohydrate control. A moderate transient increase in phosphorylation of the mammalian target of rapamycin (mTOR), the key regulator of translation, was observed. As well as large to very large increases in phosphorylation of mTOR's downstream signalling proteins, eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) and ribosomal protein S6 (RPS6), but trivial increase in p70 ribosomal protein S6 kinase (p70S6K) at 3 h with protein-rich nutrition. Post-exercise protein and carbohydrate ingestion following prolonged exercise has

previously been shown to result in increased phosphorylation of several proteins involved in translational control (Anthony, et al. 1999; Gautsch, et al. 1998; Kammer, et al. 2009; Morifuji, et al. 2009a; Morrison, et al. 2008) and lead to increased skeletal muscle protein synthesis (Levenhagen, et al. 2001) compared with carbohydrate alone. Ivy and colleagues (2008) found protein-carbohydrate supplementation following 45 minutes intense cycling exercise to increase protein kinase B (AKT), mTOR, and RPS6 phosphorylation in endurance trained men compared to a non-caloric placebo. Another study by this group (Kammer, et al. 2009), showed increased phosphorylation of AKT/PBK and mTOR when cereal and milk was ingested following 2 h of moderate intensity cycling compared with a carbohydrate-matched sports beverage. However, in both of these studies the post-exercise protein-carbohydrate supplements contained greater energy than controls. Many of the signalling pathways leading to protein translation are energy dependent, for example the 5' AMP-activated protein kinase (AMPK)-mTOR-4EBP1 (Coffey, et al. 2007; Deshmukh, et al. 2008; Hawley, et al. 2006; Williamson, et al. 2006; Yan 2009; Yang, et al. 2008; Yu, et al. 2003) pathway, described below; consequently, comparing protein rich post-exercise feeding with low energy control obscures the benefit attributed to protein intake. During prolonged exercise, protein synthesis is blunted by inhibition of translation initiation through AMPK-mTOR-4EBP1 (Coffey, et al. 2007; Deshmukh, et al. 2008; Hawley, et al. 2006; Williamson, et al. 2006; Yan 2009; Yang, et al. 2008; Yu, et al. 2003). Protein synthesis likely remains blunted after exercise when fasted, until gluconeogenesis provides blood glucose and stimulates insulin-mTOR signalling (Morrison, et al. 2008), and protein breakdown provides amino acids and stimulates mTOR signalling (Norton & Layman 2006). In the current research a greater reduction in AMPK phosphorylation at 3 h was found with protein-rich nutrition, which indicates that combined protein-carbohydrate nutrition post-exercise may offset suppression of the mTOR pathway to a greater extent than isocaloric carbohydrate. The attenuation of AMPK activity might have resulted from leucine stimulation of glutamate dehydrogenase (Zhou & Thompson 1996), and increased flux through the tricarboxylic acid cycle as exhibited in pancreatic cells (Xu, et al. 2001). Nonetheless, attenuation of AMPK provides further evidence of enhanced translation initiation as the mechanism resulting in

enhanced recovery and adaptation mediated by protein-rich nutrition following prolonged exercise. Overall, signalling to protein translation was enhanced, and inhibition of translation initiation reduced when combined protein and carbohydrate were ingested following endurance exercise compared to isocaloric carbohydrate-control. The mechanisms postulated to result in improvement of subsequent performance, with protein-rich nutrition following prolonged exercise, lie not only in enhanced transcription of mRNA profiles conducive to recovery and adaptation, but also enhanced translation to their encoded proteins.

5.1.3.3 Attenuated Muscle Damage

Data on the attenuation of muscle damage as a mechanism by which protein-rich nutrition mediates enhanced recovery and subsequent performance benefit is conflicting. On the one hand, post-exercise leucine-rich protein-carbohydrate led to a moderate-sized reduction in serum creatine kinase activity (CK), which is an indirect marker of sarcolemma disruption. In addition, transcriptomal data points towards reduced inflammatory response and increased protection from damage with protein-rich nutrition following exercise. However, on the other hand, no differential in ultra-structural damage as characterized by Z-disk disruption was manifest between conditions. Furthermore, no differences in serum lactate dehydrogenase (LDH), another marker of sarcolemma disruption, or subject's perceived pain threshold, were seen between conditions. It could be argued that differences in results of CK activity and Z-disk streaming are related to the measurement of two different indices of muscle damage. Creatine kinase is a marker of sarcolemmal disruption, albeit indirect; while Z-disk streaming is the visualisation of ultra structure damage under light microscope, and directly assesses myofilament damage (Armstrong 1990). Furthermore the proposed mechanisms of injury differ (Duncan & Jackson 1987); sarcolemmal disruption is postulated to be metabolic, while myofilament disruption is mechanical (Armstrong 1990) and unlikely to be a major contributor in mainly concentric exercise modes such as cycling (Newham, et al. 1983). The lack of a significant difference in Z-disk streaming between conditions, might be related to high variability in this measure (coefficient of variation (CV) >40%; (Beaton, et al. 2002)), combined with small sample size, contributing to an inability to detect a significant reduction in Z-disk streaming between conditions. Differences in CK and

LDH might have been related to blood collection timing; since samples were collected approximately 24 h following exercise stimulus to coincide with CK peak. Lactate dehydrogenase has been shown to peak between 24 h and 96 h post exercise (Cade, et al. 1991; Knitter, et al. 2000; Romano-Ely, et al. 2006), depending on the mode of exercise; thus, peak lactate dehydrogenase concentrations may have been missed. However this is an unlikely explanation, since Romano-Ely (2006) did observe attenuation in serum LDH activity 24 h following cycling exercise, with protein-carbohydrate compared to carbohydrate control (Romano-Ely, et al. 2006). The failure to observe a difference in LDH between conditions is unable to be explained. Nonetheless, blood creatine kinase activities were consistent with results from other studies (Cade, et al. 1991; Millard-Stafford, et al. 2005; Rowlands, et al. 2008; Rowlands, et al. 2007; Saunders, et al. 2004) and, taken together, data indicates the proposed mechanism of action might be attenuation of cell membrane disruption or more rapid reestablishment of membrane integrity, rather than attenuation of myofibrillal disruption.

5.1.3.4 Whole-Body Protein Turnover and Enhanced Muscle Glycogen Synthesis are not the Mechanisms Accounting for Improved Subsequent Endurance Performance

Despite enhanced anabolic signalling at the cellular level, no clear differences in whole body protein turnover rate between conditions over 24 h were found. Previous studies (Howarth, et al. 2009; Levenhagen, et al. 2002) have shown acute fractional skeletal muscle protein synthesis and whole body nitrogen balance (Howarth, et al. 2009; Kammer, et al. 2009) were improved with combined protein-carbohydrate ingestion following prolonged exercise. On the other hand, Greenhaff et al (2008) also found anabolic signalling was dissociated from change in muscle protein synthesis. Both the degree of phosphorylation of signalling proteins, and the temporal relationship between signalling to translation initiation and measurable accumulation of synthesised proteins are at present unknown. Furthermore, in the current research the alternate supplement was provided at the end of the day, which may have stimulated whole body protein turnover to the same degree as when provided immediately following endurance exercise, but is not likely to have resulted

in synthesis of the same early transcripts as those mediated by protein provided in close proximity to exercise.

Combined protein and carbohydrate did not enhance muscle glycogen resynthesis over short (3 h) and long (48 h) recovery compared to isocaloric high-carbohydrate control, and as such was not the likely mechanism accounting for subsequent enhanced endurance performance found in the initial study. Previously Burke et al (1995) reported that despite an initial enhanced rate of glycogen synthesis with combined protein-carbohydrate following exercise, by 24 h the total amount of glycogen storage in muscle was no different to a carbohydrate only recovery diet. Indeed, over the time frame utilized for recovery from training prior to the performance test, which in the current study was 39 h, and within a background of adequate carbohydrate intake, it was very unlikely that muscle glycogen resynthesis would be the mechanism of action enhancing performance.

5.1.4 CONCLUSION

In summary, post-exercise leucine-enriched protein-carbohydrate feeding following intense cycling on each of 3 consecutive days, enhanced subsequent repeated-sprint performance compared to isocaloric carbohydrate feeding. The improved recovery was thought to be associated with lower disruption to skeletal muscle integrity and/or enhanced recovery. Results are particularly relevant as well-trained athletes were used, the leucine-protein supplement was provided with meals consisting of normal food types and quantities, and ingested over a realistic time frame. Following on from the initial results, it was sought to determine the likely cellular mechanisms leading to improved performance, facilitated by combined protein and carbohydrate following exercise. Protein-rich post-exercise nutrition was found to mediate a refined and more specific response to exercise-induced gene expression in comparison to isocaloric carbohydrate control. In particular, protein-rich post-exercise nutrition directed a molecular programme supporting attenuation of early inflammatory response and enhanced defence, remodelling of contractile, cytoskeletal, and extracellular matrix, and later fatty acid metabolism and mitochondrial adaptive response to endurance exercise. A further mechanism by which protein-rich post-exercise nutrition might have lead to accelerated recovery

and improved performance was through enhanced protein translation, and indeed signalling to translation initiation was up-regulated early during recovery from the prolonged exercise bout. However this was dissociated from protein turnover at the whole-body level. This research provides evidence that reflects not only the importance of protein-rich nutrition in the recovery diet following intense exercise; but also provides insight into the biological processes that are likely modified by an exercise-nutrition interaction, the immediate homeostatic recovery and delayed adaptive responses, which may lead to a beneficial functional phenotype. The strength of both study designs was in the tight control of training and diet leading up to and during each study period. The alternate blinded supplement was provided at the opposite end of the day in both studies, in order to assess the true effect of post-exercise timing on performance outcome, muscle damage, signalling to translation, and gene expression. However, more research is required to determine if nitrogen status influences the efficacy of the protein-rich feeding on subsequent performance. More research is required to link the above mechanisms with performance.

5.1.5 DELIMITATIONS

The two studies described in this thesis limited subjects to males aged 19-50 years of age, although ages tended to fall into the upper and lower ends of the criteria with less subjects in their 30s. Subjects were trained cyclists, triathletes, or multisport athletes who were in regular training for 8 or more hours per week over the last 6 months, and had participated in high-intensity sessions or racing in the previous year. Every endeavour was made to ensure subjects were alike in training status, but it was found that abilities ranged from an ex pro cyclist to semi-competitive cyclists. Thus, results may not be applicable to competitive elite cyclists who generally have less scope for improvement with training and nutritional interventions than sub-elite cyclists or those not currently in the competitive phase of training. All subjects met the aerobic fitness criteria of having measured VO_{2max} greater than 55 $mL \cdot kg^{-1} \cdot min^{-1}$.

A further delimitation is that whilst the transcriptomal response presented in Chapter 4, provides insight into the biological processes likely modified under these conditions, there is large potential for post-translational regulation, and hence transcripts might only partially correlate with their encoded protein products

(Brockmann, et al. 2007). Although it is reasonable to suggest the encoded protein response is in the same direction as their transcripts (Booth & Baldwin 1996), protein half-life and degradation also play an important role in determining the relative magnitude of change leading to altered phenotype.

5.1.6 LIMITATIONS

Daily nitrogen intakes of 1.6 $g \cdot kg^{-1} \cdot d^{-1}$ were estimated to be sufficient for the intense endurance training program undertaken in the performance study, based on a well controlled nitrogen balance study of similarly trained endurance athletes (Tarnopolsky, et al. 1988). However subjects were found to be in mildly negative nitrogen balance. The cause of this mild negative nitrogen balance may have been due to the drop from habitual intakes of 2.0 $g \cdot kg^{-1} \cdot d^{-1}$. Nelson et al (2010) found no clear benefit to performance when subjects remained in positive balance (daily intakes of 1.5 and 1.9 g protein$\cdot kg^{-1} \cdot d^{-1}$), which casts doubt on the efficacy of post-exercise protein rich nutrition when used in a background of high daily protein intake. Habitual protein intakes in New Zealand cyclists of this calibre are thought to be about 2.0 $g \cdot kg^{-1} \cdot d^{-1}$ (personal communication D. Rowlands and I Perols).

Possible limitations regarding biological sampling include timing of the blood draws in the performance study (Study 1) and timing of muscle biopsy sampling in the mechanism study (Study 2). Blood sampling was timed to coincide with the peak CK activities, about 24 h following exercise stimulus; however LDH has been shown to exhibit a biphasic peak, immediately and at a later time point ranging from 24 h to 96 h (Cade, et al. 1991; Knitter, et al. 2000; Romano-Ely, et al. 2006) depending on mode of exercise, and peak lactate dehydrogenase concentrations may have been missed. Muscle tissue biopsies were taken at 3 and 48 h post exercise; however this timing schema may have missed immediate early gene expression and phosphorylation of key signalling proteins of a transient nature. For example, the transient immediate-early mRNA peak in transcription factors such as c-FOS, c-JUN, may have been missed. In addition, the rapid transient phosphorylation of p38-MAPK and ERK which has previously been demonstrated to peak and return to baseline within 30-60 minutes (Richter, et al. 2004; Widegren, et al. 1998), may have been missed.

Lastly, investigations into the delayed benefit to performance with post-exercise protein rich feeding, as proposed by Rowlands (2008), have all been generated from the same institute and collaboration of authors. Bias may be introduced to a cluster of studies generated by the same institute due to a similar approach to the problem, and similar methods, compared with large numbers of studies by independent research groups (Decombaz, et al. 2003). However, as various subjects and feeding protocols have been utilized in this group of studies, the potential for bias resulting from this clustering of studies is reduced.

5.1.7 RECOMMENDATIONS FOR FUTURE RESEARCH

Some of the limitations and delimitations discussed in relation to the 2 studies in this thesis could be addressed by future research. Firstly, the efficacy of post-exercise protein-carbohydrate ingestion on subsequent performance set in a background of high versus low daily protein intakes remains unanswered. Secondly, further research into changes in selected proteins or ideally the entire proteome under the same exercise-nutrient conditions is needed. However, the inherent difficulties involved in analysing the exercise-nutrient induced proteome, the least of which include expense, required expertise, failure to detect less abundant proteins, failure to determine protein intracellular location, and unknown correlation with cellular function make this out of range of sport science researchers.

There is room for investigation into the leucine and/or protein dose response on signalling to translation initiation and fractional protein synthesis following endurance exercise, akin to the Crozier study (2005) in rats following 18 h of fasting.

Further research is needed to determine if the enhanced recovery of homeostasis and adaptive responses found in the current study over an acute period will continue over a chronic time course to result in increased translation of encoded proteins. This is especially pertinent in light of the findings of D'Antona et al (2010) who found long term supplementation of an amino acid mixture led to greater mitochondrial capacity and improved endurance exercise capacity.

Finally, investigation into the response of other population groups is needed. For instance gene expression relating to sarcolemmal stability, such as calpain

proteolysis, and defence and immunity in female trained cyclists is warranted, bearing in mind the differences in creatine kinase levels reported by Rowlands and Wadsworth (Rowlands & Wadsworth 2010). Equally as important might be investigation of different responses in young and middle-aged athletes, particularly gene expression relating to metabolic and mitochondrial function, in part due to the findings of D'Antona et al (2010) described above, and also due to the increased interest in cycling as a sport in middle aged New Zealanders (the largest proportion of New Zealand cyclists is the 35-49 year old group) (SPARC 2009).

APPENDIX A RESEARCH PROPOSAL

Massey University | Institute of Food Nutrition & Human Health | Te Kunenga ki Pūrehuroa

RESEARCH PROPOSAL FOR PHD

Provisional Registration Date: 31/05/2006
Expected date of completion: end of 2009

The effect of combined protein-carbohydrate supplementation post-exercise on subsequent endurance performance: the delayed effect and the mechanisms involved

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ABSTRACT
Background: Recovery is an important part of an athlete's training regime, both for maintenance of cellular homeostasis, resynthesis of fuel stores, and repair of muscle damage, as well as adaptation and performance improvement. Immediately post-exercise is the most advantageous time to ingest nutrients aimed at recovery from fatiguing exercise due to increased blood flow, insulin sensitivity, and glycogen synthesis rate. Cade et al (Cade, et al. 1991) first proposed that coingestion of protein and carbohydrate may benefit muscle recovery; additionally a landmark study by Zawadzki et al (Zawadzki, et al. 1992) suggested that protein and carbohydrate act to synergistically stimulate greater insulin secretion, glucose uptake by muscle, and more rapid glycogen resynthesis. A small number of studies have aimed to quantify the effects of co-ingesting carbohydrate and protein post-exercise on subsequent performance benefits; however results are inconsistent and fail to adequately support the hypothesized benefits, particularly relating to glycogen resynthesis rate (Betts, et al. 2005; Karp, et al. 2006; Millard-Stafford, et al. 2005; Niles, et al. 2001; Romano-Ely, et al. 2006; Saunders, et al. 2004; Williams, et al. 2003). A recent study by Rowlands et al (Rowlands, et al. 2008) indicates potential benefits on other aspects of recovery, such as the promotion of anabolic environment and attenuation of muscle damage when the recovery period is extended beyond that used in previous studies, leading to improved performance of subsequent exercise.
Aims and Objectives:
To examine the expression of candidate genes and activity of specific signalling proteins likely to be involved in enhanced recovery from prolonged high intensity exercise with immediate post-exercise coingestion of protein and carbohydrate.
To determine the effect of immediate post-exercise co-ingestion of protein and carbohydrate on subsequent performance. Specifically, to define the effect attributable to post-exercise protein-carbohydrate supplementation from that of daily protein intakes (1.6 g/kg/d) recommended to endurance athletes.
To compare the effect of three different recovery formulations (protein-carbohydrate, protein-carbohydrate-leucine, and carbohydrate-leucine) on the expression of candidate genes and activity of signalling proteins involved in nutrient enhanced recovery from prolonged high intensity exercise.
Methods:
Study 1: In a single-blind crossover study, 8 trained male endurance cyclists performed a 105 minute bout of intense ergometer cycling exercise and then recovered in the lab for 3 h. Participants received carbohydrate-protein or control (carbohydrate) supplements at 0 and 1 h post-exercise and the alternative supplement at 5 h and 9 h. Blood samples were taken at 0, 30, 60, 90, 120, 180 minute, and 48 h to determine insulin, glucose and likely immune/inflammatory markers; muscle biopsies were taken at 180 minute and 48 h post-exercise to quantify the expression of select genes and activity of signalling proteins.

Study 2: In a double-blind cross-over study we will quantify the effects of post-exercise protein-carbohydrate supplementation on subsequent exercise performance in conjunction with an adequate to high protein diet. Ten to 12 male endurance cyclists will perform an intense 3-4 d ergometer cycling training program with post-exercise supplementation of protein-carbohydrate or control (carbohydrate) at 0, 30, 60 minutes during recovery in the laboratory, and the alternative supplement provided approximately 10-12 h later. Participants will return to the laboratory fasted, in the morning, approximately 36 h following the final training cycle for the performance trial. The performance trial will consist of a repeat-sprint high-intensity cycling, on a Kingcycle ergometer, in order to stimulate race conditions. Both training and diet will be controlled, and urine and sweat collected to determine Nitrogen balance. Blood will be taken 48 h after beginning the cycling training period and immediately prior to the performance test to determine plasma markers of muscle damage. Muscle soreness and wellness scales will be completed at baseline, prior to each training cycle, and immediately prior to the performance trial.

Study 3: In a double-blind cross-over study we will investigate the effect of protein-carbohydrate, protein-carbohydrate-leucine, and carbohydrate-leucine recovery formulations on gene expression and activity of signaling proteins. The expression of candidate mRNA encoding proteins relating to physiological mechanisms important for recovery, the activity of signaling proteins, and protein synthesis will be quantified. Eight to 10 male endurance cyclists will receive on 3 separate occasions, 1 of 3 recovery formulations containing protein-carbohydrate, protein-carbohydrate-leucine, and carbohydrate-leucine during recovery from prolonged high-intensity ergometer cycling exercise. Blood and muscle biopsy samples will be taken at 3 and 12 h post-exercise.

Expected Outcome: The findings generated by this research should provide better understanding of the molecular and cellular mechanisms driving nutrient-stimulated recovery from high-intensity endurance exercise and effects on subsequence performance. Furthermore, the third study should provide valuable data on the nutrient stimulated response to co-ingestion of protein-carbohydrate-leucine, carbohydrate-leucine, and protein-carbohydrate formulations during recovery from prolonged high-intensity exercise in trained men.

CONTENTS

PURPOSE

Main Research Questions

i). What are some of the mechanisms, at a cellular level, for the recovery response stimulated by post-exercise protein and carbohydrate coingestion?

ii). Is there a benefit to subsequent performance with post-exercise protein and carbohydrate coingestion, in endurance athletes already consuming recommended daily protein intakes approximating nitrogen balance?

iii). Do the protein and leucine content of post-exercise recovery supplements produce different transcriptional responses of candidate genes, expression of signalling proteins, and protein synthesis during recovery from prolonged high-intensity exercise?

Secondary Research Questions

i). Specifically, what are some of the genes and signalling proteins involved in the nutrient stimulated recovery response to prolonged high-intensity exercise?

ii). To what extent is the expression of candidate genes stimulated with post-exercise protein-carbohydrate coingestion compared to control (carbohydrate alone)?

iii). To what extent are the activities of signalling proteins changed with post-exercise protein-carbohydrate coingestion?

iv). Is there a beneficial effect of post-exercise protein-carbohydrate coingestion on markers of muscle damage compared to control (carbohydrate alone) when both treatment groups are in nitrogen balance?

v). To what extent is the expression of candidate genes and activity of signalling proteins changed in response to 3 different post-exercise recovery supplements?

vi). Is the expression of candidate genes, activity of signalling proteins, and response of muscle recovery and adaptation markers greater with the addition of leucine to carbohydrate recovery formulations compared to protein?

BACKGROUND

Recovery from a fatiguing bout of exercise is an important part of an athlete's training regime, both for maintenance and improvement of performance during the next bout of exericse when in training or competition. Without adequate recovery the athlete will fail to adapt to the training stimulus, and performance of subsequent exericse will deteriorate. Recovery from prolonged high-intensity exercise requires replenishment of fuel stores, repair of damaged muscle tissue, and the initiation of training adaptations; the body must switch from a mainly catabolic state to a mainly anabolic one (Ivy 2004; Levenhagen, et al. 2001; Roy, et al. 2002). Immediately post-exercise is the most advantageous time to ingest nutrients for recovery due to increased blood flow, insulin sensitivity, and glycogen synthesis rate. Hence, the timing of nutrient provision post-exercise has been widely studied, particularly in the case of carbohydrate (Ivy 2004; Jentjens &

Jeukendrup 2003; van Loon, et al. 2000a); and recently the addition of protein to carbohydrate recovery meals has garnered interest by various research groups.

Cade and colleagues (Cade, et al. 1991) were the first to propose a potential beneficial effect of post-exercise protein and carbohydrate coingestion on muscle recovery from prolonged high-intensity exercise. It has further been postulated that there may be a possible synergistic effect of protein and carbohydrate on protein turnover (Luden, et al. 2007; Millard-Stafford, et al. 2005). Amino acids provide precursors for protein synthesis, and essential amino acids particularly leucine, stimulate protein synthesis via cellular signalling pathways (Anthony, et al. 2001; Blomstrand & Saltin 2001; Tipton & Wolfe 2004; Williams, et al. 2003). The carbohydrate induced increase in insulin secretion and exercise-induced enhanced sensitivity to insulin (Ivy 2004) act to increase cellular uptake of amino acids, retard post-exercise protein breakdown, and may indirectly modulate muscle protein synthesis (Koopman, et al. 2004; Rennie & Tipton 2000; Tipton & Wolfe 2004; Williams, et al. 2003). There is some support for attenuation of muscle damage (Cade, et al. 1991; Flakoll, et al. 2004; Luden, et al. 2007; Romano-Ely, et al. 2006; Saunders, et al. 2004); however these findings should be viewed with caution as highly variable blood creatine kinase levels and self assessed ratings of muscle soreness were used as markers of muscle damage (Cade, et al. 1991; Flakoll, et al. 2004; Luden, et al. 2007; Millard-Stafford, et al. 2005; Saunders, et al. 2004). Further evidence is provided by Levenhagen and others (Levenhagen, et al. 2002), who found increased protein synthesis with post-exercise coingestion of protein and carbohydrate versus carbohydrate alone. In addition the importance of immediate post-exercise supplementation was illustrated in an earlier study from the same laboratory, which showed increased protein synthesis compared with delayed nutrient intake (Levenhagen, et al. 2001).

The predominant theory for benefit to recovery with post-exercise coingestion of protein and carbohydrate is the potential to synergistically stimulate greater insulin secretion, and hence increased uptake of glucose by muscle, stimulating more rapid glycogen resynthesis and recovery of fuel stores (Berardi, et al. 2006; Burke, et al. 1995; Ivy, et al. 2002; Jentjens, et al. 2001; Millard-Stafford, et al. 2005; Niles, et al. 2001; Yaspelkis & Ivy 1999). This hypothesis was first proposed by Zawadzki et al (Zawadzki, et al. 1992) who investigated the effects of carbohydrate-protein supplementation on serum substrates, hormones, and muscle glycogen storage post-endurance cycling exercise. They reported serum insulin and muscle glycogen storage were increased compared with carbohydrate alone. Results from subsequent studies are consistent, but the majority show no increase in plasma insulin (Burke, et al. 1995; Carrithers, et al. 2000; Ivy, et al. 2002; Millard-Stafford, et al. 2005; Tarnopolsky, et al. 1997; van Loon, et al. 2000a; Yaspelkis & Ivy 1999), nor muscle glycogen resynthesis rates (Berardi, et al. 2006; Burke, et al. 1995; Carrithers, et al. 2000; Jentjens, et al. 2001; Millard-Stafford, et al. 2005;

Tarnopolsky, et al. 1997; van Hall, et al. 2000; van Loon, et al. 2000b; Yaspelkis & Ivy 1999).

A less exhaustively investigated theory involves the effect on the immune system status during periods of intense endurance training. The proposed benefit of protein, specifically branched chain amino acids and glutamine, is via attenuating the immunosuppression associated with low glutamine concentrations, negative nitrogen balance, and overtraining (Flakoll, et al. 2004; Kreider, et al. 1993); in addition providing adequate carbohydrate for maximal muscle glycogen synthesis in competition with inflammatory cells (Wojcik, et al. 2001) and attenuating IL-6 elevation in response to low muscle glycogen (Rowlands, et al. 2008). There is evidence of some benefits to the immune system status from two chronic supplementation studies (Flakoll, et al. 2004; Kreider, et al. 1993), however caution should be taken in the interpretation of these results as neither study provided adequate carbohydrate content to maximize muscle glycogen synthesis, and had vastly different study design and subject populations.

Despite growing appreciation of the role of post-exercise protein or amino acid and carbohydrate on recovery process functioning, there have only been a small number of studies aimed at quantifying the associated performance benefits. Studies have found both a benefit to performance during a subsequent exercise bout (Niles, et al. 2001; Saunders, et al. 2004; Williams, et al. 2003); and no benefit (Betts, et al. 2005; Karp, et al. 2006; Luden, et al. 2007; Millard-Stafford, et al. 2005; Romano-Ely, et al. 2006) in endurance trained runners and cyclists. Again, the predominant mechanism for improvement in subsequent performance was proposed to be enhanced muscle glycogen resynthesis rates; however the evidence is inconsistent with this theory. Some studies have provided inadequate carbohydrate to optimize muscle glycogen synthesis (>1.2g·kg-1·d-1) (Betts, et al. 2005; Karp, et al. 2006; Millard-Stafford, et al. 2005; Saunders, et al. 2004; Williams, et al. 2003); a strategy biased to improve muscle glycogen resynthesis rates in the carbohydrate-protein supplemented group, although most still demonstrate no benefit to performance (Betts, et al. 2005; Karp, et al. 2006; Millard-Stafford, et al. 2005). Research groups have also examined supplements containing adequate carbohydrate contents, and don't demonstrate any performance benefits. Additional calories were provided with the addition of protein in some studies (Saunders, et al. 2004; Williams, et al. 2003), and two of these do show a benefit to performance that may be due to the protein or the additional energy content. Moreover other studies have added antioxidants to the carbohydrate-protein supplements, further masking the potential beneficial ingredient, but have not resulted in clear improvements in performance (Luden, et al. 2007; Millard-Stafford, et al. 2005; Romano-Ely, et al. 2006).

Research Proposal: The effect of combined protein-carbohydrate supplementation post-exercise on subsequent endurance performance; the delayed effect and the mechanisms involved

The majority of performance studies also seem to have focused on the initial period of glycogen resynthesis relating to subsequent performance benefits; hence short recovery periods have been used. The timing of the subsequent performance test has ranged from 2-24 h following the initial fatiguing exercise bout (Betts, et al. 2005; Karp, et al. 2006; Luden, et al. 2007; Millard-Stafford, et al. 2005; Niles, et al. 2001; Romano-Ely, et al. 2006; Saunders, et al. 2004; Williams, et al. 2003). While this may be adequate for most if not all muscle glycogen resynthesis to occur (Burke, et al. 1995; Levenhagen, et al. 2001; van Hall, et al. 2000), if alternative mechanisms are responsible for the performance benefits of carbohydrate-protein supplementation, such as increased rate of synthesis and repair of muscle protein and/or improved immune system status, a longer recovery period may be required to restore optimal performance capacity.

In a recent well controlled study by Rowlands et al (Rowlands, et al. 2008) investigated the benefits of carbohydrate-protein-fat post-exercise feeding compared with carbohydrate-fat on the recovery of performance capacity at two time points. Interestingly they found that there was no clear benefit of carbohydrate-protein feedings to performance at 15 h following the initial fatiguing exercise bout, but by 60 h there was a substantial benefit to performance compared with the carbohydrate fed group. The authors concluded that the delayed effect on performance may be related to the time required for repair and recovery. Exercise-induced gene transcription is thought to peak at 8-12 h post-exercise, then additional time is required for transcripts to be translated to their specific proteins, to facilitate repair and restoration of cellular homeostasis and adaptation.

This area of research is far from complete, there are still many unanswered questions, such as the nature of the mechanisms involved, the optimal dosage and form of amino acids (whole protein, protein hydrolysate, essential amino acids, leucine), optimal supplemental frequency, the timeline of recovery, and more definitive evidence of performance benefits are required.

FOCUS

Over the last decade or more a huge body of research has investigated carbohydrate supplementation during and following prolonged exercise. More recently, the possible advantages of adding protein to carbohydrate recovery supplements has gained more attention from both scientific and commercial groups.

Previous research in this area has narrowed its focus to only a few proposed mechanisms and has provided limited time frames for the recovery process to occur; hence there is scant evidence of benefits of post-exercise protein-carbohydrate co-ingestion, and more importantly the benefit to performance has

not been adequately proven. Previous studies have raised more questions than they have provided answers; nonetheless, the findings of a recent unpublished study by Rowland's' group are novel and propose new theories of the nature of recovery, the mechanisms driving the recovery process, and benefits to subsequent performance. In addition, scientific understanding and research is beginning to accelerate in areas of cellular regulation (Baar 2006) and gene transcription (Heller 2002) in exercise physiology, and previously unavailable techniques are now becoming more commonplace, offering new insights into the mechanisms of recovery from prolonged aerobic exercise.

This series of proposed studies will be the first body of work that we know of, to investigate the effect of specific recovery nutrition on signaling pathways and gene transcription following fatiguing aerobic exercise; and are expected to provide additional evidence confirming the performance benefits found in Rowlands and others (Rowlands, et al. 2008) initial study.

Proposed are three studies.
1. In the first study, I aim to quantify the expression of a set of genes and proteins important for the adaptive and recovery responses in muscle, the signalling processes that govern gene expression and anabolism in response to post-exercise protein-carbohydrate co-ingestion.

2. Secondly, I will investigate the effect of post-exercise protein-carbohydrate co-ingestion compared with carbohydrate only, on subsequent performance and markers of muscle damage. I aim to differentiate the effect attributed to the post-exercise protein-carbohydrate supplement from that of maintaining positive nitrogen balance.

3. Finally, I aim to quantify the effect of 3 different recovery supplements, control (carbohydrate), carbohydrate-protein, and carbohydrate-protein-free leucine, on the expression of candidate genes involved in nutrient-stimulated recovery from 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 is possible that the present recommendations of 1.2-1.6 g·kg-1·d-1 may be adequate for N balance, but not optimal for performance. In addition the recovery period immediately post-exercise is proving especially important for the timing of nutrient intake, and the co-ingestion of protein and carbohydrate may maximize restoration of fuels, recovery and repair processes, and cellular signalling and gene transcription induced adaptation to endurance training.

Research Proposal: The effect of combined protein-carbohydrate supplementation post-exercise on subsequent endurance performance; the delayed effect and the mechanisms involved

METHODS

Study 1: Gene Expression, Signalling Pathways, and Protein Turnover during Recovery from Prolonged High-Intensity Cycling: Effect of Protein-Carbohydrate Recovery Supplement

Data Collection and Collaboration

The data collection for this study was conducted June-October 2006 at McMaster University Hospital with my supervisor Dr David Rowlands and his collaborator Prof. Mark Tarnopolsky. Prof. Tarnopolsky performed the muscle biopsies, and gene analysis is presently being done at McMaster and by Nestec (Switzerland). I was responsible for the recruitment and organization of the subjects, VO_{2max} testing, controlled diets, provision of supplements, and data collection. The study is funded by Massey University Research Fund, Sport and Recreation New Zealand, Life Science Nutritional (Canada), and Nestec (Switzerland).

Statement of the Problem

A recent study by Rowlands et al, (Rowlands, et al. 2008) showed that a post-exercise protein-enriched feeding intervention enhanced subsequent (60 h later) prolonged high-intensity exercise performance. However the cellular mechanisms involved in this nutrient-recovery interaction are unknown.

Aims

To examine the expression of specific genes and the activity of specific signalling proteins postulated as most likely to be involved in the process relating to the enhanced recovery response to a protein-carbohydrate supplement. The genes and signalling factors were chosen on the basis of an in depth assessment of current literature in muscle exercise and amino acid molecular physiology, and inferences from the results of recent performance studies.

Hypotheses

1. The protein-carbohydrate recovery supplement will stimulate the expression of the selected mRNA species involved in modulating functional adaptation, anabolism (protein synthesis), and recovery/repair from prolonged high-intensity exercise.
2. The protein-carbohydrate recovery supplement will stimulate signalling the activation of proteins favouring the promotion of protein synthesis, gene expression, cell growth, and glucose and lipid metabolism.

Study Design

Eight trained male endurance cyclists (road, mountain, triathletes) participated in a single-blind, randomized, crossover design, with recovery supplementation of protein-carbohydrate and control beverages following prolonged intense ergometer cycling exercise. There were 2 experimental blocks with at least 1 week washout between; details are illustrated in Figure 1 and described below.

During Visit 1 participants' VO_{2max} were determined; participants were screened based on high aerobic fitness (pre-testing maximal oxygen capacity of 59.8 ± 5.95 $mL \cdot kg^{-1} \cdot min^{-1}$) and training histories, and then a familiarization ride was performed. During Visit 2 a standardized ride was performed and a controlled diet was provided to participants to up to Visit 3, the baseline muscle biopsy. From day -7 until day -2 before the experiment day participants consumed a standardised diet. During Visit 4 (day -2), a standardized training ride was performed and again a controlled diet provided until the experiment day, Visit 5. During Visit 5 participants arrived fasted, a catheter was inserted in a forearm vein for drawing blood, participants received a snack, and then performed an intense 105 minute intermittent-intensity cycle protocol, representative of a hard training session or race. Following the cycle protocol, participants recovered in the laboratory for 3 h, and received recovery supplements (high-protein or control) immediately and at 1 h post-exercise, blood was taken at times 0, 30, 60, 90, 120, and 180 minutes post-exercise, and the muscle biopsy from the Vastus lateralis at time 180 minutes post-exercise. To balance total protein and carbohydrate intake and hence to isolate the immediate post-exercise effects, the alternate supplement was provided at 5 h and 9 h post-exercise. Approximately 48 h post-exercise, during Visit 6, final blood samples and muscle biopsy from the Vastus lateralis were taken. Visits 4, 5, and 6 were repeated with the alternative treatment (high-protein or control supplements).

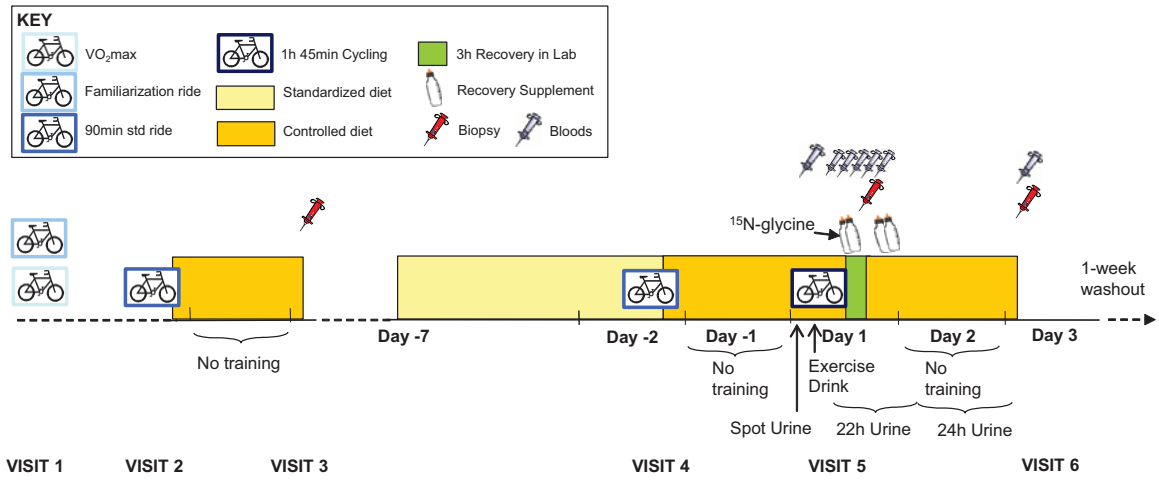

Figure 1: Experimental design and procedures

Duration

Sample collection was done over 15 weeks during 2006, and an additional 15 months is required for analysis.

Research Proposal: The effect of combined protein-carbohydrate supplementation post-exercise on subsequent endurance performance; the delayed effect and the mechanisms involved

Methods

Supplement Formulation:

1. Intervention: The high protein formulation contained 0.4 g·kg-1·h-1 protein (whey isolate), 1.2 g·kg-1·h-1 carbohydrate (maltodextrin and fructose), and 0.2 g·kg-1·h-1 fat (dried canola oil) content, aimed at saturating post-exercise rates of protein and glycogen synthesis.
2. Control: The carbohydrate only control formulation contained 0.03 g·kg-1·h-1 protein, 1.6 g·kg-1·h-1 carbohydrate (maltodextrin and fructose), and 0.2 g·kg-1·h-1 fat (dried canola oil) content, aimed at saturating post-exercise rates of glycogen synthesis. These values were taken from interpretation of current literature on post-exercise carbohydrate supplementation of 1.2 g·kg-1·h-1 to maximise glycogen resynthesis rate (Ivy 2004; Jentjens & Jeukendrup 2003; Tarnopolsky, et al. 2005).

Muscle biopsies:

Needle biopsy samples were taken from the Vastus lateralis approximately 15-20 cm proximal to the lateral knee joint under local anesthesia (1% xylocaine, Astra Zeneca, 20 mL, PROD# 012, and 1% xylocaine, Astra Zeneca, 20 mL) using a Bergström needle (5 mm diameter Bergström-Stille muscle biopsy cannula) with manual suction. All biopsies were taken from separate incisions on alternating legs, several cm apart from previous incisions, to minimize the potential effect of the biopsy itself on gene expression.

1. The expression of candidate genes will be assessed via mRNA quantification by RT-PCR (real time reverse transcriptase polymerase chain reaction). To date the following mRNA have been analysed; mitochondrial (PGC-1α, PDK4), BCAA metabolism (BCOADkinase), transcription factors (FOXO1, NFκB), cell growth, development & maintenance (IGF1), calcium signalling and cell growth (DSCR1/MCIP1/Adapt78). The housekeeping gene used in this study was Beta 2 microglobulin (B2M), and has been previously validated as a gene which doesn't change with exercise or differ between the treatment groups used in this study. Further analysis has paused pending the outcome of a whole genome transcriptome screen via Illumina DNA array.
2. The expression and phosphorylation of candidate signalling proteins will be assayed by gel electrophoresis and immunoblot techniques. The following signalling proteins important for exercise and nutritive signalling, will be analysed; mTOR (role in regulation of protein translation and ultimately cell size), AKT (regulate cell survival and metabolism), AMPK (part of a signalling cascade influencing glucose and lipid metabolism, gene expression and protein synthesis), p70S6K (part of signalling pathway linking amino acid sufficiency to control of peptide chain initiation).

Outcomes

This project will be the first to quantify the expression of candidate genes and the activity of signalling proteins representing factors that we believe are most likely to be involved in the specific recovery responses to the high-protein supplement. To date, RT-PCR analysis shows significant increase in expression of PDK4 and PGC-1α in both treatments at 3 h post-exercise, and significantly higher expression of PGC-1α and a trend for increase in PDK4 in the protein-carbohydrate treatment group at 48 h post-exercise compared to the carbohydrate only. In addition the expression of FOXO1, DSCR1, & NFκB increased at 3 h post-exercise in both treatment groups, and there was a trend for increase in FOXO1 by 48 h. However, there were no significant differences found in pre- and post-exercise or between treatment groups for BCOADkinase, or IGF1 mRNA expression.

Study 2: Effect of Post-Exercise Protein-Carbohydrate Supplementation on Subsequent Performance during maintenance of Nitrogen Balance

Statement of the Problem

A recent study by Rowlands et al. (Rowlands, et al. 2008) , showed a benefit with post-exercise protein-enriched food intake on subsequent (60 h) prolonged high-intensity exercise performance. This study also found that the control group was in negative nitrogen balance compared with the positive nitrogen balance of the protein-enriched diet group during post-exercise recovery. This suggests that improving nitrogen balance may be a factor in the benefits of post-exercise protein-enriched food intake on subsequent exercise performance.

Aims

1. Determine the effect of post-exercise protein-carbohydrate supplementation on subsequent performance.
2. Define the effect attributed to the post-exercise protein-carbohydrate supplementation from that of adequate to high daily dietary protein (maintaining nitrogen balance) on subsequent performance.
3. Quantify the effect of post-exercise protein-carbohydrate supplementation on muscle damage following prolonged intense cycling exercise over 3-4 d.

Hypotheses and Postulates

1. The post-exercise protein-carbohydrate recovery supplementation protocol will benefit subsequent prolonged cycling exercise performance relative to control (isocaloric carbohydrate) supplement.
2. The post-exercise protein-carbohydrate recovery supplementation protocol will benefit subsequent prolonged cycling exercise performance when both treatment groups are maintaining positive nitrogen balance.
3. Markers of muscle damage will be reduced with post-exercise protein-carbohydrate supplementation.

Research Proposal: The effect of combined protein-carbohydrate supplementation post-exercise on subsequent endurance performance; the delayed effect and the mechanisms involved

Duration
Preliminary and pilot work will be done over 10-12 weeks prior to the experiment. Data collection and analysis will be done over 12 months starting August 2007.

Study Design
Ten to 12 trained male endurance cyclists (road, mountain, triathletes) will be recruited to participate in this study. A double blind crossover design will be used with at least a 2 week washout period between each experimental block. This study will replicate the recovery supplement formulation used in Study 1. To isolate the effects of immediate post-exercise supplementation from that of altered total energy and protein intakes, daily energy and macronutrient intakes will be balanced by provision of the alternative supplement at the other end of the day. To separate the mechanism driving the effects of post-exercise supplementation from that of positive nitrogen balance, athletes in both treatment groups will receive a diet high in protein.

During Visit 1 participants' VO_{2max} will be determined; screening of participants is based on high aerobic fitness (pre-testing maximal oxygen capacity of 55 mL·kg-1·min-1 or greater), training histories, and general health. Visit 2 will be a familiarization trial of both the Muscle Soreness and Wellness questionnaires, and the performance test protocol. In addition, baseline blood samples will be taken. During Visit 3 participants will arrive in the morning fasted, and resting metabolic rate will be determined. After provision of a meal, participants will undergo a series of sub-maximal rides to determine metabolic rate at varying power intensities. From Day -9 participants will record their daily dietary intakes and training volumes and intensities over 4 days, to be repeated during the second block of the experiment. During Visits 4-7, participants will arrive in the laboratory in late afternoon and begin the laboratory-based training and recovery protocol; the recovery supplementation will be taken immediately and at 30 minute intervals during the 1 h recovery period. During Visit 4 sweat collection will be done whilst the participants complete the training ride. During Visits 5 and 7 immediately prior to the training ride, the Muscle Soreness and Wellness questionnaires will be completed, and blood samples taken. Participants will follow the laboratory-based training protocol and controlled diet, and 24 h urine samples will be collected throughout over 4 days. During Visit 8 participants will arrive in the morning fasted, then will receive a calorie and protein controlled meal, complete the Muscle Soreness and Wellness questionnaires, final blood samples will be taken, and then participants will carry out the performance test protocol. Following at least 1 week washout period Days -9 to Day 1 of the experiment block will be repeated on the alternative treatment.

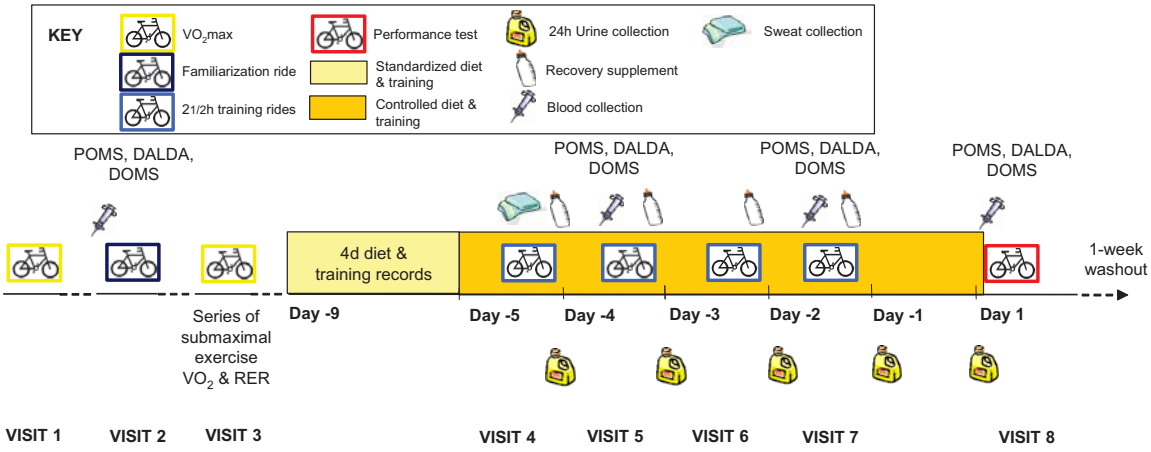

Figure 2: Experimental procedures; standardized diet from Day -9, and controlled diet and training from Day -5 prior to Performance Test.

Methods

Performance Test:

Performance will be assessed using a tested and validated repeat-sprint protocol and high-intensity exhaustion tests to simulate race conditions via Kingcycle.

Experimental Conditions:

1. Treatment Condition: post-exercise protein-carbohydrate supplement
2. Control: Iso-caloric carbohydrate control supplement.

Controlled Diet:

1. Total daily energy intake for the control diet will be calculated as follows: Participants resting metabolic rate will be determined in the morning fasted, via the Douglas bag technique of indirect calorimetry. Then participants will undergo a series of sub-maximal rides to determine VO2 at varying power intensities. A regression curve of workload Vs VO2 will be plotted to determine energy expenditure at a given cycling exercise workload. Correction factors for daily activity and the thermic effect of food will be added to the equation to determine individual energy expenditure over Days -4 to 1 of the experimental block.
2. Dietary protein intake for the control diet will be based on averaged protein intakes from dietary data of well trained/sub-elite New Zealand cyclists during a period of regular training (from the literature or pilot survey). Dietary protein intakes for the control diet will be ≥1.5 g·kg-1·d-1 to maintain neutral or positive nitrogen balance. This figure is based on a recent study by Rowland's et al (Rowlands, et al. 2008), where a regression plot of nitrogen balance data provided an average neutral nitrogen balance for well-trained cyclists under a similar experimental model at protein intakes of 1.5 g·kg-1·d-1.

3. To balance total energy and macronutrient intake and hence to isolate the immediate post-exercise effects, the alternate supplement will be provided to the participants in the morning meal.

Nitrogen Balance:
Urine and sweat will be collected, and a controlled diet provided (any food not consumed will be returned and measured) for 4 days leading up to the performance test, to determine Nitrogen balance.

Plasma and Urine Biochemistry:
Plasma marker of muscle damage (creatine kinase) and urine marker of muscle damage (3-methylhistidine) will be measured. Maybe FABP will be measured – time line will have to change (peaks at 2-6hrs after stress) – not good for training (long-term) effect only single bout (acute) muscle damage measure

Muscle Soreness and Wellness Scales:
Wellness and muscle soreness scales (Short Profile of Mood States questionnaire (Short POMS), Daily Analysis of Life Demands of Athletes questionnaire (DALDA), and muscle soreness scale) will be used prior to the familiarization ride, on Days -4 and -2 of the experiment block, and prior to the performance test on Day 1 of the experiment.

Outcomes
This project will quantify the effect attributed to immediate post-exercise protein-carbohydrate supplementation on subsequent exercise performance, from that positive nitrogen balance provided by high daily protein intake.

Study 3: Gene Expression and Activity of Signalling proteins during Recovery from Prolonged High-Intensity Cycling: Response to Three Different Recovery Supplements

Statement of the Problem
It has already been established that certain recovery formulations aid recovery from exercise, for instance protein and specific amino acids have been shown to increase protein synthesis following prolonged aerobic exercise in rat studies (Anthony, et al. 1999), and following resistance exercise in human studies (Koopman, et al. 2005; Rasmussen, et al. 2000). In addition to the supply of amino acid substrates for protein synthesis, several other components of whey protein are thought to have roles in recovery from the stress of intense exercise, such as antioxidant activity and benefits to immune function (Ha & Zemel 2003; Walzem, et al. 2002), and specific amino acids have been shown to increase protein synthesis, in part due to stimulation of mRNA translation via mTOR signalling pathway (Anthony, et al. 2001).

Data from rat and other studies indicate that leucine is a key nutrient signal regulating protein synthesis by activation of the mammalian target of rapamycin (mTOR) pathway (Anthony, et al. 2001). In fact, 0.135 g·kg-1·h-1 free leucine effectively stimulates skeletal muscle protein synthesis in rats following fasting conditions (Crozier, et al. 2005; Kimball & Jefferson 2006b), 0.02-0.12 g·kg-1·h-1 free leucine added to protein-carbohydrate supplements stimulates skeletal muscle protein synthesis (Koopman, et al. 2005; Rasmussen, et al. 2000) in humans following resistance exercise, and 0.02 g·kg-1·h-1 free leucine content of a branched chain amino acid supplement stimulates phosphorylation of the signalling protein P70S6K following resistance exercise (Karlsson, et al. 2004). In addition to promoting global translation of mRNA, leucine mediates selective initiation of mRNA translation (Jefferson & Kimball 2003).

The optimal formulation to maximise the recovery processes from prolonged aerobic exercise is unknown. It is possible that leucine may act synergistically with the other constituents of whey protein, and with carbohydrate on the rapid recovery of fuel stores and cellular homeostasis, and stimulate the response of muscle recovery and adaptation mechanisms.

Aims

The optimal formulation to maximise the recovery processes from prolonged aerobic exercise is unknown, therefore the aim of this study is to determine the effect of increasing the leucine content of a carbohydrate-protein recovery formulation on the expression of candidate genes, activity of signalling proteins involved in recovery processes, and the response of muscle recovery and adaptation markers following prolonged high intensity exercise.

Hypotheses and Postulates

The comparison of 1 of 3 recovery formulations will allow us to investigate the effects of adding leucine and whey protein to carbohydrate on the expression of genes and the response of signalling proteins involved in recovery processes from prolonged high intensity exercise. We will determine whether increasing the leucine content acts synergistically with bioactive peptides and other ingredients of whey protein when added to carbohydrate recovery formulations, compared to carbohydrate-leucine and protein-carbohydrate formulations on nutrient-stimulated recovery from prolonged high-intensity exercise in males.

Duration

Data collection will be done over 12 months in 2008.

Study Design

Eight to 10 trained male endurance cyclists (road, mountain, triathletes) will receive, on 3 separate occasions, 1 of 3 recovery formulations at times 0, 30, 60 and 120 minutes following prolonged intermittent high-intensity endurance cycling. A double-blind triple-crossover design will be used, with at least a 1 week washout period between each experimental block.

Prior to the experiment blocks participants will be screened for health, training experience, and maximal oxygen capacity, and will perform an ergometer cycle familiarization trial. Leading up to each of the 3 experimental blocks, participants will follow controlled diet and training regimes. During each block, participants will arrive in the laboratory during the afternoon, and will perform a prolonged intermittent high-intensity ergometer cycling protocol. During 1 h recovery in the lab participants will receive 1 of 3 recovery formulations consisting provided immediately and at 30 minute intervals post-exercise. A muscle biopsy will be taken at 3 h post-exercise. Participants will return to the lab the following morning, fasted and a further muscle biopsy will be taken. Following a 1 week washout the experiment block will be repeated with the alternative treatments.

Methods

Supplement Formulation:

1. Control: The control formulation will contain 1.6 g·kg-1·h-1 carbohydrate (maltodextrin and fructose), 0.4 g·kg-1·h-1 protein (whey isolate, and 0.2 g·kg-1·h-1 fat content.
2. Intervention 1: The protein-carbohydrate formulation will contain 0.28 g·kg-1·h-1 protein (whey isolate), 1.2 g·kg-1·h-1 carbohydrate (maltodextrin and fructose), 0.2 g·kg-1·h-1 fat, and 0.12 g·kg-1·h-1 free leucine content (leucine content will be about 0.14 g·kg-1·h-1 in total).
3. Intervention 2: The protein-leucine formulation will contain 1.2 g·kg-1·h-1 carbohydrate (maltodextrin and fructose), 0.4 g·kg-1·h-1 free leucine content, and 0.2 g·kg-1·h-1 fat content.

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 (5 mm diameter Bergström-Stille muscle biopsy cannula) with manual suction.

The expression of candidate genes (will be assessed via mRNA quantification by RT-PCR (real time reverse transcriptase polymerase chain reaction). Key signalling proteins and translation products (e.g. mitochondrial enzymes, electrolyte transporters, and regulatory kinases) will be determined by Immunoblot (Western blot).

Outcomes

This project will be the first to investigate the effect of 3 nutrient recovery strategies on the expression of candidate genes involved in the recovery responses to intense endurance exercise.

Significance

The evaluation of protein-carbohydrate, protein-carbohydrate-leucine, and carbohydrate-leucine recovery formulations will allow further definition of the formulations to maximally stimulate the expression of candidate genes, activity of signalling proteins involved in recovery processes, and the response of muscle recovery and adaptation markers following prolonged high intensity exercise. Enhanced nutrient-stimulated recovery is of importance not only to the athletes themselves, but will also be of relevance to nutrition experts and supplement companies.

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 is set to 5%, and Type 2 error set at 25%.

Data derived from each study will be analysed with paired sample t test or Wilcoxon signed rank test if non-parametric using SPSS software (SPSS 14.0 for Windows, SPSS Inc. Chicago) and a published spreadsheet (Hopkins 2006). Analysis of covariates, correlations, and regression modelling will be conducted once initial analysis of data is suggestive of a relationship or trend.

I will report the size of the effect and associated 90 or 95% confidence limits rather than null-hypothesis testing. The *p*-values and statistical significance contain no information about the precision of the result, whereas the confidence limits give an indication of precision of estimated effect and also contain information about the *p*-values within the confidence interval. 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. 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

2006	
June	1 June begin provisional registration. Read around topics relevant to Study 1, Thesis Proposal and Thesis Literature Review.
July	End of July travel to McMaster University, Canada. Begin Study 1 data collection.
August	Canada. Study 1 data collection. Observe and learn something about the techniques to be used for the analysis of your samples. Continue reading if there is time.
September	Canada. Study 1 data collection. Observe and learn more on the techniques to be used for the analysis of your samples. Continue reading if time.
October	Canada. Finish up enough Study 1 data collection to begin some analysis. Continue reading and begin any writing of Thesis Chapter/Paper if time. First 6 month Report is due 14th October.
November	Return to New Zealand. Prepare and give presentation to Sport and Medicine Conference on the 18th. Begin to write up Thesis Methods on Study 1 Canada. Begin meetings with supervisors on Planning Study 2. Work on Pedometer meta-analysis journal article
December	Continue to write Methods. Continue meetings with supervisors and Planning study 2.
2007	
January	Continue to write Thesis Methods on Study 1. Begin to write Thesis Proposal.
February	Continue planning for Study 2. Work on Pedometer Meta-analysis Journal Article. Continue on Thesis Proposal.
March	Begin writing PhD Thesis Literature Review. Continue planning for Study 2 and prepare Study 2 Ethics Proposal.

April	Continue Planning and Ethics Proposal for Study 2. Present Thesis Proposal to IFNHH students and staff. DRC 3 due on the 14th.
May	Apply for Full Registration, complete DRC 13, by 04/05/07 (confirmation due date 30/04/05). Work on PhD Thesis Literature Review. Background research on study 3, in order to write some of the MURF application. Begin writing methods & results for scientific paper on data generated from study 1.
June	Work on PhD Thesis Literature Review. Continue writing scientific paper on Study 1.
July	Commence Study 2. Work on PhD Thesis Literature Review. Begin tutoring 194.101 labs. Enrol in 151.709 Biometrics paper.
August	Study 2 data collection. Work on PhD Thesis Literature Review.
September	Study 2 data collection.
October	Study 2 data collection. Discussions on Study 3. DRC 3 due around this time.
November	Study 2 data collection. Discussions on Study 3.
December	Work toward completion of Study 2. Planning for Study 3 and Ethics for Study 3.
2008	
January	When Study 2 is complete begin data analysis and write up. Continue planning and ethics for Study 3.
February	Continue data analysis and write up of study 2
March	Continue data analysis and write up of study 2
April	DRC 4 due around this time
May	Commence Study 3
June	Study 3 data collection
July	Study 3 data collection
August	Study 3 data collection
September	Study 3 data collection

October	Work toward completion of Study 3. DRC 3 due around this time.
November	When Study 3 is complete begin data analysis and write up.
December	Continue data analysis and write up of study 3
2009	
January	Write up papers for studies and Submit Papers to Journals. Convert papers to Thesis Chapters and Appendices. Paper drafts to supervisors as they are written.
February	
March	Work on Thesis General Discussion.
April	Tidy up chapters/appendices. Submit final draft to supervisors.
May	
June	Submit thesis for examination.
July	Write up papers for studies and Submit Papers to Journals.
August	Work on any changes to thesis.
September	Exam preparation
October	Oral examination.
November	
December	Graduation.

RESOURCE REQUIREMENTS

Indicative Budget for Proposed Research: Study 2

Item	Unit Cost $	Total Cost $
Cycling Protocols		
VO_{2max} Testing	$75	-
Performance Testing Trials	$150	-
Transporting Kingcycles to Auckland		$195 IFNHH
Supplements		

Whey Protein	$350	$350 acquired
Control Ingredients	$100	$100 acquired
Blood and Urine Biochemistry		
Consumables (cannulae, vaccutainers, syringes, gloves, saline, swabs, IV taps, bench wrap, site preparation, tape, etc)	$33.96	$407.52 acquired
Blood and Urine transport for analysis		????
Urine urea	$49.5	$594
Urine creatinine	$49.5	$594
Blood Creatine Kinase	$31.5	$378
Miscellaneous		
Subject Controlled Diet	$20/d	$2400
Participant Reimbursement	$50	$600
Journal Page Charges	$820	$820 IFNHH
TOTAL	$10,092.52	
IFNHH	$3715.00	
Total Funding Sought	$5927.52	

Indicative Budget for Proposed Research: Study 3

Item	Unit Cost $	Total Cost $
Cycling Protocols		
VO_{2max} Testing	$75	$750 IFNHH
Study Trials	$150	$1,500 IFNHH
Supplements		
Whey Protein	$350	$350 IFNHH

L-Leucine	1kg at $751.14	$751.14
Control Ingredients	$100	$100 IFNHH
Muscle, and Blood Biochemistry		
Doctor Fee for biopsy procedure		$2000
Purchase of Bergstrom Biopsy Needles	$2000	$2000
Biopsy Consumables (liquid nitrogen, autoclave, etc)	variable	?
General Consumables (cannulae, vaccutainers, syringes, gloves, saline, swabs, IV taps, bench wrap, site preparation, tape, etc)	$33.96	339.60
RT-PCR mRNA Analysis (Labour, transport, storage, consumables)	$150-200 per gene, $150 per probe	?
Miscellaneous		
Subject Controlled Diet	$20/d	$800
Participant Reimbursement	$200	$2000
Journal Page Charges	$820	$820 IFNHH
TOTAL	$20,000-30,000	
IFNHH	$3520.00	
Total Funding Sought		

RESEARCH ETHICS

Ethical approval will be obtained from the applicable research ethics bodies for the location of proposed research (Massey University Palmerston North Ethics Committee; and Hamilton Health Sciences/McMaster University, Hamilton, Ontario). 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
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
2.4	American Journal of Sports Medicine	Basic science relevant to clinical sports medicine, including the subjects of anatomy, biomechanics, and cell biology; Sport specific subjects such as	

		soccer, baseball and football issues	
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	
2.3	Clinical Nutrition	Scientific information on nutritional and metabolic care and the relationship between nutrition and disease	
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
1.1	Journal of Science and Medicine in Sport	Exercise physiology, sports nutrition and aspects of sports science with applications to sports and exercise	$US208-$312 /colour

1.7	Journal of Sports Sciences	Aspects of sports sciences including biochemistry, human responses to exercise, etc	
1.1	Journal of Strength and Conditioning Research	Original research including exercise physiology, nutrition, and underlying biological basis for performance.	
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

Presented the paper 'Pedometer utility and efficacy as a tool for initiation and maintenance of physical activity behaviours' at the NZ Sports Medicine and Science Conference in November 2006.

Conferences that will be considered include those organised by Sports Medicine New Zealand, the Nutrition Society of New Zealand, New Zealand Dietetic Association, and possibly the Physiological Society of New Zealand. Submitted abstracts for presentation and conference attendance will depend on conferences and topics of relevance to the area of research, occurring within the relevant timeframe in terms of generating results and forming conclusions to the scientific questions we are asking, and those occurring within New Zealand (or Australia) and therefore more economically viable to attend.

APPENDIX B ETHICS APPLICATIONS

Massey University

OFFICE OF THE ASSISTANT
TO THE VICE-CHANCELLOR
(Ethics & Equity)
Private Bag 11 222
Palmerston North
New Zealand
T 64 6 350 5573/350 5575
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humanethics@massey.ac.nz
animalethics@massey.ac.nz
gtc@massey.ac.nz
www.massey.ac.nz

27 August 2007

Ms Jasmine Thomson
IFNHH
ALBANY

Dear Jasmine

Re: HEC: Southern A Application – 07/25
Effect of post-exercise protein-carbohydrate supplementation on subsequent performance during maintenance of nitrogen balance

Thank you for your letter dated 27 August 2007.

On behalf of the Massey University Human Ethics Committee: Southern A, I am pleased to advise you that the ethics of your application are now approved. Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely

J. O'Neill

Professor John O'Neill, Chair
Massey University Human Ethics Committee: Southern A

cc Dr David Rowlands
IFNHH
WELLINGTON

Dr Ajmol Ali
IFNHH
ALBANY

Prof Richard, Archer, HoI
IFNHH
PN452

McMaster University Health Sciences

Hamilton Health Sciences

RESEARCH ETHICS BOARD

AMENDMENT REQUEST

REB Project #: 06-245

Locally Responsible Investigator: Dr. Mark Tarnopolsky

Title of Study: Gene Expression, Protein Synthesis, and Anabolic Signalling during Recovery from Prolonged High-Intensity Cycling: Effect of a Protein-Enriched Recovery Formulation.

Document(s) Amended with version # and date:

- Other (Specify:) - Revised Poster

Research Ethics Board Review

(this box to be completed by REB Chair only)

[X] **Amendment approved as submitted**

[ ] **Amendment approved conditional on changes noted in "Conditions" section below**

[ ] **New enrolment suspended**

[ ] **Study suspended pending further review**

Level of Review:

[X] Expedite

[ ] Full Research Ethics Board

[X] Research Ethics Board Executive Committee

Conditions:

The Hamilton Health Sciences/McMaster Health Sciences Research Ethics Board operates in compliance with the ICH Good Clinical Practice Guidelines and the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans.

F. Jack Hollland, MD, FRCP, FRCP(C), Chair
Hamilton Health Sciences/McMaster Health Sciences Research Ethics Board

25 July, 2006
Date

All Correspondence should be addressed to the REB Chair and forwarded to:
REB Coordinator, Hamilton Health Sciences
1057 Main Street West, Suite 1, Hamilton ON L8S IB7
Telephone: 905-521-2100, ext. 42013
Fax: 905-577-8379

APPENDIX C PARTICIPANT RECRUITMENT


Massey University
Institute of
Food Nutrition & Human Health
Recovery & Performance Study
Are you an experienced cyclist, mountain biker,
triathlete, or multisport athlete?
Are you male, between 19-50 years of age?
How fit are you?
We test your VO_2max
We analyse your training diet
We provide an intense training programme
We feed you
And then we test your performance!
Call Jasmine on 414 0800 ext. 41177 (wk) or 021 210 6269 (mob) or email cyclestudy@gmail.com
Cycle Performance Study
Ph. Jasmine 414 0800 ext. 41177

McMaster University

Inspiring Innovation and Discovery

Hamilton Health Sciences

MALE VOLUNTEERS NEEDED

- **Cyclists, triathletes, mountain bikers**
- 20 to 50 years of age
- Non-smokers

FOR RESEARCH ON...

- The influences of protein and carbohydrate supplementation on exercise training and muscle recovery
- Under the supervision of Dr. Mark Tarnopolsky

TESTS INCLUDE...

- Blood
- Urine
- Muscle Biopsies

You will be financially compensated for your time. For more information phone **905-521-2100 ext 76371**, or email **alissaaboud@hotmail.com** or **emepearce@hotmail.com**

Protein/CHO Recovery study	521-2100 ext.76371	alissaaboud@hotmail.com
Protein/CHO Recovery study	521-2100 ext.76371	emepearce@hotmail.com
Protein/CHO Recovery study	521-2100 ext.76371	alissaaboud@hotmail.com
Protein/CHO Recovery study	521-2100 ext.76371	emepearce@hotmail.com
Protein/CHO Recovery study	521-2100 ext.76371	alissaaboud@hotmail.com
Protein/CHO Recovery study	521-2100 ext.76371	emepearce@hotmail.com
Protein/CHO Recovery study	521-2100 ext.76371	alissaaboud@hotmail.com
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APPENDIX D INFORMATION SHEETS

Institute of Food,
Nutrition and Human Health
Massey University
Private Bag 102-904
North Shore Mail Centre
Auckland

EFFECT OF POST-EXERCISE PROTEIN-CARBOHYDRATE SUPPLEMENTATION ON SUBSEQUENT PERFORMANCE DURING MAINTENANCE OF NITROGEN BALANCE

LUCOPRO CYCLE STUDY INFORMATION SHEET

The Institute of Food, Nutrition and Human Health
The Institute of Food, Nutrition and Human Health includes a large team of lecturers and scientists who are interested in human nutrition, sport and exercise science, physiology, and health. The lead researcher, Mrs. Jasmine Thomson, is conducting this project as part of the requirements for her Doctorate in Nutritional Science. Her supervisors on this project are Dr. David Rowlands, Dr Ajmol Ali, and Associate Professor Welma Stonehouse. Ms Helen Ryan and Dr Andrew Foskett will also be involved.

Why Are We Doing This Study?
Recovery from hard exercise is an important part of a cyclist's training regime, both to maintain training volume and to improve performance during the next bout. Without adequate recovery the body may not adapt to training and performance during the next training session or competition may deteriorate. Recovery from prolonged high intensity exercise requires refuelling muscle energy stores, repair of damaged tissue, and adaptation to training.

Immediately post-exercise is the best time to eat nutrients for recovery due to the increased blood flow and muscle cell uptake of nutrients for fuel stores. A new area of research is the addition of protein to carbohydrate recovery meals and sports drinks, and there is some indication of benefit to later performance. However, most studies have focused on the effects of post-exercise protein and carbohydrate on rate of glycogen synthesis (muscle carbohydrate stores) in relation to later performance. Recovery periods were relatively short, and cycling performance tested 2-24 h after initial hard exercise, which may not be enough time for proper recovery and regeneration to occur. A recent study by Massey University researchers showed a benefit with post-exercise protein-carbohydrate meal on performance 60 h later. The researchers also found that the group fed a high-carbohydrate recovery meal were in negative protein balance (a protein breakdown state when the loss of protein as waste is greater than the dietary intake) compared with the group fed the protein-carbohydrate meal who were in positive protein balance (a rebuilding state when the dietary intake of protein is greater than loss and there is an increase in the total body pool of protein).

It is still unknown how post-exercise combined protein and carbohydrate intake affects other aspects of recovery such as muscle soreness, muscle damage, and adaptation to training, which likely take longer (24h or more) to recover performance ability. Therefore, the aim of this study is to determine the effect of a post-exercise protein-carbohydrate supplement drink on recovery of cycling performance when subjects consume enough daily protein to maintain neutral protein balance (a balance between dietary intake of protein and its use by the body and excretion as waste) during 4 days of training. Results may provide evidence to support the use of protein-carbohydrate recovery supplements, by endurance athletes in optimizing recovery and adaptation to hard training or cycle tour, without the need to eat excessive amounts of protein, which is not only expensive but not very healthy.

Research aims:

1. Determine the effect of post-exercise protein-carbohydrate drink on later performance when included within the recommended daily dietary protein intakes for endurance athletes.
2. Assess the effect of the post-exercise protein-carbohydrate drink using diet to maintain neutral protein balance during training.
3. Specifically, determine if protein-carbohydrate recovery drinks consumed immediately post-exercise have a benefit over the same amount consumed much later in the day.
4. Quantify the effect of post-exercise protein-carbohydrate drink on reducing muscle soreness and damage, and improving psychological and physiological recovery.

Participant Recruitment

To become a subject you should be:

Male, 19-50 years of age.

Doing regular cycling training for your sport, whether it is road cycling, mountain biking, triathlon, multisport, etc. Regular cycling training is defined as 8 hours or more per week for at least the last 6 months. You should also have experienced high-intensity training or competed in races in the past year.

Aerobically fit, your VO_{2max} (which we will measure) should be at least 55 mL/kg/min.

Healthy, you should have no history of heart disease, diabetes, kidney trouble, or uncontrolled asthma.

Responsible, committed to the project, and willing to give your best efforts in the performance tests.

For most people physical activity does not pose a risk, however there may be a small number of people for whom physical activity might not be appropriate. Therefore, you should be healthy, have high aerobic fitness, specifically in cycling to ensure you are physically able to perform the training program. Also, if you have previously competed you will have a good understanding of how hard you can push yourself during the performance test.

What is involved?

The ergometer-cycling training regime is designed to replicate the first few days of a cycle tour or back-to-back hard training. The harder days are interspersed with easier days and a day off to recover before the performance test. Visits will be arranged to occur in the evenings after work and weekends for your convenience. Each visit involves cycling, so please make sure you are ready to exercise and bring your normal cycling clothes, shoes, and pedals.

Visit 1: Preliminary. An explanation of study protocols and tour of lab and equipment is given. Informed consent is required if you would like to participate. Your suitability for this study will be evaluated using health and training histories, and analysis of your lactate threshold, maximal aerobic fitness (VO_{2max}) and peak power. The lactate threshold test involves riding the cycle ergometer for approximately 30 minutes, the workload increases by 20W every 5 minutes until you reach blood lactate of 4.5mmol/L. The VO_{2max} test involves riding the cycle ergometer for 10-12 minutes, with increasing workload of 25 W every minute, until you cannot continue, while expired air is collected for analysis. Please ensure you have not done long hard training or competition 2 days before, or eaten a large meal just before this visit. Time requirement is about 1.5h.

Visit 2: Metabolic Rate. Arrive in the morning fasted, i.e. no food, coffee or alcohol for 12 h beforehand (not even breakfast). We ask that you drive or are driven to the lab to keep your pulse rate low, and then lie quietly on a bed for 30 minutes prior to testing. Your resting and exercise metabolic rate are measured (how fast you convert stored fuels into energy), this involves lying prone on a bed while your resting metabolic rate is measured, and then riding the cycle ergometer for 3 x 10 minutes stages at intensities of 40-60% maximum power for exercise metabolism measures. Time requirement is about 1.5 h.

Visit 3: Familiarization. You are asked to complete questionnaires on mood and stress, and location of sore muscles. A pressure algometer is used to determine extent of muscle soreness, and then you complete your first cycle-ergometer performance test. Time requirement is about 3 h all inclusive.

After this visit we ask you to record everything you eat and all training (cycling plus other forms of exercise) for 4-5 days leading up to the first experiment block. A training log and diet diary will be provided to help you remember exactly what you ate and the exercise you did, then you simply repeat your diet and training leading up to the second experiment block. This is very important for our research outcomes, to ensure you start each experiment block with similar muscle fuel (glycogen and fat) stores.

Visits 4-7: Controlled Training and Recovery Supplementation. Arrive at the lab at an agreed time around 4:00-5:30pm. You are asked to complete questionnaires on mood and stress, and location of sore muscles. Blood samples are taken, and then you are weighed and sweat collection patches placed on your chest, and stomach, before performing the cycle-ergometer training ride. Following the ride you are given your recovery drinks, and get to relax and recover in the lab for 1 h during which time you may shower. Following your training ride, we ask you to collect all urination in a sample container provided, up until we see you the following day. At the end of the visit you will receive your pre-packaged individualized diet, it is very important that you eat only the food we provide for you, and any food you don't eat is returned so the remaining amounts can be measured. Each training ride

involves 2-2.5 h ergometer cycling interval sessions based on your peak power established during visit 1. We have a stereo, TV and video/DVD player for entertainment. Each Visit takes 4 h, remember 2-2.5 h of this is indoor cycling training, a bonus on cold, wet, and windy nights.
Visit 8: This is the main testing day; therefore it is important to have a rest from training and do only light everyday activities the day before this visit. Arrive in the morning fasted, i.e. no food, coffee or alcohol for 12 h beforehand (not even breakfast). Breakfast is provided, and you are again asked to complete questionnaires on mood and stress, and location of sore muscles. Blood samples are taken, followed by your performance test. The cycle-ergometer performance test involves a warm-up followed by the performance test itself (20 minutes of 50%, then blocks of 4 x sprint intervals, repeated 3 times) to stimulate race conditions. Visit 8 will last 3.5 h.
After at least 1 week Visits 4-8 will be repeated with the alternative recovery drink.

Are any of the Procedures Harmful or Painful?
Blood Sampling
Trained phlebotomists will take blood samples, and 2 researchers with current first aid qualifications will be present whenever blood is taken. You will lie down on a bed while blood is taken to avoid risk of injury if you faint. Bruising is rare, and the chance of bruising is further reduced by using a small bore needle, and applying slight pressure on the incision hole with cotton gauze for a couple minutes until clotted. Blood is taken 8 times over the entire study duration, the amount of blood taken each time will be about 5-10 ml, which should not have any harmful effects. The blood will be stored in a -70ºC freezer for up to 24 months during which time biochemical analysis will be conducted. Some samples may be sent to the Massey University laboratory in Palmerston North, or to commercial medical laboratories in Auckland.

Exercise
There is often some physical and psychological discomfort associated with intense exercise. Even among healthy athletes who exercise intensely and regularly, there is a small risk of sudden death due to heart failure. This is rare, but can occur in people who may have an undiagnosed condition. If you have any reason to suspect that you may have a heart problem, please see your physician and get an ECG before you agree to participate. If symptoms such as shortness of breath, pain in your chest, arms, upper back, and fatigue occur, then you should immediately stop exercise, you will be seated or may lie down, and further assessment made. If symptoms continue, medical attention will be sought via 111, first aid (CPR) and defibrillation administered if necessary by staff trained in this technique. You will be required to complete a Health Screening Questionnaire prior to doing any physical activity for this research project. However, if you have any additional medical concerns associated with this project, please contact your GP, or discuss with the researcher.

What if I Suffer a Personal Injury?
If physical injury results from your participation in this study, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and

entitlements are not automatic and your claim will be assessed by ACC in accordance with the Injury Prevention, Rehabilitation and Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlements, and must help you access those entitlements. 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. If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim.

Benefits to Participants

You will learn your VO_{2max} and may have a follow up test provided free of charge if desired, these tests are normally worth $150 each. Your current training diet is assessed, you get 2 weeks of supervised high-quality training in the lab on scientific cycle ergometers, food is provided while on the study, and you get the chance to learn more about how post-exercise nutrition strategies may benefit your own cycling performance. We will send you a report summarizing the main findings of the project and your individual results once the data is analyzed. You will also receive $50 in MTA vouchers for participating in the study.

Participant's Rights

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:
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 are Interested in Taking Part
Contact: Mrs. Jasmine Thomson
Institute of Food, Nutrition and Human Health
Massey University
Private Bag 102 904
North Shore Mail Centre
Auckland
Phone: 413 0800 ext. 41177 (wk)
Phone: 021 210 6269 (mob)
Email: cyclestudy@gmail.com

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 07/25. If you have any concerns about the conduct of this research, please contact Professor John O'Neill, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 350 5799 x 8771, email humanethicsoutha@massey.ac.nz

Hamilton Health Sciences

McMaster University

Inspiring Innovation and Discovery

Letter of Information/Consent Form

Gene Expression, Protein Synthesis, and Anabolic Signalling during Recovery from Prolonged High-Intensity Cycling: Effect of a Protein-Enriched Recovery Formulation.

Dr. M.A. Tarnopolsky, MD, PhD, FRCP (C)1
David S. Rowlands, PhD
[1]Dept. of Pediatrics, McMaster University

PURPOSE

You are invited to participate in a research project aimed at investigating muscle recovery. Endurance exercise is very stressful to human skeletal muscle, but it is this damage that helps the cells adapt and prevents future damage. Muscle damage after exercise is common if the exercise is intense or if someone over-trains. Often, people take anti-inflammatory agents; however, these can have serious side effects (GI upset and bleeding) and may ultimately impair the muscle recovery process. Nutritional strategies such as taking a carbohydrate and protein drink immediately after exercise can speed recovery and is not associated with side effects. Although effective, the biochemical and molecular processes that are involved in this recovery technique are not known. Rapid recovery from competition and heavy training is important for future performance and adaptive responses to training in many sports. Based on our research and study of the literature, we have selected genes, proteins, substrates, and signalling factors most likely to be involved in the repair and recovery processes leading to stronger, more prepared muscle. We aim to measure the activity and amount of these factors to determine those most important for muscle recovery following protein and carbohydrate ingestion after exercise. The results will provide new information to the scientific and sporting communities on the cellular mechanisms that determine recovery. It may give us evidence to support the use of protein-enriched recovery food by endurance athletes, and stimulate coach, athlete, and provider interest in optimising nutrition strategies to enhance adaptation to training and performance.

The research is sponsored jointly by the Natural Sciences and Engineering Research Council of Canada (Dr Tarnopolsky), and grants from the Massey University Research Fund and Sport and Recreation New Zealand (Dr Rowlands). Additional funds for some aspects of the analysis have been sought from and Nestle Research Centre, Switzerland.

PROCEDURE

As a participant you will be required to come into the lab a total of 9 times, with three 30 min only. All guidelines set forth by the McMaster University Health Sciences Human Research Ethics Board will be strictly adhered to.

Visit #1: During this visit you will have the study explained to you in detail, and have the opportunity to become accustomed to the experimental apparatus. At this point you will be required to provide informed consent if you would like to participate in the study. This initial visit will also be used to determine your pre-testing maximal oxygen capacity (VO_{2max}). This procedure requires you to ride a bicycle for 10-12 minutes with the workload increasing every 2.5 minutes until you cannot continue or start to feel any pain. You should not have had a large meal close to riding. Additionally, after a rest and time to recover and stretch, you will do a familiarization ride. This procedure requires you to do a ride of similar protocol to the experiment day intervals but of shorter period, so that you are more prepared for what to expect on experiment day. Based on initial VO_{2max} all subjects will be matched and randomly assigned to one of two different supplement groups in a single-blind crossover study design. Each time you come to the lab bring in your cycling shorts, top, shoes and pedals, and your bike set-up measurements or bike so that we may adjust the lab bike to fit.

After this visit you will need to record your diet for four days (1 weekend day and 3 weekdays), so that we can design a controlled diet, and provide you with controlled food intake leading up to important testing visits, based on your normal energy intake.

Visit #2: On an afternoon to be arranged, you will come into the lab ~17:00 h to ride for 90 min with blocks at 50 and 70% of your maximum. This is a standardizing ride two days before the baseline testing day. After this ride we will give you your controlled diet to eat for that evening and the entire following day. You must eat only the food items that we provide for you. The day before baseline testing, you have the day off from training and should do only light everyday activities e.g. biking or walking to work. It is very important that you follow these procedures so that the baseline testing is controlled for.

Visit #3: Return to the lab in the morning ~7:30-8:30am fasted (no breakfast) for a baseline muscle biopsy. A small amount (0.10 - 0.15 g) of wet muscle sample will be taken from the outside of the thigh (vastus lateralis). This visit will take about 30 min. You will be asked to record your diet and training program for 5 days leading up to experiment day, which will be repeated for the second block of the study, to insure your muscles enter the experiment in consistent condition.

Visit #4: You will come into the lab ~17:00 h to repeat the same ride as on Visit #2. After this ride we will give you your food to eat that evening and for the entire following day. On the day before Experiment Day you must eat only the food items that we provide for you. You have the day off from training and should do only light everyday activities e.g. biking or walking to work. It is very important that you are well rested before the first day of the experiment and follow the procedures carefully.

Visit #5: You will arrive for the first experiment day in the early morning, fasted (no breakfast). Wednesday is the best day to schedule this due to often conflicting patient scheduling, however if this is impossible for you, feel free to discuss this with us and something else can be arranged. Once in the laboratory, a catheter for blood sampling is placed in a forearm vein. We will then give you a snack bar and a drink and you will then ride an intermittent-intensity protocol for 1h 45min to simulate an interval session or race, this will reduce muscle glycogen and cause exercise stress in the muscles representative of a hard training session. You will receive a sports drink during exercise. Following the cycle, you may have a shower, and will recover in lab watching videos or reading, and commence with the experimental treatment of either the protein-enriched carbohydrate formulation or placebo. Three hours after exercise, another muscle biopsy will be taken from the outside of the thigh. Blood will also be taken prior to, during, and in the recovery period post cycling. We will provide a control diet which you must eat for the remainder of the day and for the day following

Visit #6: 48 hours post cycling, you return to the laboratory in the morning fasted (no breakfast) for the final phase of testing. One muscle biopsy will be taken from the outside of the thigh, and blood will also be taken at this time point.

Visits #4, 5, and 6 will be repeated on the alternative supplement/placebo following a washout period of 1-2 weeks and the five days of standardized diet and training that you recorded leading up to the first block of the experiment.

During both phases of the experiment you will have to refrain from consuming alcohol. Additionally, you must refrain from consuming caffeine on the days of testing.

POTENTIAL RISKS

Pharmacological intervention. Protein-enriched carbohydrate formulation will be used for this study that will contain a blend of whey and casein as the protein source (this is the same as in milk) and carbohydrate from maltodextrin. There is no risk associated with this intervention.

Blood sampling. Either Dr. Tarnopolsky or Dr. Rowlands will do all blood sampling. A single needle stick will be used, and a plastic catheter will be inserted. Blood will be drawn through a rubber attachment on the catheter. The potential for mild discomfort exists, and transient bruising at the sampling site may occur. The total amount of blood taken at each testing point will be approximately 30-60 ml (2-4 tablespoons), which should not have any deleterious effects.

Muscle biopsies. All biopsies will be performed by Dr. Tarnopolsky, who has performed over 10000 in both patients and healthy subjects ranging from 1 week to >90 years old with the following complications:

4/10000 with a local skin infection

6/10000 with a fibrous lump at the site of the biopsy (connective tissue), all of which disappeared within 1 week.

4/10000 with a small patch of numbness just past the incision (approximately the size of a quarter), due to the cutting of a small sensory nerve patch. In all cases complete recovery occurred within 3 months.

Most subjects experience transient dull muscle aching for 24-48 hours following biopsy, which is markedly reduced with ice and Tylenol.

It is possible that the biopsy could damage a small motor branch of the muscle on the outside of the thigh (vastus lateralis) and partially weaken the lower aspect of the muscle. This should not affect the function for this muscle (knee extension), as it is one of four muscles involved. Furthermore, Dr. Tarnopolsky has never observed this in any patients or research subject's biopsies.

Cycling. There is a risk of approximately 1/100 000 of major cardiac events during strenuous exercise in non-cardiac patients in all age groups combined. This risk is markedly reduced in our age group of interest. All subjects will have continuous heart rate monitoring during exercise. Our lab has not had a significant problem in over a decade of conducting exercise studies.

COMPENSATION

You will receive $300 upon completing the study to compensate you for your time. At any time during the course of the study you will be free to withdraw your participation without any negative repercussions. In the event that you withdraw from the study for personal reasons, the compensation will be adjusted to reflect the amount of time you spent in the study. If you choose to withdraw due to a complication arising from the study, you will be provided with full compensation.

CONFIDETIALITY AND RIGHTS

All data will be stored in a locked filing cabinet under the supervision of Dr. Tarnopolsky and David Rowlands; information will not be released to any other party without the written consent of the subject. If the results of this study are published, the subject's identity will remain confidential. Should you have any questions or concerns regarding your rights as a research participant, you may contact the Hamilton Health Sciences Patient Relations Specialist at 905-521-2100, Ext. 75240

FUTURE RESEARCH

If at the completion of data analysis there is any remaining sample, we may wish to store the sample for use in a future study. Researchers wishing to use the remaining sample will be required to apply for ethical approval before analyses of the remaining sample commences. All records identifying you will remain confidential, and information about you will not be released. Once again, if the results of the study are published, your identity will remain confidential.

FINAL CONSENT

I have had the study explained to me, and I understand the risks and benefits involved. I may withdraw from the study at any time with no prejudice. I will also receive a signed copy of this form to keep at home in case I have any questions during the study.

Dr. Mark A Tarnopolsky: (905) 521-2100 x76593 (Work)
(905) 521-2100 x75226 (Pager 2888)

Dr. David Rowlands (905) 521-2100 x76931 (Daytime)
(905) 522-3781 (Evening)
d.s.rowlands@massey.ac.nz

_____	_____	_____
Participant	Signature	Date
_____	_____	_____
Person Obtaining Consent	Signature	Date
Dr. Mark A Tarnopolsky	_____	_____
Investigator	Signature	Date

APPENDIX E INFORMED CONSENT

Institute of Food, Nutrition and Human Health
Massey University
Private Bag 102-904
North Shore Mail Centre
Auckland

LUCOPRO CYCLE STUDY

PARTICIPANT CONSENT FORM

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

I have read the information sheet, and had details of the study explained to me.

My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I understand the risks and benefits involved. Should an emergency arise during testing, I understand that there is an emergency plan to follow, and if I feel I have been injured in the course of this study I am happy to contact Mrs. Jasmine Thomson (414 0800 ext 41177 or email cyclestudy@gmail.com) with my concerns.

I understand that I am free to withdraw from the study at any time with no prejudice.

I agree to my blood being stored after collection for later biochemical analysis.

I understand all of my personal data will be kept confidential.

I understand the requirements for participation, and hereby agree to participate in this study under the conditions set out in the information sheet.

Participant Signature _____ Date _____

Full Name *Printed* _____

Researcher _____ Researcher Signature _____

"This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 07/25. If you have any concerns about the conduct of this research, please contact Professor John O'Neill, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 350 5799 x 8771, email humanethicsoutha@massey.ac.nz."

APPENDIX F INITIAL SCREENING

Institute of Food, Nutrition and Human Health
Massey University
Private Bag 102-904
North Shore Mail Centre
Auckland

LUCOPRO CYCLE STUDY

HEALTH QUESTIONNAIRE

NAME _____ DATE _____

ADDRESS _____

_____

PHONE _____ (HM) _____ (MOB)

Occupation _____

Current GP _____ Phone _____

Address _____

Person to contact in case of _____ Phone _____

Please answer the following questions. For most people physical activity should not pose a hazard. This questionnaire has been designed to identify the small number of persons (18-50 years of age) for whom physical activity might be inappropriate and to provide the researchers with descriptive information about the participant. If you have any doubts or difficulty answering the questions, please ask the investigator for guidance. These questions help us to determine whether the proposed physical activity requirement of this study is appropriate for you. Your answers will be kept strictly confidential.

HEALTH HISTORY

Has your GP advised you not to perform vigorous exercise?	Yes ☐	No ☐
Have you been diagnosed with a heart condition?	Yes ☐	No ☐
Have you been diagnosed with high blood pressure?	Yes ☐	No ☐
Have you been diagnosed with a heart condition?	Yes ☐	No ☐
Have you been diagnosed with high cholesterol?	Yes ☐	No ☐
Have you been diagnosed with diabetes?	Yes ☐	No ☐
Do you feel chest pain while doing physical activity?	Yes ☐	No ☐
In the past month, have you had chest pain while not doing physical	Yes ☐	No ☐
Do you lose balance due to dizziness or loss of consciousness?	Yes ☐	No ☐
Do you have a back or joint pain that may be made worse with vigorous exercise?	Yes ☐	No ☐
Do you suffer from asthma?	Yes ☐	No ☐
If yes, do you control it with medication?	Yes ☐	No ☐
Do you suffer from a blood borne contagious disease?	Yes ☐	No ☐

Do you currently smoke?			Yes ☐	No ☐
If yes, please indicate average packs per day?	<0.5 ☐	0.5-1 ☐	1.5-2 ☐	>2 ☐
Are you currently taking medication?			Yes ☐	No ☐

PHYSICAL ACTIVITY & TRAINING HISTORY

Have you previously done a maximal exercise test?	Yes ☐	No ☐
Over the last 6 months, have you been performing vigorous exercise	Yes ☐	No ☐
How long have you purposely trained in a sport involving cycling?		years
Which		
Over the past 6 months, what was your average volume of aerobic		h/wk
Average volume of cycling training over this period?		h/wk

DIET RELATED

Over the last month, has your diet been stable?	Yes ☐	No ☐
Do you have any food allergies?	Yes ☐	No ☐
If yes, to which		
Do you have any food intolerances/dislikes?	Yes ☐	No ☐
If yes, which foods don't you		
Are you Vegetarian?	Yes ☐	No ☐
Do you eat milk, milk products, and eggs?	Yes ☐	No ☐

I have completed the questionnaire to the best of my knowledge, and any doubts or difficulty I had answering questions were addressed by the researcher to my satisfaction.

Participant Signature _____ DATE _____

APPENDIX G TIME SCHEDULE

Day -4 (Ride 1)

Pre-Visit

Weighed Food
Extra Food Items (apples, bananas)
Urine collection containers and 6M HCl
Sports Drinks
Supplement Drinks
Subway
Venepuncture & Cannulation trays
Plasma Tubes x 2 (labelled)
EDTA Tubes x 5 (labelled)
Microtubes (labelled)
Disposable pipettes
Ice
Bike Specifications
Program Bike
TV & DVD player
Organise Phlebotomist
Organise Research Assistant
Data Collection Sheets
Instruction Sheets
Cook pasta

During Visit

POMS (profile of mood state)
DALDA (daily Life demands of athletes)
Muscle soreness diagram and scale
Cereal Bar + 250mL water (15-min before ride)
Blood Venepuncture (2 sample into plain)
Urination into toilet
Body weight

During Cycle

Start training ride
Start fans
RPE and GRS scales during ride
Sports Drink during ride

Post Cycle

Urination in toilet
Body weight (body weight after toilet as urine is fluid loss [like sweat]).
Cannulation (5-samples into EDTA)
Blood 1 (t=0)
Supplement 1 (t=0)
Start collecting urine into collection Day -4 (t=0)
Blood 2 (t=30)
Supplement 2 (t=30)
Blood 3 (t=60)

Supplement 3 (t=60)
Blood 4 (t=90)
Supplement 4 (t=90)
Blood 4 (t=120)
Give food, instructions, send subject home
Spin blood samples

Day -3 (Ride 2)
Pre-Visit
Weighed Food
Extra Food Items
Urine collection containers and 6M HCl
Sports Drinks
Supplement Drinks
Subway
Venepuncture Tray
Sweat Tray
Plasma Tubes x 2 (labelled)
50mL x 2 Sweat Centrifuge tubes (labelled)
15mL x 2 Sweat Centrifuge tubes (labelled)
15mL x 5 Urine Total Nitrogen and Creatinine Centrifuge tubes (labelled)
Microtubes (labelled)
Disposable pipettes
Bike Specifications
Program Bike
Pre-Weigh sweat patches
TV & DVD player
Organise Phlebotomist
Organise Research Assistant
Data Collection Sheets
Instruction Sheets
Cook Pasta

During Visit
POMS (profile of mood state)
DALDA (daily Life demands of athletes)
Muscle soreness diagram and scale
Blood venepuncture
Urination into collection Day -4
Nude Body weight
Sweat patch application

During Cycle
Start training ride
Start fans
RPE and GRS scales during ride
Sports Drink during ride
Start training ride

Post Cycle
Remove sweat patches
Weigh sweat patches
Nude Bodyweight
Weigh remaining drink (there should be none remaining)
Urination in collection Day -4
Supplementation 1 (t=0)
Urination into collection Day -3
Supplementation 2 (t=30)
Supplementation 3 (t=60)
Supplementation 4 (t=90)
Go over Diet Records with Subject
Give food, instructions, go home.

Day -2 (Ride 3)
Pre-Visit
Weighed Food
Extra Food Items
Urine collection containers and 6M HCl
Sports Drinks
Supplement Drinks
Subway
Venepuncture Tray
Sweat Tray
Plasma Tubes x 2 (labelled)
50mL x 2 Sweat Centrifuge tubes (labelled)
15mL x 2 Sweat Centrifuge tubes (labelled)
15mL x 5 Urine Total Nitrogen and Creatinine Centrifuge tubes (labelled)
Microtubes (labelled)
Disposable pipettes
Bike Specifications
Program Bike
TV & DVD player
Organise Phlebotomist
Organise Research Assistant
Data Collection Sheets
Instruction Sheets
Cook Pasta

During Visit
POMS (profile of mood state)
DALDA (daily Life demands of athletes)
Muscle soreness diagram and scale
Blood venepuncture
Urination into collection Day -3
Nude Body weight
Weigh sweat patches

Sweat patch application

During Cycle
Start training ride
Start fans
RPE and GRS scales during ride
Sports Drink during ride
Start training ride

Post Cycle
Remove sweat patches
Weigh sweat patches
Nude Bodyweight
Weigh remaining drink (there should be none remaining)
Urination in collection Day -3
Supplementation 1 (t=0)
Urination into collection Day -2
Supplementation 2 (t=30)
Supplementation 3 (t=60)
Supplementation 4 (t=90)
Give food, instructions, go home.

Day 1 (Performance Ride)
Pre-Visit
Get the room to 20°C and 40-50% humidity
Breakfast (within 60-min and snack within 15-min)
Sports Drinks
Venepuncture Tray
Plasma Tubes x 2 (labelled)
15mL x 10 Urine Total Nitrogen and Creatinine Centrifuge tubes (labelled)
Microtubes (labelled)
Disposable pipettes
Bike Specifications
Program Bike
Stop Watch
Timer
Polar Watch and HR (downloadable)
Organise Phlebotomist
Organise Research Assistant
Data Collection Sheets
Instruction Sheets

During Visit
Breakfast (60-mins prior)
POMS (profile of mood state)
DALDA (daily Life demands of athletes)
Muscle soreness diagram and scale
Muscle Soreness by Pressure Algometer

Snack (15-mins prior)
Blood venepuncture
Body weight (USE BODYWEIGHT FROM METABOLIC RATE CALCULATIONS)

During Ride
Start Warm-up Ride
Provide Warm-up sports drink
Start Fans
Do normal stretching only (repeat of last time)
Start performance ride
Provide Performance ride sports drink (3 equal batches)
Start polar watch (hit red button 2 times to start, hit stop button to stop)
Start timer clock and split timer/stop watch
RPE and GRS scales during ride

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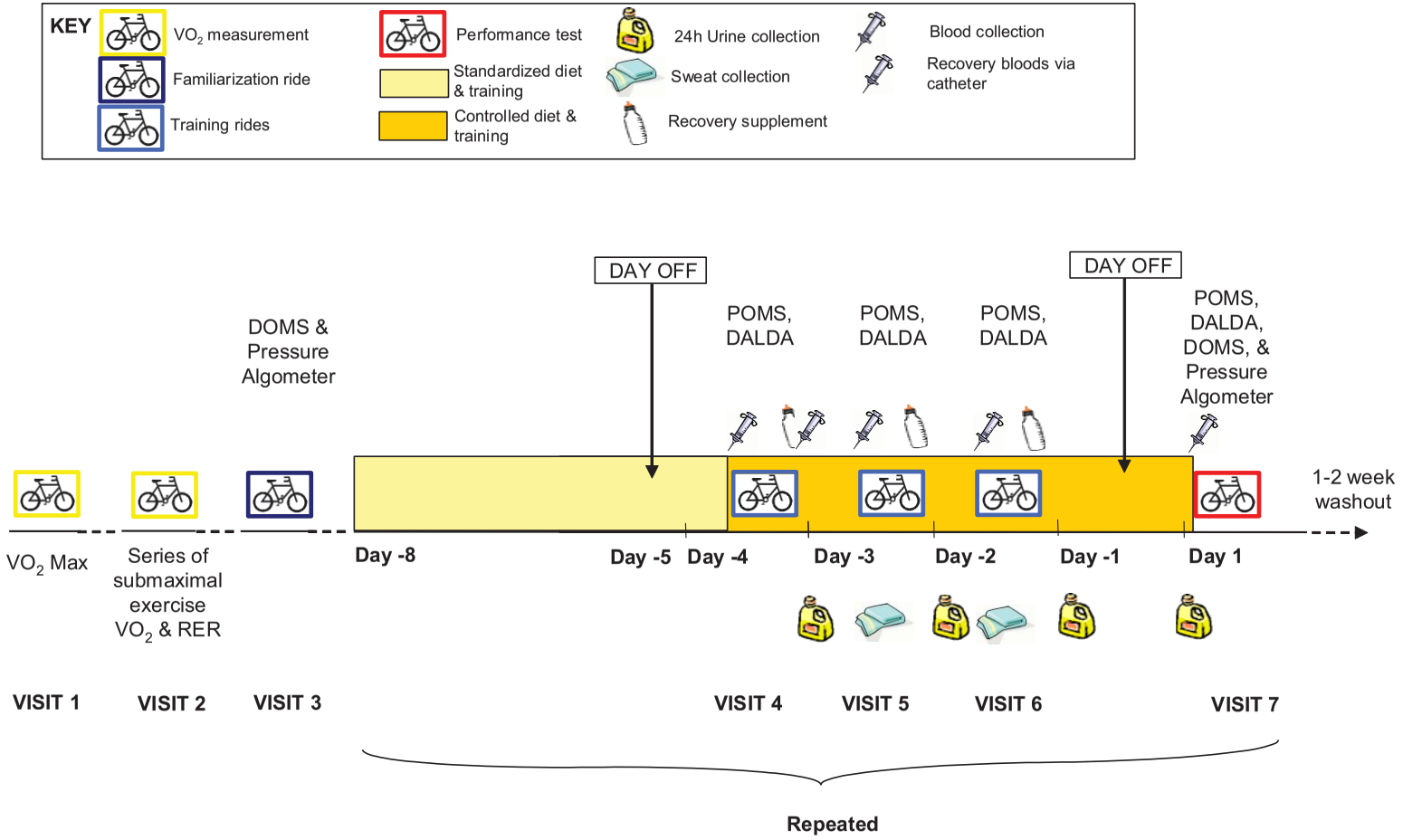
LUCOPRO CYCLE STUDY SCHEDULE

BLOCK 1							
VISIT	VISIT 1	VISIT 2	VISIT 3	VISIT 4	VISIT 5	VISIT 6	VISIT 7
DATE							
EVENT	VO_2max & Health Questionnaire	Resting & Sub-Maximal Metabolic Rate	Practice Pressure Algometer & Performance Test, RPE & Psychometric scales,	Bloods, DOMS scale, POMS, DALDA, Training ride, RPE & Psychometric scales, Bloods, Urine Collection	Bloods, DOMS scale, POMS, DALDA, Sweat patches, Training Ride, RPE & Psychometric scales, Urine Collection,	Bloods, DOMS scale, POMS, DALDA, Sweat patches Training Ride, RPE & Psychometric scales, Urine Collection	Bloods, DOMS scale, Pressure Algometer, POMS, DALDA, Performance Test (all-out effort!), RPE & Psychometric scales,
DAY				Day -4	Day -3	Day -2	Main Test Day
TIME	1.5h	1.5h	3h	5h	4h	4h	3.5h
BLOCK 2							
DATE							
EVENT				Bloods, DOMS scale, POMS, DALDA, Training ride, RPE & Psychometric scales, Bloods, Urine Collection	Bloods, DOMS scale, POMS, DALDA, Sweat patches, Training Ride, RPE & Psychometric scales, Urine Collection,	Bloods, DOMS scale, POMS, DALDA, Sweat patches Training Ride, RPE & Psychometric scales, Urine Collection	Bloods, DOMS scale, Pressure Algometer, POMS, DALDA, Performance Test (all-out effort!), RPE & Psychometric scales,
DAY				Day -4	Day -3	Day -2	Main Test Day
TIME				5h	4h	4h	3.5h
NOTES	• Bring cycle clothes, shoes, pedals, & bike specifications	• No food before visit • Drive to lab • Cycle clothes, shoes, pedals	• Cycle clothes, shoes, pedals • Start recording training, daily activity, & diet from Day -8 • DAY OFF Day -5	• Cycle shoes, pedals • Start controlled training & diet	• Fresh cycle clothes • Empty containers • Urine samples • Controlled training & diet • Fluid Intake diary	• Fresh cycle clothes • Empty containers • Urine samples • Cycle clothes, shoes, pedals • Controlled training & diet • DAY OFF Day -1	• No food before visit • Empty containers • Urine samples • Cycle clothes, shoes, pedals


KEY
VO2 measurement
Familiarization ride
Training rides
Performance test
Standardized diet & training
Controlled diet & training
24h Urine collection
Sweat collection
Recovery supplement
Blood collection
Recovery bloods via catheter
DAY OFF
DAY OFF
DOMS & Pressure Algometer
POMS, DALDA
POMS, DALDA
POMS, DALDA
POMS, DALDA, DOMS, & Pressure Algometer
1-2 week washout
VO2 Max
Series of submaximal exercise VO2 & RER
Day -8
Day -5
Day -4
Day -3
Day -2
Day -1
Day 1
VISIT 1
VISIT 2
VISIT 3
VISIT 4
VISIT 5
VISIT 6
VISIT 7
Repeated

INSTRUCTIONS AND SCHEDULE

PROCEDURE

This is a crossover design study; you are required to come into the lab for 3 initial visits, and then 3 testing visits during block 1. Visits 4, 5, & 6 will be repeated during block 2, with the alternative supplement, after a 1-week washout period, see diagram on page 3. Your dietary intake and training schedule leading up to the testing visits of block 1 will be replicated for block 2.

Visit #1:

~2 h duration. Arrive at the lab fasted for 3-4 h

This visit will establish your pre-testing maximal oxygen capacity (VO_{2max}). This involves riding for 10-15 minutes with workload increasing every 2.5 minutes until you cannot continue

You will also perform a familiarization ride, which will be similar to the experiment day protocol but of shorter duration.

You will be required to keep a diet record over 4 days (1 weekend day & 3 weekdays) for design of your controlled diet

Visit #2:

~2 h duration. Arrive at the lab at ~17:00

You will perform a standardizing ride for 90 min at 50-70% of your maximum

You will receive food to eat for the evening and following day. It is important you eat only the food we provide to maintain a controlled diet

The following day you should not train; complete only your normal everyday activities.

Visit #3:

~ ½ h duration. Arrive at the lab at ~07:00-09:00, fasted overnight

A muscle biopsy will be taken

From Day -7 to Day -2, you will keep a diet and training diary

Visit #4:

~2 h duration. Arrive at the lab at ~17:00

You will perform a standardizing ride for 90 min at 50-70% of your maximum

You will receive food to eat for the evening and following day. It is important you eat only the food we provide to maintain a controlled diet

On day -1 you should not train; complete only your normal everyday activities. It is important that you are well rested before the experiment

Visit #5:

~ 6 h duration. Arrive at the lab at ~06:00-09:00, fasted overnight

A catheter will be placed in a forearm vein

You will receive a snack bar before, and sports drink during the ride

You will perform an interval protocol at 50-90% of your maximum for 1h 45min

After the ride, you receive your first serving of the supplement /placebo, then you may have a shower, relax and recover in the lab

You will receive supplement 2 at 1h, supplement 3 at 5h, & supplement 4 at 9h after exercise.

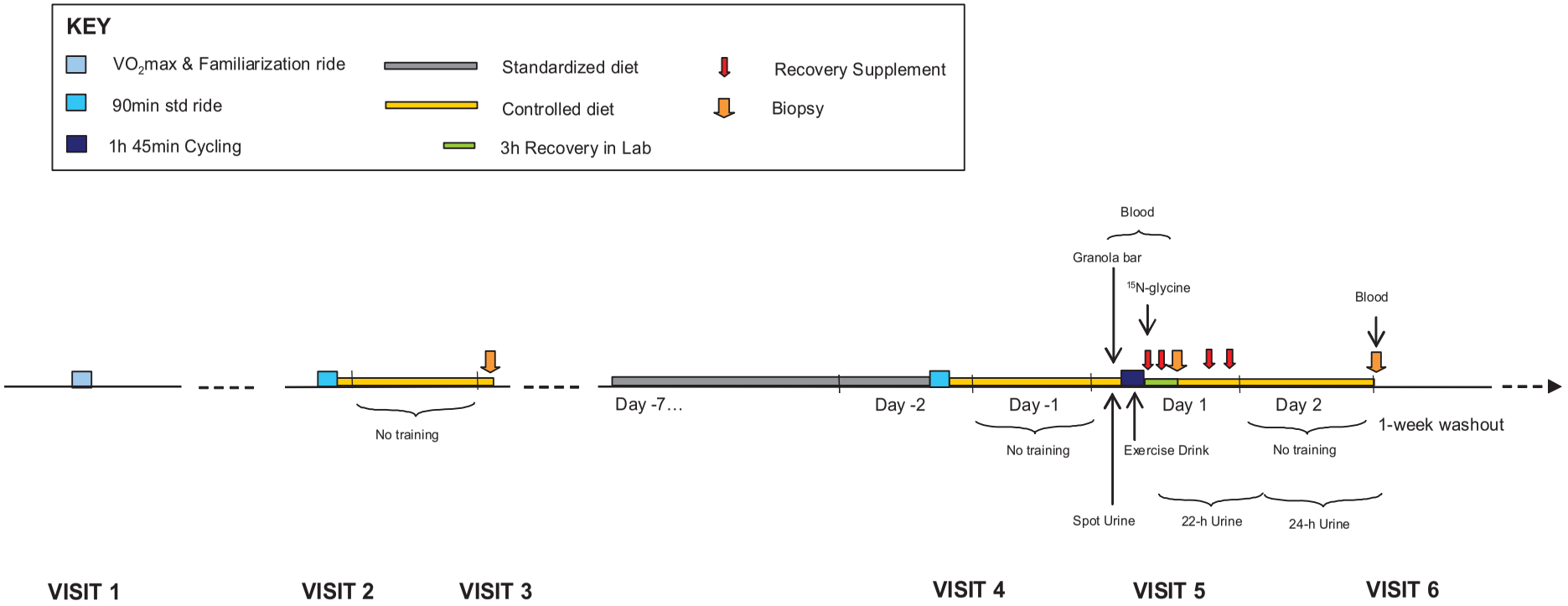
3 h after exercise a muscle biopsy will be taken

Blood will be taken before, during exercise, and recovery

Continue to eat only the food we provide throughout days 1 and 2
On day 2 you should not train
Urine will be collected following the ride and for the next 48 h
Visit #6:
~½ h duration. Return to the lab 48 h later, at ~06:00-09:00, fasted overnight
A muscle biopsy and blood will be taken

SCHEDULE

BLOCK 1						
VISIT	Visit 1	Visit 2	Visit 3	Visit 4	Visit 5	Visit 6
EVENT	VO_{2max} & Familiarization Ride	Standardizing Ride	Baseline Biopsy	Standardizing Ride	Experiment Day	48 h Sample
DAY				Day -2	Day 1	Day 3
DATE						
TIME						
BLOCK 2						
EVENT				Standardizing Ride	Experiment Day	48 h Sample
DATE				Day -2	Day 1	Day 3
TIME						
NOTES	Bring cycle clothes, shoes, pedals, & bike specifications. Start standardized training & diet		Fasted overnight	Fasted for 3-4 h. Bring cycle clothes, shoes, & pedals. Start controlled diet	Fasted overnight. Bring cycle clothes, shoes, pedals, & 7-d training/diet diaries. Continue controlled diet	Fasted overnight. Urine Samples


KEY
VO2max & Familiarization ride
90min std ride
1h 45min Cycling
Standardized diet
Controlled diet
3h Recovery in Lab
Recovery Supplement
Biopsy
Blood
Granola bar
15N-glycine
Blood
No training
Day -7…
Day -2
Day -1
Day 1
Day 2
1-week washout
No training
Exercise Drink
No training
Spot Urine
22-h Urine
24-h Urine
VISIT 1
VISIT 2
VISIT 3
VISIT 4
VISIT 5
VISIT 6

APPENDIX H PRELIMINARY TESTING

PRELIMINARY TESTING

Preliminary Testing

Aerobic Fitness

Fitness was assessed prior to initiation of the study using a maximal oxygen uptake (VO_{2max}) test. Subjects were asked to avoid strenuous exercise 12-h and fasted for 3-h prior to the exercise test. The exercise test consisted of a continuous graded test to exhaustion, a modification of the test described previously (Thornburn, et al, 2006), on an electromagnetically-braked cycle ergometer (Velotron, Racermate, Seattle, WA in Study 2) equipped with aero bars and adjusted so as to replicate the subjects own bicycle saddle height and handlebar position. Briefly, subjects started with a 10-min warm-up at 100 W, followed by the Lactate threshold protocol which starts at 150 W and increases by 20W every 5 minutes. Blood lactate concentration is taken at 4-min into every stage. Once blood lactate reaches approximately 4.5 mmol, exercise intensity is reduced, and the athlete given 10-min recovery at 50% of power output where 4mmol [La-] was achieved. The incremental exercise test used to predict VO_{2max}, starts at a workload of 3 $W \cdot kg^{-1}$ body mass and increasing in steps of 25 W every 150 s until exhaustion. Exhaustion was considered to have occurred when at least two of the following criteria was met; i) volitional fatigue, ii) the subject could no longer maintain pedal cadence of 60 rpm despite fervent verbal encouragement, iii) heart rate within 10 beats/min of HRmax (220 - age), and/or iv) a respiratory exchange ratio > 1.05. Subjects were asked to continue cycling for a few minutes cool down at about 50-100W before dismounting.

VO_{2max} was measured with the Douglas bag method and taken as the highest attained oxygen uptake taken over the last 60 s of each 150 s stage. W_{max} was determined by the following formula adapted from Kuipers et al. (1987).

$$W_{max} = W_{out} + (t/150)*25$$

Where W_{out} is the final completed stage and t is the time into the final stage. Peak power outputs were used to determine the power output settings in all subsequent cycle ergometer protocols. Heart rate (HR) was recorded by a radiotelemetry HR monitor (Polar Vantage NV, Kempele, Finland) every 10-sec and then averaged over the last 60-s of each 150-s stage.

Before each test, the gas meter (Servomex 1440, Applied Instruments Auckland, New Zealand used in *Study 1*), was calibrated high calibration gas (26% O_2 and 4% CO_2) and ambient air.

Resting Metabolic Rate

Resting metabolic rate was determined with open-circuit indirect calorimetry via the classic Douglas Bag method under standard conditions. Standardized conditions for measurement of resting metabolic rate (RMR) adapted from Haugen et al (2003) were defined as following a 12h fast and abstinence from exercise, caffeine, and alcohol, in a darkened isolated room, while subjects rested quietly in supine position, covered by a blanket, at controlled temperature of 21-24 °C. Further, all measurements were made in the morning between 0700-1000 hours, subjects drove or were driven to the laboratory, and rested quietly for 25 minutes prior to

connection to the collection apparatus. Subjects were asked to breath normally into the mouthpiece connected to a Harvard Apparatus 2-way valve (Harvard Apparatus, Holliston, MA) with nose-clip for 5 minutes, to allow for familiarization (allows subject to re-establish normal breathing pattern) prior to opening valve of Douglas bag (Harvard Apparatus, Holliston, MA) and to ensure all dead space in the valves and tubing was flushed with expired gas. Douglas bag collections of expired gases were made for 6-10 minutes and heart rate collected for the final 1 minute.

Metabolic Rate during Steady-state Sub-maximal Exercise

To calculate RER and energy expenditure for three loads of cycle ergometer exercise, following a 10 minute warm-up each subject cycled for 3 consecutive 10 minutes stages at intensities of 40, 50, and 60% of their peak power measured previously. During the final 1 minute of each stage expired air was again collected into Douglas bags.

RER Determinations

The individual Douglas bags were emptied using a vacuum pump, and the volume of air measured with a dry gas meter (Harvard, Biolab, New Zealand). Expired gas fractions were analysed using a Servomex gas analyser (Servomex 1440, Applied Instruments Auckland, New Zealand), which was calibrated with blank (99.9% N_2), high calibration gas (26% O_2 and 4% CO_2) and ambient air prior to each session. Inspiratory volume (VI) was corrected to expiratory volume (VE) by use of the Haldane transformation (Wilmore & Costill, 1973). Oxygen consumption and RER were calculated in a spreadsheet containing standard formulae (Egan 1999), however further adapted to calculate rates of substrate utilization and energy expenditure using the stoichiometric equations of Jeukendrup and Wallis (2005) and Péronnet and Massicotte (1991), with the assumption that protein oxidation during exercise was negligible, below:

Resting Stoichiometric Equations

Rate of fat metabolism (g/min)

$1.695 \times VO_2 - 1.701 \times VCO_2$ (Péronnet and Massicotte, 1991)

Where $C_{17.2702}H_{32.7142}O_2$ stoichiometry is used to represent the weighted average of 13 fatty acids considered to make up 99% of human adipose tissue (Péronnet and Massicotte, 1991).

Rate of carbohydrate metabolism (g/min)

$4.5852 \times VCO_2 - 3.2260 \times VO_2 - 0.40 \times N$ (Jeukendrup & Wallis, 2005)

Where glucose stoichiometry is used to represent carbohydrate oxidation since glucose is considered to be the main carbohydrate utilised as fuel at rest (Ferannini, 1988; Jeukendrup & Wallis, 2005).

Rate of energy expenditure (kJ/min)

$18.6647 \times VO_2 - 2.3642 \times VCO_2$ (Ferannini, 1988; Jeukendrup & Wallis, 2005)

Low Intensity Exercise Stoichiometric Equations

Rate of fat metabolism (g/min)

$1.695 \times VO_2 - 1.701 \times VCO_2$ (Péronnet and Massicotte, 1991)

Rate of carbohydrate metabolism (g/min)

$4.3438 \times VCO_2 - 3.0561 \times VO_2 - 0.40 \times N$ (Jeukendrup & Wallis, 2005)

Where low intensity exercise is considered to include intensities of 40-50% VO_{2max}, and a mixture of glucose and glycogen stoichiometry (assumed 50% carbohydrate oxidation derived from glucose and 50% from glycogen) has been used (Jeukendrup & Wallis, 2005)

Rate of energy expenditure (kJ/min)

$18.7021 \times VO_2 - 2.3077 \times VCO_2$ (Ferannini, 1988; Jeukendrup & Wallis, 2005)

Moderate to High Intensity Exercise Stoichiometric Equations

Rate of fat metabolism (g/min)

$1.695 \times VO_2 - 1.701 \times VCO_2$ (Péronnet and Massicotte, 1991)

Rate of carbohydrate metabolism (g/min)

$4.210 \times VCO_2 - 2.962 \times VO_2 - 2.37 \times N$ (Jeukendrup & Wallis, 2005)

Where moderate to high intensity exercise is considered to include intensities of 50-75% VO_{2max}, and a mixture of glucose and glycogen stoichiometry (assumed 20% carbohydrate oxidation derived from glucose and 80% from glycogen) has been used (Jeukendrup & Wallis, 2005).

Rate of energy expenditure (kJ/min)

$18.7311 \times VO_2 - 2.2742 \times VCO_2$ (Ferannini, 1988; Jeukendrup & Wallis, 2005)

Conversion factors of 15.65 $kJ \cdot g^{-1}$ for glucose utilised during rest, 16.51 $kJ \cdot g^{-1}$ for carbohydrate (50:50 mix of glucose and glycogen assumed) utilised during low intensity exercise, 17.02 $kJ \cdot g^{-1}$ for carbohydrate (20:80 mix of glucose and glycogen assumed) utilised in moderate to high intensity exercise (Ferannini, 1988; Jeukendrup & Wallis, 2005) for CHO and 40.79 $kJ \cdot g^{-1}$ for fat oxidation (Péronnet and Massicotte, 1991) were used to estimate contribution to energy expenditure. In the above calculations it is assumed that protein oxidation was negligible as no measure of urinary nitrogen excretion, N, was taken during metabolic rate measurements. The error that is introduced when estimating energy metabolism is from VO_2 and VCO_2 alone is thought to be about 4% in fasting metabolic rate calculations, and to decrease as metabolic rate increases, such as with exercise (Ferrannini, 1988).

Preliminary testing record sheets are given over the page, and the spreadsheet of metabolic rate calculations is provided in the appendix titled ‘Metabolic Rate Calculations’ in the appendices on the CD in the back cover of this thesis.

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LUCOPRO CYCLE STUDY LACTATE THRESHOLD & VO_{2MAX}

CALIBRATING THE GAS ANALYSER

At the gas analyzer (Servomex 1440, Applied Instruments Auckland, New Zealand), first check the granular desiccant (calcium sulphate, Drierite) in the gas tubing; if the colour has turned pink it will need to be changed with fresh blue desiccant. Turn on the pump; the gas analyzer should already be on. Check the LPM air valve 'bubble' (ball-bearing) of the pump is at 4 (we will be reading for 1.5 minutes, 4 * 1.5 = 6L).

Calibrate on the blank gas (Oxygen-free nitrogen, 99.9% N2 or equivalent). Turn the black gas cylinder tap on first (the one closest to the cylinder), then turn the red valve to open flow though the tube to the gas analyzer. Let the analyzer read for 90s for both O2 and CO2. If the analyzer does not read zero for either O2 or CO2, then using tweezers adjust zero drift control behind flip down slot on the front panel. Once adjusted, read the gas again for another 30s. Turn off the red valve tap and the black gas cylinder tap. Next calibrate the span gas, the higher concentration gas (containing 26% O2 and 4% CO2), following the same procedure, however this time adjust the span drift control to 26.0 for O2 and/or 4.00 for CO2, if adjusting was required read for another 30s. Ensure the red valve tap and black gas cylinder tap are off/closed. Finally read ambient air (containing 20.9 O2 and 0.03% CO2), if the CO2 reads a little high this is due to the effect of the air-conditioning unit, so ignore. Turn off the pump. Record all calibration values in the log book. The gas analyzer generally stays on constantly for 2 reasons, it takes an hour to warm-up, and it has previously been found there are fewer problems with the calibration when the analyzer is allowed to remain on.

Lactate Threshold and VO_{2max}

Valves, mouthpieces, nose clips etc are found in the Douglas bag equipment cupboard or on the drying rack beside the sink. Timers and heart rate monitors/straps are found in the trolleys, which should be rolled over to where you are testing each subject. Douglas bags can be found hanging on the racks in the corner of the room. Use the Dry Gas Meter (method below) to evacuate any air from all Douglas bags and turn the valves shut. Ensure the rubber bungs are tightly inserted in the narrow tubes, so the Douglas bags are air tight. When attaching the valve, the arrow indicates direction of air flow, so must point to the tube heading to the Douglas bag. The mouthpiece is secured to the side of the valve, the white/clear mouthpieces are the larger size.

No strenuous exercise 12h prior to testing.

Briefly explain the protocol to the subject, they can stop at any time but are encouraged to last as long as they can.

Set up the bike to the subject's specifications, provide subject with Polar monitor strap and watch, take subject details such as BW, Ht, age etc (see over page), turn on the fan, and ask the subject to warm-up for 10-min.

While subject is warming up, program the Lactate protocol into the Lode or Velotron.

The Lactate threshold protocol starts at 150 W and increases by 20W every 5 minutes. Blood lactate concentration is taken at 4-min into every stage. Once blood lactate reaches approximately 4.5 mmol, exercise intensity is reduced, and the athlete given 10-min recovery at 50% of power output where 4mmol [La-] was achieved.

Start the Lode or Velotron protocol, timer, and polar watch simultaneously.

To get HR you will have to periodically check the Polar Watch, especially as the subject begins to tire. Heart rate should be measured every 10-sec and average over the last 1-min of each block. Keep a close watch on the heart rate monitor to ensure it measures what you expect (i.e. it goes up with and increase in workload is at an appropriate level, and is beating regularly).

During the 10-min active recovery, program the VO_{2max} protocol into the Lode or Velotron.

The VO_{2max} protocol starts at **3W/kg bodyweight**, and increases by 25 W every 150s until the subject is exhausted, around 10-15 min.

You will use 2 timers, one for duration of entire test, the other for collection time into each Douglas bag.

Start polar watch. Start the Lode or Velotron protocol, and timer.

To get HR you will have to periodically check the Polar Watch, especially as the subject begins to tire. Heart rate should be measured every 10-sec during the final 1-min of each 2.5-min block. Keep a close watch on the heart rate monitor to ensure it measures what you expect.

Turn on the Douglas bag valve and collect breath for the final 1-min of each 2.5-min stage.

Ensure you accurately time the breath collection into each bag. i.e. turn on timer & immediately open Douglas bag, turn off timer, and immediately close Douglas bag, Tell the Recorder the time. Use Douglas bags in order, each 1-min breath sample (representative of final 1-min of each stage) in a new bag.

Recommended pedalling frequency or cadence, especially at ≥200W is a high as possible/comfortable (> 80-90 rpm), and test will be stopped if cadence of ≥60 rpm is unable to be maintained.

Inform the subject just prior to each incremental increase in work-rate. The subject should continue until exhaustion. Encourage subjects to continue even if they feel they cannot last another 150s at a higher power as some valuable data may still be obtained.

Exhaustion is defined as a drop in pedalling rate of <60rpm, volitional fatigue, Hr within 10 beats/min of HRmax (220-age). Once calculations are done, RER should be >1.1 to indicate VO_{2max}.

Stop the timer at point of subject exhaustion. Note the total time completed and the partially completed Stage. Stop the Polar Watch. Get the subject

to continue riding for a few minutes cool down at about 50-100W before dismounting.
Peak power output is calculated using the last completed work rate in W plus the fraction of time spent in the final non-completed work rate multiplied by 25 W.

MEASURING GAS CONCENTRATION
At the gas analyzer, first note the arrow directions on the red valve tap indicating which direction the gas flow is directed to, set this to O2; also ensure the red tap valve is airtight and not coming loose. Pinch off the tube containing the rubber bung leading from the Douglas bag, attach this to the attachment and tubing leading into the gas analyzer, turn on the pump, release the pinched tube, and turn on the timer all at the same time. Let the analyzer read for 90s for O2. Note the O2 concentration down on your record sheet, at 1min 30s, immediately switch the red valve tap to read CO2, note the CO2 concentration down on your record sheet. At 3mins, pinch off the tube leading from the Douglas bag, replace the bung and turn off the pump. Turn off the timer. The gas volume samples for both O2 and CO2 analysis is 6L (when pump LPM air valve is set to 4, then 4 * 1.5 = 6L).

MEASURING DOUGLAS BAG VOLUME
At the Dry Gas Meter (Harvard, Biolab, New Zealand), turn on the meter and the thermometer (attached to the hosing). Attach the hosing to the Douglas bag valve; twist the valve at the same time as turning the dust buster on. Watch the thermometer, and take note of the expired air (collected in the Douglas bag) temperature. When the volume reading has stopped changing i.e. the Douglas bag is empty, turn off the Douglas bag valve, then turn off the dust buster, make note of the volume reading on the gas meter, and then press the reset button on the gas meter. Turn off the Dry Gas Meter if no further use.

Check the weather gauge for barometric pressure (measures inches of Hg therefore multiply by 25.4 to get mmHg), percent humidity, and temperature (gas temperature is what we need, however this is invariably close to ambient to we use ambient to approximate this).

CLEAN-UP
The nose-clips are rinsed, and then set aside to dry, while the mouthpieces and valves are rinsed, then placed in the bucket containing Milton. The tubes are rinsed, and hung to dry.

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LUCOPRO CYCLE STUDY LACTATE & VO_{2MAX}

CYCLE ERGOMETER DIMENSIONS

SUBJECT ID _____ DATE _____ TIME _____

Age _____ BW (kg) _____ Ht _____

Cycle _____ Handlebar Extn _____ Handlebar Ht _____

Seat Extn (cm) _____ Seat Ht (cm) _____ Seat-Pedal (cm) _____

Crank Length (cm) _____

Warm up at ~100W or load comfortable for cyclist. Start at 150W.

LACTATE THRESHOLD

Work Rate (W)										
Total Time										
Lactate mmol										
Collection Time		4-min	9-min	14-min	19-min	24-min	29-min	34-min	39-min	44-min
HR (bpm)	0s									
HR (bpm)	10s									
HR (bpm)	20s									
HR (bpm)	30s									
HR (bpm)	40s									
HR (bpm)	50s									
HR_{Av} (bpm)										

Active recovery at ~50% of load that 4mmol/l lactate was achieved. Start VO_{2max} at 3W/kg.

VO_{2max} CALCULATIONS

Work Rate (W) 3W/kg										
Stage	2.5	5.0	7.5	10.0	12.5	15.0	17.5	20.0	22.5	25.0
Douglas Bag										
Collection Time (s)										
TimeTotal (min:s)										
$t_{final\ stage}$ (s)										
W_{max} (W)										**$W_{max} = W_{final} + (t_{final\ stage}/150)*25$**
Barometric Pressure (mmHg)										**mmHg = InHg*25.4**
Humidity (%)										
Temperature (°C)										
Temp Exp Air (°C)										
a). Vol. Sample CO_2										
b). Vol. Sample O_2										
c). Vol. Gas Meter										
Total Vol. [$V_{E(ATPS)}$] (L)										
VE(STPD) (L/min)										
F_EO_2 (%)										
F_ECO_2 (%)										
VO_2 (L/min)										
VCO_2 (L/min)										
VO_2 (mL/kg/min)										
RER										
HR (bpm)	0s									
HR (bpm)	10s									
HR (bpm)	20s									
HR (bpm)	30s									
HR (bpm)	40s									
HR (bpm)	50s									
1-min HR_{Av} (bpm)										
HR_{max} (bpm)										

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LUCOPRO CYCLE STUDY MEATBOLIC RATE

CALIBRATING THE GAS ANALYSER

At the gas analyzer (Servomex 1440, Applied Instruments Auckland, New Zealand), first check the granular desiccant (calcium sulphate, Drierite) in the gas tubing; if the colour has turned pink it will need to be changed with fresh blue desiccant. Turn on the pump; the gas analyzer should already be on. Check the LPM air valve 'bubble' (ball-bearing) of the pump is at 4 (we will be reading for 1.5 minutes, 4 * 1.5 = 6L).

Calibrate on the blank gas (Oxygen-free nitrogen, 99.9% N2 or equivalent). Turn the black gas cylinder tap on first (the one closest to the cylinder), then turn the red valve to open flow though the tube to the gas analyzer. Let the analyzer read for 90s for both O2 and CO2. If the analyzer does not read zero for either O2 or CO2, then using tweezers adjust zero drift control behind flip down slot on the front panel. Once adjusted, read the gas again for another 30s. Turn off the red valve tap and the black gas cylinder tap. Next calibrate the span gas, the higher concentration gas (containing 26% O2 and 4% CO2), following the same procedure, however this time adjust the span drift control to 26.0 for O2 and/or 4.00 for CO2, if adjusting was required read for another 30s. Ensure the red valve tap and black gas cylinder tap are off/closed. Finally read ambient air (containing 20.9 O2 and 0.03% CO2), if the CO2 reads a little high this is due to the effect of the air-conditioning unit, so ignore. Turn off the pump. The gas analyzer generally stays on constantly for 2 reasons, it takes an hour to warm-up, and it has previously been found there are fewer problems with the calibration when the analyzer is allowed to remain on.

RESTING METABOLIC RATE (OPEN CIRCUIT DOUGLAS BAG METHOD)

Valves, mouthpieces, nose clips etc are found in the Douglas bag equipment cupboard or on the drying rack beside the sink. Timers and heart rate monitors/straps are found in the trolleys, which should be rolled over to where you are testing each subject. Douglas bags can be found hanging on the racks in the corner of the room. Use the Dry Gas Meter (method below) to evacuate any air from Douglas bag (number 1) and turn the valve shut. Ensure the rubber bung is tightly inserted in the narrow tube, so the Douglas bag is air tight.

When attaching the valve, the arrow indicates direction of air flow, so must point to the tube heading to the Douglas bag. The mouthpiece is secured to the side of the valve, the white/clear mouthpieces are the larger size.

The subject should **not perform exercise training, or ingest food, coffee or alcohol for 12 h beforehand.** The subject will drive or be driven to the lab in the morning, heart rate strap and watch are provided, then have the subject lie supine in isolation in the anthropometric room, temperature controlled between **21-24 °C**, for 25 minutes. Get the subject to breath through the mouthpiece for 5 minutes to allow for familiarization (allows subject to re-establish normal breathing pattern) prior to opening valve of Douglas bag i.e. taking actual measurement.

At 30 minutes, turn on the Douglas bag valve at the same time as setting the timer, and collect air for **6-10 minutes**, while the subject continues to rest supine on the massage table. At 6-10 minutes immediately turn off the Douglas bag valve, and allow the subject to remove the mouthpiece. The tubing, mouthpiece, and nose-clip may be put aside for later.

METABOLIC RATE DURING STEADY-STATE SUBMAXIMAL EXERCISE

To calculate RER and energy expenditure for cycle ergometer exercise, following a 10 minute warm-up the subject will cycle for 3 x 10 minutes stages at intensities of 40, 50, and 60% of their peak power measured previously. During the final 1 minute of each stage expired air will be collected into Douglas bags (numbers 2, 3, 4).

MEASURING GAS CONCENTRATION

At the gas analyzer, first note the arrow directions on the red valve tap indicating which direction the gas flow is directed to, set this to O_2; also ensure the red tap valve is airtight and not coming loose. Pinch off the tube containing the rubber bung leading from the Douglas bag, attach this to the attachment and tubing leading into the gas analyzer, turn on the pump, release the pinched tube, and turn on the timer all at the same time. Let the analyzer read for 90s for O_2. Note the O_2 concentration down on your record sheet, at 1min 30s, immediately switch the red valve tap to read CO_2, note the CO_2 concentration down on your record sheet. At 3mins, pinch off the tube leading from the Douglas bag, replace the bung and turn off the pump. Turn off the timer. The gas volume samples for both O_2 and CO_2 analysis is 6L (when pump LPM air valve is set to 4, then 4 * 1.5 = 6L).

MEASURING DOUGLAS BAG VOLUME

At the Dry Gas Meter (Harvard, Biolab, New Zealand), turn on the meter and the thermometer (attached to the hosing). Attach the hosing to the Douglas bag valve; twist the valve at the same time as turning the dust buster on. Watch the thermometer, and take note of the expired air (collected in the Douglas bag) temperature. When the volume reading has stopped changing i.e. the Douglas bag is empty, make note of the volume reading on the gas meter (you must do this BEFORE anything off otherwise it will be lost), then turn off the Douglas bag valve and dust buster, and then press the reset button on the gas meter. Turn off the Dry Gas Meter if no further use.

Check the weather gauge for barometric pressure (measures inches of Hg therefore multiply by 25.4 to get mmHg), percent humidity, and temperature.

CALCULATIONS

Enter data into the Excel spreadsheet on the building 60 computers, and use the output to calculate energy expenditure.

CLEAN-UP

The nose-clips are rinsed, and then set aside to dry, while the mouthpieces and valves are rinsed, then placed in the bucket containing Milton. The tubes are rinsed, and hung to dry.

METABOLIC RATE CALCULATIONS

Work Rate (W)							
Douglas Bag							
Collection Time (s)							
Barometric Pressure (mmHg) InHg*25.4							
Humidity (%)							
Temperature (°C)							
Start Time							
Date							
BW (kg)							
Temp Expired Air (°C)							
a). Vol. Sample CO_2							
b). Vol. Sample O_2							
c). Vol. Gas Meter							
Total Vol. [$V_{E(ATPS)}$] (L)							
VE(STPD) (L/min)							
F_EO_2 (%)							
F_ECO_2 (%)							
VO_2 (L/min)							
VO_2 (mL/kg/min)							
VCO_2 (L/min)							
VCO_2 (mL/kg/min)							
RER							
V_E/VO_2							
Collection Time	10	20	30	40	50	60	HRAv
Resting HR (bpm)							

REFERENCE

Haugen, H. A., Melanson, E. L., Vu Tran, Z., Kearney, J. T., & Hill, J. O. 2003. Variability of measured resting metabolic rate. Am J Clin Nutr 78: 1141–4.

PRELIMINARY TESTING

Preliminary Testing

Aerobic Fitness

Fitness was assessed prior to initiation of the study using a maximal oxygen uptake (VO_{2max}) test. Subjects were asked to avoid strenuous exercise 12-h and fast for 3-h prior to the exercise test. The exercise test consisted of a continuous graded test to exhaustion, a modification of the test described previously (Thornburn et al, 2006), on an electromagnetically-braked cycle ergometer (Excalibur Sport V2, Lode BV Groningen, The Netherlands) equipped with aero bars and adjusted so as to replicate the subjects own bicycle saddle height and handlebar position. Briefly, subjects started with a 10-min warm-up at 100 W, followed by the incremental exercise test, starting at a workload of 3 W·kg-1 body mass and increasing in steps of 25 W every 150 s until exhaustion. Exhaustion was considered to have occurred when at least two of the following criteria was met; i) volitional fatigue, ii) the subject could no longer maintain pedal cadence of 60 rpm despite fervent verbal encouragement, iii) heart rate within 10 beats/min of HRmax (220-age), and/or iv) a respiratory exchange ratio > 1.05. Subjects were asked to continue cycling for a few minutes cool down at about 50-100W before dismounting.

VO_{2max} was measured online with a calibrated (Moxus Modulator VO_2 system; AEI Technologies, Pittsburgh, PA) and taken as the highest attained 20 s average oxygen uptake. W_{max} was determined by the following formula adapted from Kuipers et al. (1987).

$$W_{max} = W_{out} + (t/150)*25$$

Where W_{out} is the final completed stage and t is the time into the final stage. Peak power outputs were used to determine the power output settings in all subsequent cycle ergometer protocols. Heart rate (HR) was recorded by a radiotelemetry HR monitor (Polar Vantage NV, Kempele, Finland) continuously in *Study 2* and every 10 s and then averaged over the last 60 s of each 150 s stage in *Study 1*.

Before each test, the gas meters (Moxus Modulator VO_2 system; AEI Technologies, Pittsburgh, PA), were calibrated (with high calibration gas (5% CO_2), low calibration gas (16.7% O_2) and ambient air.

VO2 MAX & FAMILIARIZATION RIDE

PROCEDURE

Two researchers are required in the lab when testing subjects
Switch on Pump Power and MOXUS computer ½ an hour before use
Assemble Mouth Piece
Require HR Strap, Nose Clip, Towel, Water, and Gatorade
Calibrate MOXUS (Cart 2)

Turn on calibration Port → go into MOXUS home icon → Set up Calibration→ wait for average readings to come on screen → tick O2 mix → High Cal →wait until it goes blank → tick CO2 mix → Low Cal → wait until it goes blank→ Turn on red knob at gas cylinder → attach hose to calibration gas port → tick O2 mix → Low Cal →wait for it to say calibrated → tick CO2 mix → High Cal → wait until it says calibrated → Save → Exit → Remove hose from calibration gas port → turn off calibration port → turn off red knob

Or calibrate MOXUS (Cart 1)

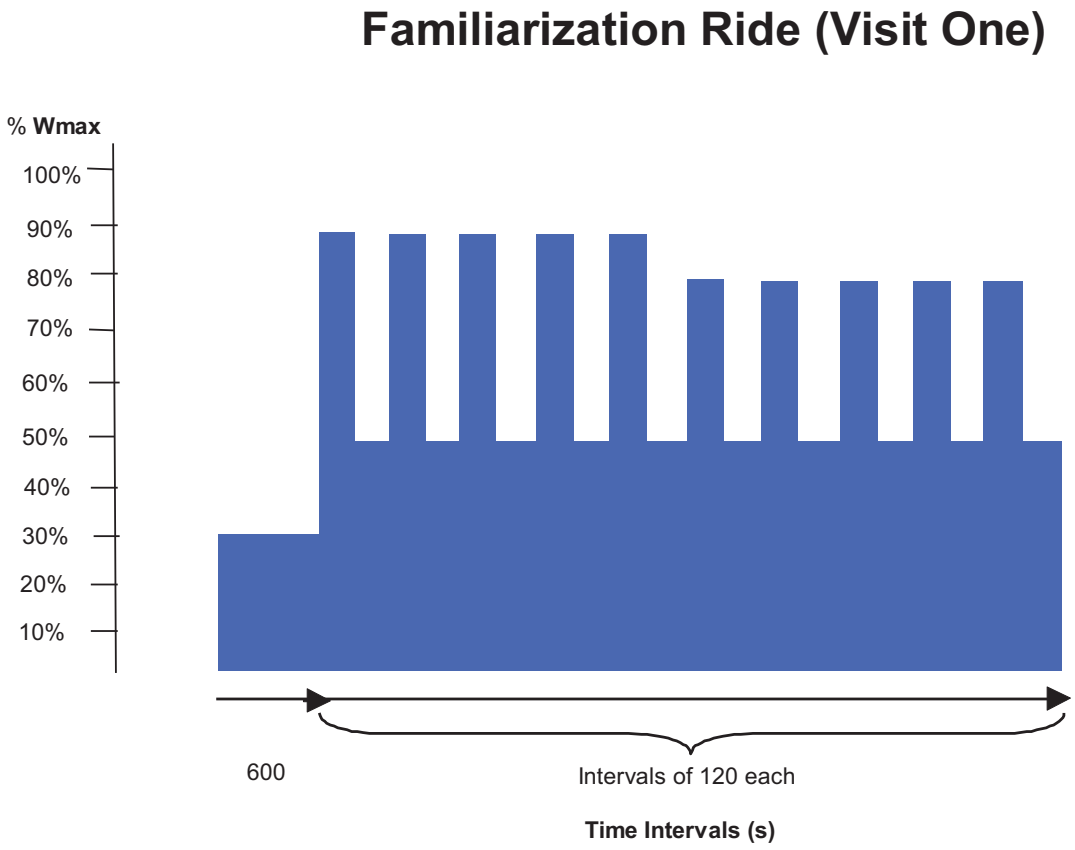
Turn on calibration Port → go into VO2 PDI icon → Check calibration port is working (see flow control ball bearings) → Setup → Enter → Calibration→ Enter → Perform calibration → Turn on red knob at gas cylinder → attach hose to calibration gas port → O2 mix using gas is low calibration (~20) → remove hose → O2 mix using air is high calibration (~16.7) → Enter → Perform Calibration → CO2 mix using air is low calibration (~0.02-0.03) → attach hose → CO2 mix using gas is high calibration (~5) → Enter → Enter → Remove hose from calibration gas port → turn off calibration port → turn off red knob → Subject Data Entry → Esc → Acquire → Begin Test

Complete Subject Contact, Weight, Height, Age, Bike Setup Details of Record Sheet
Enter Subject Information & Program MOXUS
Warm-up for 10-20 minutes
Start Protocol, Timer, and Polar Watch simultaneously (Cart 1 doesn't record HR, you will have to periodically check the Polar Watch, especially as the subject begins to tire)

Protocol starts at power of 3*BW Watts and increases 25W every 2.5min (150s) until subject is exhausted, around 10-15 min. Protocol name is PROGVO2 (subject number).

Stop the stop watch at point of subject exhaustion.
Note Total Time completed and partially completed Stage
Complete VO_{2max} Record Sheet
Stop Polar Watch
Print → right click → export to excel → PROGENE folder → then check short cut to make sure it has been saved
Switch off Pump Power
Give out Subject Handouts and explain study requirements

Book Subject for Remaining Tests
Exercise Day must be on a Wednesday (preferably) or Monday
Clean bike with 10% Ethanol
Pull apart mouthpiece, rinse quickly under tap, then 10 min Cydex, 10 min Bleach, and 5 min mouth wash
Book lab room and bike on lab calendar, book biopsies with Dolores, Erin, and Alissa. Find biopsy assistant, person able to take bloods, and insert catheter.
Write details in your calendar
Ring subjects the evening before each appointment.



Subject Details - VO2 max

Subject Details:					
Name				ID	
e-mail					
Phone	work		Phone		cell
Phone	home				
Medical History					
Do you have any known medical conditions that would affect your participation in this study?					
Do you have any known blood disorders or blood borne diseases?					
Comments:					
Training History					
Years of purposefully training in a sport involving cycling:					
Average cycling training volume over the previous 6 months?					
Comments:					
Diet History:					
Are you currently following a specific diet?					
Do you have any food allergies (for example nuts, dairy, gluten):					
Comments:					
Tests:					
Age			Body Weight		kg
Height	cm				
Start Power			*Start Power men 3.0*wt (ω/kg)*		
VO2 max	/kg		VO2 max		
HRmax			RERmax		
Total Time	min		Wout (completed stage)		
Stages					
t (final stage)	s				
W_{max}			$W_{max} = W_{out} + (t/150)*25$		
Bike Dimensions:					
Bike #			Handlebar Height		
Seat Height			Handlebar Extension		
Seat Extension			Pedal Extension		

APPENDIX I DIET RECORDS

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LUCOPRO CYCLE STUDY DIETARY RECORD

INSTRUCTIONS:
Please record foods soon after they are eaten so you don't forget and list only one food item per line.

Record your diet intake for 4.5 d leading up to the training block **including the day of your first session**.

Be as specific as possible when describing each food/meal, the way it was prepared or cooked (if it was cooked), and the amount that you ate. Include methods used to prepare the food, for example: fresh, frozen, stewed, fried, baked, canned, broiled, raw or braised. For canned foods indicate the liquid in which it was canned and the amount of this that you ate or if you drained off the liquid, for example: heavy syrup, light syrup, fruit juice, spring water, vegetable oil, brine. Record the amounts of visible fats you add and use in cooking for example: butter and spreads, vegetable oils, salad dressing, etc

Remember to report only the amount that you actually ate, not the total amount prepared. Record all amounts in household measures, for example: g, ml, tablespoon (tbsp) (= 15ml), teaspoon (tsp) (=5ml), cups (=250ml), slices or units (described on the packet). Include brand names whenever possible, or bring in the Nutrition Label from pre-packaged meals. Do not alter your normal diet during the time you keep this diary

You should include enough detail so that you can **repeat each meal and snack on the days leading up to the second block of the study!**

SUBJECT ID: _____ Day of the _____ Date: _____

TIME	FOOD ITEM and METHOD of PREPARATION	AMOUNT EATEN

CONTROL DIETS

Study 1 Controlled Diet

A pre-weighed, pre-packaged animal flesh-free diet was provided on Days -4 to Day 1 of each study block, following the first controlled cycling training ride on Day -4. Total daily energy intake for the controlled diet was estimated using previously determined resting metabolic rate, previously measured VO_2 over 3 loads of steady-state sub-maximal exercise, and with correction factors for energy expenditure in daily activity estimated using the daily physical activity diaries. Described further in Appendix Q.

Dietary protein intakes for the control diet were approximated to 1.6 $g \cdot kg^{-1} \cdot d^{-1}$ to maintain neutral or positive nitrogen balance. This figure was based on a recent study by Rowland's et al (Rowlands, et al. 2008), where regression plots of nitrogen balance data provided an average neutral nitrogen balance for well-trained male cyclists under a similar experimental model at protein intakes of 1.5 $g \cdot kg^{-1} \cdot d^{-1}$. Although averaged protein intakes from dietary data of well-trained sub-elite NZ cyclists during a period of regular training have been found to be greater than this Ingrid Perols (personal communication) found New Zealand cyclists averaged 1.9 ± 0.1 $g \cdot kg^{-1} \cdot d^{-1}$ David Rowlands found in the same study described above habitual diets of cyclists were 2.1± 0.4 $g \cdot kg^{-1} \cdot d^{-1}$ (Rowlands, et al. 2008).

Dietary carbohydrate intakes were 7-10 $g \cdot kg^{-1} \cdot d^{-1}$ designed for optimal replenishment of intramuscular glycogen concentrations for endurance athletes (Burke, et al. 2001b). The remainder of the estimated energy requirement was provided by fat intake.

Immediately and 30 min following exercise subjects received supplemental beverage and high-carbohydrate snack (cereal bar [Kellog's LCM's] and orange and mango juice), and at 1 h and 1.5 h post-exercise subjects received further servings of of supplemental beverage and high-carbohydrate snack (pasta spirals and bolognese sauce). The remainder of the diet consisted of milk, flavoured milk, cheese, dried fruit and nuts, cereal bars, muesli, wholemeal bread and buns, spreads (jam, peanut butter, margarine), sports drink and commercially prepared standard vegetarian salad roll (Subway Veggie Delite).

All food and beverage portions were measured using an electronic balance to the nearest 0.01 g (Sartorius CP 4202S, Sartorius, Göttingen, Germany). Subjects received written instructions to consume only the controlled diet and to refrain from all stimulants (caffeine, coffee, energy drinks, and carbonated beverages), alcohol, and supplements (multivitamins, antioxidant vitamins, minerals, sports supplements, and herbal remedies) during the controlled diet. To accommodate unknown variation in hunger, subjects had the option of consuming "extra food items" consisting of low protein foods (1 apple, 1 banana, 2 servings of cookies [Arnotts Farmbake Hokey Pokey Cookies, North Strathfield, Australia]) but were required to record all food items eaten; or if subjects were too satiated to eat the entire diet, must return any food items not consumed. Diet check sheets were

returned on a daily basis and any “extra items” consumed or uneaten food was accounted for.

Controlled diet instruction sheets are given over the page, and the spreadsheet where daily energy requirements and dietary intakes were estimated is provided in the appendix titled ‘Daily Energy Requirement, control Diet and supplement Calculations’ on the CD in the back cover of this thesis.

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LUCOPRO CYCLE STUDY CONTROLLED DIET BLOCK 1

During the controlled diet please refrain from all stimulants (caffeine, coffee, energy drinks, soft drinks), alcohol, and supplements (multivitamins, antioxidant vitamins, minerals, sports supplements, and herbal remedies). Please notify the researcher of any prescribed medications you are taking.

The controlled diet provides your calculated energy requirement, including protein and carbohydrate for recovery from the training we provide and normal daily activities. If you are totally full, and could not possibly eat another bite, you should keep the leftovers and return to us so we can measure remains. If you are absolutely starving hungry, and feel we have not provided enough food for you on the calculated diet, you may eat the extra items provided (1 banana, 1 apple, 4 cookies).

Tick off food as you consume it and please return instruction sheet, any leftover food (including extra items), and plastic containers on your next visit to the lab.

The purpose of the controlled diet is so we can measure the energy and protein you eat, as well as protein waste products (urine and sweat nitrogen), and therefore calculate the amount of protein your body is using.

Enjoy!

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LUCOPRO CYCLE STUDY CONTROLLED DIET BLOCK 2
Day _____

The controlled diet provides your calculated energy requirement, including protein and carbohydrate for recovery from the training we provide and normal daily activities.

The controlled diet for this block of the study should exactly replicate what you ate during block 1, therefore we have provided the **same food and portions** that you ate during the first block of the study.

It is VERY IMPORTANT that you eat ALL of the food provided to replicate what you ate during the first block of the study.

The purpose of the controlled diet is to exactly replicate what you ate during the first block, so that the only difference to affect performance in the time trial will be the recovery supplement. This is very important for the integrity of our data; this will make all of your tests and collections worthwhile.

Enjoy!

CONTROL DIETS

Study 2 Controlled Diet

A pre-weighed, pre-packaged animal flesh-free diet was provided by researchers on Days 1 and 2 of each study block, following the controlled cycling protocol on Day 1. The controlled diet was designed to equal usual energy intakes based on the food diaries, but with macronutrient content approximating 55% carbohydrate, 16% protein, and 20% fat. Immediately following exercise subjects received 1.9 $g \cdot kg^{-1}$ (dry weight) of supplemental nutritional powder made up to 500 mL with water and at 1 h post-exercise. The first meal was provided 5 h post-ride and consisted of the alternate supplement and a commercially prepared standard vegetarian salad roll (Subway Veggie Delite). The next meal was consumed 9 h post ride and consisted of the second alternate supplement plus egg omelette, mixed grain bread, and supermarket packaged salad and dressing. The remainder of the diet consisted of flavoured milk, orange juice, cheese, dried fruit and nuts, bananas, granola bars, breakfast cereal, and additional mixed grain bread. All food and beverage portions were measured using an electronic balance scale (Denver Instrument Top loading Balance 600g/0.1g, Fisher Scientific). The subjects received written instructions to consume only the controlled diet, no additional foods, vitamin or mineral supplements, caffeinated or alcoholic beverages, and to return any uneaten food to be accounted for by the researchers.

Diet recording sheets are given over the page, and a spreadsheet representing the control diets provided to subjects can be found on the CD in the back cover of this thesis. The appendices are titled ‘Control Diets’ and ‘Day 1 Diets’.

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
Include brand names whenever possible, or bring in the Nutrition Label from pre-packaged meals
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: heavy syrup, light syrup, fruit juice, spring water, vegetable oil, brine
Do not alter your normal diet during the time you keep this diary
Remember to record the amounts of visible fats you add and use in cooking
– For example: butter and spreads, vegetable oils, salad dressing, etc

INSTRUCTIONS FOR THE TRAINING DIARY

Please record your training as specified
Note the type of training you performed
– For example: cycling, mountain biking, running, swimming, weight training, etc
Time of day you performed your training
Intensity of training from the following 5-point scale:
1 = light/easy
2 = light-medium
3 = medium/moderate
4 = medium-heavy
5 = high/heavy
If training twice a day, include both sessions
– For example: cycling, running; 06:00, 17:30; 1hr:30min, 30min; light-medium, medium-moderate
If you have races or competitions you have to record them as well
This is all necessary, because you need to **repeat your training schedule on the days leading up to the second block of the study!**

INSTRUCTIONS FOR THE DIET DIARY

List all meals and snacks, and times that food is eaten
Include enough detail so that you can repeat each meal and snack on the days leading up to the second block of the study!

INSTRUCTIONS FOR URINE SAMPLES

Following the experiment cycling protocol, all but your first urination will need to be collected for analysis in the lab
You will be given labelled containers for collection, and bags to seal the containers inside to transport them to the lab
Please keep all samples refrigerated until you bring them to the lab

DIET DIARY

Name:		
Day of the Week:		Date:
TIME	**FOOD ITEM and METHOD of PREPARATION**	**AMOUNT EATEN**

APPENDIX J ACTIVITY AND TRAINING RECORDS

TRAINING RECORDS AND DAILY ACTIVITY

Physical Activity Record

A physical activity record over 3 days (2 weekdays and 1 weekend day) was used to estimate daily energy expenditure. This was a modification of that used previously by Bouchard and colleagues (1983) and more recently by Bratteby et al (1997) whereby records for each day were divided into 96 15-min periods and subjects were asked to write down the dominant activity over that time period. Physical Activity diaries have previously found to correlate with the more accurate doubly labelled water method, however variability is large (Bratteby, et al, 1997). Subjects were asked to record entries as close to the time of completing the activity as possible, and to be as precise as possible when describing the activity type (for example, showering, personal hygiene, seated or standing while eating meals, driving your car or passenger in car, sitting at a desk, walking about at work, etc…). Following completion of the Physical Activity Records an experienced interviewer edited the records for clarity and accuracy with the participants if required. Each activity was assigned a code from the Compendium of Physical Activity to indicate activity type and intensity in MET (a ratio of metabolic rate of activity in min·day-1 compared to resting metabolic rate). The activity scores were then summed over the entire day, and daily estimates averaged over the 3-day time period excluding intentional training and/or exercise, to get an estimate of non-intentional daily energy expenditure. Although the Compendium of Physical Activities Ainsworth, et al 2000a) was not developed specifically to determine the energy cost of daily physical activity in individuals, due to unknown differences in metabolic rate with body mass, fat mass, age, gender, efficiency of movement, altitude, and temperature they have been used for this purpose previously Ainsworth, et al, 2000b).

Daily Activity recording sheets are given over the page, and the spreadsheet of incidental daily energy expenditure and estimated energy expenditure for the controlled training are provided in the CD appendices titled ‘Daily Energy Requirement, Control Diet and Supplement Calculations’ and ‘Controlled Training Ride Calculations’ found in the back cover of this thesis.

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LUCOPRO CYCLE STUDY DAILY ACTIVITY RECORD

Instructions:

Please complete the physical activity diary below for all daily activities for two week days and one weekend day (e.g. Thursday, Friday, and Saturday), including time spent training (although specific details of your training will be recorded elsewhere). Record the main activity, (for example, showering, personal hygiene, seated or standing while eating meals, driving your car or passenger in car, sitting at a desk, walking about at work, etc…) for each 15min block over each 24h period.

SUBJECT ID: Example

Date: 1/10/2007 Start Time: 9:00am

HOUR	Physical Activity (15 min Blocks)			
1	Snack, seated	Computer	Computer	Computer
2	Walk to Lecture	Lecture	Lecture	Lecture
3	Walk to office	Dress	Training	Training
4	Training	Training	Shower	Drive home
5	Prepare dinner	Prepare dinner	Prepare dinner	Eat, seated
6	Prepare lunches	Prepare lunches	Watch TV	Watch TV
7	Snack, seated	Watch TV	Shower	Prepare for bed
8	Sleep	Sleep	Sleep	Sleep
9	Sleep	Sleep	Sleep	Sleep
10	Sleep	Sleep	Sleep	Sleep
11	Sleep	Sleep	Sleep	Sleep
12	Sleep	Sleep	Sleep	Sleep
13	Sleep	Sleep	Sleep	Sleep
14	Sleep	Sleep	Sleep	Sleep
15	Sleep	Sleep	Sleep	Sleep
16	Sleep	Sleep	Sleep	Sleep
17	Shower	Dress	Prepare breakfast	Eat, standing
18	Pack bag	Drive to Uni	Computer	Computer
19	Computer	Computer	Computer	Computer
20	Snack, seated	Computer	Computer	Computer
21	Walk to library	Photocopying	Seated, reading	Seated, reading
22	Walk to office	Lunch, seated	Lunch, seated	Lunch, seated
23	Computer	Computer	Computer	Computer
24	Walk to lab	Lab work	Lab work	Lab work

SUBJECT ID: _____

Date: _____ Start Time: _____

HOUR	Physical Activity (15 min Blocks)			
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
11				
12				
13				
14				
15				
16				
17				
18				
19				
20				
21				
22				
23				
24				

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LUCOPRO CYCLE STUDY TRAINING

TRAINING LOG

Please record all of your exercise training from the Friday before your first study training block up until you come to the laboratory for the Tuesday session. Include enough detail so that you can repeat each exercise session leading up to the second block of the study. The activity refers to the type of exercise, for example weight training, running, cycling, etc. The route indicates the course you cycled or ran over, for example Albany to Riverhead. There is room to record two exercise sessions per day, if needed. If you have races or competitions you must record them also. You will be required to replicate your training schedule leading up to the second block of the study!

SUBJECT ID ________________

Date	Time	Duration (min)	Activity Type	Route	Discipline	Intensity
					Endurance ☐ Intervals ☐ Strength ☐ Skill ☐	Light-Easy ☐ Light-Medium ☐ Medium-Moderate ☐ Medium-Heavy ☐ High-Heavy ☐
					Endurance ☐ Intervals ☐ Strength ☐ Skill ☐	Light-Easy ☐ Light-Medium ☐ Medium-Moderate ☐ Medium-Heavy ☐ High-Heavy ☐
					Endurance ☐ Intervals ☐ Strength ☐ Skill ☐	Light-Easy ☐ Light-Medium ☐ Medium-Moderate ☐ Medium-Heavy ☐ High-Heavy ☐
					Endurance ☐ Intervals ☐ Strength ☐ Skill ☐	Light-Easy ☐ Light-Medium ☐ Medium-Moderate ☐ Medium-Heavy ☐ High-Heavy ☐

TRAINING DIARY Standardized

5-point training intensity scale:
1 = light/easy
2 = light-medium
3 = medium/moderate
4 = medium-heavy
5 = high/heavy

DAY -7	Name:	Date:

Type	Time	Duration	Intensity
Type of training	Time of Day	h + min	Scale of 1-5
Comments:			

DAY -6	Date:

Type	Time	Duration	Intensity
Type of training	Time of Day	h + min	Scale of 1-5
Comments:			

DAY -5	Date:

Type	Time	Duration	Intensity
Type of training	Time of Day	h + min	Scale of 1-5
Comments:			

DAY -4	Name:	Date:

Type	Time	Duration	Intensity
Type of training	Time of Day	h + min	Scale of 1-5

Comments:			

DAY -3

Date:

Type	Time	Duration	Intensity
Type of training	Time of Day	h + min	Scale of 1-5
Comments:			

APPENDIX K TREATMENT BEVERAGES

RECIPES

Amount of emulsifier suggested (0.3-0.35% emulsifier) in relation to the total beverage.

CHO-ONLY SUPPLEMENT

Add 269.70 g maltodextrin, 127.26 g sugar, 55.74 g canola oil, 4.55 g splenda, 18.18 vanilla essence, 1100.98 g water).

Mix using the Silverson mixer and emulsifier head, mix until powders are fully mixed in solution.

Place entire mixture on the on hotplate. At 65 degrees, add 5.25g Palsgaard (0.35% total volume subtracted from amount of canola oil). When mixture has reached 75 degrees, mix again using the Silverson, at 8000rpm for 5 minutes, cool in ice bucket, and refrigerate. Makes 1500 g supplement.

LUCOPRO SUPPLEMENT

Add 82.7 2g milk protein concentrate, 50.90 g whey protein isolate, 34.09 g L-Leucine, 94.99g maltodextrin, 127.26 g sugar, 22.73 g splenda, 18.18 g vanilla essence, and 1007.99 g water.

Mix using the Silverson mixer and emulsifier head, mix until powders are fully mixed in solution.

Place 56.49 g canola oil in glass beaker on the on hotplate. At 65 degrees, add 4.5 g Palsgaard (0.3% total volume subtracted from amount of canola oil). When mixture has reached 75 degrees, add to remaining mixture and mix again using the Silverson, at 8000 rpm for 5 minutes, cool in ice bucket, and refrigerate. Makes 1500 g supplement.

SPORTS DRINK

The recommended nutrition guidelines for sports drinks during endurance activity (800mL, 0.4g CHO per hour and 1.17 NaCl per litre) have been standardized relative to "average" PPO of 360W based on the cyclists own PPO, and hence energy expenditure. Example for 75 kg male with PPO 360W for 1h ride, sports drink will provide 0.8 $L \cdot h^{-1}$ fluid, 60 $g \cdot h^{-1}$ carbohydrate, 0.4 $g \cdot L^{-1}$ citric acid, and 500-700 mg·L-1 sodium.

To make a 2000 mL serving, in a 2.5 or 3 L beaker weigh out 1858.98 g water, add 10.55 g lime juice. In smaller beakers weigh out 63.33 g maltodextrin, 63.33 g caster sugar, 0.68 g citric acid, and 3.15 g salt. Add to the water and lime, and mix with the industrial hand-held mixer. Make up 10 L and store in the sports drink Jerry can.

References

Maughan & Murray (2000) Sports drinks: Basic Science and Practical Aspects

Shi et al (1995) Effects of carbohydrate type and concentration and solution osmolarity on water absorption. Med & Sci in Sports & Exercise

Convertino et al (1996). ACSM Position Stand:Exercise and fluid Replacement. Med Sci Sports Ex; 28(10).

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

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Dairy for life

Typical Amino Acid Profile

Milk Protein Concentrates / Milk Protein Isolates

NUTRITION BULLETIN

NB. 049
Version 3.0307

The typical value is an average of representative samples taken from across the manufacturing season.

AMINO ACID	TYPICAL AMOUNT PER 100GM OF PROTEIN
ESSENTIAL AMINO ACIDS	
Isoleucine	5.3 g
Leucine	9.6 g
Lysine	8.4 g
Methionine	2.8 g
Phenylalanine	4.9 g
Threonine	4.5 g
Tryptophan	1.4 g
Valine	6.4 g
NON-ESSENTIAL AMINO ACIDS	
Histidine (essential for infants)	2.7 g
Alanine	3.3 g
Arginine	3.7 g
Aspartic acid	7.8 g
Cysteine/cystine	1.2 g
Glutamic acid	21.6 g
Glycine	1.9 g
Proline	9.8 g
Serine	5.6 g
Tyrosine	5.3 g

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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

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Typical Amino Acid Profile
Whey Protein Isolate 894

NUTRITION BULLETIN

NB 082
Version 1. 1006

The typical value is an average of representative samples taken from across the manufacturing season.

AMINO ACID	TYPICAL AMOUNT PER 100GM OF PROTEIN
ESSENTIAL AMINO ACIDS	
Isoleucine	7.2 g
Leucine	11.3 g
Lysine	10.3 g
Methionine	2.4 g
Phenylalanine	3.3 g
Threonine	7.4 g
Tryptophan	1.9 g
Valine	6.4 g
NON-ESSENTIAL AMINO ACIDS	
Histidine (essential for infants)	1.9 g
Alanine	5.6 g
Arginine	2.4 g
Aspartic acid	11.3 g
Cysteine/cystine	2.9 g
Glutamic acid	19.0 g
Glycine	1.9 g
Proline	6.4 g
Serine	5.1 g
Tyrosine	3.4 g

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ACROS ORGANICS

Product Specifications

Product 12512
L-Leucine,99%

General Product Data

Version	2
CAS No	61-90-5
Molecular weight	131.17
Molecular formula	C6 H13 N O2
Linear formula	

Product Specifications

Appearance	white crystals or crystalline powder
Infrared spectrometry	authentic
Assay Total base	98.5 to 101.5 %
Loss on drying	<0.2 % (105°C, 3 hrs)
Heavy metals (as Pb)	<10 ppm
Sulfated ash	<0.1 %
Other amino acids	<0.5 % (TLC)
Specific optical rotation	+14.9° to +16° (20°C, 589 nm) (c=4, 6 N HCl)
Trace analysis	Type: Cl measure < 200 ppm Type: SO4 measure < 200 ppm Type: NH4 measure < 200 ppm Type: Fe measure < 10 ppm
Transmittance	>95 % (c=10, 2 N HCl, 430 nm) 1 cm cell
pH	5.5 to 6.5 (2.5% aq. soln. at 25°C)

Research Study formula Protein version					B batch		
29/07/2006							
Vanilla	Amount per		amount per unit		Carbs	Protein	Fat
500 ml (16.5 oz)	serving		**50.00**				
Water	500.00	ml					
Maltodextrin	44.51	gm	2225.50	gm	44.51		
Fructose	40.00	gm	2000.00	gm	40.00		
Canola oil dry	21.30	gm	1065.00	gm	3.23	2.16	14.48
MPC 80	19.81	gm	990.50	gm	1.09	15.85	0.40
Whey Isolate	13.33	gm	666.65	gm	0.07	12.00	0.13
Vanilla 442	1.00	gm	50.00	gm	1.00		
Salt	0.22	gm	10.75	gm			
Total	**140.17**	**gm**	**7008.40**	lb	89.89	30.00	15.01
			3181813.60				

Research Study formula NO Protein version					A Batch		
29/07/2006							
Vanilla	Amount per		amount per unit		Carbs	Protein	Fat
500 ml (16.5 oz)	serving		**50.00**				
Water	500.00	ml					
Maltodextrin	76.66	gm	3833.00	gm	76.66		
Fructose	40.00	gm	2000.00	gm	40.00		
Canola oil dry	22.08	gm	1103.90	gm	3.34	2.23	15.01
MPC 80	0.00	gm	0.00	gm	0.00	0.00	0.00
Whey Isolate	0.00	gm	0.00	gm	0.00	0.00	0.00
Vanilla 442	1.00	gm	50.00	gm	1.00		
Salt	0.22	gm	10.75	gm			
Total	**139.95**	**gm**	**6997.65**	lb	121.00	2.23	15.01
			3176933.10				

APPENDIX L EXERCISE PROTOCOLS

CONTROL TRAINING

Each training ride protocol was pre-programmed into the ergometer software and intensities were based on peak power (W_{max}) established during preliminary testing. Subjects were provided with a choice of their own DVD or music compilations, and conditions replicated during the second block of the study. Cycling training rides were modified from a previous study (Rowlands, et al. 2008) following pilot trials, and were categorized as very hard, moderate, and hard loading intensities aimed to provide a demanding but achievable block of training for most cyclists.

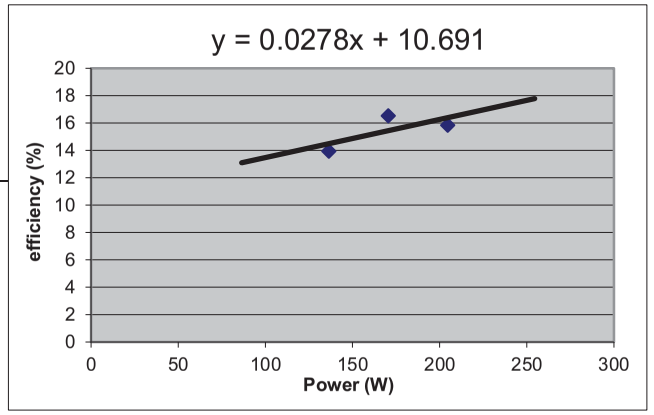
Day -4 cycling protocol consisted of a warm-up period of 15 min at 30%, 10 min at 40%, and 5 min at 50% W_{max}; then 5 blocks of 5 x 2 min intervals (1 x 90%, 2 x 80%, and 2 x 70%) interspersed with 2 min recovery intervals at 50%, and each block separated by 5 min recovery at 50% W_{max}; followed by 10 min cool-down at 30% W_{max}, for a total of 150 min cycling.

Day -3 cycling protocol consisted of a warm-up period of 15 min at 30%, 10 min at 40%, and 5 min at 50% W_{max}; then 8 x 5 min intervals at 70% interspersed with 5 min recovery intervals at 50%; followed by 10 min cool-down at 30% W_{max}, for a total of 120 min cycling.

Day -2 cycling protocol consisted of a warm-up period of 15 min at 30%, 10 min at 40%, and 10 min at 50% W_{max}; 5 blocks of 5 x 1 min intervals (2 x 90%, 2 x 80%, and 1 x 70%) interspersed with 2 min recovery intervals at 40%, and each block separated by 5 min recovery at 40% W_{max}; followed by 10 min cool-down at 40% W_{max}, for a total of 130 min cycling.

Record sheets taken during each of the training days are given over the page. The spreadsheet version of these training ride metabolic calculations and further calculations of mass change due to substrate use and water loss (as in Maughan et al, 2007) can be found on the CD appendices

LUCOPRO CYCLE STUDY TRAINING RIDE Day -4



METABOLIC CALCULATIONS

Subject _____ Condition _____ Date _____ Start Time _____

Pre BW (kg) _____ Post BW (kg) _____ Finish Time _____

Ext E % PPO	Int E kJ/min	Ext E kJ/min	Effic %	Power W
40% PPO	58.7	8.18	13.94	136.4
50% PPO	61.9	10.23	16.53	170.5
60% PPO	77.5	12.28	15.84	204.6
DAY -4 HARD-VERY HARD DAY				
PPO		341		5.3541

Stage	% max	Power (W)	Time (s)	Time (min)	Time	Ext Work (kJ)	Adj Int Work (kJ)	Efficiency Factor	RPE	Overall Tiredness	Leg Tiredness	Muscle Soreness	Leg Strength
1	0.3	102	900	15	15	92.07	680.24	13.53494					
2	0.4	136	600	10	25	81.84	565.08	14.48292					
3	0.5	171	300	5	30	51.15	331.48	15.4309					
4	0.9	307	120	2	32	36.83	191.58	19.22282					
5	0.5	171	120	2	34	20.46	132.59	15.4309					
6	0.9	307	120	2	36	36.83	191.58	19.22282					

7	0.5	171	120	2	38	20.46	132.59	15.4309						
8	0.9	307	120	2	40	36.83	191.58	19.22282						
9	0.5	171	120	2	42	20.46	132.59	15.4309						
10	0.9	307	120	2	44	36.83	191.58	19.22282						
11	0.5	171	120	2	46	20.46	132.59	15.4309						
12	0.9	307	120	2	48	36.83	191.58	19.22282						
13	0.5	171	300	5	53	51.15	331.48	15.4309						
14	0.8	273	120	2	55	32.74	179.13	18.27484						
15	0.5	171	120	2	57	20.46	132.59	15.4309						
16	0.8	273	120	2	59	32.74	179.13	18.27484						
17	0.5	171	120	2	61	20.46	132.59	15.4309						
18	0.8	273	120	2	63	32.74	179.13	18.27484						
19	0.5	171	120	2	65	20.46	132.59	15.4309						
20	0.8	273	120	2	67	32.74	179.13	18.27484						
21	0.5	171	120	2	69	20.46	132.59	15.4309						
22	0.8	273	120	2	71	32.74	179.13	18.27484						
23	0.5	171	300	5	76	51.15	331.48	15.4309						
24	0.8	273	120	2	78	32.74	179.13	18.27484						
25	0.5	171	120	2	80	20.46	132.59	15.4309						
26	0.8	273	120	2	82	32.74	179.13	18.27484						
27	0.5	171	120	2	84	20.46	132.59	15.4309						
28	0.8	273	120	2	86	32.74	179.13	18.27484						
29	0.5	171	120	2	88	20.46	132.59	15.4309						
30	0.8	273	120	2	90	32.74	179.13	18.27484						
31	0.5	171	120	2	92	20.46	132.59	15.4309						
32	0.8	273	120	2	94	32.74	179.13	18.27484						

33	0.5	171	300	5	99	51.15	331.48	15.4309						
34	0.7	239	120	2	101	28.64	165.32	17.32686						
35	0.5	171	120	2	103	20.46	132.59	15.4309						
36	0.77	263	120	2	105	31.51	175.14	17.990446						
37	0.5	171	120	2	107	20.46	132.59	15.4309						
38	0.7	239	120	2	109	28.64	165.32	17.32686						
39	0.5	171	120	2	111	20.46	132.59	15.4309						
40	0.7	239	120	2	113	28.64	165.32	17.32686						
41	0.5	171	120	2	115	20.46	132.59	15.4309						
42	0.7	239	120	2	117	28.64	165.32	17.32686						
43	0.5	171	300	5	122	51.15	331.48	15.4309						
44	0.7	239	120	2	124	28.64	165.32	17.32686						
45	0.5	171	120	2	126	20.46	132.59	15.4309						
46	0.7	239	120	2	128	28.64	165.32	17.32686						
47	0.5	171	120	2	130	20.46	132.59	15.4309						
48	0.7	239	120	2	132	28.64	165.32	17.32686						
49	0.5	171	120	2	134	20.46	132.59	15.4309						
50	0.7	239	120	2	136	28.64	165.32	17.32686						
51	0.5	171	120	2	138	20.46	132.59	15.4309						
52	0.7	239	120	2	140	28.64	165.32	17.32686						
53	0.3	102	600	10	150	61.38	453.49	13.53494						
							10420.24							

CYCLE STUDY TRAINING RIDE Day -3

Subject _____ Condition _____ Date _____ Start Time _____

Pre BW (kg) _____ Post BW (kg) _____ Fluid Req _____

DAY -3 MODERATE DAY

PPO 341

Stage	% max	Power (W)	Time (s)	Time (min)	Time	Ext Work (kJ)	Adj Int Work (kJ)	Efficiency Factor	RPE	Overall Tiredness	Leg Tiredness	Muscle Soreness	Leg Strength
1	0.3	102	900	15	15	92.07	680.24	13.53494					
2	0.4	136	600	10	25	81.84	565.08	14.48292					
3	0.5	171	300	5	30	51.15	331.48	15.4309					
4	0.7	239	300	5	35	71.61	413.29	17.32686					
5	0.5	171	300	5	40	51.15	331.48	15.4309					
6	0.7	239	300	5	45	71.61	413.29	17.32686					
7	0.5	171	300	5	50	51.15	331.48	15.4309					
8	0.7	239	300	5	55	71.61	413.29	17.32686					
9	0.5	171	300	5	60	51.15	331.48	15.4309					
10	0.7	239	300	5	65	71.61	413.29	17.32686					
11	0.5	171	300	5	70	51.15	331.48	15.4309					
12	0.7	239	300	5	75	71.61	413.29	17.32686					
13	0.5	171	300	5	80	51.15	331.48	15.4309					
14	0.7	239	300	5	85	71.61	413.29	17.32686					
15	0.5	171	300	5	90	51.15	331.48	15.4309					
16	0.7	239	300	5	95	71.61	413.29	17.32686					
17	0.5	171	300	5	100	51.15	331.48	15.4309					
18	0.7	239	300	5	105	71.61	413.29	17.32686					
19	0.5	171	300	5	110	51.15	331.48	15.4309					

20	0.3	102	600	10	120	61.38	453.49	13.53494	
							7988.42		

CYCLE STUDY TRAINING RIDE Day -2

Subject _____ Condition _____ Date _____ Start Time _____

Pre BW (kg) _____ Post BW (kg) _____ Fluid Req _____

DAY -2 HARD DAY

PPO 341

Stage	% max	Power (W)	Time (s)	Time (min)	Time	Ext Work (kJ)	Adj Int Work (kJ)	Efficiency Factor	RPE	Overall Tiredness	Leg Tiredness	Muscle Soreness	Leg Strength
1	0.3	102	900	15	15	92.07	680.24	13.53494					
2	0.4	136	600	10	25	81.84	565.08	14.48292					
3	0.5	171	600	10	35	102.30	662.96	15.4309					
4	0.9	307	60	1	36	18.41	95.79	19.22282					
5	0.4	136	120	2	38	16.37	113.02	14.48292					
6	0.9	307	60	1	39	18.41	95.79	19.22282					
7	0.4	136	120	2	41	16.37	113.02	14.48292					
8	0.9	307	60	1	42	18.41	95.79	19.22282					
9	0.4	136	120	2	44	16.37	113.02	14.48292					
10	0.9	307	60	1	45	18.41	95.79	19.22282					
11	0.4	136	120	2	47	16.37	113.02	14.48292					
12	0.9	307	60	1	48	18.41	95.79	19.22282					
13	0.4	136	300	5	53	40.92	282.54	14.48292					
14	0.9	307	60	1	54	18.41	95.79	19.22282					
15	0.4	136	120	2	56	16.37	113.02	14.48292					
16	0.9	307	60	1	57	18.41	95.79	19.22282					
17	0.4	136	120	2	59	16.37	113.02	14.48292					
18	0.9	307	60	1	60	18.41	95.79	19.22282					
19	0.4	136	120	2	62	16.37	113.02	14.48292					

20	0.9	307	60	1	63	18.41	95.79	19.22282						
21	0.4	136	120	2	65	16.37	113.02	14.48292						
22	0.9	307	60	1	66	18.41	95.79	19.22282						
23	0.4	136	300	5	71	40.92	282.54	14.48292						
24	0.8	273	60	1	72	16.37	89.57	18.27484						
25	0.4	136	120	2	74	16.37	113.02	14.48292						
26	0.8	273	60	1	75	16.37	89.57	18.27484						
27	0.4	136	120	2	77	16.37	113.02	14.48292						
28	0.8	273	60	1	78	16.37	89.57	18.27484						
29	0.4	136	120	2	80	16.37	113.02	14.48292						
30	0.8	273	60	1	81	16.37	89.57	18.27484						
31	0.4	136	120	2	83	16.37	113.02	14.48292						
32	0.8	273	60	1	84	16.37	89.57	18.27484						
33	0.4	136	300	5	89	40.92	282.54	14.48292						
34	0.8	273	60	1	90	16.37	89.57	18.27484						
35	0.4	136	120	2	92	16.37	113.02	14.48292						
36	0.8	273	60	1	93	16.37	89.57	18.27484						
37	0.4	136	120	2	95	16.37	113.02	14.48292						
38	0.8	273	60	1	96	16.37	89.57	18.27484						
39	0.4	136	120	2	98	16.37	113.02	14.48292						
40	0.8	273	60	1	99	16.37	89.57	18.27484						
41	0.4	136	120	2	101	16.37	113.02	14.48292						
42	0.8	273	60	1	102	16.37	89.57	18.27484						
43	0.4	136	300	5	107	40.92	282.54	14.48292						
44	0.7	239	60	1	108	14.32	82.66	17.32686						
45	0.4	136	120	2	110	16.37	113.02	14.48292						

46	0.7	239	60	1	111	14.32	82.66	17.32686						
47	0.4	136	120	2	113	16.37	113.02	14.48292						
48	0.7	239	60	1	114	14.32	82.66	17.32686						
49	0.4	136	120	2	116	16.37	113.02	14.48292						
50	0.7	239	60	1	117	14.32	82.66	17.32686						
51	0.4	136	120	2	119	16.37	113.02	14.48292						
52	0.7	239	60	1	120	14.32	82.66	17.32686						
53	0.4	136	600	10	130	81.84	565.08	14.48292						

STANDARDIZING RIDE

BEFOREHAND

Schedule for the evening around 5pm

Day Before:

Purchase fresh food

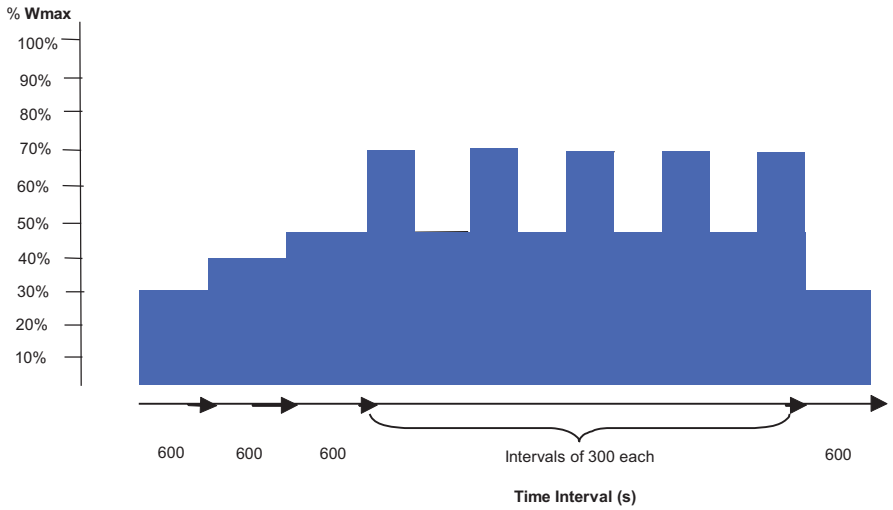
Measure out food for evening and following day

PROCEDURE

Switch on MOXUS ½ an hour before use

Calibrate

Standardizing Ride (Visit Three)



APPENDIX M EXPERIMENT DAY

Massey University Institute of Food Nutrition & Human Health Te Kunenga ki Pūrehuroa

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LUCOPRO CYCLE STUDY PERFORMANCE TEST

REPEATED SPRINT PERFORMANCE TEST

All exercise tests will be performed in the same environmental conditions of 20°C, 50-60% humidity, and cyclists will be cooled with fans to minimize thermal distress. The exercise-performance test consists of a 30-min warm-up of 40% W_{max} for 15-min then 50% for 15-min. The warm-up will be performed on the same cycle as used for the training rides (Lode or Velotron).

The performance test following the warm-up will consist of 10 sprints alternated with 10 recovery periods at 40% W_{max}. The external work done in the sprints and recovery periods are based on kcal required to complete 0.125 x W_{max} i.e. if W_{max} is 408 W then kcal required during each sprint and recovery period is 51kcal. This will take the cyclists approximately 2–3 min to complete each sprint at around 100% W_{max} and allow approximately 5–6 min recovery.

The performance test will be performed in the simulated-gears mode of the Velotron. Gearings of a 39- or 48-tooth front chain ring and a 10-sprocket, 21- to 11-tooth rear cluster programmed into the Velotron software. The ergometer has been modified with an up-or-down switch positioned on the front of the right handlebar brake hood for gear changing. **To change gear hold the switch up or down for 2 seconds to move the front chain rings, but short clicks up or down will move the back gear rings.** Cyclists may self select cadence and gearing, but should sprint as fast as possible until required work done, displayed in calories is completed, this will be timed. No verbal encouragement is permitted, except for countdown for start and finish of each sprint (20, 10, 5, 4, 3, 2, 1 calories to go).

Cyclists are not permitted to see power output or heart rates during sprints; laptop screen will be covered at these times, except for expended calories. <u>Stress to the subject that the only feedback they will receive is calorie count down. However we are able to tell them number of sprints to go, gears or gearing.</u>

Accurately time the sprints. During recovery collect Psychometric scale and RPE data (following sprints 2, 4, 6, 8, and 10). Time the entire trial. Push F1 to stop the trial, and FN and F1 together to save. Down load the gearing and power outputs from Velotron software. Download average heart rates from Polar watch.

EQUIPMENT

Warm or cool room to 20°C
Open or close doors, windows, and use fan to get humidity to 40-50%
Lap-top & Screen Cover
Velotron and/or Lode cycle specifications
Fan
Pressure Algometer
GRS Scales
RPE Scales (from lab)
Towel
Blood collection (tubes, needles, phlebotomist, ice bucket, ice, protocol)
Sports Drink (give 1 bottle at warm-up, the next prior to sprints 1-3, another prior to sprints 4-6, and the final drink prior to sprints 7-10)
Breakfast
2 x Stopwatches
Polar Watch (S610i) and Strap (check watch has sufficient memory).

PERFORMANCE TEST

Breakfast consists of 2 slices of white toast, 1 x 10g margarine, 2 x 14g jam, and 200mL sports drink, 1h prior to performance test.
Muscle Soreness Scale (performance test), Pressure Algometer (performance test & Familiarization), POMS (performance test), DALDA (performance test), then Blood Collection (performance test).
Ceral (Kellog's LCM) bar and 100mL water 15 minutes (timed) before starting warm-up.
Provide calculated amount of sports drink for warm-up, and calculated amount of sports drink for performance test (divided evenly into thirds, first bottle for the 1st-3rd sprint, second bottle for 3rd-6th sprint, and final bottle 6th-10th sprint).
Warm-up on the Lode or Velotron depending on which cycle erg was used for the training sessions.
Switch Velotron cycle ergometer off and then back on just before loading program. Always ask cyclist if the set power (W) feels as it should. Watch the blue arrow indicating cadence, moves when it should.
To use the Velotron select Start → Charts → Ergo Courses → Performance Test_Warm-up → Open → Press F1
Once the warm-up is finished allow the cyclist to stretch (this must be standardised between trials – if the cyclist stretches, tell, them to repeat stretches – no heavy static stretches required), and then get them onto the Velotron for the performance test.
Source → New → Create New User → Enter subject name and BW from day of familiarization test (important for calorie calculations) → OK → XX0x.vdf → Save → Yes → OK
Select Start → Charts → Courses →PC1 → Performance Test_Test → Open → **Press F1, start timer, and start polar watch at the same time.**
The cyclist may select their own gears, and must keep to 40%PPO (W) for set number of calories (calculated as 0.125 x W_{max}). Give them the countdown from 20, 10, 5, 4, 3, 2, 1, to go, GO! Immediately start the second timer to time the sprint.

Cover the computer screen during sprints. The cyclist must sprint for the same calories.

The cyclist should be changing gears a lot, breathing hard, and RPE should be near maximum each sprint.

Give them the countdown from 20, 10, 5, 4, 3, 2, 1, to go, RECOVERY! Immediately stopping the timer, write this value down to the 10th of the second, reset, ready for next sprint.

Show the cyclist the RPE scale immediately following the sprint, GRS scales following the 2nd, 4th, 6th, 8th, and last sprints.

Immediately stop both timers following the last sprint.

To stop the Velotron program press F1 → Fn & F1 at the same time → Save as → XX0x_testx → OK

To Export go to File → Export Options → select the outcome variables you want (Time, Speed, Power, RPM, HR, GEAR, Front Teeth, Rear Teeth, Comma Delimited, Toss = 166 (every 5 seconds) → OK → File → Export → select the file you want → Open → OK → open up Excel → File → Open → change Files of Type to 'All Files' → My Computer → Local Disk (C:) → Program Files → Velotron CS 1.5 → Perf Files → select the file you want → select Delimited → Next → Select Semicolon, Comma, Space → Next → Finish.

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LUCOPRO CYCLE STUDY PERFORMANCE TEST

Subject _____ Cond _____ BW (kg) _____ Date/Day _____ Time _____ BW _____

Start Temperature _____ Finish Temperature _____ Barometric Pressure _____

Start Humidity _____ Finish Humidity _____

Modeled on PPO _____ Factor 0.125 kcal 55 work @ 25% efficiency (kcal)

Stage	% PPO	Power (W)	Work (kcal)	Time	RPE	Overall Tiredness	Leg Tiredness	Musscle Soreness	Leg Strength
2nd sports drink									
1	0.4	176	55						
2	max	max	110						
3	0.4	176	165						
4	max	max	221						
5	0.4	176	276						
6	max	max	331						
3rd sports drink									
7	0.4	176	386						
8	max	max	441						
9	0.4	176	496						
10	max	max	551						
11	0.4	176	606						
12	max	max	662						
4th sports drink									
13	0.4	176	717						
14	max	max	772						
15	0.4	176	827						
16	max	max	882						
17	0.4	176	937						
18	max	max	992						
19	0.4	176	1047						
20	max	max	1103						
	Total Time (min:s)								

Recovery 40% PPO 176.4

SPRINT		START CALORIES	FINISH CALORIES
1	MAXIMAL	55	110
2	MAXIMAL	165	221
3	MAXIMAL	276	331
4	MAXIMAL	386	441
5	MAXIMAL	496	551
6	MAXIMAL	606	662
7	MAXIMAL	717	772
8	MAXIMAL	827	882
9	MAXIMAL	937	992
10	MAXIMAL	1047	1103

	RECOVERY		SPRINT
Gearing	_____	Gearing	_____
Gear	_____	Gear	_____

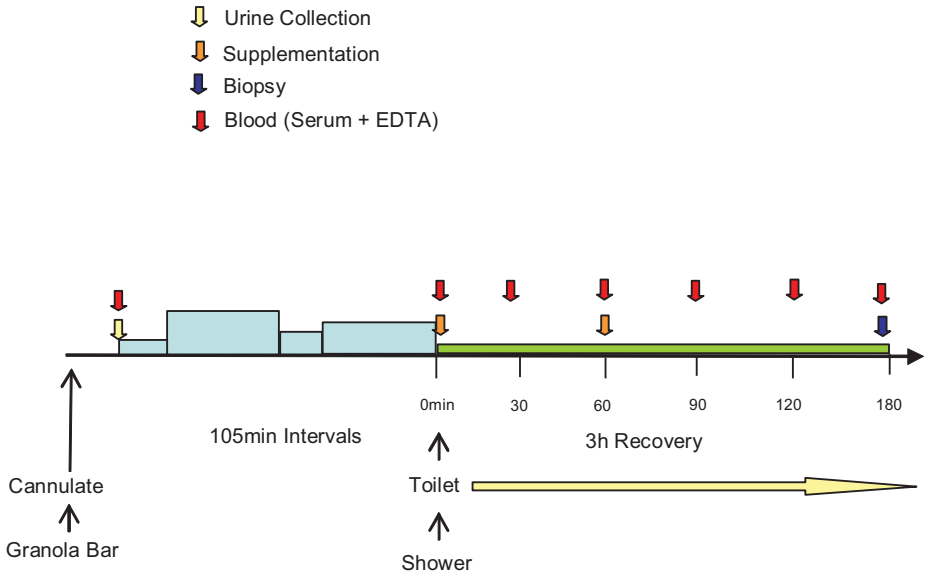
EXPERIMENT DAY

BEFOREHAND

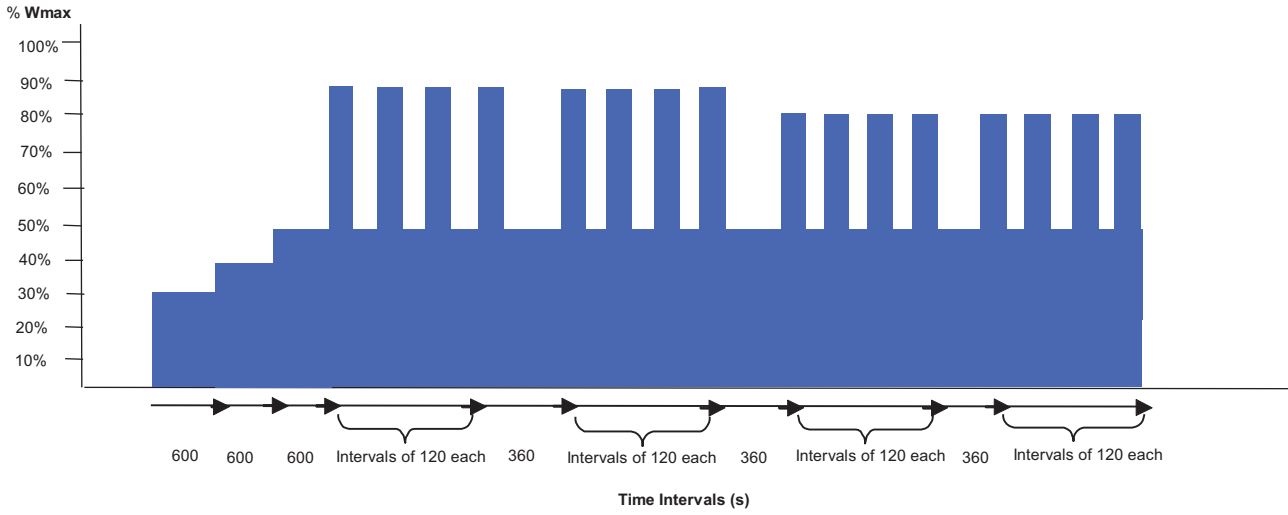
Day Before:
Biopsy Tray
Blood Tray
Day 1, Day 2 Food
Biopsy eppindorfs, EDTA, serum, urine tubes
Measure 10mL 10M HCl into 22 and 24h urine containers, and 5mL of 10M HCl into spot urine container preservative (preserve urea). Bag and label urine spot, 22h, and 24h containers
Sports Drink (800mL/h for 75kg male)
15Nglycine added to supplement 1 (2mg/kg)
Supplements 2, 3, and 4 (140g serving of supplement for 75 kg male)
Morning of:
Liquid Nitrogen
EM ice and gluteraldehyde
Set up bikes for each subject

PROCEDURE

Before Exercise:
Granola Bar
Spot Urine
Cannulate
Fast EDTA, Serum
During Exercise:
Provide sports drink
As soon as final interval is finished and subject is off the bike, start timing recovery period.
Recovery Period:
Toilet
Provide Supplement 1 at time 0
Shower
Provide supplement 2 at time 60
Time 0, 30, 60, 90, 120, 180 EDTA, and Serum samples
Time 180 Biopsy
Begin 22h urine collection up to & including first urination of Day 2
Provide Day 1, Day 2 food, and supplements 3, and 4
Subway for Day 2
After Experiment:
Measure and record volume of spot urine, decant into urine tubes, discard remaining into toilet. Wash spot urine containers with bleach and several rinses with water
EDTA goes on ice, and is spun immediately
Serum sits for ½ h at room temperature, and then is spun down
Clean up used bottles and food containers in kitchen



Experiment Ride (Visit Four)



APPENDIX N PSYCHOMETRIC QUESTIONNAIRES AND SCALES

Massey University Institute of Food Nutrition & Human Health Te Kunenga ki Pūrehuroa

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LUCOPRO CYCLE STUDY POMS

SUBJECT ID _____ BLOCK/BEVERAGE _____

DATE _____ TIME _____ DAY _____

PROFILE OF MOOD STATES-SHORT FORM (POMS-40)

Refer to the definitions below. Consider how you are feeling right now, when CIRCLING the appropriate response beside each item. Please check to make sure you have responded to all the items.

FATIGUE	NOT AT ALL	A LITTLE	MODERATELY	QUITE A BIT	EXTREMELY
Worn Out	0	1	2	3	4
Weary	0	1	2	3	4
Bushed	0	1	2	3	4
Fatigued	0	1	2	3	4
Exhausted	0	1	2	3	4

ANGER	NOT AT ALL	A LITTLE	MODERATELY	QUITE A BIT	EXTREMELY
Peeved	0	1	2	3	4
Bitter	0	1	2	3	4
Resentful	0	1	2	3	4
Grouchy	0	1	2	3	4
Angry	0	1	2	3	4
Furious	0	1	2	3	4
Annoyed	0	1	2	3	4

VIGOR	NOT AT ALL	A LITTLE	MODERATELY	QUITE A BIT	EXTREMELY
Cheerful	0	1	2	3	4
Powerful	0	1	2	3	4
Full of Pep	0	1	2	3	4
Active	0	1	2	3	4
Energetic	0	1	2	3	4
Lively	0	1	2	3	4

TENSION	NOT AT ALL	A LITTLE	MODERATELY	QUITE A BIT	EXTREMELY
Restless	0	1	2	3	4
Nervous	0	1	2	3	4
On-edge	0	1	2	3	4
Tense	0	1	2	3	4
Uneasy	0	1	2	3	4
Anxious	0	1	2	3	4

ESTEEM	NOT AT ALL	A LITTLE	MODERATELY	QUITE A BIT	EXTREMELY
Embarrassed	0	1	2	3	4
Ashamed	0	1	2	3	4
Proud	0	1	2	3	4
Competent	0	1	2	3	4
Satisfied	0	1	2	3	4

CONFUSION	NOT AT ALL	A LITTLE	MODERATELY	QUITE A BIT	EXTREMELY
Bewildered	0	1	2	3	4
Forgetful	0	1	2	3	4
Confused	0	1	2	3	4
Unable to concentrate	0	1	2	3	4
Uncertain about things	0	1	2	3	4

DEPRESSION	NOT AT ALL	A LITTLE	MODERATELY	QUITE A BIT	EXTREMELY
Hopeless	0	1	2	3	4
Helpless	0	1	2	3	4
Sad	0	1	2	3	4
Worthless	0	1	2	3	4
Miserable	0	1	2	3	4
Discouraged	0	1	2	3	4

Grove, J.R., Prapavessis, H. Preliminary evidence for the reliability and validity of an abbreviated Profile of Mood States. International Journal of Sport Psychology. 1992 Apr-Jun Vol 23(2) 93-109.

Halson, S. L. Bridge, M. W., Meeusen, R.,Busschaert, B., Dleeson, M., Jones, D. A., and Jeukendrup, A. E. Time course of performance changes and fatigue markers during intensified training in trained cyclists. J Appl Physiol 93: 947–956, 2002.

Morgan W. P., Brown D. R., Raglin, J. S., O'Connor, P. J., and Ellickson K. A. Psychological
monitoring of overtraining and staleness. Br J Sports Med 21: 107–114, 1987.

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LUCOPRO CYCLE STUDY GRAPHIC RATING SCALES (GRS)

INSTRUCTIONS

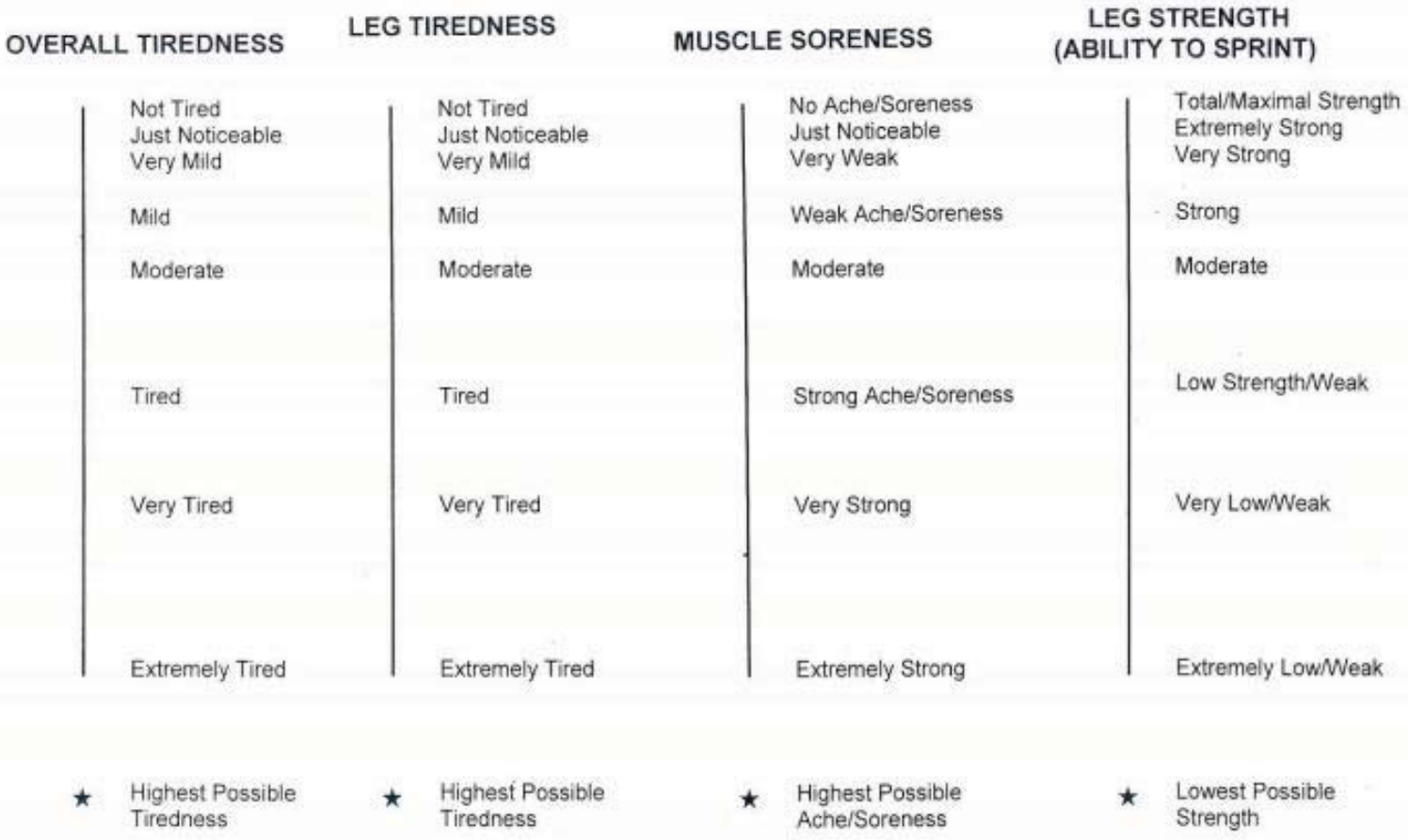
The scale measures from nothing at all (no tiredness, muscle ache/soreness, strength) to absolute maximum (tiredness, muscle ache/soreness, strength). Extremely tired, strong represents the strongest feeling the subject has ever experienced. However it is possible that the subject is experiencing a magnitude stronger than he has ever experienced before, represented by absolute maximum (★). Extremely mild, weak, low indicates the feeling is only just noticeable, on the boundary of just being able to be perceived.

Ask the subject how they felt during the sprint interval just completed

Ask the subject to first read the descriptions, and then to mark on the line with a fine-tip white-board marker pen where their feelings of fatigue, limb tiredness, muscle ache and strength is in relation to the scales. They may choose anywhere on the line, if their perception is halfway or partway between the descriptions.

Reinforce to your subject that they should answer as honestly as possible, not to over or underestimate the intensities; they must answer how they feel, not how they or we think they should feel. Remind them to start looking at the description, and then to mark a point on the line.

Ask questions in order. Give subject an example such as how sour do you think a lemon tastes? How sweet is a ripe banana?


OVERALL TIREDNESS
Not Tired
Just Noticeable
Very Mild
Mild
Moderate
Tired
Very Tired
Extremely Tired
★ Highest Possible Tiredness
LEG TIREDNESS
Not Tired
Just Noticeable
Very Mild
Mild
Moderate
Tired
Very Tired
Extremely Tired
★ Highest Possible Tiredness
MUSCLE SORENESS
No Ache/Soreness
Just Noticeable
Very Weak
Weak Ache/Soreness
Moderate
Strong Ache/Soreness
Very Strong
Extremely Strong
★ Highest Possible Ache/Soreness
LEG STRENGTH (ABILITY TO SPRINT)
Total/Maximal Strength
Extremely Strong
Very Strong
Strong
Moderate
Low Strength/Weak
Very Low/Weak
Extremely Low/Weak
★ Lowest Possible Strength

APPENDIX O MUSCLE SORENESS

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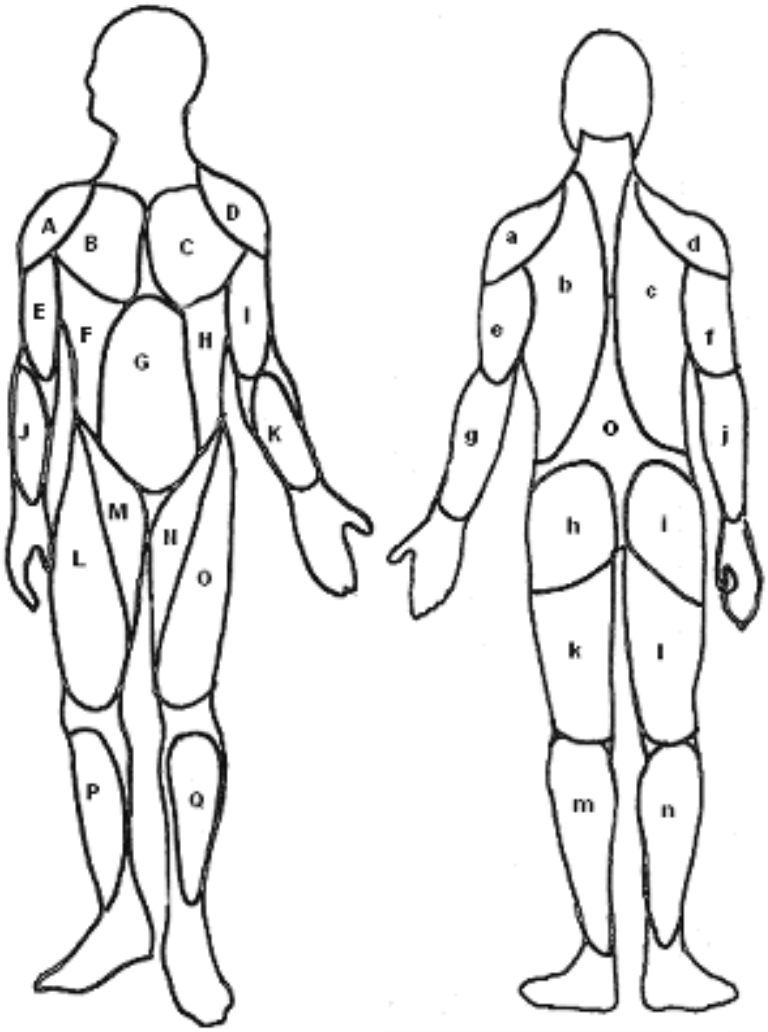
LUCOPRO CYCLE STUDY MUSCLE SORENESS

SUBJECT ID _____ TIME _____ DATE _____

BLOCK/BEVERAGE _____ DAY _____

LOCATION OF SORENESS

Using the pen provided please circle the letter representing the parts of the body where you feel muscle soreness.

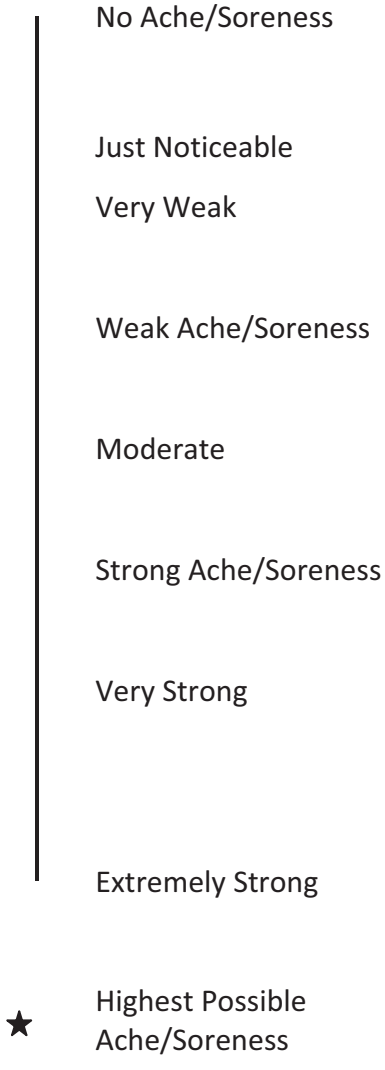


Researcher Use															
A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q
a	b	c	d	e	f	g	h	i	j	k	l	m	n	o	Other	Other

RATINGS OF SORENESS

Please rate the intensity of muscle ache or soreness by marking anywhere on the line (at or between the descriptors) on the scale below, of each body part circled over page (A to Q and a to n).

MUSCLE SORENESS



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LUCOPRO CYCLE STUDY MUSCLE SORENESS

SUBJECT ID _____ VISIT _____ DATE _____

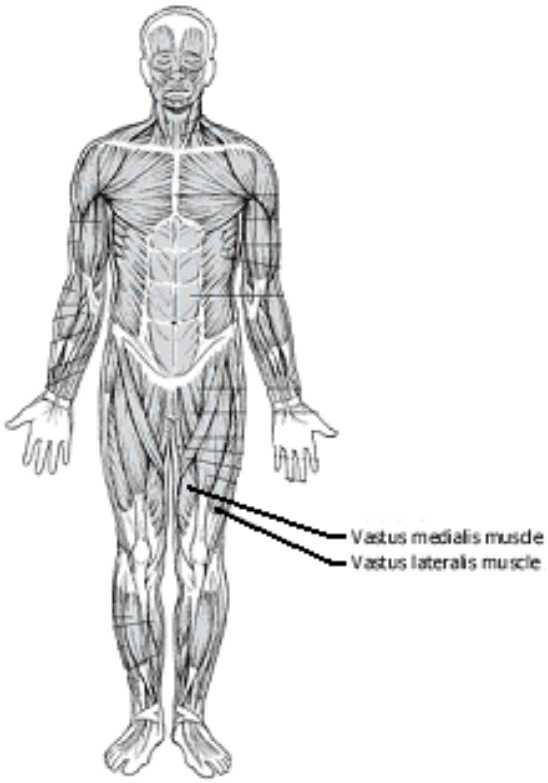
Quantification of Muscle Soreness (Pressure Algometer)

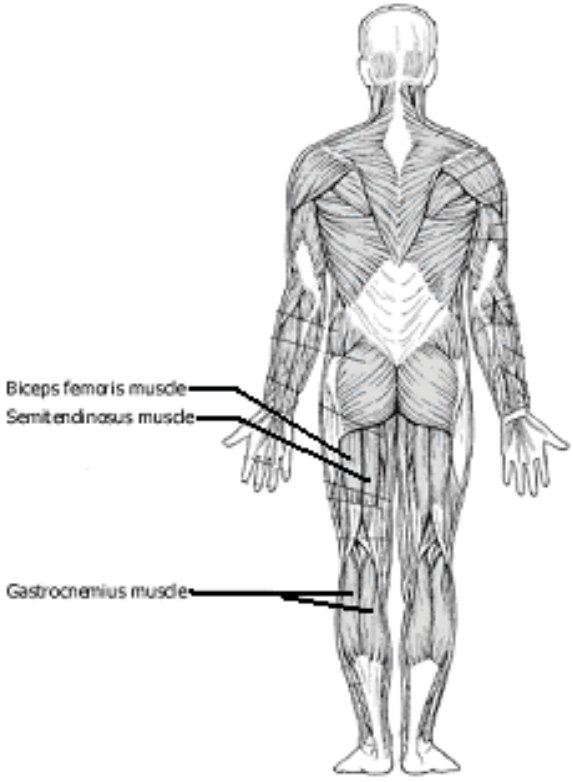
The muscle belly of the Vastus lateralis, medial aspect of the Vastus medialis, Belly of Biceps femoris, and medial and lateral heads of gastrocnemium muscles are located via palpation and marked with a permanent marker pen.

While subject is lying prone on the massage table, apply the pressure algometer to each marked site (both sides of the body) two times (and a third taken if the first two are not within 10%) with 10-s break between. Apply force at a rate of 1kg/cm2 per second. Instruct the subject to tell you when the pressure starts to become uncomfortable/pain by saying “yes”; record that force. If no discomfort is indicated up to a force of 11kg/cm2, then conclude that no soreness is present.

Average the readings epresentative of pain threshold for each muscle (take the mean of values between 3.0 and 11kg [device sensitivity is 3kg and upper limit cut-off of 11kg due to experimentally induced tenderness thought to be caused above this pressure value]). Total Pressure Pain Threshold (PPT) is summed soreness pressures divided by number of sites with soreness (no soreness present if force required exceeds 11kg).

Subjects are advised not to take pain medication to alleviate muscle soreness should it occur.





MUSCLE SITE	FORCE (kg)						
	Right Side			Left side			Av
Vastus lateralis (20 cm proximal to patella)							
Vastus Medialis (medial aspect)							
Biceps Femoris (middle of hamstrings)							
Gastronemius medial head							
Gastromemius lateral head							
Pain Threshold							

Reference:
Nussbaum, E. T., & DownesL. (1998). Reliability of clinical pressure-pain algometric measurements obtained on consecutive days. Physical therapy 78: 160-169.
Fischer, A. A. (1987). Pressure algometry over normal muscles. Standard values, validity and reproducibility of pressure threshold. Pain 30, 115-126.
Ohrbach R., & Gale, E. N. (1989). Pressure pain thresholds in normal muscles: reliability, measurement effects, and topographic differences. Pain 37, 257-263.

APPENDIX P BIOLOGICAL SAMPLE COLLECTION

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LUCOPRO CYCLE STUDY URINE COLLECTION

PREPARATION

Add 15mL 6M HCl into the 4L urine collection containers, affix warning labels to the bottle as potentially corrosive, and advise the subject not to pour this out. To make the 6M HCl used for the urine sample, follow directions below:

Concentrated HCl 36% is 11.64M
M1V1 = M2 x V2
V1 = M2 x V2 / M1
V1 = 6 x 250 / 11.64
V1 = 129 mL
i.e. pipette (not by mouth) 129mL and make up to volume in 250 mL volumetric flask. Add the HCl slowly to about 100 mL, then make up to volume.

Concentrated HCl 38% is 12.39M
M1V1 = M2 x V2
V1 = M2 x V2 / M1
V1 = 6 x 250 / 12.39
V1 = 121 mL

TIMED 24-H URINE COLLECTION

Avoid alcoholic beverages, vitamins, and other medications (if possible) for at least 24h prior to starting.

Once the training ride is finished ask subjects to urinate in the toilet and flush this urine.
Label the container with subject ID, date, and start time. Collect all urine, day and night, in the collection container, for the next 24h.
Urinate into the container or another collection device and add it to the container. Keep collection container in the refrigerator or on ice.
At the end of the collection period, empty bladder directly into container or add to container, and note ending time.
Mix urine well to ensure pH and analyte concentrations are homogenous throughout sample.
Total volume of the urine collection must be measured prior to removing any aliquots for analysis.
Remove 5 10-15mL aliquots in duplicate for creatinine and triplicate for total urine analysis.
Store in the -80°C freezer.

SUBJECT ID _____ BLOCK/BEVERAGE _____

Sample	Date	Start Time	Finish Time	Urine Volume	HCl Volume
DAY -4 24h					
DAY -3 24h					
DAY -2 24h					
DAY -1 ~12h					

SUBJECT ID _____ BLOCK/BEVERAGE _____

Sample	Date	Start Time	Finish Time	Urine Volume	HCl Volume
DAY -4 24h					
DAY -3 24h					
DAY -2 24h					
DAY -1 ~12h					

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LUCOPRO CYCLE STUDY SWEAT COLLECTION

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

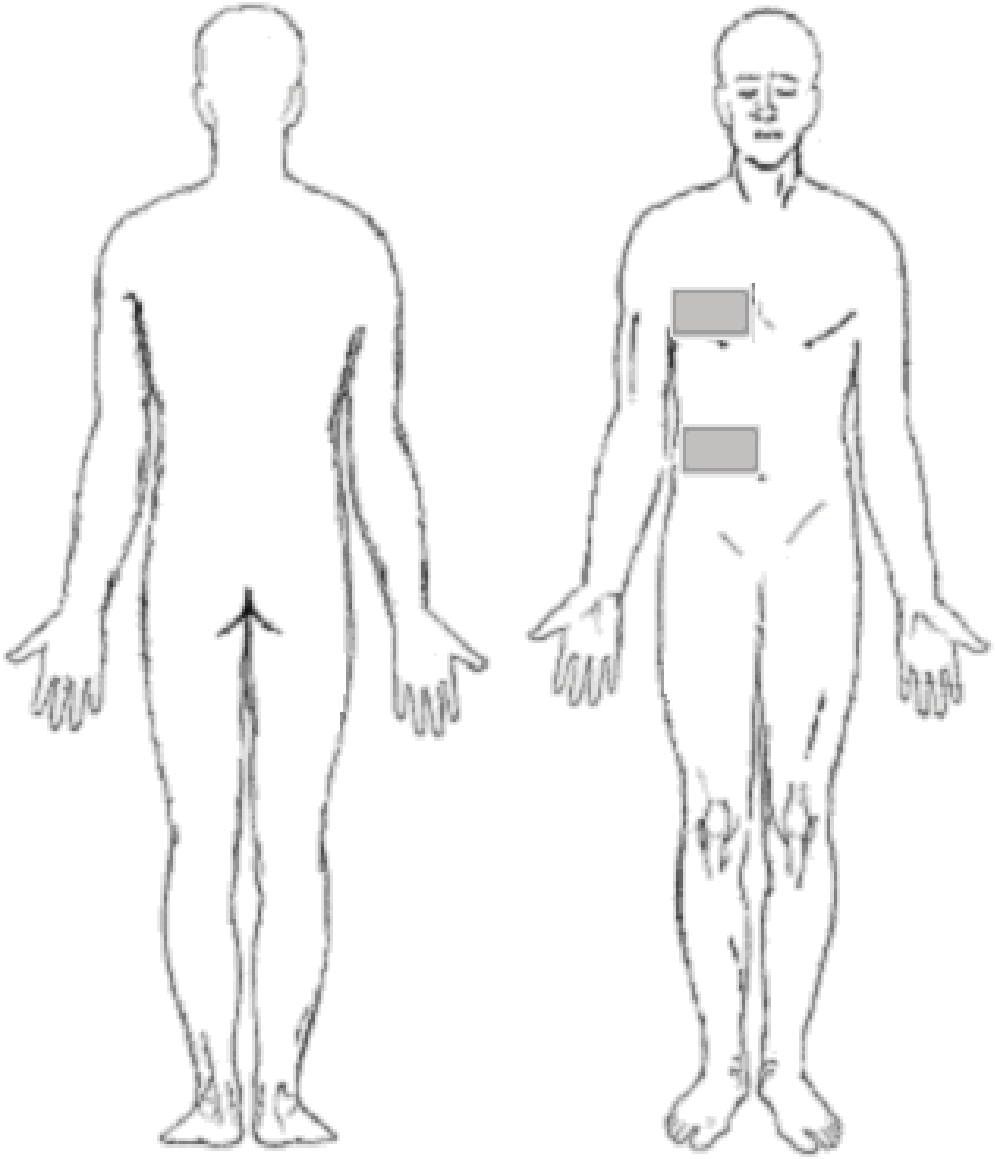
REGIONAL SWEAT COLLECTION METHOD

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 4th 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, 1st & 2nd 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 3000 rpm and 4 °C. Pipette representative samples into spare 50 mL centrifuge tube and mix well, then pipette into 15mL centrifuge tubes (leave a 1-2 cm 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 H2O
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 H2O
Centrifuge Tube with lid, Gauze, & Sweat	Abdominal				
	Chest				

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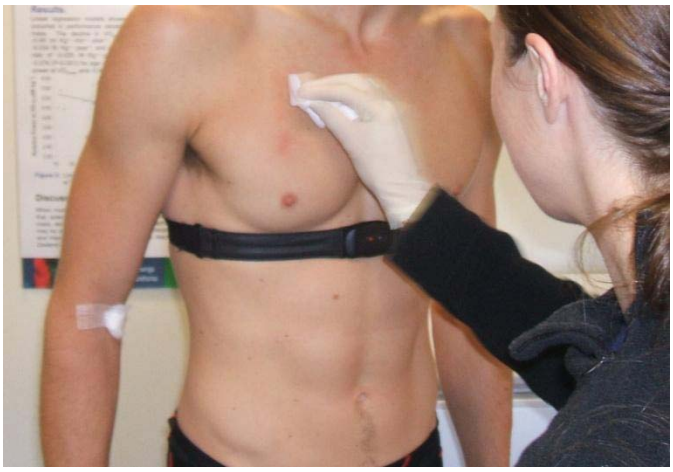
2. 
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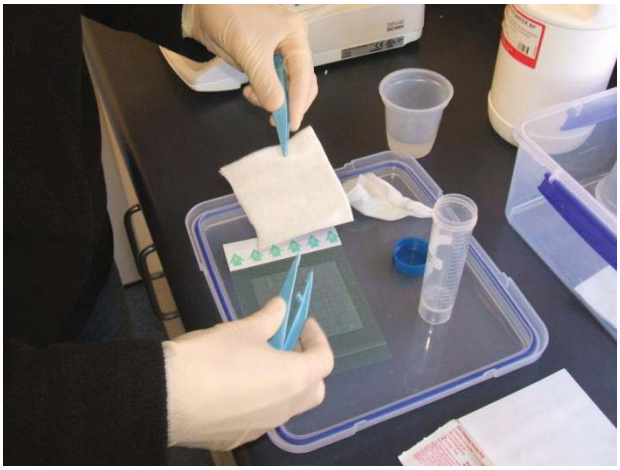
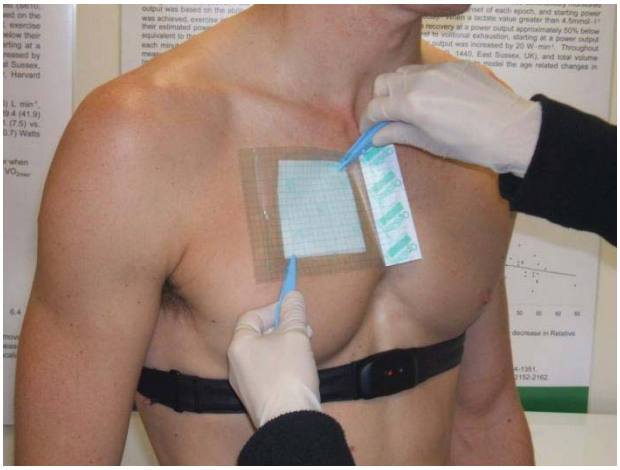
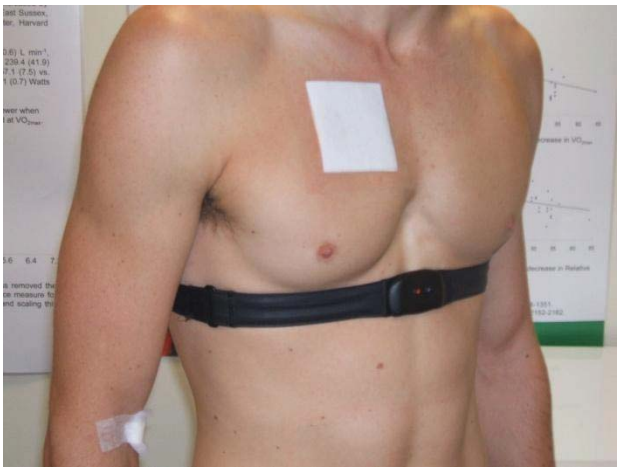
4. 

5. 

6. 

Institute of Food, Nutrition and Human Health
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Auckland

LUCOPRO CYCLE STUDY BLOOD COLLECTION

BLOOD COLLECTION

Venepuncture Equipment

Evacuated Collection Tubes (Plain x 2 [1 for CK and 1 for LDH])
Needles. The gauge number indicates the bore size i.e. the larger the gauge number, the smaller the needle bore (22-guage needles).
Tube Holder/Adapter.
Tourniquet.
Alcohol Wipes (70% isopropyl alcohol).
Gauze or cotton balls.
Folded tissues x 2
Surgical Tape.
Plaster
Needle disposal unit. Needles should NEVER be broken, bent, or recapped. Needles should be placed in a proper disposal unit IMMEDIATELY after their use.
Gloves.
Test tube rack
Disposable Transfer pipettes.
Microtubes.

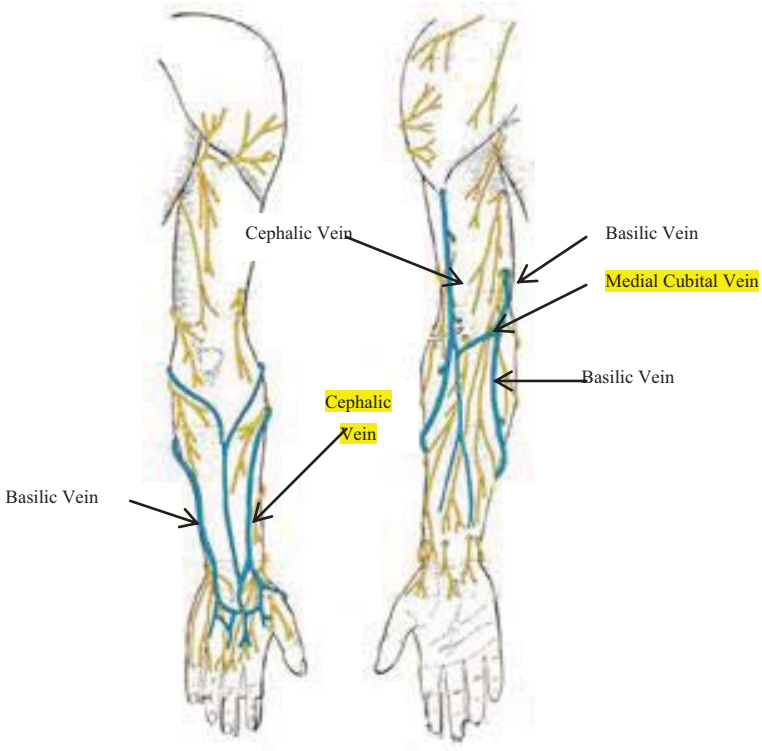
Cannulation Equipment
In addition to the above on Visit 4/Day -4/Training Ride 1 you will need
Cannulation Needles
Tegaderm
Luer-Loc adapter tap
Tourniquet
Alcohol Wipes (70% isopropyl alcohol).
Gauze or cotton balls.
Folded tissues x 4
Surgical tape
Plaster
Razor
3mL syringes
5mL Syringes x 5
Saline x 5 (break seal to open)
Evacuated Collection Tubes EDTA (5 tubes [t=0, t=30-min, t=60-min, t=90-min, and t=120-min]
Ice bucket and ice.

Order of the draw

Blood collection tubes must be drawn in a specific order to avoid cross-over contamination of additives between tubes. The recommended order of draw is serum tubes (2 tubes pre-exercise), then tubes containing other additives i.e. EDTA tubes (5 tubes post-exercise).

Venepuncture

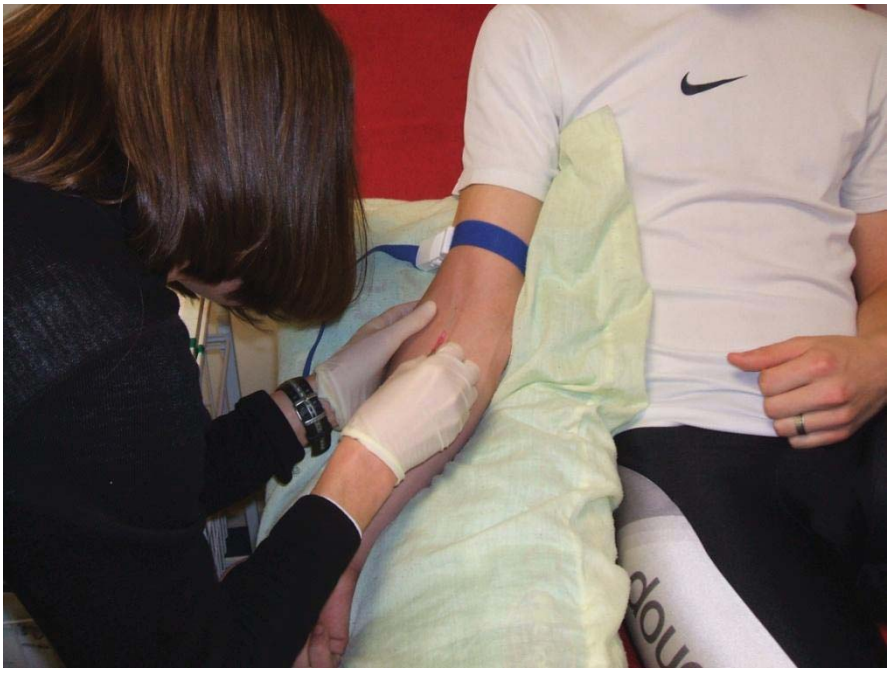
Wash your hands.
Make sure the subject is lying on the bed in the wet lab, sleeves rolled up, with a cushion supporting the arm you will be drawing blood from.
Make sure the area is well lit.
Explain the procedure to the subject.
Apply the tourniquet approximately 10cm above the proposed site.
After 30-60 s the main veins should be sufficiently engorged so that you can locate them.
Palpate the site, veins should be bouncy and will not pulsate with heart beat (nerves are not bouncy and not as superficial; arteries pulsate, and are not as superficial as veins).
Examine the veins for the best venepuncture site. The **median cephalic** is generally the vein of choice, but median cubital and basilic are also satisfactory. **Avoid areas with extensive scarring, a hematoma area or previous venepuncture sites.**



Put on gloves.
Cleanse the site with alcohol wipe, moving the wipe in expanding circles.
Attach the needle to tube holder/adaptor, uncap needle, needle bevel should be uppermost, so that the sharp point can be inserted into the skin
Fix the vein by pulling down on the skin below the proposed site with the thumb of your left hand.
Insert the needle, bevel uppermost into the skin, at an angle of 30º, as the needle enters the vein, there will be a sensation of resistance followed by easy penetration. Once in the vein lower the needle angle to 15º and advance a short distance into the vein following the direction of the vein.
Attach the vacutainer while stabilising the needle.
Withdraw required amount of blood.
Release the tourniquet (the tourniquet should not be left on for longer than 1-2 minutes).
Remove the needle.
Apply gauze to the site and ask the subject to apply pressure for about 2 minutes, while keeping the arm straight to reduce bleeding and bruising.
Discard the needle and tube holder/adaptor into the sharps box.
Apply plaster or surgical tape over the gauze.

Cannulation Instructions
Wash your hands.
Make sure the subject is lying on the bed in the wet lab, sleeves rolled up, with a cushion supporting the arm you will be drawing blood from.
Make sure the area is well lit.
Explain the procedure to the subject.

Apply the tourniquet approximately 10cm above the proposed site.
After 30-60 seconds the main veins should be sufficiently engorged so that you can locate them.
Palpate the site, veins should be bouncy and will not pulsate with heart beat (nerves are not bouncy and not as superficial; arteries pulsate, and are not as superficial as veins).
Examine the veins for the best cannulation site. The cephalic vein of the forearm is large, easily stabilised and superficial.
Put on gloves.
Shave the site if required (subject's choice).
Cleanse the site with alcohol wipe, moving the wipe in expanding circles.
Fix the vein by pulling down on the skin below the proposed site with the thumb of your left hand.
Insert the needle, bevel uppermost into the skin, at an angle of 15-20º more fragile veins require lower angle of insertion, as the needle enters the vein, there will be a sensation of resistance followed by easy penetration.
Stop as blood is seen in the flashback chamber.
Lower angle of insertion to almost flush with subject's skin. Hold the needle while advancing the cannulae approximately 5mm into the vein.
Place gauze under the cannulae apparatus, withdraw needle and quickly attach adaptor tap.
Tape firmly into position using tegaderm and surgical tape.
Open tap to the 3 o'clock position, insert tube holder, insert EDTA tube and draw 5-7mL blood. Remove vacutainer, close tap, remove tube holder.
Using pre-prepared saline syringe (air bubbles tapped out), turn tap to 3 o'clock position to open, take up slightly and then empty saline into the cannulae, close tap. Warn the subject they will experience a cold sensation in their arm.
For each subsequent sample, first use waste syringe to remove any saline etc, then draw blood, then flush with saline.



Vacutainer Clotting Instructions

BD vacutainer plus serum tubes are coated with silicone and micronized silica particles to accelerate clotting. Particles in the white film on the interior surface activate clotting when tubes are mixed 5 times by inversion. The silicon coating on the walls of the serum tubes reduces adherence of red cells to tube walls. Do not shake. Vigorous mixing may cause haemolysis. Insufficient mixing or delayed mixing in serum tubes may result in delayed clotting and incorrect results. Tubes should be allowed to clot at room temperature, allow blood to clot thoroughly before centrifugation i.e. 20-30 minutes in silicon coated or glass, and 60 minutes for plastic serum tubes. Separation of serum or plasma from cells (centrifugation and pipetting into microtube) should take place within 2 hours of collection to prevent erroneous results.

Anticoagulation Instructions

PRIOR TO COLLECTION, CHILL BLOOD TUBES AND EPPINDORF MICROTUBES ON ICE. For EDTA filled additive tubes invert 8-10 times. Do not shake. Vigorous mixing may cause haemolysis. Insufficient mixing or delayed mixing in anticoagulant containing tubes may result in platelet crumpling, clotting and incorrect results. EDTA tubes then go onto ice until centrifugation.

Handling/Storage

Centrifuge the tubes for 5-10 minutes at 3500rpm. The serum/plasma can then be transferred to microtubes using disposable pipettes and stored upright in freezer boxes, generally in the -80°C freezer (LabPlus recommends -20°C for 4 weeks for CK and -20°C for 6 weeks for LDH). Send samples in batches of 20-30 samples at a time to LabPlus for analysis. Set Centrifuge to 4°C for EDTA tubes.

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APPENDIX

If blood fails to enter the vacutainer tube:

Change positions of needle e.g. push forward slightly, pull back slightly, adjust the angle of the needle to the skin, and/or rotate the needle a half turn.

Try another tube, the first tube may not have enough vacuum.

Loosen the tourniquet for a few seconds then reapply.

Fix the vein by pulling down on the skin below the proposed site with the thumb of your left hand.

Try a different vein and/or the other arm.
If blood stops flowing into the vacutainer tube:
The vein may have collapsed, resecure the torniquet.
The needle may have pulled out of the vein, remove needle and try another vein and/or other arm.
Other problems:
If a haematoma should form under the skin of the puncture site, immediately remove the needle and apply firm pressure with gauze.
If blood is bright red (arterial blood), immediately remove needle, and apply firm pressure for more than 5 minutes.

BIOPSY

PROCEDURE

Day Before: Preparing Biopsy Tray
Do this aseptically i.e. do not touch any of the items
Most equipment will be in the biopsy cabinet
On a clean plastic tray place a green sterile towel (in the bottom shelf of the biopsy cabinet is a green material package of green towels). Carefully open green material and using the tip of your forefingers and thumbs remove by one corner, a green towel, allow unfolding on its own, and drape over tray.
Place the following items on the towel, with tip of forefinger and thumbs, opening away from yourself, and without touching, drop onto tray:
1). 10cc syringe
2). Surgical blade
3). Silk sutures (Johnson & Johnson Medical Products, Ethicon, 30" (75cm) Met. 2.0, Taper SH 26mm, 3Dz sterile, K832H)
4). 26 G ½ brown needle
5). 22 G 1½ black needle
6). 18 G 1½ pink needle
7). O-ring
8). Biopsy Needle Pack (Bergström-Stille muscle biopsy cannula 119-29187-50)
Cover with another green towel
On top of the second green towel, place;
1). Dressing trays (MED Rx Wound Care Kit, Re-order# 85-2002)
2). 60cc Syringe (reusable)
3). Elastic Bandage
4). Ansell gloves
5) Stitch Removal Kit
Label all eppindorfs and EM containers.

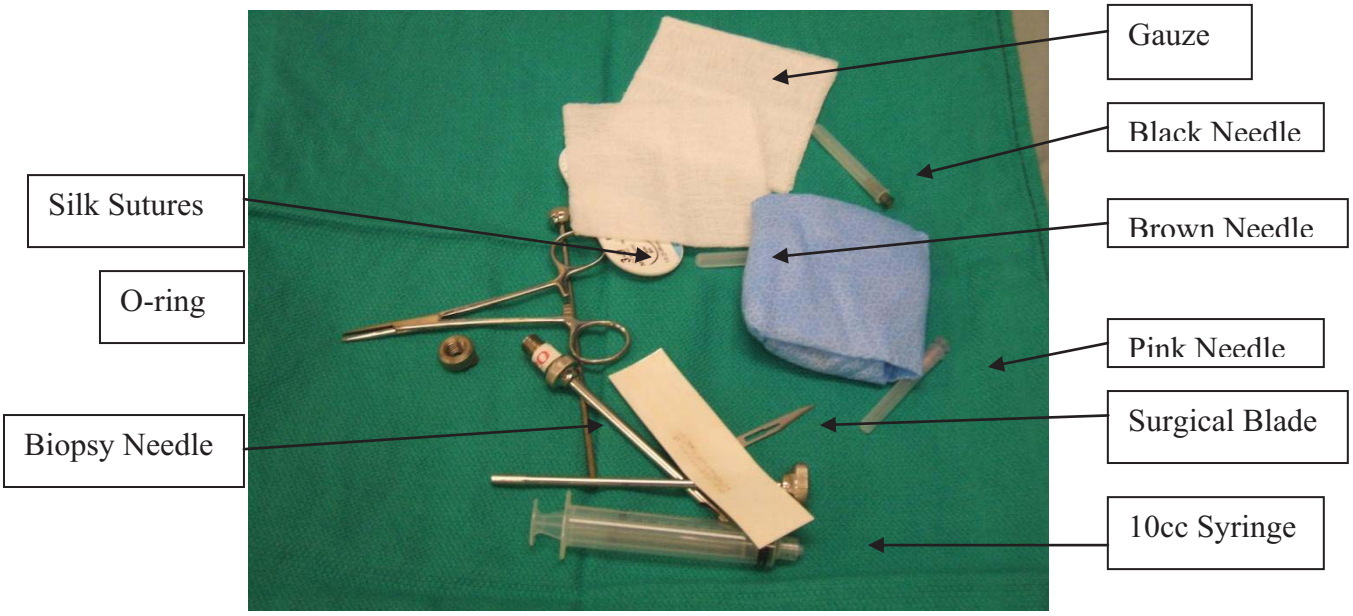
Morning of:
You will need:
1). Liquid Nitrogen
2). Gluteraldehyde (2% Gluteraldehyde in Sodium Cacogylate) from fridge

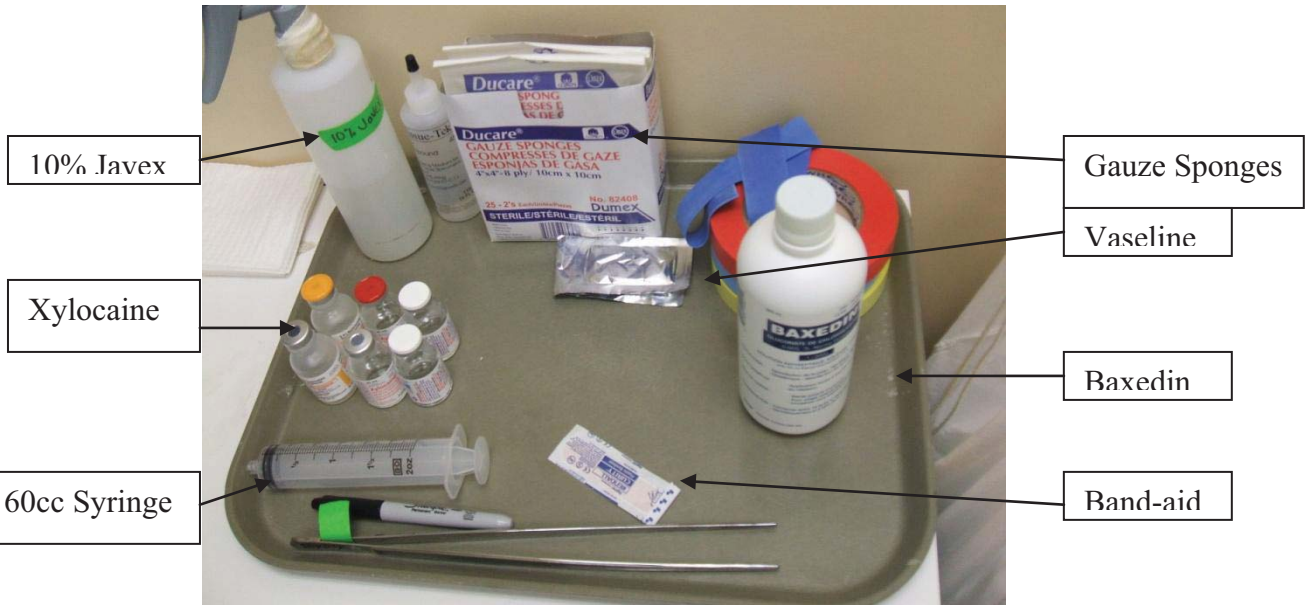
During the Procedure: Assisting with Biopsy Procedure
You will need sterile muscle biopsy apparatus, sterile gloves, and Vaseline.
Mark will open the sterile muscle biopsy apparatus
Hand Mark the sterile gloves unopened
Mark needs to place Vaseline around the seal of the muscle biopsy syringe/needle. With the tip of forefingers and thumb open the Vaseline pack aseptically (without touching). Allow Mark to take some Vaseline. Close mouth of pack and put in a clean place, it can be reused
Have two bottles of Xylocaine ready (1% xylocaine, Astra Zeneca, 20mL, PROD# 012, and 1% xylocaine, Astra Zeneca, 20mL, PROD# 016). If using new bottles, take off coloured plastic cover and wipe top with alcohol. If bottles have previously been

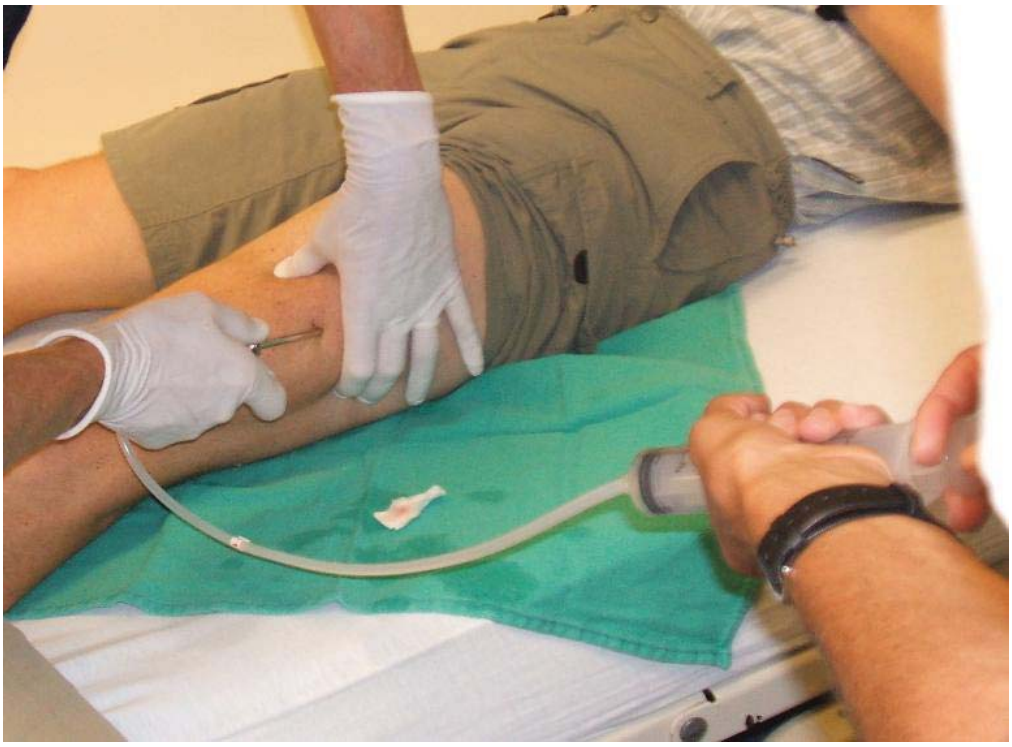
used, dip top in Baxedin (0.05% Chlorohexidine Gluconate, Omega, 500mL, DIN 00788422 CODE L0000001). Hold steady for Mark

During the biopsy, upon Marks request, connect the 60-cc syringe to tubing and follow Mark's instructions. Mark will tell you when to suction. Take care not to pull the plunger out of the syringe

Following removal of the muscle tissue, the blade is used to slice the tissue and place in eppindorfs in the following order; PCR slice, microarray slice, protein, and extra, all go into liquid N. EM goes into the plastic container, tap the container to ensure the muscle tissue goes into the solution at the bottom, and then goes on ice. Always keep pressure on the incision site. Bandage and pin including bag of ice at the site.







BLOOD/IV CATHETER

PROCEDURE

Day Before: Preparing Catheter Tray

Most equipment will be in the blood cabinet

Label a clean plastic tray with study name, subject name, subject ID number, and supplement condition.

Add the following pieces of equipment to the tray;

1). 4 x 10cc syringes
2). 4 x 18 G 1½ pink needle
3). 8 x green vacutainers for waste
4). 8 x green capped vacutainer single use needles
5). Alcohol wipes
6). Small Gauze
7). Tegaderm (3M Healthcare 1624W 6cm x 7cm)
8). 0.11mL Luer-Loc PRN Adapter (BD REF385111)
9). Catheter (blue) (BD Insyte Autoguard REF 381423 35mL/min 0.9 x 25mm)
10). Tape
11). Blue Tourniquet strap
12). Yellow vacutainer case (label with subject name and ID)
13). Plaster

In a tube holder, put out subjects EDTA, Serum tubes, and Urine tubes, and EDTA, and Serum eppindorfs.

Day Before: Preparing Blood Tray

1). Yellow vacutainer case (label with subject name and ID)
2). Plaster
3). Blue Tourniquet strap
4). Green capped vacutainer single use needles
5). Alcohol wipes
6). Small Gauze

APPENDIX Q CONFERENCE ABSTRACTS

LEUCINE-PROTEIN SUPPLEMENTED CARBOHYDRATE RECOVERY FEEDING ENHANCES SUBSEQUENT CYCLING PERFORMANCE IN WELL-TRAINED MEN

Jasmine S. Thomson[1], Ajmol Ali[2], & David S. Rowlands[3]

Institute of Food, Nutrition and Human Health, Massey University, Palmerston North[1], Auckland[2], and Wellington[3], New Zealand

Background and Aim: Recovery from intense prolonged exercise requires not only fuel replenishment, but also nutrient substrates to facilitate muscle tissue repair and training-induced adaptations. Protein ingestion, and particularly the amino acid leucine, has potent stimulatory effects on muscle protein synthesis following exercise, however the effects when co-ingested with carbohydrate on deferred subsequent performance are unknown. The purpose of this study was to determine whether a leucine-protein rich high-carbohydrate post-exercise feeding regimen and practical recovery period could improve subsequent cycling performance, mechanistic markers of muscle damage, and psychometric markers of recovery state relative to control feeding.

Methods: In a crossover, 10 male cyclists performed 2-2.5 h interval training bouts on 3 consecutive evenings. Following exercise they ingested either leucine-protein high-carbohydrate (0.1/0.4/1.2/0.2 $g \cdot kg^{-1} \cdot h^{-1}$ leucine/protein/carbohydrate/fat) or isocaloric carbohydrate control (0.06/1.6/0.2 $g \cdot kg^{-1} \cdot h^{-1}$ protein/carbohydrate/fat) feedings immediately and every half hour for 1.5 h. Diet was controlled, energy and macronutrient intake balanced, and protein intake clamped at 1.6 $g \cdot kg^{-1} \cdot h^{-1}$ over the training and recovery period. The effect of post-exercise nutrition was isolated by providing the alternate supplement the morning after each training bout. Thirty nine hours after the final training bout, performance was evaluated in a repeat-sprint protocol comprising of 10 maximal sprints interspaced with recovery periods standardized external work done.

Results: Co-ingestion of leucine-protein and carbohydrate improved mean sprint power by 2.5% (99% confidence limits, ±2.6%; P = 0.013) and reduced perceived overall tiredness during the sprints by 13% (90% confidence limits, ±9.2%), however effects on leg tiredness and soreness were trivial.

Markers of muscle damage, serum creatine-kinase and lactate dehydrogenase concentrations were decreased by 19% (90% confidence limits, ±18%) and trivial respectively, relative to control. Perceived muscle soreness and further quantification by pressure-pain threshold were also trivial compared to control. Over the training and testing period there was a small reduction in anger (25% ±18), but change in other moods states and global mood scores were trivial. Net nitrogen balance was mildly negative in both conditions and estimated protein requirement was 1.70 $g \cdot kg^{-1} \cdot h^{-1}$.

Conclusion: In summary, supplementing post-exercise high-carbohydrate meals with a leucine-protein rich beverage during a block of intense cycling is likely to enhance subsequent high-intensity performance relative to isocaloric carbohydrate feeding. Daily energy, leucine, and macronutrient intake was balanced suggesting post-exercise timing of the supplement is effectual. More research is required to determine if the training to rest ratio or daily nitrogen balance influences the efficacy of the post-exercise supplement on subsequent performance.

The study was supported by a Post Graduate Support Grant from the Institute of Food, Nutrition and Human Health.

ACUTE EFFECTS OF PROTEIN-CARBOHYDRATE FEEDING ON GENE EXPRESSION DURING RECOVERY FROM TRAINING IN CYCLISTS

David S. Rowlands[1], Jasmine S. Thomson[2], Brian Timmons[3], Gordon Tsang[3], Mark Tarnopolsky[3]

Institute of Food, Nutrition and Human Health, Massey University, Wellington[1] and Auckland[2] New Zealand; [3]Department of Pediatrics, McMaster University, Hamilton, Ontario, Canada L8N 3Z5;

Background and Aim: The ingestion of high protein-carbohydrate feedings following hard endurance exercise was previously seen to enhance subsequent cycling performance. However, the expression of selected mRNA species involved in processes relating to the enhanced recovery such as modulating functional adaptation, and recovery/repair are relatively uncertain. Therefore, our aim was to examine the expression of candidate genes possibly involved in enhanced recovery from prolonged high-intensity cycling and regulated by dietary protein.

Methods: Eight well-trained cyclists participated in a randomised crossover involving post-exercise ingestion of protein-carbohydrate (PRO: 1.2/0.4/0.2 $g \cdot kg^{-1} \cdot h^{-1}$ carbohydrate/protein/fat) or carbohydrate control (CHO: 1.6/0.2 $g \cdot kg^{-1} \cdot h^{-1}$) carbohydrate/fat) immediately and 1-h following 1.75-h high-intensity interval cycling. Muscle biopsies from the Vastus lateralis muscle were collected pre, 3-h and 48-h post cycling, and then analysed via real-time RT-PCR and fluorescence to amplify and quantify mRNA expression of PGC-1α, PDK4, SIRT (mitochondrial function); BCOAD kinase (BCAA metabolism), transcription factor C/EBP or CHOP (amino acid absence and stress response), transcription factor SREBP-2 (cholesterol homeostasis); DSCR1 (calcium signalling and cell growth), IGF1 (cell growth, development and maintenance), transcription factor FOXO1 (cell cycle, cell death, DNA repair, energy homeostasis), transcription factor NFκβ (redox sensitive stress response gene). The housekeeping gene used in this study was β-2-microglobulin, previously validated as a gene which doesn't change with exercise or differ between the dietary conditions.

Results: The post-exercise PRO condition lead to moderate very likely increase in FOXO1 [effect size (ES); ± 90% confidence limits: 0.95 ±0.77], small to moderate likely increase in PGC-1α [0.58 ±0.73], PDK4 [0.59 ±0.65], and CHOP [0.89 ±0.90], likely trivial increase in IGF-1 [0.04 ±0.23] at 3 h; and moderate likely increase in PGC-1α [0.62 ±0.72] and PDK4 [0.66 ±0.65] mRNA by 48 h compared to the CHO placebo condition. The prolonged high-intensity cycling protocol induced very-large almost certain increases in PGC-1α [32 ±7], FOXO1 [5.8 ±1.5], and CHOP [4.4 ±1.4], and a likely trivial increase in IGF1 [0.03 ±0.16] at 3 h; small likely increase in FOXO1 [0.41 ±0.48] and likely trivial increase in IGF1 [0.05 ±0.16] mRNA persisting at 48 h.

Conclusion: The transcriptional response suggests that the ingestion of high protein-carbohydrate food following high-intensity endurance exercise may expedite cellular recovery and adaptation processes and facilitate the metabolic and mitochondrial adaptive responses to the exercise stress. Such as, the important role of transcription factor FOXO1 in the regulation of cell cycle, DNA repair, and energy homeostasis; more specifically, regulation of PDK4 expression possibly contributing to glucose sparing and increased fat utilization during recovery and subsequent exercise, and the sustained up-regulation of the major regulator of lipid metabolism and mitochondrial biogenesis and activity PGC-1α. Additionally, the nutrient-responsive gene CHOP regulates some cell responses to the endoplasmic reticulum stress cascade thought to regulate programmed cell death and possibly cellular regeneration. The autocrine-anabolic signaling action of IGF-1 through the

insulin/IGF-1 receptor pathway and parameters relating to oxidative stress may not be involved in the protein-nutrient induced myocellular response.

Funding by SPARC and Massey University (MURF)

APPENDIX R PUBLISHED MANUSCRIPT

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: Jasmine Sarah Thomson

Name/Title of Principal Supervisor: David Rowlands

Name of Published Paper: Leucine-protein Supplemented recovery feeding enhances subsequent cycling performance in well-trained men

In which Chapter is the Published Work: Chapter 3

What percentage of the Published Work was contributed by the candidate: 80%

Candidate's Signature — 22/12/2010 Date

Principal Supervisor's signature — 12/1/11 Date

GRS Version 2- 1 December 2010

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