PROTEIN FEEDING AND EXERCISE RECOVERY

By

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A thesis submitted to

The School of Sport and Exercise Sciences

The University of Birmingham

For the degree of

DOCTOR OF PHILOSOPHY

Human Performance Laboratory

School of Sport and Exercise Sciences

University of Birmingham

June 2009

Abstract

The importance of protein feeding for maximising the anabolic effect of resistance exercise is well established. Ingestion of amino acids or intact protein sources with and without carbohydrate during exercise recovery further stimulates muscle protein synthesis. Less clear is the impact of an acute bout of resistance exercise on the protein synthetic rate of muscle already stimulated by food intake. This thesis demonstrates that an acute bout of resistance exercise further augments the protein synthetic rate of muscle already stimulated by food intake. Simulating everyday practice, whereby resistance exercise is typically performed in the fed state, an exercise-induced elevation in muscle protein synthesis was accompanied by an increased phosphorylation status of signaling proteins downstream of mammalian target of rapamycin (mTOR).

Recent studies advocate the potential role for protein feeding in improving subsequent performance following acute bouts of fatiguing endurance-type exercise. However, previous studies have focussed upon carbohydrate nutrition, rather than examining the role of protein feeding for exercise recovery in the context of an intense period of endurance training. Increasing dietary protein intake partially countered the blunted minimal mobilisation of antiviral lymphocytes during exercise following intensified training. In addition, the number of negative symptoms of psychological stress experienced following intensified training was attenuated with additional dietary protein intake. The mechanism(s) underpinning the suggestion that a high protein diet may potentiate a better maintainence of endurance performance following intensified training could not be definitively elucidated from our experimental design. The most likely explanation appears to be related to psychological status.

Acknowledgements

First and foremost I would like to thank Dr Kevin Tipton for allowing me the opportunity to work alongside him. His supervision over the past three years is greatly appreciated and without his support none of this research would have been possible.

In addition, I thank Professor Asker Jeukendrup and Dr Jos Bosch for their advice and assistance throughout my postgraduate studies. Dr Rene Koopman, Dr Luc van Loon, Milou Beelen, Michael Tieland, Bart Goen, Jos Stegen and Joan Senden are thanked for their contribution towards the first experimental chapter of this thesis. It was a great experience working alongside Rene, Luc and Milou at the Human Nutrition Laboratory, Maastricht University. Dr Juul Achten, Dr Carl Hulston, Dr Edward Chambers, Dr Chris Shaw, Dr Kate Edwards, Dr Vikki Burns, Dr Matthew Bridge, Dr Michelle Venables, Dr Toby Mundell, Steve Faulkener, Lucy Walton, Hannah Cox and Mhairi Keil are thanked for their contribution to chapters 3-5.

I would also like to acknowledge the support I received from my fellow PhD students, especially Sarah Jackman. Natalie Riddell, Leila Anane, Rachel Turner, Nick Hurren, John Campbell and Beate Pfeiffer are also thanked. I thank all undergraduate students whom assisted in data collection for each study. I am also extremely grateful for the financial support we received from DSM Foods, The Netherlands. Of course, without the commitment of each subject, none of this work would have been possible.

As part of my PhD process, I was fortunate enough to spend periods of time at Maastricht University, The Netherlands, University of Texas Medical Branch, Galveston, University of Birmingham, Alabama and Northumbria University, Newcastle. I am very grateful to Tara Cocke, Stephanie Blaze, Chris Danesi, Dr Marcus Bammann, Dr David Mayhew, Dr Dave Kosek, Dr Paula Ansley-Robson and Ian Walshe for sharing their expertise with me.

Finally, I would like to thank my family and friends for their continuous love and support.

List of publications

Published

- **Oliver C Witard,** Michael Tieland, Milou Beelen, Kevin D Tipton, Luc JC van Loon and Rene Koopman. Resistance exercise increases post-prandial muscle protein synthesis in Humans. *Med Sci Sports Ex.* 2009; 41(1) 144-54.
- Kevin D Tipton and **Oliver C Witard**. Protein requirements and recommendations for athletes: relevance of ivory tower arguments and practical recommendations. *Clinics Sports Med.* 2007;26(1): 17-36.

Submitted

- Sarah R Jackman, **Oliver C Witard**, Asker E Jeukendrup and Kevin D Tipton. Branched chain amino acid supplementation may ameliorate muscle soreness following eccentric exercise. *Med Sci Sports Ex. In review.*

In preparation

- **Oliver C Witard**, Sarah R Jackman, Jos A Bosch, Asker E Jeukendrup and Kevin D Tipton. Intensified training attenuates the exercise-induced mobilisation of effector memory CD8+ T lymphocytes. *In preparation*.
- **Oliver C Witard,** Sarah R Jackman, Jos A Bosch, Asker E Jeukendrup and Kevin D Tipton. Increased protein intake restores the mobilisation of cytotoxic CD8+ T lymphocytes during intensified training. *In preparation.*
- **Oliver C Witard**, Sarah R Jackman, Asker E Jeukendrup and Kevin D Tipton. Increasing dietary protein intake may help maintain in endurance performance during intensified periods of training in cyclists. *In preparation*.
- **Oliver C Witard,** Sarah R Jackman, Jos A Bosch, Asker E Jeukendrup and Kevin D Tipton. Effect of whey protein or carbohydrate supplementation on markers of muscle damage, muscular dysfunction and leg soreness. *In preparation.*

Abstracts

- **Oliver C Witard,** Sarah R Jackman, Jos A Bosch, Asker E Jeukendrup and Kevin D Tipton. Effect of whey protein or carbohydrate supplementation on markers of muscle damage, muscular dysfunction and leg soreness. Proceedings of the FASEB Journal, Experimental Biology. 2008.
- **Oliver C Witard,** Sarah R Jackman, Jos A Bosch, Asker E Jeukendrup and Kevin D Tipton. Increased protein intake restores the attenuated mobilisation of cytotoxic lymphocytes during intensified training. Proceedings of the 13th annual congress of the European College of Sports Science. 2008.
- Sarah R Jackman, **Oliver C Witard**, Asker E Jeukendrup and Kevin D Tipton. Branched chain amino acid supplementation may ameliorate soreness following eccentric exercise. Proceedings of the 13th annual congress of the European College of Sports Science. 2008.

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CHAPTER 1 – General Introduction

1.1 Exercise Recovery and Nutrition

Recovery after exercise poses an important challenge to athletes (19). Major perturbations in cellular homeostasis occur during and following exercise (67). These include changes in numerous physiological, biochemical, metabolic, hormonal and immunological parameters, which ultimately result in an acute impairment in exercise performance (62). Nutritional manipulations, aimed at restoring cellular homeostasis, are advantageous to exercise recovery.

Nutritional manipulations have previously been investigated in relation to exercise recovery within many different exercise contexts. Carbohydrate (CHO) nutrition has received most attention as a recommended strategy for the enhancement of recovery from exercise consisting predominantly of an endurance component (20). However, in the context of maximising the anabolic response to resistance exercise, the primary focus has been on protein/amino acid manipulations (80,122,138,143,164,165). Indeed, a number of investigations demonstrate the efficacy of ingesting protein sources for optimising the response of muscle protein synthesis to an acute bout of resistance exercise (16,136,142,144). Less obvious is the role of protein/amino acid feeding for recovery from endurance exercise. Amino acids provide a precursor for the synthesis of new proteins. In addition, the breakdown of damaged proteins liberates amino acids which may be used to synthesize new functional proteins. Alternatively, amino acids are degraded and subsequently oxidised to provide an energy substrate for muscle contraction (53,120,122,146,154,155,165). The predominant fuels catabolised for energy provision during endurance exercise are carbohydrates and lipids (52)Given that both aforementioned fuel sources become depleted during prolonged endurance exercise (139)and the role played by amino acids for intermediary muscle metabolism (122), protein feeding has recently been advocated as a potential strategy to

attenuate the fatiguing effects of an acute bout of intense endurance exercise (97,131). Further rationale for why additional protein intake may potentiate an improved recovery from endurance based exercise are three-fold. These mechanisms include the breakdown and resynthesis of previously damaged proteins (91,128,132), the modulation of the balance between protein synthesis and protein breakdown during postexercise recovery (78,90) and the initiation of an insulin-mediated accelerated rate of glycogen repletion following exercise (151). Thus, protein nutrition may be considered important in optimising exercise recovery from both resistance and endurance-based exercise activities. The overall aim of this thesis is to examine the role of practical protein feeding manipulations for exercise recovery within the contexts of an acute bout of resistance exercise and an intensified period of endurance training.

1.2 Response of muscle protein synthesis to resistance exercise and nutrition

Muscle mass is critical for human health, physical activity and athletic performance (166). Like all body proteins, muscle proteins are constantly and concurrently being synthesised and degraded (referred to as muscle protein turnover). The metabolic basis for change in muscle mass is net muscle protein balance (NBAL), i.e. the difference between muscle protein synthesis (MPS) and muscle protein breakdown (MPB). To accrue muscle proteins, rate of MPS must exceed MPB, resulting in a positive NBAL. Exercise, above a threshold intensity, provides a powerful stimulus to promote MPS in the post exercise period (18,92,116). Studies that have measured NBAL acutely following resistance exercise have unequivocally found that, whilst MPS is markedly elevated, NBAL remains negative in the absence of nutrient supply (6,116,117). Provision of an amino acid source combined with exercise is required to switch NBAL from negative to positive (7,119,144). This switch is

largely due to increases in MPS rather than reduced MPB (123). Thus skeletal muscle hypertrophy, a common goal for competitive athletes (striving to improve strength for physical prowess), habitual exercisers (health and /or aesthetic) and the elderly (wishing to enhance the functionality of muscle) depends on the interaction between exercise and nutrition.

The interactive effect of exercise and nutrition on the skeletal muscle protein synthetic response to exercise has been systematically investigated in the past 10-15 years (143) (Table 1.1-1.3). Intravenous infusion (7) or the more practical oral administration (144) of amino acids following exercise increases extracellular amino acid availability and transport into the muscle, resulting in a positive NBAL post exercise (7,17,144). The co-ingestion of CHO with amino acids has been shown to further augment the anabolic response to exercise (99,119,145), likely via an insulin-mediated attenuation in MPB (8,16,129). These investigations greatly enhanced our understanding of the control of protein metabolism after exercise, demonstrating that provision of crystalline amino acids alone or in combination with CHO following exercise effectively stimulates muscle anabolism in the post exercise period (Table 1.1). However, since crystalline amino acids are not particularly palatable, or readily available in food form for either athletes or exercisers, the practicality of these studies may be questioned. Subsequent studies have demonstrated that ingestion of intact proteins following resistance exercise also results in muscle anabolism. Net protein balance is increased when whey (136,141,142), casein (142), milk (162), soy (162) or egg (101) proteins are ingested alone or co-ingested with CHO post exercise (Table 1.2). Taken together, these data underline the anabolic effect of protein sources when ingested post exercise.

The anabolic effect of nutrition has previously been shown to be enhanced by exercise. Investigations have compared the anabolic effect of exercise and nutrition verses nutrition

alone (7,101,115,136,137,161) (Table 1.3). Several of these studies (101,115,136,137,161) used a unilateral exercise model, whereby the contralateral leg served as a non-exercised control. A single leg experimental design enables one to attribute acute changes in MPS to the respective feeding and/or exercise stimuli rather than to inter-individual variation on a single occasion. Repeated ingestion of a mixed macronutrient drink during exercise recovery (115,137,161), or the single bolus administration of whey protein (101) or whey protein co-ingested with CHO (136) immediately following resistance exercise further stimulates muscle protein synthetic rates compared with a control leg stimulated by nutrient intake only. Taken together, these data clearly demonstrate that exercise followed by intake of a nutritional supplement combine to elicit a greater anabolic response compared with nutrient intake alone.

Many previous studies provide a sound scientific framework in which to base our understanding of the control of protein metabolism after exercise with nutrition. Regulation of the post exercise response of net muscle protein balance to nutrient ingestion is known to be dependent upon multiple factors (147), including the type (43,142) and amount of amino acids (17,99,144) or protein (101), protein digestive properties (142), timing of nutrient intake in relation to exercise (50,142,145) and the concurrent ingestion of other nutrients (16,99,145). Most studies have implemented necessary (due to inherent principles of stable isotope methodology), but impractical, experimental designs whereby resistance exercise is performed in the fasted state or in combination with small (~6-20g) repeated bolus ingestion of nutrients, rather than in the fed state (17,144). Hence, the practical applications of these studies which combine the interactive effect of exercise and nutrition on muscle protein synthesis to exercising populations aiming to increase muscle anabolism remains unclear, at least in the context of normal dieting patterns.

In typical everyday practice, exercise activity is commonly performed in the fed state, following meal ingestion. The impact of resistance exercise on the protein synthetic rate of muscle already stimulated by food intake has yet to be fully elucidated. **Chapter 2** describes an investigation whereby untrained males performed an acute bout of unilateral resistance exercise following ingestion of a standardised protein-rich breakfast. The primary goal of this practically-based study was to differentiate between the impact of exercise and nutrition on muscle protein synthesis in a more realistic situation whereby exercise was preceded by intake of a meal. We hypothesised that an acute bout of resistance exercise would increase postprandial muscle protein synthetic rates in humans.

Table 1.1: Additive effect of ingesting crystalline amino acid sources post resistance-based exercise compared with resistance exercise alone on muscle protein synthesis

Authors	Participants	Exercise Model	Group Comparisons	Muscle protein synthetic response
Tipton *et al*. (1999) (144)	UT males (n=6)	75% 1 RM leg press (5x10) 75% 1 RM duo-squat (4x8) 75% 1 RM leg curl (4x8) 75% 1 RM leg extension (4x8)	REx REx + MAA (40g) REx + EAA (40g)	↑ MPS by ~70% in REx + MAA vs. REx (fasted) ↑ MPS by ~50% in REx + EAA vs. REx (fasted)
Biolo *et al.* (1997) (7)	UT males (n=6)	12 RM leg press (5x10) 10 RM duo-squat (4x8) 10 RM leg curl (4x8) 10 RM leg extension (4x8)	Basal IV AA (100mg/mL) REx + IV AA (100mg/mL)	↑ MPS by 53% in IV AA vs. basal (fasted) ↑ MPS by 119% in REx + IV AA vs. basal (fasted)
Miller *et al.* (2003) (99)	UT males (n=6) & females (n=4)	75% 1 RM leg press (10x10) 75% 1 RM knee extensions (8x8)	REx + AA REx + AA+ CHO	↑ MPS by ~61% in REx + AA + CHO vs. REx + AA
Dreyer *et al.* (2008) (38)	UT males (n=16)	70% 1 RM leg extension (10x10)	REx REx + Leu-enriched EAA + CHO	↑ MPS by ~78% in REx + Leu-enriched EAA + CHO vs. REx (fasted)

UT = untrained; RM = repetition maximum; REx = resistance exercise; MAA = mixed amino acids; EAA = essential amino acids; CHO = carbohydrate; Leu = leucine IV = intravenous; MPS = muscle protein synthesis

Table 1.2: Additive effect of administering intact proteins post resistance-based exercise compared with resistance exercise alone on muscle protein synthesis.

Authors	Participants	Exercise Model	Group Comparisons	Muscle protein synthetic response
Roy *et al*. (2000) (129)	T males (n=10)	80% 1 RM bench press (3x10) sit-ups (20) 80% 1 RM knee extension (6x10) 80% 1 RM latissmus pull downs (3x10) 80% 1 RM bicep-curls (3x10) 80% 1 RM leg press (3x10) 80% 1 RM triceps press (3x10) 80% 1 RM military press (3x10)	REx + PLA REx + CHO/PRO/FAT	↑ MPS by ~ 41% in REx CHO/PRO/FAT vs. REx + PLA
Tipton *et al.* (2004) (142)	UT males and females (n=23)	80% 1 RM Leg extension (10x8)	REx REx + Casein (20g) Rex + Whey (20g)	↑ MPS by ~ 85% in REx + Casein vs. REx (fasted) ↑ MPS by ~ 65% in REx + Whey vs. REx (fasted
Koopman *et al.* (2005) (81)	UT males (n=8)	80% 1 RM leg press (8x8) 80% 1 RM leg extension (8x8)	REx + CHO REx + CHO + PRO REx + CHO + PRO + LEU	↑ MPS by ~ 35% in REx + CHO + PRO vs. REx + CHO ↑ MPS by ~ 46% in REx + CHO +PRO + LEU vs. REx + CHO ↑ MPS by ~ 8% in REx + CHO + PRO + LEU vs. REx + CHO + PRO
Koopman *et al.* (2007) (77)	UT males (n=10)	40% BW chest press (3x10) 40% BW shoulder press (3x10) 50% BW front pull down (3x10) 75% 1RM leg press (8x10) 75% 1 RM leg extension (8x10)	REx + PRO REx + PRO + LCHO REx + PRO + HCHO	↑ MPS by ~ 9% in REx + PRO + LCHO vs. REx + PRO ↑ MPS by ~ 18% in REx + PRO + LCHO vs. REx + PRO
Tang *et al.* (2007) (136)	T males (n=8)	Unilateral 80% 1 RM leg press (4x8-10) 80% 1 RM leg extension (4x8-10)	REx + CHO REx + WP + CHO	↑ MPS by ~ 24% in REx + WP + CHO vs. REx + CHO

UT = untrained; T = trained; RM = repetition maximum; BW = body weight; REx = resistance exercise; PLA = placebo; CHO = carbohydrate; LCHO = low carbohydrate; HCHO = high carbohydrate; PRO=protein; LEU = leucine; MPS = muscle protein synthesis

Table 1.3: Additive effect of amino acid/ protein sources and resistance-based exercise compared to nutrition alone on muscle protein synthesis

Authors	Participants	Exercise Model	Group Comparisons	Muscle protein synthetic response
Biolo *et al.* (1997) (7)	UT males (n=6)	12 RM leg press (5x10) 10 RM duo-squat (4x8) 10 RM leg curl (4x8) 10 RM leg extension (4x8)	IV AA (100mg/mL) REx + IV AA (100mg/mL)	↑ MPS by ~44% in Ex + IV AA vs. IV AA
Phillips *et al.* (2002) (115)	UT males (n=23)	Unilateral 80% 1 RM leg press (2x10) 80% 1 RM leg extension (6x10)	REx +MMN MMN	↑ MPS by ~26% in REx + MMN vs. MMN
Tang *et al.* (2007) (136)	T males (n=8)	Unilateral 80% 1 RM leg press (4x8-10) 80% 1 RM leg extension (4x8-10)	REx + WP + CHO WP + CHO CHO	↑ MPS by ~89% in REx + WP +CHO vs. WP + CHO ↑ MPS by ~130% in REx + WP + CHO vs. CHO
Wilkinson *et al.* (2008)	UT males (n=10)	Unilateral 80% 1 RM leg extension (5x8-10)	MMN MMN + REx	↑ MPS* by ~ 91% in MMN + REx vs. MMN
Moore *et al.* (2009) (102)	T males (n=7)	Unilateral 8-10 RM leg press (5x8-10) 8-10 RM leg extension (5x8-10)	REx + WP WP	↑ MPS* by ~ 43% in WP + REx vs. WP
Tang *et al.* (2008) (137)	UT males (n=10)	Unilateral 80% 1 RM knee extenseion (6x8-10)	MMN MMN + REx	↑ MPS by ~ 108% in MMN + REx vs. MMN

UT = untrained; T =t rained; RM = repetition maximum; REx = resistance exercise; IV = intravenous infusion; AA = amino acid; WP = whey protein; CHO = carbohydrate; MMN = mixed macronutrient intake; MPS = muscle protein synthesis; * denotes synthetic rate of myofibrillar proteins

1.3 Control of muscle protein synthesis via anabolic signaling

Translation initiation and elongation are considered rate limiting steps in the synthesis of proteins (159). Intracellular signalling proteins control the rate of translation initiation and elongation. Particularly important to the regulation of translation initiation appears to be the activation of the mammalian target of rapamycin (mTOR) signaling cascade (118), including the downstream proteins eukaryotic initiation factor (eIF) 4E-Binding Protein 1 (4E-BP1), p70-S6 protein kinase (S6K1) and ribosomal protein S6 (rpS6).

The ability to measure changes in phosphorylation status (indicative of activation) of mTOR-associated signaling proteins has enabled researchers to explore potential mechanisms underpinning the modulated protein synthetic response to exercise and/or nutrients. Rodent models have generated the majority of information examining the interaction of nutrition and exercise on translation initiation (2,14,15,44,87). Only recently have studies investigated the response of intracellular signaling proteins to exercise and nutrition in humans (for review see (41) (Table 1.4-1.6)). Amino acid feeding with (49,59) and without (66,71) additional CHO enhances the phosphoylation status of mTOR and its downstream effectors involved in the initiation (S6K1, 4E-BP1) and elongation (eukaryotic elongation factor-2 (eEF2)) of mRNA translation (Table 1.4). In the absence of nutrient intake, previous authors reported an increased activation of mTOR (39,48), S6K1 at serine and threonine residues 424 and 421 ($S^{421/}T^{424}$) (42,58,82) and ribosomal protein S6 (rpS6) (58) in skeletal muscle following acute bouts of resistance exercise performed in the fasted state (Table 1.5). Nutrients and exercise synergistically activate the mTOR signaling cascade (Table 1.6). Ingestion of protein (79) or essential amino acids (EAA) (38,76) augmented the activation status of mTOR, S6K1 and 4E-BP1 following exercise. Thus, exercise and/or amino acid/protein ingestion activate

intracellular signalling proteins known to regulate translation initiation and muscle protein synthesis.

Recent investigations (33,38,39,40,48,49,50,101,161) have determined the activation status of mTOR-associated signaling proteins alongside measurements of muscle protein synthesis; however, in human studies, this association remains problematic. Acute bouts of both high-intensity (39) and low-intensity blood flow restricted (48) resistance exercise performed in the fasted state have been shown to stimulate muscle protein synthesis and also activate components of the mTOR signaling pathway. Dreyer *et al.* (39) findings revealed an increased phosphorylation status of mTOR and S6K1 2 h after exercise and a 41% increase in fractional synthetic rate, a direct measure of mixed muscle protein synthesis, measured over a 2 h recovery time period. Indeed, the augmented S6K1 activation 6 h following exercise has been shown to correlate with muscle hypertrophy following training in both rats (3) and humans (140). An augmented anabolic hormone response, or more likely an increased muscle fiber recruitment provide possible explanations underpinning the increased rate of muscle protein synthesis and activation of the mTOR-signalling cascade induced by low-intensity resistance exercise during blood flow restriction (48). Two studies (38,101) recently explored potential cellular mechanisms responsible for the enhanced synthesis of muscle proteins when nutrients are ingested following exercise. Augmented muscle protein synthetic rates were accompanied by enhanced mTOR-signaling when leucine enriched EAA+CHO solutions (38) or whole egg protein sources (101) were administered post exercise. Taken together, these studies provide useful information with reference to molecular mechanisms which may be important in modulating changes in muscle mass. However, in human studies caution must prevail. Due to ethical constraints, a paucity of data exist examining the time course of activation of MPS and of phosphorlyation of mTOR-associated signaling proteins in humans.

These data would provide useful information underpinning the regulation of muscle protein accretion. Alternatively, in **Chapter 2**, we took two snapshots of the phosphorylation status of mTOR-associated signaling proteins measured immediately after exercise and following 6 hours of recovery together with measurements of fractional synthetic rate of muscle in an attempt to explain any additive effects of exercise performed in the fed state on mixed muscle protein synthesis.

Table 1.4: Response of intracellular signaling proteins to nutrient intake

Authors	Participants	Group Comparisons	Biopsy Times	Intracellular signalling response
Greiwe *et al*. (2001) (60)	UT males (n=10) and females (n=8)	- Leucine infusion - Insulin infusion - Leucine + Insulin infusion	Pre Immediately post feeding	 ↑ S6K1 ↑ S6K1 ↑ S6K1
Fujita *et al*. (2007) (49)	UT males (n=14)	Basal Leucine-enriched EAA CHO	Pre 1 h post nutrition	 ↑ mTOR, ↑S6K1, ↑ 4E-BP1, ↓ AMPK, ↓Akt/PKB, eEF2
Greenhaff *et al*. (2008) (59)	UT males (n=8)	Intravenous infusion of amino acids	1 h post feeding 3 h post feeding	 ↑PKB, ↑S6K1, ↑mTOR, ↑4E-BP1, ↑GSK3ß
Glover *et al*. (2008) (58)	T males (n=9)	Rest-fasted Rest-fed	Pre 6 h Post Ex 6 h Post Ex	 ↑PKB/Akt

UT = untrained; T =t rained; RM = repetition maximum; REx = resistance exercise; MPS = muscle protein synthesis.
AMPK = AMP kinase; mTOR = mammalian target of rapamycin; S6K1 = p70 S6 kinase; 4E-BP1 = eukaryotic initiation factor 4E binding protein 1; PKB=protein kinase B; eEF2=eukaryotic elongation factor 2; GSK 3ß=glycogen synthase kinase

Table 1.5: Response of intracellular signaling proteins to resistance exercise

Authors	Subject	Exercise Model	Group Comparisons	Biopsy Times	Intracellular signalling response
Cuthbertson *et al.* (2006) (33)	UT males (n=8)	Step ups (12 min)		Pre 3 h post Ex 6 h post Ex 24 h post Ex	 ↑PKB, ↑ S6K1 (T^{389}) in Ex vs. rest ↑PKB, ↑ S6K1 (T^{389}) in Ex vs. rest ↑PKB, ↑ S6K1 (T^{389}) in Ex vs. rest
Dreyer *et al.* (2006) (39)	UT males & females (n=11)	70% 1 RM leg extension (10x10)	Basal REx	Rest Immediately following REx (T0) 1 h post REx (T1) 2 h post REx (T2)	 ↑ S6K1(S^{424}/T^{421}), ↓4E-BP1 in REx vs. basal ↑ S6K1 (S^{424}/T^{421}), ↑ AMPK, ↑Akt, ↑mTOR, ↓ 4E-BP1, ↓ eEF2 in REx vs. basal ↑ S6K1 (S^{424}/T^{421}), ↑ AMPK, ↑mTOR, ↓ 4E-BP1, ↓ eEF2 in REx vs. basal
Eliasson *et al.* (2006) (42)	UT males (n=16)	4x6 maximal eccentric contractions vs. 4x6 concentric contractions	Basal Concentric exercise Eccentric Exercise	Pre Immediately post Ex 1 h post Ex 2 h post Ex Pre Immediately post Ex 1 h post Ex 2 h post Ex	 ↑S6K1 (S^{424}/T^{421}), ↑S6K1 (T^{389}), ↑rpS6, ↑mTOR ↑ S6K1 (S^{424}/T^{421}), ↑S6K1 (T^{389}) ↑ S6K1 (S^{424}/T^{421}), ↑S6K1 (T^{389}) ↑rpS6 ↑S6K1 (T^{389}), ↑rpS6 ↑rpS6
Koopman *et al.* (2006) (82)	UT males (n=8)	75% 1 RM Leg press (8x10) 75% 1 RM leg extension (8x10)	75%1RM leg press (8x10) 75% 1RM leg extension (8x10)	Pre Immediately post Ex 0.5 h post Ex 2 h post Ex	 ↑ AMPK, ↑ S6K1 (S^{424}/T^{421}), ↑ rpS6, ↓ 4E-BP1 in REx vs rest ↑ S6K1 (S^{424}/T^{421}),, ↑ rpS6, in REx vs rest ↑ S6K1 (S^{424}/T^{421}),, ↑ rpS6, ↓ 4E-BP1 in REx vs rest
Fujita *et al.* (2007) (48)	UT males (n=6)	Blood flow restricted low intensity REx 20% 1 RM (75 reps)	Blood flow restricted REx	Pre 3 h post Ex	 ↑S6K1 (S^{424}/T^{421}), ↓eEF2
Glover *et al.* (2008) (58)	T males (n=9)	Unilateral leg press (4x10) @ 10 RM Unilateral leg extension (4x10) @ 10 RM	Basal REx	Pre 6 h Post Ex	 ↑Akt, ↑S6K1(T^{389}), ↑rpS6

UT = untrained; T =t rained; RM = repetition maximum; REx = resistance exercise; PKB = protein kinase B; AMPK = AMP kinase; mTOR = mammalian target of rapamycin; S6K1 = p70 S6 kinase; rpS6 = ribosomal protein S6; 4E-BP1 = eukaryotic initiation factor 4E binding protein 1; eEF2 =e ukaryotic elongation factor 2.

Table 1.6: Response of intracellular signaling proteins to resistance exercise and nutrition

Authors	Participants	Exercise Model	Group Comparisons	Biopsy Times	Intracellular signalling response
Fujita *et al.* (2008) (50)	UT males (n=13) & females (n=9)	70% 1 RM leg extension (10x10)	REx EAA/CHO + REx	1 h Pre REx Pre REx Post REx 1 h REx 2 h post REx	 ↑Akt/PKB, mTOR, 4E-BP1, S6K1 (T^{389}) , ↓eEF2 EAA/CHO+REx vs. REx ↑Akt/PKB, 4E-BP1, S6K1 (T^{389}), ↓eEF2 EAA/CHO+REx vs. REx ↑Akt/PKB, 4E-BP1 EAA/CHO+REx vs. REx
Karlsson *et al.* (2004) (76)	T males (n=7)	75% 1 RM leg Press (8x10) 75% 1 RM leg extension	REx REx + BCAA	Pre 0 1 2	 S6K1 (S^{424}/T^{421}) ↑rpS6, S6K1 (S^{424}/T^{421}), S6K1 (T^{389}) REx +BCAA vs. REx ↑rpS6, S6K1 (S^{424}/T^{421}), S6K1 (T^{389}) REx +BCAA vs. REx
Dreyer *et al.* (2008) (38)	UT males (n=16)	70% 1RM leg extension (10x10)	REx REx + Leu-enriched EAA + CHO	Basal Immediately Pre Immediately Post 1 h Post 2 h Post	 ↑Akt ↑TSC, ↑mTOR, ↑4E-BP1, ↑S6K1 (S^{424}/T^{421}) REx + Leu-enriched EAA+CHO
Moore *et al.* (2009) (101)	T males (n=6)	Bilateral 8-10 RM leg press (4x8-10) 8-10 RM leg extension (4x8-10) 8-10 RM leg curl (4x8-10)	REx + WP REx	1 h Post Ex 4 h Post Ex	↔ S6K1 (S^{424}/T^{421}), S6K1 (T^{389}), eIF2Bε ↔ S6K1 (S^{424}/T^{421}), S6K1 (T^{389}), eIF2Bε

UT = unrained; T = trained; RM = repetition maximum; REx = resistance exercise; EAA = essential amino acids; CHO = carbohydrate; BCAA = branched chain amino acids; Leu=leucine; WP = whey protein; PKB=protein kinase B; AMPK = AMP kinase; mTOR = mammalian target of rapamycin; S6K1 = p70 S6 kinase; rpS6 = ribosomal protein S6; 4E-BP1 = eukaryotic initiation factor 4E binding protein 1; eEF2=eukaryotic elongation factor 2; TSC=tuberous schlerosis complex; eIF2Bε=eukaryotic initiation factor 2 beta eepsilon.

1.4 Protein feeding and recovery from endurance exercise

The importance of protein intake with reference to recovery from endurance exercise has recently received attention, almost entirely in an acute setting (131). Recent acute studies suggest a potential role for protein ingestion during and after exercise for the improvement of subsequent exercise performance (131). The addition of protein to sub-optimal CHO intake has been shown to elicit an insulin-mediated increased resynthesis of muscle glycogen (72) and claimed post exercise amelioration of muscle soreness and creatine kinase responses to high intensity exercise (91,98,128,132). Ultimately, marked improvements (>40%) in exercise capacity during a subsequent bout of exercise have also been reported when protein was co-ingested with CHO (132,163). However, despite promising rationale for acute based studies, the impact of protein manipulations on exercise recovery from an intensified period of endurance training is yet to be investigated.

Ideally, longitudinal studies, lasting ≥12 wk, would be conducted whereby the protein content of the diet is manipulated during a chronic period of excessive training, with multiple indices of recovery monitored. However, inherent difficulties may be associated with such study designs. Innumerable variables, including nutritional intake, training quantification, would need to be controlled. The financial cost of such studies also requires consideration (139,147). Short-term periods of intensified training are commonly associated with a decline in immune function (64,94) and perturbations in physiological (74), biochemical (148) and endocrine responses (149) during and following exercise and an altered psychological status (i.e. mood state) (47,150). Ultimately, exercise performance capacity is acutely impaired (61,74). In this thesis, as an alternative to a longitudinal experimental study design, we examined the impact of manipulating dietary protein intake on immune function, psychological status and exercise performance following a short-term period of intensified

training in cyclists. The trafficking pattern of anti-viral lymphocytes marked immune status. Symptoms of training-related stress were quantified together with mood state to monitor psychological status. Exercise performance was assessed with a previously validated time trial on the cycle ergometer (73).

1.5 Overview of the Immune System

The body is constantly under attack from infectious agents, including viruses, bacteria and parasites. A fundamental characteristic of the immune system is that it involves multiple functionally different cell types, which permits a large variety of defense mechanisms (54). Leukocytes, or white blood cells consist of the granulocytes (60-70% of circulating leukocytes), monocytes (10-15%) and lymphocytes (20-25%). Various subsets of the latter, including B cells, T cells and natural killer (NK) cells can be identified by the use of fluorescent-labelled monoclonal antibodies to identify cell surface markers (known as clusters of differentiation, CD). These cells have diverse functions in immune defence (54).

Attempts of infectious agents to penetrate the body activate the innate immune system to mount an immediate defence response. This first line of defence comprises multiple mechanisms which share the principal goal to restrict entry of micro-organisms into the body. Physical and chemical barriers of the innate system include the skin, tears, saliva and acidity of the gut. Specialised phagocytic cells, such as neutrophils and monocytes/macrophages ingest and then kill invading micro-organisms. Equally significant are natural killer (NK) cells which exhibit the capacity to destroy infected cells (35), hence are critical for protection against invading pathogens (9). These lymphocytes (which are considered part of the innate immune system) have the propensity to swiftly migrate out of the blood into peripheral tissues, thus are among the first immune cells to arrive at sites of tissue damage and infection (134).

Failure of innate immunity to protect its host results in infection. Subsequently, an adaptive immune response is initiated which aids recovery from infection.

The adaptive immune system responds with a proliferation of T and B lymphocyte sub-populations. Receptors on the cell surface of lymphocytes that recognise the antigen engender specificity and memory, enabling the immune system to mount an augmented response when the host is re-infected by the same pathogen. Two types of T lymphocytes exist. CD4+ T lymphocytes (CD4+TL) promote the activation of both macrophages and B lymphocytes, thus eradicate both intracellular and extracellular pathogens. CD8+ T lymphocytes (CD8+TL) eliminate antigens by direct cell-to-cell contact, killing off infected or malignant cells that express viral or tumor antigens (160). Antigens may be located anywhere in the body, hence sufficient numbers of CD8+TL must preferentially migrate to organs where antigens persist (153). Innate and adaptive immune systems function synergistically to protect against, recognise, attack and destroy elements which are foreign to the body.

Lymphocyte cells differ in maturation status, reflecting their history of antigen exposure and cell division. Recent methodological advances in immunology allow for the division of T lymphocyte and NK cell sub-populations into functionally and phenotypically distinct sub-sets (26,30,65,130). Four broad subsets of T lymphocytes may be discerned; naïve (NA); central memory (CM); effector memory (EM); and effector memory T lymphocytes that have re-expressed the naïve marker CD45RA (RA+EM) (65,89,130). NA T lymphocytes, which have yet to encounter their cognate antigen, are programmed to home continuously from the blood to lymph nodes and other secondary lymphoid tissues (i.e. lymph nodes, spleen, peyers patches and tonsils) (160). The homing behaviour of NA cells is relatively homogenous within a sub-population (93). An encounter with an antigen induces

the proliferation of T cell clones into memory subsets. Most memory cells are short-lived, however the few which survive are sub-divided into two subsets on the basis of their migratory ability (153). CM cells express homing molecules similar to that of NA cells, and therefore preferentially migrate to lymphoid organs. EM, and to a greater extent RA+EM, cells are characterised by high effector potential and express receptors that allow heterogenous access to selected peripheral tissues and possible sites of inflammation (i.e. intestinal lamina propria, pulmonary interstitium, inflamed skin and joints). RA+EM cells, which have an extensive history of activation and cell division, exhibit a particularly strong effector potential (65,89). Effector CD4+TL and CD8+TL produce the cytokines, interferon γ (IFN- γ), interleukin (IL)-4 and IL-5 within hours following antigenic stimulation (130). Highly cytotoxic RA+EM CD8+TL carry large amounts of perforin (130), hence this cell subset exhibits the capacity to immediately destroy infected cells (75). Expression of CD45RA in combination with CD27 is typically used to differentiate between T lymphocyte subsets (Fig. 1.1). The differentiation of cell subsets from NA to fully functional effector cells requires several days.

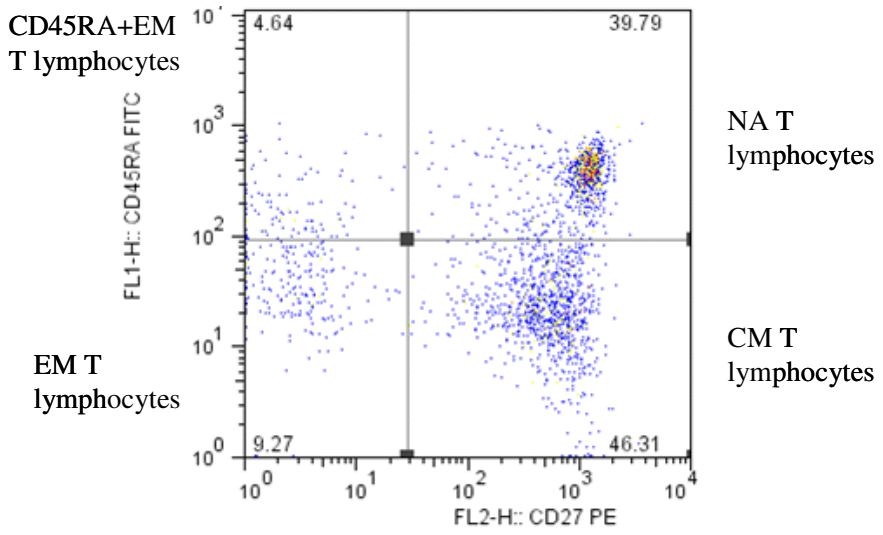

Figure 1.1: Flow cytometric gating for T lymphocyte subsets.

Naïve (NA) T lymphocytes (CD27+CD45RA+) (top right quadrant), Central Memory (CM) T lymphocytes (CD27+CD45RA-) (bottom right quadrant), Effector Memory (EM) T lymphocytes (CD27-CD45RA-) (bottom left quadrant) & RA+EM T lymphocytes (CD27-CD45RA+) (top left quadrant). CD, cluster of differentiation; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

Analogous to T lymphocytes, NK cells can also be divided into functionally distinct subsets that exhibit distinct migratory patterns and cytotoxic potential. Two main groups of NK cells exist. The majority of NK cells (~90%) express relatively low levels of CD56 (CD56lo) and function as cytotoxic cells that sample peripheral tissues such as the lungs, gut and upper respiratory tract, for the presence of infected cells (31,32). Functionally (e.g. immediate cytolytic ability and migratory potential) these cells show a strong resemblance to the effector CD8+TL subsets. A smaller proportion of NK cells (~10%), characterised by high expression of CD56 (CD56hi) exhibit a predominantly immuno-modulatory role through the release of cytokines, and preferentially re-circulate between the blood and the secondary lymphoid tissues (32). Typically, flow cytometric analyses identify CD56hi and CD56lo cells by combining CD56+ and CD16- antibodies and isolating the two fluorescently distinct sub-

populations of CD56+ cells using appropriate electronic gates (Fig. 1.2). Characterising the trafficking pattern, i.e. mobilisation into the bloodstream and subsequent efflux, of functionally distinct T lymphocyte and NK cell subsets in response to acute exercise/chronic training may establish a better understanding of how the immune system adapts to physiological stress.

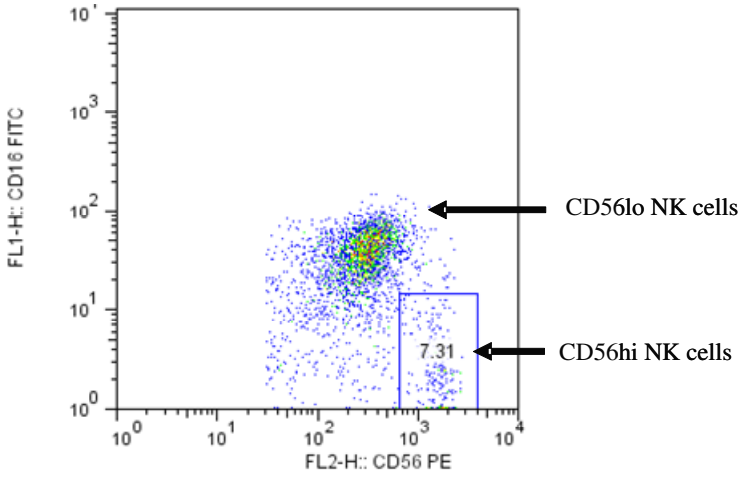

Figure 1.2: Flow cytometric gating for CD56hi and CD56lo Natural Killer (NK) cells.

CD56lo, NK cells with low expression of CD56; CD56hi, NK cells with hi expression of CD56; CD, cluster of differentiation; PE, phycoerythrin; FITC, fluorescein isothiocyanate.

1.6 Intensified Training and Immune Status

Endurance athletes engaged in intense periods of endurance training appear to be susceptible to minor viral infections of the upper respiratory tract, such as sore throats and the common cold (54). The relationship between exercise training and susceptibility to infection has been modelled in the form of a 'J' shaped curve (105) (Fig. 1.3). This model suggests that although the risk of upper respiratory tract infections (URTI) may decrease to below that of a sedentary individual when one engages in moderate exercise training, risk may rise above average during periods of excessive high-intensity exercise. Recent epidemiological evidence supports the contention that incidence of URTI is reduced in moderately active individuals. A

recent (n=547) observational study conducted in a large cohort of healthy adults revealed regular performance of ~2 h of moderate exercise per day to be associated with a ~30% reduction in risk of URTI compared with a sedentary lifestyle (111). In contrast, athletes engaged in intensive periods of endurance training appear to be more susceptible to minor infection (107). A dose-response relationship between training load and infection risk has been demonstrated in both runners (68) and speed skaters (46). Incidence of minor infection poses a significant concern to the athlete, as it is generally recognised that even minor illness adversely affects exercise performance and impairs the ability to sustain heavy training (124). Thus, interest in the relationship between chronic exercise training and changes in immune parameters has received attention over the last 40 years (94).

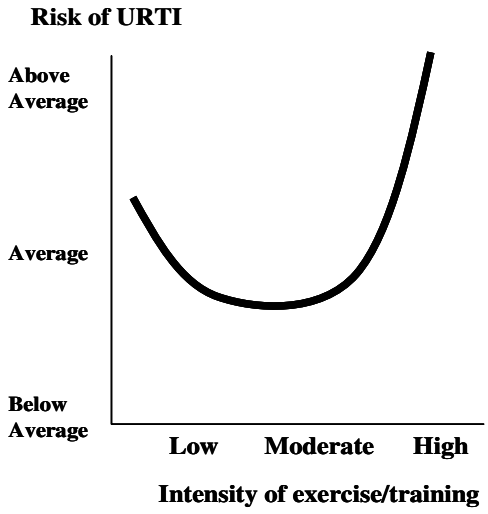

Figure 1.3: The J-shaped model of the relationship between risk of upper respiratory tract infection (URTI) and exercise/training volume. Adapted from Nieman (1994) (105).

Several direct indices of leukocyte function are sensitive to increased training loads. Numerous studies have investigated the effects of short periods of intensified training on indices of immune function measured at rest (for review see (94)) (Table 1.7). Functional aspects of the innate immune system, including degranulation of neutrophils (125) and

cytotoxic activity of NK cells (135) are depressed. Marked reductions in adaptive immunity, including mitogen-stimulated lymphocyte proliferation and activation (21), salivary immunoglobulin A (S-IgA) concentrations (56,57) and IFN-γ (a cytokine critical to anti-viral defence) production from T cells (88) also appear prevalent following chronic periods of heavy training. Intuitively, an impaired immune status sustained over an extended period would render athletes more susceptible to minor illnesses and infection. In fact, the J-shaped curve may be extended by suggesting that if regular moderate exercise lowers infection risk, it should be accompanied by enhanced immuno-surveillance (107) (Fig. 1.4). On the other hand, when an athlete engages in unusually heavy exercise workloads, infection risk should be related to diminished immuno-surveillance. Interestingly, with the exception of mucosal immunity (i.e. S-IgA), to date very few studies have established an association between perturbations in resting surrogate blood measures of immune function following heavy exertion and infection incidence in athletes. Thus, the search continues to fully elucidate direct functional markers of immune status that may be used as a diagnostic tool to predict impending infection.

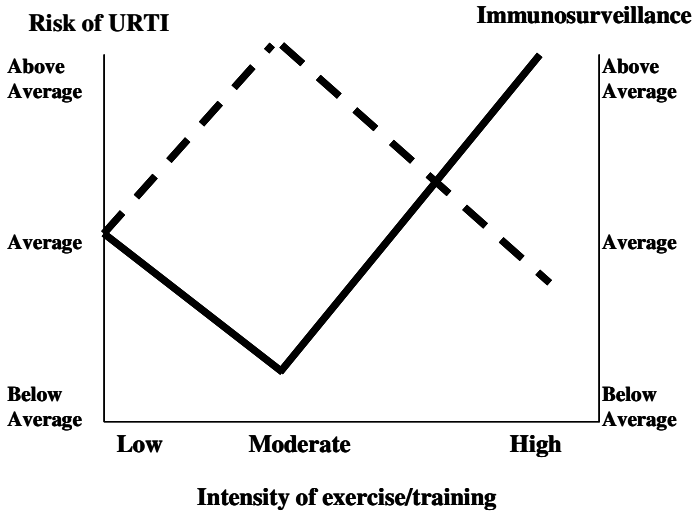

Figure 1.4: Proposed theoretical relationship between exercise/training workload, risk of upper respiratory tract infection and immunosurveillance. ─ = risk of upper respiratory tract infection (URTI), --- = immunosurveillance. Adapted from Nieman *et al.* (2000) (107).

The magnitude of change from baseline in circulating leukocyte counts measured in the blood, both during and after exercise may provide a useful marker of immune status. Acutely, exercise causes profound changes in the number and relative distribution of leukocyte subsets in the circulation; the most defined of these in terms of relative change from baseline being lymphocytes. The rapid increase in peripheral blood lymphocytes during exercise, predominantly of CD8+TL and NK cell sub-populations (both cytotoxic), is one of the most robust findings in the exercise immunology literature (54,84). Subsequently, a rapid but reversible state of lymphocytopenia ensues, whereby lymphocyte counts reach a nadir for between 3 and 72 h post exercise (51,133). Traditionally, this post exercise efflux of lymphocytes is often termed the 'open window.' This 'open window' concept is thought to represent the most vulnerable period for an athlete, providing an opportunity for viruses and bacteria to gain a foothold and ultimately elicit a sub-clinical infection (114) (Fig. 1.5a). On the otherhand, it was recently proposed that transient decreases in blood lymphocyte numbers are critical to the regulation of the immune system (22,36,153). Previous rodent model based studies have demonstrated a decline in blood lymphocyte numbers and concomitantly increased prevalence of lymphocytes in the skin, lymph nodes, and bone marrow following exercise (83) or psychological stress (36,37). This redeployment of lymphocytes is thought to represent a direct homing of lymphocytes from the blood to particular tissues such as the lungs, upper respiratory tract or the gut, thus influencing the nature of local immune and inflammatory responses (22,36) (Fig. 1.5b). A systematic characterisation of how chronic periods of intensified training affect this continuous trafficking of lymphocytes during and after exercise may thus provide informative data concerning the increased number of infectious episodes typically experienced by athletes.

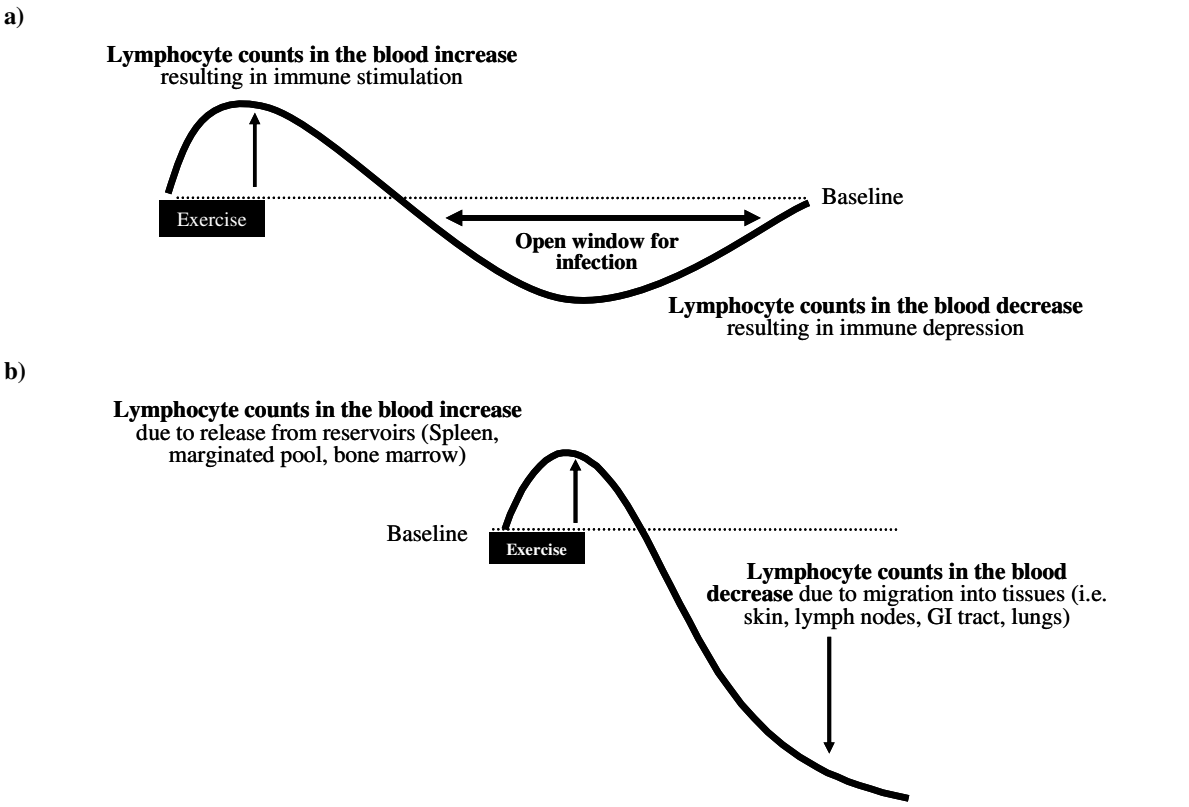

Figure 1.5: Schematic representation of the exercise-induced pattern of lymphocyte trafficking interpreted as, a) a post exercise 'open window' hypothesis for infection derived from the decline in lymphocyte counts to below baseline post exercise (figure adapted from Pedersen *et al.*, 1997 (114)), b) an adaptive response essential to the maintenance of an effective immune defence network derived from the post exercise redistribution of lymphocytes to potentially virally infected peripheral tissues (figure adapted from Dhabhar *et al.*, 1995 (36).

The utility of enumerative data to monitor immunological perturbations associated with intensified training may be further enhanced by the determination of specific subsets of anti-viral lymphocyte sub-populations. Infections of the upper respiratory tract, which impose the greatest threat to athletes involved in intense periods of training, are viral in nature (54). Whereas direct evidence is lacking, intuitively, antiviral lymphocytes, i.e. CD8+TL and NK cells are likely to play significant roles in protecting the athlete from illness. Previous research has shown a preferential mobilisation of the effector CD45RA+ EM CD8+TL (27) and

CD56lo NK cells (135) into the blood during brief bouts of exercise. Modulating the proportion of circulating effector CD8+TL and NK modifies the functional potential of the blood and thus may be considered essential to the maintenance of an effective immune defence network. To date, no study has examined the effect of an intensified period of training on the trafficking pattern of functionally distinct lymphocytes. In **Chapter 3,** we measured the trafficking pattern of functionally distinct subsets of CD8+TL and NK cells during and after exercise following a period of intensified training in trained cyclists.

Table 1.7: Impact of increased training load on selected direct markers of leukocyte function

Authors	Participants	Training model	Measurement parameter of immune function	Effect of intensified training
Gedge *et al.* (1997)	Males swimmers (n=8)	4 wk intensified training period	NKCA	↓
Suzui *et al* (2004) (135)	Female volleyball players (n=8)	1 month heavy pre-season training (5 h/day volleyball drills 6 days/wk	NKCA	↓
Lancaster *et al.* (2004) (88)	Endurance trained cyclists (n=7)	6 day intensified training period	Circulating % and number of IFN-γ+(type I) T cells measured at rest Concentration of IFN-γ produced by stimulated T cells post exercise	↓ ↓
Gleeson *et al.* (1995) (56)	Australian Institute of Sport (AIS) swimming team (15 males, 11 females)	7 months pre national championships 20-25 pool training/wk, 5 h dry land training.	sIgA sIgG sIgM	↓ ↔ ↓
Gleeson *et al.* (1999) (57)	Australian Institute of Sport (AIS) swimming team (15 males, 11 females)	7 months pre national championships 20-25 pool training/wk, 5 h dry land training.	sIgA	↓
Bury *et al.* (1996) (21)	15 professional footballers	Football season (~8 months)	T lymphocyte proliferation Neutrophil oxidative burst activity	↓
Smith *et al.* (1990)	Males (n=11)	T vs. UT	Neutrophil microbial activity	↓
Hack *et al.* (1994)	Male long distance runners (n=8)	Moderate vs. intense training	Neutrophil phagocytic activity	↓
Pyne *et al.* (1995)	Elite male swimmers	12 wk intensified training period	Neutrophil oxidative burst activity	↓
Robson-Ansley *et al.* (2006)	Male endurance trained runners (n=8)	2 wks intensive interval running training	IL-6 Neutrophil function	↑ ↓

wk =weeks; T=trained; UT=untrained; NKCA = natural killer cell cytotoxic activity; IFN-γ=interferon gamma; sIg=salivary immunoglobulin; IL=interleukin

1.7 Nutrition, Intensified Training and Immune Function

Nutritional interventions may be effective for the amelioration of immune perturbations associated with intensified training, thus minimising risk of infection (108). Virtually all nutrients in the diet play a crucial role in the synthesis and regulation of immune factors (152), ultimately maintaining an 'optimal' immune response (25). To maintain immune function, athletes are advised to eat a well-balanced diet with sufficient energy intake to maintain energy balance (54). Nutrient deficiencies are implicated in the aetiology of exercise-induced immune depression and subsequent increased infection risk (24). Likewise, diets that are excessively high in some nutrients (e.g. omega-3 polyunsaturated fatty acids) also have the potential to be detrimental to immune function (11). Thus, appropriate nutrition should be considered as one important strategy to attenuate the potentially immuno-suppressive effects of intensified periods of training.

1.7.1 Carbohydrate Nutrition and Immune Function

CHO feeding has received the most attention as a potential nutritional counter-measure to the immuno-suppressive effects of acute bouts of intense exercise or chronic periods of excessive training. CHO beverage ingestion during exercise was shown to preclude an exercise-induced decline in neutrophil function (10) and attenuate the decrement in mitogen-stimulated T lymphocyte proliferation (69) following prolonged exercise. Furthermore, Bishop and colleagues (10) demonstrated that consuming CHO (30-60 g/h) during 2.5 h of strenuous cycling precluded the post exercise suppression of IFN-γ production and attenuated the release of inflammatory cytokines (IL-6, IL-10 and IL-1 receptor agonist) during exercise (11). Reduced blood glucose levels during prolonged bouts of strenuous exercise initiate the activation of the hypothalamic-pituitary-adrenal (HPA) axis and

subsequent increase in blood concentration of the stress hormone cortisol, - a well known mediator of immune suppression (103). Consumption of CHO during high-intensity (~75-80% VO_2max) exercise bouts lasting >90 min attenuates the increase in plasma cortisol, and has been suggested to mediate an attenuation of the immuno-suppressive effects of intense exercise (106).

The immune response to acute CHO feeding has been well studied, however less convincing are data which have investigated the impact of manipulating dietary CHO intake on the immune response to exercise. Studies (12,13,55,100) have typically employed experimental designs whereby individuals exercised after 2-3 days on diets low in CHO (<10% of dietary intake from CHO) or high in CHO (>70% of dietary intake from CHO). Increasing dietary CHO intake failed to attenuate declines in mitogen-stimulated lymphocyte proliferation (100) and neutrophil degranulation (12) following prolonged bouts of intense exercise. Neither acute CHO supplementation, nor dietary CHO manipulations appear effective in countering post exercise decrements in NK cell cytotoxic activity (NKCA) (110) or S-IgA output (109). Furthermore, no evidence exists that any beneficial effects of CHO feeding on immune responses to high intensity (~75-80% VO_2max) exercise lasting >90 min translates into a reduced incidence of URTI after prolonged exercise. Collectively, these data suggest that CHO nutrition acts only as a partial counter-measure to the immuno-suppressive effects of intense exercise. Many nutritional components, in addition to CHO, have the potential to serve as counter-measures to immune dysfunction in athletes. Ongoing research addressing the value of alternative nutritional strategies which specifically target the functional capacity of antiviral cells (i.e. CD8+TL and NK cells) may benefit endurance athletes at increased risk of viral infection during heavy training periods.

1.7.2 Protein/Amino Acid Intake and Immune Function

Whereas the effect of CHO nutrition on immune status has been extensively investigated in an exercise setting, the impact of protein manipulations for immune function has been largely restricted to a clinical setting (25). Perhaps surprising is the lack of studies examining the impact of supplementing the habitual diet with complete protein sources or manipulating the protein content of the diet in an attempt to counter the immuno-suppressive effects of heavy physical exertion. Interestingly, a recent study conducted by the late Paul Flakoll and colleagues (45) reported improvements in informative, albeit crude markers of immune status, such as 33% fewer medical visits, a reduced incidence of bacterial/viral infection and 37% fewer medical visits due to muscle/joint problems when United States marine recruits consumed nutritional supplements which included protein immediately following each training session of a basic 54 day training period. However, to date the impact of manipulating dietary protein intake on direct measures of immune status following exercise training is yet to be examined.

1.7.3 Glutamine and Immune Function

On the other hand, the impact of supplementing the diet with free amino acid sources on immune function has been well investigated. In the search for an alternative supplementation strategy to CHO in countering the immuno-suppressive effect of heavy exertion, glutamine has attracted attention from investigators with mixed findings (95,126,127). Glutamine, the most abundant amino acid in human muscle and plasma, is an important substrate for cells of the immune system, in particular lymphocytes and macrophages (104). Plasma glutamine levels fall in response to prolonged exercise, mediated by increased plasma cortisol and/or glucagon levels and increased tissue requirement of glutamine for gluconeogenesis. Nearly 30 years ago, Rennie and colleagues (121) reported

that plasma glutamine levels decline from 557 μmol/L at rest to 391 μmol/L 2 h following 3 h 45 min of cycling at 50% VO_2max. Whether exercise-induced reductions in plasma glutamine are linked to impaired immunity and host protection in athletes remains somewhat controversial, but the majority of studies do not favour such a relationship (112).

The suggestion that glutamine supplementation may reverse the immuno-suppressive effects of heavy exercise and training is largely based on in vitro experiments which demonstrate that a depletion of glutamine has a direct effect in lowering proliferation rates of T and B lymphocytes (70). Castell *et al.* (29), have to date, provided the only evidence for a prophylactic effect of oral glutamine supplementation on the occurrence of infection, concluding that provision of two glutamine drinks (5 g glutamine in 330mL water) in the first two hours following exercise decreased the incidence of infection in the week after ultra-marathon/marathon events. Plasma glutamine concentrations were not determined in this study (29), however a follow-up study by the same research group suggested that the administration of this dose of glutamine was unlikely to prevent the post-exercise fall in plasma glutamine concentrations (28). In contrast, previous studies demonstrated that although glutamine feeding before/during and after exercise prevented the fall in plasma glutamine concentrations, lymphocyte proliferation (126), NKCA (85), neutrophil degranulation (157) and concentrations of S-IgA (86) remained blunted. Hence, a general consensus has been reached that it is unlikely that in vivo plasma glutamine levels following prolonged exercise decline below threshold values depicted by in vitro studies to be detrimental to immune function (158). Thus, directly supplementing the diet with glutamine to preclude such moderate exercise-induced declines in plasma glutamine concentrations fails to counter the immuno-suppressive effects of physical exertion. Increasing the availability of

multiple amino acids may be a more effective nutritional counter-measure to the immune suppressive effects of intensified training.

1.7.4 Branched Chain Amino Acid Supplementation and Immune Function

Preliminary data provide promising, albeit inconclusive evidence that branched chain amino acid (BCAA) supplementation may attenuate the immuno-suppressive effects of intense exercise training. Recent studies have reported that supplementation (6 g/day for 15 days) with a BCAA mixture (60% leucine, 20% isoleucine and 20% valine, i.e., 3:1:1) prior to a triathlon/30-km run precluded the ~40% decline in mitogen-stimulated lymphocyte proliferation (4,5) and increased production of IL-1 (4), IL-2 (4,5) tumor necrosis factor-α (4) and IFN-γ (4,5), thus diverting the pattern of cytokine production towards a T helper 1 (denoted Th-1) cell-mediated immune response, which is typically compromised with physical exertion. However, these papers (4,5) pooled results from both triathlon and running modes of exercise, thus statistical analyses performed in these studies may be questioned. Hence, the uncontrolled nature of these studies lends uncertainty to results.

The relative contribution of BCAA as an energy source for immune cells compared with glucose or glutamine is small. Hence, any protective effect of BCAA administration on immune function is unlikely mediated by their role as substrates for energy production. The immune system has a high dependence upon protein synthesis, since mounting an immune response requires generation of new cells and the synthesis of antigen-presenting machinery such as immunoglobulins, cytokines, cytokine receptors and acute phase proteins. In vitro observations show that the omission of a single BCAA from the medium of cultured lymphocytes results in complete abolition of protein synthesis (156). In particular, leucine is known as an activator of the mTOR signalling pathway that regulates protein synthesis and degradation in cells (96). Thus, it seems most likely that the essentiality of BCAA for immune

cells relates to protein synthesis. Clearly more research is needed to fully elucidate the effectiveness of BCAA in countering the immuno-suppressive effects of high intensity training.

Provision of all amino acids in a high protein diet may be useful in countering the immuno-suppressive effect of intensified training. The majority of immune defences involve cell replication or the production of proteins with specific functions (34). Thus, it may be proposed that the provision of all amino acids, rather than glutamine alone or BCAA, may be effective for the optimisation of protein synthesis required to maintain the immune status of athletes during periods of intensified training (23). **Chapter 4** describes a study in which we examined the effectiveness of increasing the dietary protein intake of trained cyclists as a potential nutritional counter-measure to the immuno-suppressive effects of intensified cycle training.

1.8 Protein Feeding, Endurance Training and Exercise Performance

The influence of protein feeding on post exercise immune function may be translated into the wider context of overall exercise recovery. Recent acute studies suggest a potential role of protein ingestion during and after exercise for the improvement of subsequent exercise performance (131). The co-ingestion of protein with CHO alone (78) or CHO and fat (90) immediately after prolonged, glycogen-depleting exercise has been demonstrated to maintain a positive whole-body (78,90) and muscle (90) net protein balance in the early post exercise period. Furthermore, the addition of protein to sub-optimal CHO intake has been shown to elicit an insulin-mediated increased resynthesis of muscle glycogen (72) and claimed post exercise amelioration of muscle soreness and creatine kinase responses to high intensity exercise (91,98,128,132). Marked improvements (>40%) in exercise capacity during a subsequent bout of exercise have also been shown when protein was co-ingested with CHO

(132,163). However, these data should be considered with caution in the context of exercise performance. When exercise performance has been measured with a time trial (113) and/or an isocaloric CHO recovery drink is compared with CHO + protein (128), no beneficial effect of protein has been reported, nor were rates of muscle glycogen resynthesis enhanced (151). Thus, observations of improved performance and/or muscle glycogen resynthesis observed after the co-ingestion of protein + CHO may be attributed to greater energy intake per se, rather than any proven physiological effect (67). Taken together, these preliminary data provide promising, albeit inconclusive evidence that protein feeding may play a role in recovery from acute bouts of endurance exercise.

Nutrient intake plays an important role for the maintenance of exercise performance within the context of an intensified period of training. Recent studies conducted in our laboratory reported an attenuated impairment in endurance performance when athletes supplemented their habitual diet for eight days with CHO before and after each intense training session (63) or strictly adhered to a high (8.5 g·kg body mass $(BM)^{-1}\cdot day^{-1}$ = ~65% total energy intake) compared to a low (5.4 g·kg $BM^{-1}\cdot day^{-1}$ = ~41% total energy intake) CHO diet (1) before and during periods of increased training volume. Better maintenance of endurance performance and psychological mood state with CHO feeding during intensified training was manifested with attenuated exacerbations in endocrine responsiveness to exercise (63) and maintained rates of muscle glycogenolysis (1). However, in both studies (1,63), the protein content of the diet remained constant. Thus, it remains unknown what impact manipulating dietary protein intake has on the development of fatigue following a period of intensified training. Therefore, the main purpose of **Chapter 4** and **Chapter 5** was to examine the impact of manipulating the dietary protein intake of trained cyclists during a week of intensified training on exercise recovery. Health status and performance are ultimately the

most important outcomes to the athlete. Thus, we measured immune status **(Chapter 4),** psychological mood state **(Chapter, 4-5)** and time trial performance **(Chapter 5)** as primary endpoints to practically assess the effectiveness of a high dietary protein intake (i.e. 3 g·kg BM^{-1}·day^{-1}) during intensified periods of training.

1.9 Scope of the Thesis

This thesis describes a series of studies which investigated the effectiveness of protein feeding strategies for exercise recovery. **Chapter 2** describes a study in which we determined the protein synthetic response to an acute bout of resistance exercise performed in the fed state. We simultaneously measured the phosphorylation status of signalling proteins, known to play a key role in regulating translation initiation, in an attempt to provide a molecular mechanistic explanation for the modulated response of muscle protein synthesis to exercise when preceded by food intake. **Chapters 3-5** focus on exercise recovery from a period of intensified training in endurance athletes. **Chapter 3** describes a study which utilised recent methodological advances in immunology to measure the impact of a short-term period of intensified training on the trafficking pattern of functionally distinct lymphocyte sub-populations. **Chapter 4** investigated the effect of increasing the dietary protein intake of trained cyclists during a period of intensified training on the subset-specific pattern of lymphocyte trafficking. **Chapter 5** describes a study in which we examined the effect of dietary protein intake on endurance performance decrements, mood disturbances and physiological perturbations associated with a period of intensified training in trained cyclists. Finally, **Chapter 6** discusses the results of aforementioned studies, providing an overview of main conclusions. The practical implications of the performed research are discussed and subsequent aims for future research are provided.

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CHAPTER 2 - Resistance Exercise Increases Postprandial Muscle Protein Synthesis in Humans

Published article

Oliver C Witard, Michael Tieland, Milou Beelen, Kevin D Tipton, Luc JC van Loon and Rene Koopman. Resistance exercise increases post-prandial muscle protein synthesis in Humans. *Med Sci Sports Ex.* 2009; 41(1) 144-54.

MT assisted with data collection, MB and LvL collected performed muscle biopsy procedures, KT, LvL and RK assisted with the design of the study, statistical analysis and writing of the manuscript. Joan Senden, Jos Stegen and Annemie Gijsen assisted with muscle and blood analysis.

2.1 Abstract

Purpose: We examined the impact of an acute bout of resistance-type exercise on mixed muscle protein synthesis in the fed state. **Methods:** Following a standardized breakfast, 10 untrained males completed a single, unilateral lower-limb resistance-type exercise session. A primed, continuous infusion of L-[ring-$^{13}C_6$]phenylalanine was combined with muscle biopsy collection from both the exercised (Ex) and non-exercised (NEx) leg to assess the impact of local muscle contractions on muscle protein synthesis rates following food intake. Muscle biopsies were collected ~10 min post exercise and 6 h following post exercise recovery, enabling the determination of the rate of incorporation of amino acids into bound protein over a 6 h recovery period. Western blotting with phospho-specific and pan antibodies was used to determine phosphorylation status of AMP-activated protein kinase (AMPK), 4E-binding protein (4E-BP1), mammalian target of rapamycin (mTOR), and p70-S6 protein kinase (S6K1), ribosomal S6 protein (S6) 10 min following exercise cessation and after 6 h recovery. **Results:** Muscle protein synthesis rates were ~20% higher in EX compared with NEx (0.098±0.005 vs 0.083±0.002 %·h^{-1}, respectively, $P<0.01$). In the fed state, resistance type exercise did not elevate AMPK phosphorylation. However, the phosphorylation status of 4E-BP1 was ~20% lower following cessation of exercise in Ex compared with NEx ($P<0.05$). Conversely, 4E-BP1 phosphorylation was significantly higher in Ex compared with NEx after 6h of recovery ($P<0.05$) with no changes in mTOR phosphorylation. S6 phosphorylation was greater in Ex vs NEx after cessation of exercise ($P<0.05$), despite the fact that S6K1 phosphorylation at T^{389} was not up-regulated ($P>0.05$). **Conclusion**: We conclude that resistance-type exercise performed in a fed state further elevates post-prandial muscle protein synthesis rates, which is accompanied by an increase in S6 and 4E-BP1 phosphorylation state.

Keywords: skeletal muscle, food intake, translation initiation, local muscle contraction

2.2 Introduction

Skeletal muscle protein synthesis is accelerated following an acute bout of resistance-type exercise (9,28). However, in the absence of nutrient intake, net muscle protein balance (synthesis – breakdown) remains negative (3,28,29). Numerous studies unequivocally demonstrate that the ingestion of essential amino acids (8,35) and/or intact protein (22,34) can further stimulate muscle protein synthesis rates following resistance type exercise performed in a fasted state, resulting in net muscle protein accretion. However, with the experimental set-up of these studies, it is not possible to differentiate between the exercise vs diet induced protein synthetic response.

The application of a unilateral leg exercise protocol, with the contra-lateral leg as a control, has been shown to provide an appropriate tool to study the additive effect of exercise on muscle protein synthesis (29,32). Using such an approach, it has been shown that resistance exercise training results in an altered response to an acute bout of resistance exercise followed by food intake (32). However, to date, no study has examined the impact of resistance-type exercise on muscle protein synthesis following meal ingestion using the unilateral exercise model.

As changes in muscle protein synthesis occur before changes in muscle mRNA content (38), it is commonly accepted that muscle protein synthesis is largely controlled on a post-transcriptional level. The initiation of mRNA translation, which includes the binding of the initiator methionyl-tRNA and mRNA to the ribosomal subunits, is generally regarded to be rate limiting (18). Much recent research has been focused on the role played by the mammalian target of rapamycin (mTOR) signal pathway, i.e. activation of mTOR and its downstream signaling proteins p70 ribosomal protein S6 kinase (S6K1) and eukaryotic initiation factor (eIF) 4E-binding protein (4E-BP1), in regulating translation initiation (18).

Both S6K1 and 4E-BP1 modulate translation initiation and control the binding of mRNA to the 40S ribosomal subunit. 4E-BP1 can bind to the initiation factor eIF4E, thereby acting as a translational repressor, thus preventing the formation of the eIF4F scaffolding complex which is necessary for efficient binding of the 40S ribosomal subunit to mRNA (18). Via mTOR-mediated phosphorylation of 4E-BP1, 4E-BP1 is released from the initiation factor eIF4E, allowing the formation of the active eIF4F complex that mediates binding of mRNA to the 40S ribosomal unit, consequently allowing the translation-initiation process to occur (18). Another mechanism regulating the binding of mRNA to the 40S ribosomal subunit involves the phosphorylation of ribosomal protein S6 (S6), which is controlled by the activity of S6K1. Activation of S6K1 leads to the phosphorylation of S6 on the 40S subunit, located in near proximity to the eIF's. As a consequence, the interaction of the ribosomal subunit protein with the mRNA molecule promotes mRNA translation.

Pioneering studies in rodents demonstrate increased phosphorylation of both 4E-BP1 and S6K1 in response to electrical stimulation of the muscle (1,4,26), resistance-type exercise (24) and protein and/or leucine intake (18). Only a few studies have measured muscle protein synthesis and intracellular simultaneously following exercise and nutrition in humans (10,12,13,16). Therefore, evidence linking accelerated protein synthetic rates following exercise and nutrition in humans with the activation of the mTOR pathway remains limited.

The aim of the present study is to determine the impact of resistance-type exercise on muscle fractional synthetic rate (FSR) and mTOR-associated signaling proteins in a setting in which resistance-type exercise is performed in the fed state. A single-leg exercise protocol was performed to allow differentiation between the muscle protein synthetic response to local muscle contraction and the systemic changes in substrate availability and hormonal response.

2.3 Methods

2.3.1 Participants

Ten healthy male volunteers with no history of participating in any regular exercise program were recruited to participate in the present study. Subjects' characteristics are provided in Table 2.1. All subjects were informed on the nature and possible risks of the experimental procedures, before written informed consent was obtained. This study was approved by the Medical Ethics Committee of the Academic Hospital Maastricht. All volunteers were instructed to refrain from any sort of heavy physical exercise and to consume a normal diet for 3 days prior to the experiment.

Table 2.1 Subjects' characteristics

Subjects	
Age (yrs)	22.4 ± 0.7
Weight (kg)	76.8 ± 2.3
Height (m)	1.85 ± 0.02
BMI (kg.m-2)	22.4 ± 0.5
% bodyfat (%)	17.7 ± 1.7
Basal glucose (mmol.L-1)	5.1 ± 0.1
Basal insulin (mU.L-1)	10.3 ± 0.8
Leg volume (L)	8.8 ± 0.4
Single leg 1RM leg press (kg)	127 ± 7
Single leg 1RM leg extension (kg)	63 ± 4

Values are expressed as means±SE (n=10). 1RM, one-repetition maximum

2.3.2 Pretesting

Body composition was assessed using the hydrostatic weighing method in the morning following an overnight fast as described previously (23). Thereafter, maximum single-leg strength was estimated using the multiple repetitions testing procedure for leg press and leg

extension (23). In an additional exercise session, at least one wk before the first experimental treatment, the subjects' single-leg one repetition maximum (1-RM) was determined (23).

2.3.3 Protocol

The evening prior to the experiment, subjects consumed a standardized meal (32±2 kJ·kg^{-1} body weight, consisting of 55 energy% (En%) carbohydrate, 25 En% protein and 30 En% fat). A schematic overview of the study protocol is provided in Fig. 2.1. At 8.00 a.m., following an overnight fast, subjects arrived at the laboratory by car or public transportation. A Teflon catheter was inserted into an antecubital vein for stable isotope infusion. A second Teflon catheter was inserted into a heated dorsal hand vein of the contra-lateral arm, and placed in a hot-box (60°C) for arterialized blood sampling. After basal blood sample collection, subjects consumed a standardized breakfast (31±1 kJ·kg^{-1} body weight, consisting of 52 energy% (En%) carbohydrate, 34 En% protein and 14 En% fat), containing 50 g breakfast cereal (Quaker Cruesli, PepsiCo, Utrecht, Netherlands), 500 g curd cheese (Zaanse Hoeve, Campina, Veghel, Netherlands) and 10 mL strawberry flavoring (Karvan Cévitam, Heinz, Utrecht, Netherlands). Thereafter (t=-120 min), a single intravenous dose of L-[ring-$^{13}C_6$]phenylalanine (2 μmol·kg^{-1}) was administered to prime the phenylalanine pool. Subsequently, continuous tracer infusion was started (infusion rate (IR) 0.049±0.001 μmol·kg^{-1}·min^{-1}) for L-[ring-$^{13}C_6$]phenylalanine. Subjects rested in a supine position for 1 h before engaging in the resistance exercise protocol. After a 5 min warm-up on a cycle ergometer (~75 W), subjects completed a session of uni-lateral lower-limb exercises, consisting of 8 sets of 10 repetitions on the leg press and leg extension machines (Technogym BV, Rotterdam, The Netherlands), both performed at 70% of their individual 1-RM, with 2-min rest intervals between sets. All subjects were verbally encouraged during exercise and the entire exercise protocol required ~45 min to complete. At the end of the exercise protocol (t=0 min), subjects

rested supine and an arterialized blood sample and muscle biopsies from the *vastus lateralis* muscle of the exercised (Ex) and non-exercised (NEx) leg were collected. Arterialized blood samples were collected at t= 60, 120, 180, 240, 300, and 360 min with additional muscle biopsies taken at t=360 min from both the Ex and NEx limb, each from a new incision.

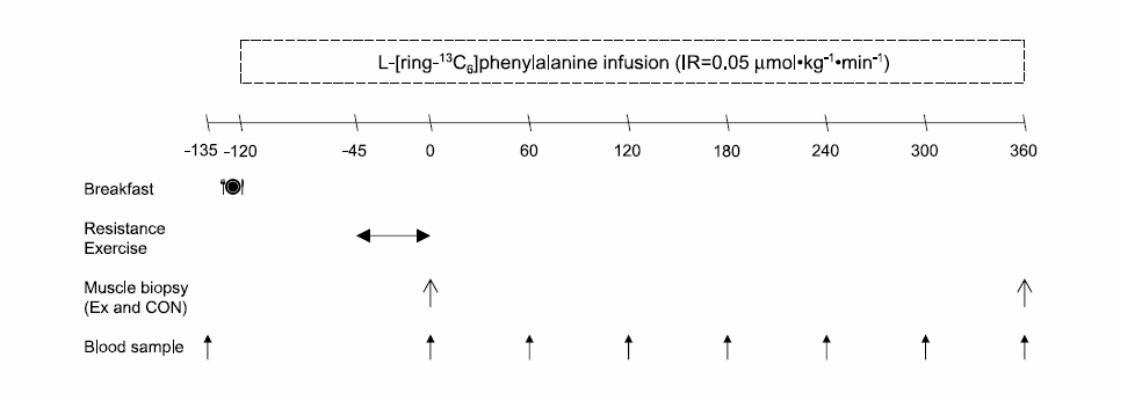


Figure. 2.1: Schematic representation of the experimental protocol. Muscle biopsies were collected from the exercised (Ex) and non-exercised (NEx) leg immediately after cessation of exercise and after 6 h of post-exercise recovery.

Time is expressed in min before or after the exercise bout. IR = infusion rate.

Biopsies were obtained from the middle region of the *vastus lateralis* muscle (~15 cm above the patella) and ~3 cm below entry through the fascia using the percutaneous needle biopsy technique (2). Muscle samples were dissected carefully, and freed from any visible non-muscle material. The muscle sample was immediately frozen in liquid nitrogen and stored at –80°C until further analysis. Blood samples were collected in EDTA containing tubes and centrifuged at 1000*g* and 4°C for 5 min. Aliquots of plasma were frozen in liquid nitrogen and stored at –80°C until further analyses.

2.3.4 Plasma sample analyses

Plasma glucose (Uni Kit III, 07367204, Roche, Basel, Switzerland) concentrations were analyzed with the COBAS-FARA semi-automatic analyzer (Roche). Insulin was

analyzed by radio immunoassay (Insulin RIA kit, LINCO Research Inc., St. Charles, MO, USA). Plasma (100µL) was deproteinized using 5-sulphosalicylic acid and free amino acid concentrations were measured using an HPLC technique, after precolumn derivatization with *o*-phthaldialdehyde (36). For measurement of plasma phenylalanine enrichment, plasma phenylalanine was derivatized to its t-butyldimethylsilyl (TBDMS) derivative and ^{13}C enrichment was determined by electron ionization gas chromatography-mass spectrometry (GC-MS, Agilent 6890N GC/5973N MSD Little Falls, DE, USA) using selected ion monitoring of masses 336 and 342 for unlabeled and labeled phenylalanine, respectively (22). We applied standard regression curves in all isotopic enrichment analysis to assess linearity of the mass spectrometer and to control for loss of tracer.

2.3.5 Muscle sample analyses

For measurement of L-[ring-$^{13}C_6$]phenylalanine enrichment in the free amino acid pool and mixed muscle protein, 55 mg of wet muscle was freeze-dried. Collagen, blood, and other non-muscle fiber material were removed from the muscle fibers under a light microscope. The isolated muscle fiber mass (10 mg) was weighed and eight volumes (8x dry weight of isolated muscle fibers x wet/dry ratio) of ice-cold 2% perchloric acid (PCA) were added. The tissue was then homogenized and centrifuged. The supernatant was collected and processed in the same manner as the plasma samples, such that intracellular free L-[ring-$^{13}C_6$]phenylalanine enrichments could be measured using their TBDMS derivatives on a GC-MS (22) and free amino acid concentration could be measured using HPLC technique (36).

The protein pellet was washed with 3 additional 1.5 ml washes of 2% PCA, dried and the proteins were hydrolyzed in 6M HCl at 120°C for 15-18h. The hydrolyzed protein fraction was dried under a nitrogen stream while heated to 120°C, then dissolved in a 50% acetic acid solution, and passed over a Dowex exchange resin (AG 50W-X8, 100-200 mesh hydrogen

form, Biorad, Hercules, CA, USA) using 2M NH_4OH. Thereafter, the eluate was dried and the purified amino acids were derivatized to their *N(O,S)*-ethoxycarbonyl ethyl esters for the determination of $^{13}C/^{12}C$ ratios of muscle protein-bound phenylalanine (19). Thereafter, the derivative was measured by GC-IRMS (Finnigan MAT 252, Bremen, Germany) using HP Ultra I GC-column (#19091A-112, Hewlett-Packard, Palo. Alto, CA), combustion interface II and monitoring of ion masses 44, 45 and 46. By establishing the relationship between the enrichment of a series of [ring-$^{13}C_6$]phenylalanine standards of variable enrichment and the enrichment of the *N(O,S)*-ethoxycarbonyl ethyl esters of these standards, the muscle protein-bound enrichment of phenylalanine was determined (Table 2). We applied standard regression curves to assess linearity of the mass spectrometer and to control for loss of tracer. The coefficient of variance (CV) for the measurement of L-[ring-$^{13}C_6$]phenylalanine enrichment in mixed muscle protein averaged 4.3±0.6%.

Another portion of the muscle samples was treated and homogenized using a previously described buffer containing several protease inhibitors (7,20,23). Primary phospho-specific antibodies (anti-phospho AMPK (T^{172}), anti-phospho 4E-BP1 (T^{37}) anti-phospho eEF2 (T^{56}), anti-phospho mTOR (S^{2448}), anti-phospho S6K1 (T^{421}/S^{424}), anti-phospho S6K1 (T^{389}), anti-phospho S6 ($S^{235}/^{236}$), anti-phospho eIF4B (T^{422})) and anti-AMPK, anti-4E-BP1, anti-eEF2, anti-mTOR, anti-S6K1, anti-S6, anti-eIF4B were purchased from Cell Signaling Technologies (Beverly, MA, USA). Quantification of phosphorylation status of AMPK, 4E-BP1, eEF2, mTOR, S6K1, S6, and eIF4B was performed using western blotting with phospho-specific and a-specific antibodies as previously described using α-actin as a loading control (20,23). Phosphorylation was expressed relative to the total amount of each protein.

2.3.6 Calculations

Fractional rate of mixed muscle protein synthesis (FSR) was calculated by dividing the increment in enrichment in the product, i.e. protein-bound L-[ring-$^{13}C_6$]phenylalanine, by the enrichment of the precursor. Free muscle L-[ring-$^{13}C_6$]phenylalanine enrichment was used as precursor pool to calculate fractional synthesis rate of mixed muscle protein (22).

2.3.7 Statistics

The present study was designed to assess the impact of single-leg exercise on muscle protein synthesis during recovery. All data are expressed as means±SE. Two-way analyses of variance (ANOVA) for repeated measures were applied to determine differences in phosphorylation status in the proteins of interest over time between treatments. In case of significant interaction between time and treatment effects, student paired t-tests were applied to locate differences between treatments and biopsies taken immediately post-exercise and following 6 h of recovery. Statistical significance was set at $P<0.05$. All calculations were performed using StatView 5.0 (SAS Institute inc., Cary, NC, USA).

2.4 Results

2.4.1 Plasma insulin, glucose and amino acid concentrations

Plasma insulin, glucose, phenylalanine, and BCAA (leucine, isoleucine and valine) concentrations reached maximal values after cessation of exercise (~2h after breakfast) and returned to baseline values within the next 2-4 h (Fig. 2.2).

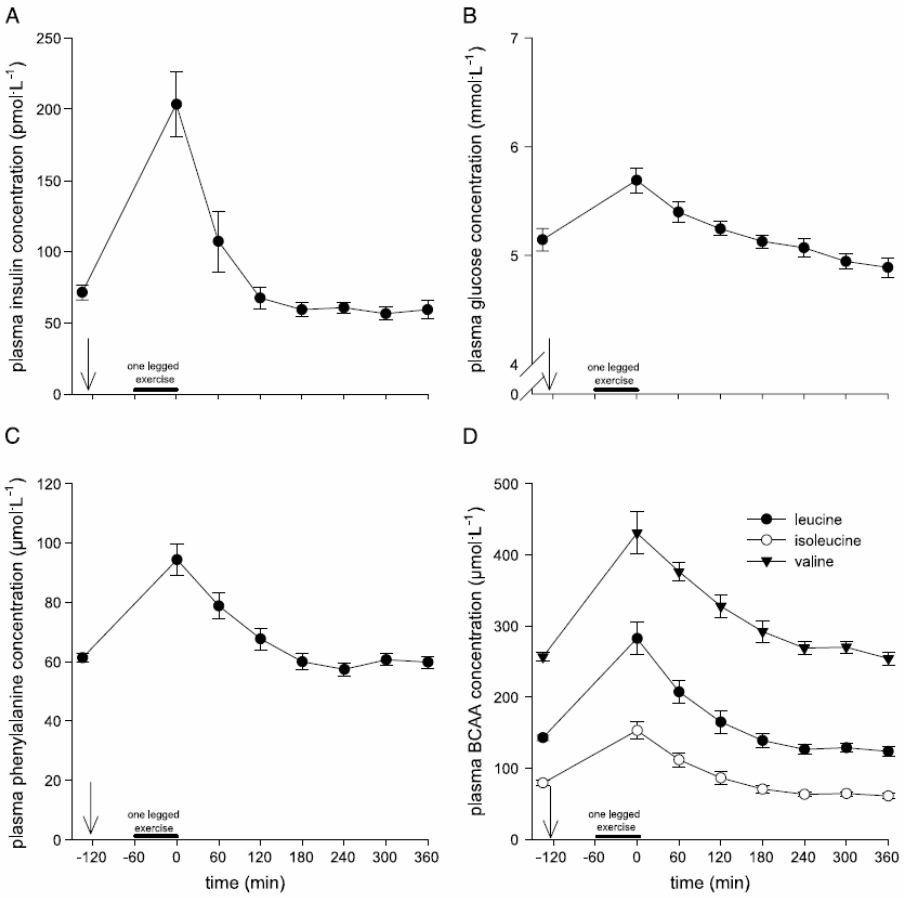


Figure 2.2: Plasma insulin (A), glucose (B), phenylalanine (C) and leucine, isoleucine and valine concentrations (D) during the entire test period. The horizontal lines in the graphs indicate time-period of the single-legged exercise.

Values represent means±SE (n=10). Arrows indicate timing of the breakfast.

2.4.2 Muscle phenylalanine enrichment and amino acid concentrations

Muscle free L-[ring-$^{13}C_6$]phenylalanine enrichment and L-[ring-$^{13}C_6$]phenylalanine incorporation were significantly higher in the exercised (Ex) leg compared to the non-exercised (NEx) leg (Table 2.2). Free muscle phenylalanine, leucine, isoleucine and valine concentrations as determined in the 0 and 6 h post-exercise muscle biopsy did not differ between the Ex and NEx leg (Table 2.3).

Table 2.2: Plasma and muscle tracer L-[ring-$^{13}C_6$]phenylalanine enrichments during post-exercise recovery

	Plasma (n=10)	**Exercised leg (n=10)**	**Non-exercised leg (n=10)**
Enrichment during recovery (TTR)	0.0687±0.0026	0.0507±0.0020*	0.0438±0.0019
Δ enrichment muscle protein (TTR)	NA	0.00030±0.00001*	0.00022±0.00001

Values represent means±SE. Tracer enrichments are expressed as tracer/tracee ratio (TTR). NA = not applicable.
* Significantly different from non-exercised leg (P<0.05).

Table 2.3: Muscle free amino acid concentrations during post-exercise recovery

	Non-exercised leg (n=10)			Exercised leg (n=10)		
	t=0	**t=360**	**(0-360)**	**t=0**	**t=360**	**(0-360)**
GLU	2536±327	2465±319	-71±470	1939±298	3449±407	1510±267 *
ASN	208±14	152±11	-55±14	221±15	201±18	-20±17
SER	542±22	440±13	-102±24	541±33	540±31	-1±30 *
GLN	9468±830	7362±410	-2106±939	8746±720	10403±1094	1658±705 *
HIS	393±34	316±15	-77±31	356±27	428±407	72±35 *
GLY	836±65	784±135	-52±153	831±74	887±87	56±46
THR	518±38	398±19	-120±34	557±43	536±58	-21±41
CIT	84±9	70±6	-14±7	84±8	103±13	19±7 *
ARG	311±28	220±190	-91±32	278±26	254±44	-24±31
ALA	1824±178	971±109	-853±245	1589±142	1116±115	-473±175 *
TYR	143±11	87±13	-56±12	139±14	78±7	-61±15
VAL	279±17	236±18	-43±15	305±23	245±21	-59±22
MET	30±9	14±3	-17±6	26±6	16±4	-13±5
ILE	100±9	70±11	-30±21	111±12	67±10	-39±21
PHE	68±7	61±8	-7±3	72±7	50±5	-22±8
TRP	16±2	11±3	8±2	15±2	17±7	3±6
LEU	184±12	139±15	-45±12	199±18	132±12	-68±19
ORN	171±13	146±10	-24±15	142±11	164±15	21±11 *
LYS	184±12	139±15	-45±12	199±18	132±12	-168±19

Values represent means±SE. Data are expressed in $nmol \cdot g^{-1}$ wet muscle weight. * Significantly different from non-exercised leg ($P<0.05$).

2.4.3 Muscle protein synthesis

Mixed muscle protein fractional FSR, with the average free L-[ring-$^{13}C_6$]phenylalanine enrichment in muscle tissue collected after exercise (t=0 min) and 6 h into recovery (t=360 min) as the precursor pool, averaged 0.083±0.002 and 0.098±0.005 $\%\cdot h^{-1}$ in the NEx and Ex leg, respectively (Fig. 2.3; $P<0.05$).

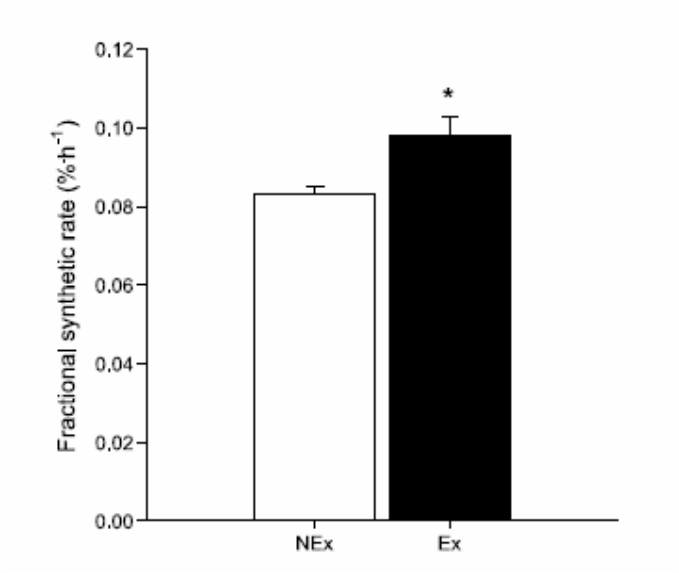


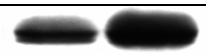
Figure 2.3: Mean (±SE) fractional synthetic rate (FSR) of mixed muscle protein during post-exercise recovery in the non-exercised (NEx) and exercised (Ex) leg in lean, young males (n=10) with the use of plasma phenylalanine enrichment as a precursor.
* Significantly different from non-exercised leg, P<0.05.

2.4.4 Western blotting results

Representative blots of phosphorylated and total protein content of the signaling proteins are presented in Table 2.4. The changes in the phosphorylation status of AMPK (T^{172}), 4E-BP1 (T^{37}), eEF2 (T^{56}) and mTOR (S^{2448}) during recovery from single-legged resistance type exercise in the Ex and NEx leg are presented in Fig. 2.4. No significant differences in phosphorylation status of AMPK, eEF2 and mTOR were observed between the NEx and Ex leg after cessation of exercise or following 6h of post-exercise recovery. 4E-BP1 phosphorylation was significantly lower in the Ex leg compared to the NEx leg immediately after exercise (P<0.05). Over time, a reduction in 4E-BP1 phosphorylation was observed in the NEx leg, whereas no changes over time were observed in the Ex leg (Fig. 2.4B). As a

result, 4E-BP1 phosphorylation was significantly higher in muscle taken from the Ex leg at 6 h post-exercise when compared to the NEx leg ($P<0.05$).

Table 2.4: Representative blots of phosphorylated and total protein content

		post		6h post	
		NEx	**Ex**	**NEx**	**Ex**
AMPK	phospho (T^{172})				
	total				
4E-BP1	phospho (T^{37})				
	total				
eEF2	phospho (T^{56})				
	total				
mTOR	phospho (S^{2448})				
	total				
S6K1	phospho (T^{421}/S^{424})				
	total				
S6K1	phospho (T^{389})				
	total				
S6	phospho ($S^{235/236}$)				
	total				
eIF4B	phospho (T422)				
	total				

NEx, non-exercised leg; Ex exercised leg

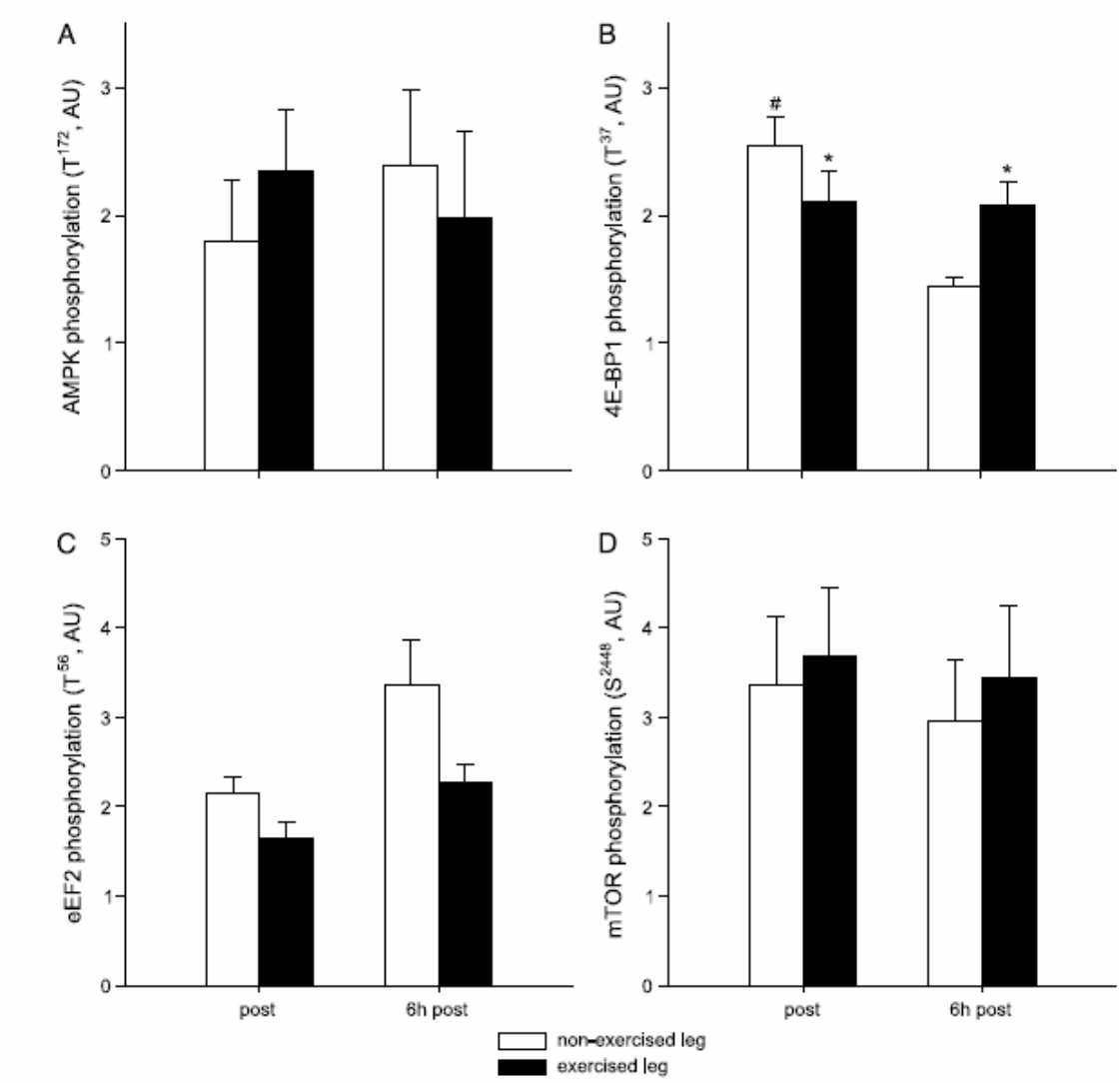


Figure 2.4: AMPK phosphorylation at T172 (A), 4E-BP1 phosphorylation at T37 (B), eEF2 phosphorylation at T56 (C), mTOR phosphorylation at S2448 (D) immediately after cessation of exercise and following 6 h of post-exercise recovery in the exercised and non-exercised leg. Representative immunoblots are shown (top).

Values represent means±SE (n=10). *Significantly different from the non-exercised leg, P<0.05. # Significantly different from values at 6 h post, P<0.05.

The changes in the phosphorylation status of S6K1 (T^{421}/S^{424} and T^{389}), S6 ($S^{235/236}$), eIF4B (S^{422}) during recovery from single-legged resistance exercise in the Ex and NEx leg are presented in Fig. 2.5. Two-way ANOVA for repeated measures revealed a significant time

effect ($P<0.05$) and a trend for a treatment effect ($P=0.06$), without significant interaction ($P=0.12$) for S6K1 phosphorylation status (T^{421}/S^{424}). No significant changes in phosphorylation status of S6K1 (T^{389}), and eIF4B (S^{422}) were observed between the NEx and Ex leg. S6 phosphorylation status was significantly higher immediately after cessation of exercise in the Ex leg compared to the NEx leg ($P<0.05$). During recovery, the S6 phosphorylation status declined in both the NEx and Ex leg (-42±7 vs -26±10%, respectively, P=NS). No significant differences in S6 phosphorylation were observed following 6 h of recovery.

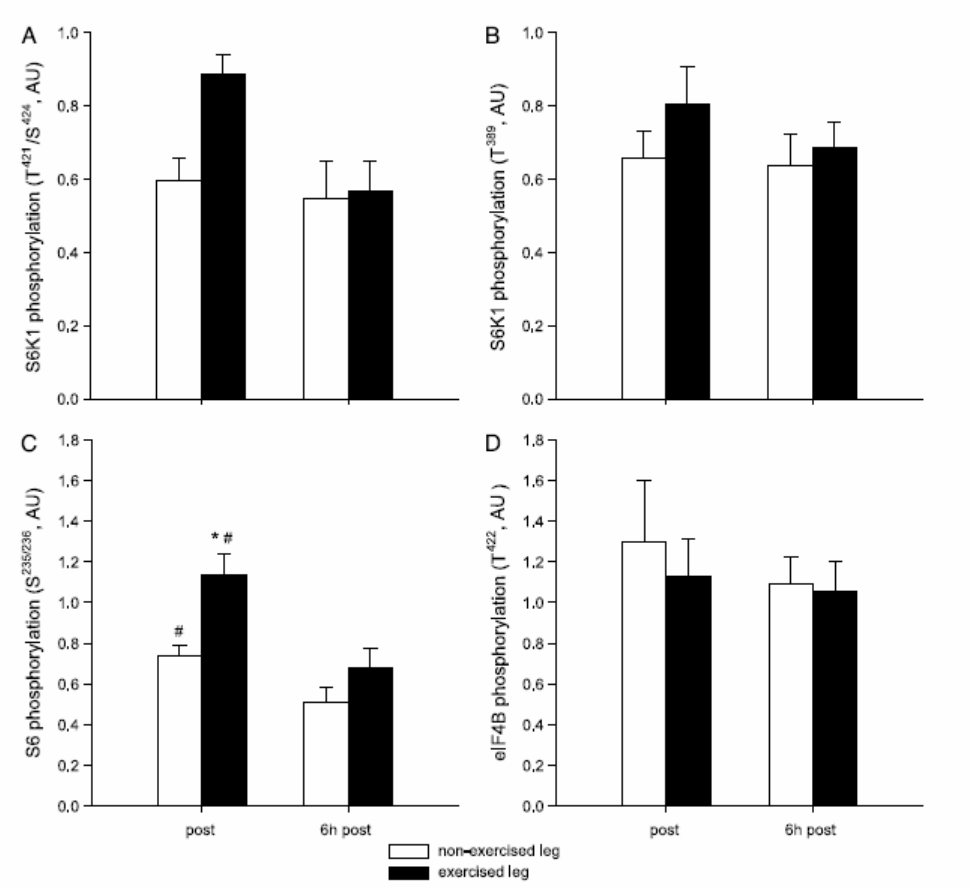


Figure 2.5: S6K1 phosphorylation at T421/S424 (A), S6K1 phosphorylation at T389 (B), S6 phosphorylation at S235/236 (C), eIF4B phosphorylation at S422 (D) immediately after cessation of exercise and following 6 h of post-exercise recovery in the exercised and non-exercised leg. Representative immunoblots are shown (top).
Values represent means±SE (n=10). *Significantly different from non-exercised leg, P<0.05. # Significantly different from values at 6 h post, P<0.05.

2.5 Discussion

In the present study, we assessed the impact of resistance exercise on skeletal muscle protein synthesis rates when exercise is performed in the fed state. The application of a single-legged exercise protocol allowed differentiation between the impact of exercise and the systemic changes in substrate availability and hormonal responses due to the intake of food. In addition, we determined the impact of exercise on the phosphorylation status of a number of signaling proteins known to play a key role in the regulation of muscle protein synthesis at two time points following exercise and/or food intake. Resistance exercise substantially increases muscle protein synthesis rates, accompanied by an elevated phosphorylation state of both 4E-BP1 and S6.

Resistance exercise has been reported to stimulate muscle protein synthesis for up to 48 h post-exercise (28). Moreover, it has been well established that net protein balance following exercise remains negative until protein and/or amino acids are administered (3,28). Numerous studies have demonstrated that the ingestion of intact protein (21,22,33,34) or amino acids (12,16,25,30,35) further stimulate muscle protein synthesis during post-exercise recovery. However, these studies compared the interventions to the basal, i.e. fasted, resting, condition. Few previous studies have compared the additive effect of exercise + nutrition vs nutrition only. In these studies, resistance exercise was carried out in the fasted state followed by the infusion of amino acids (3) or the continuous administration of small amounts of a mixed macronutrient drink (27,31,32). Both scenarios are atypical of normal everyday practice, in which exercise activities are commonly performed following feeding. Furthermore, the pattern of amino acid availability in the blood is different following a bolus ingestion of protein (34) vs. continuous infusion or feeding (3,11,27) and this difference

might modulate the protein synthetic response (6). The impact of resistance exercise on the protein synthetic rate of muscle already stimulated by food intake has yet to be elucidated.

In the present study, we applied a study design in which untrained males performed an acute bout of unilateral resistance-type exercise 75 min after ingesting a standardized breakfast. Our data demonstrate that mixed muscle protein synthesis rates were significantly higher (~20%) in the Ex vs NEx muscle (Fig. 3). The observed FSR values are in a range with previously reported protein synthesis rates following amino acid infusion (3) or repeated ingestion of a mixed macronutrient drinks (27,32) during post-exercise recovery. We acknowledge that it is not everyday practice to remain fasted during 6h of post-exercise recovery. However, this study design was a prerequisite to allow us to differentiate between the impact of muscle contraction and nutrition on muscle protein synthesis following exercise performed in the fed state. The logical extension to this study design is to add ingestion of protein following exercise, such as is common practice among those participating in resistance exercise programs.

Resistance exercise performed in the fasted state has previously been demonstrated to induce a 100-150% increase in FSR compared to a basal (i.e. resting and fasted) situation (28). In the present study, we did not measure basal mixed muscle protein synthetic rate. However, using similar measurement techniques (i.e. tracer incorporation method and choice of IC as precursor) to the present study, previous papers commonly report basal FSR values to be ~0.04-0.06%/h (28,37). Thus, the reported FSR values in the present study would correspond with an ~80-150% increase from basal muscle protein synthesis rates, values similar to previously published data (28). Therefore, the present study clearly shows that resistance exercise substantially stimulates mixed muscle protein synthesis in conditions where food intake is followed by resistance exercise.

Over the past few years, it has been well established that the mTOR signal-transduction pathway plays a key regulatory role in the control of muscle protein synthesis (18). Downstream targets of mTOR, e.g. S6K1 and 4E-BP1, control the rate of translation initiation by regulating the binding of mRNA to the ribosomal subunits (18). Although it has been demonstrated in humans that increases in muscle protein synthesis following either resistance exercise (12,13) or amino acid administration (10,16) are accompanied by the activation of the mTOR signaling pathway, a paucity of data examining the effect of exercise and nutrition on both FSR and molecular signaling currently exists. In our study design, muscle biopsies were collected from Ex and NEx muscle immediately following exercise and after 6h of post exercise recovery, corresponding to 2 and 8h following food intake (Fig. 1). This approach allowed us to determine potential differences in phosphorylation status of AMPK, 4E-BP1, eEF2, mTOR, S6K1, S6, and eIF4B due to food intake and food intake in combination with exercise at these time-points. The present study design is restricted by the number of biopsies that were collected. No biopsies were collected prior to exercise and/or throughout the different stages of post-exercise recovery. Therefore, the present data are not representative of the time-course of the changes in mixed muscle protein synthesis and/or skeletal muscle signaling.

It has previously been shown that a single bout of high-intensity resistance-type exercise performed in the fasted state results in skeletal muscle AMPK phosphorylation (13,23). The latter has been associated with a decline in 4E-BP1 phosphorylation and an inhibition of muscle protein synthesis (7,13,23). Therefore, it has been proposed that AMPK plays an important role in the regulation of muscle protein synthesis. However, in the present study, in which exercise was performed in the post-prandial state, we did not observe a significant increase in AMPK phosphorylation in the Ex compared to the NEx leg (Fig. 4A).

These observations suggest that AMPK is not further activated when resistance type exercise is performed in a fed state, which would be in line with previously published data showing that feeding reduces AMPK phosphorylation (16). Interestingly, we did observe a significantly lower 4E-BP1 phosphorylation state following exercise in the Ex muscle compared to the NEx leg, despite the fact that AMPK and mTOR phosphorylation were similar in muscle tissue from both legs (Fig. 4). The latter is not surprising as this would be in line with previous findings showing that the impact of nutrition is of greater importance in the up-regulation of mTOR phosphorylation when compared to exercise (12,16). Ingestion of essential amino acids has previously been shown to substantially enhance 4E-BP1 phosphorylation in humans (16). Combined with recent observations (20), it seems reasonable to assume that the marked decrease in 4E-BP1 phosphorylation generally observed after exercise (12,23) is ameliorated when exercise is performed in a fed, as opposed to a fasted state. In the present study, the phosphorylation status of 4E-BP1 was significantly higher (~40%) in the Ex leg after 6 h of post-exercise recovery compared to the NEx leg (8h following meal ingestion) (Fig. 4B). The latter tends to be in line with the observation that exercise lead to a more prolonged stimulation of muscle protein synthesis (28) when compared with the protein synthetic response to food intake (5).

The present study shows that phosphorylation of S6K1 at T421/S424 tended to be higher following local muscle contraction when compared to the impact of food intake only (Fig. 5). These data confirm that the T421/S424 phosphorylation status of S6K1 is predominantly influenced by exercise (23), and is not greatly affected by mere changes in circulating insulin and/or amino acid concentrations (20). Phosphorylation of S6K1 at T421/S424 is not necessarily associated with increased activity of the kinase (S6K1). This notion is supported by the observation that exercise only does not increase the

phosphorylation status of S6K1 at its activation site (T389) during the early stages of post-exercise recovery (14,17,23). Amino acid availability has been suggested to be essential to augment S6K1 phosphorylation at T389 following exercise (12,17,20). In the present study, skeletal muscle S6K1 phosphorylation at T389 did not differ between the exercised leg at 0 and 6h of post-exercise recovery and the non-exercised leg (2 and 8h following food intake, Fig. 5). Despite the lack of significant changes in S6K1 phosphorylation at T389, we did observe greater S6 phosphorylation after cessation of exercise in the active limb (Fig. 5). The latter might be attributed to the inability to sample muscle tissue more frequently, preventing the observation of a peak in S6K1 phosphorylation following exercise and food intake. Alternatively, S6 phosphorylation could also be mediated by another anabolic signaling pathway in addition to the mTOR signaling cascade, i.e. MAP-dependant kinase (MAPK) signaling (15,39). Recent studies in rodents (15) and humans (39) have shown activation of MAPK signaling proteins (ERK 1/2, p90RSK, Mnk 1, P38 MAPK and JNK/SAPK) following resistance type exercise. However, based on the paucity of currently available data in humans, it is difficult to speculate on the potential role of MAPK signaling proteins in augmenting protein synthesis following muscle contraction.

In the present study, we simultaneously measured protein FSR and the phosphorylation state of key signaling proteins in the mTOR pathway in exercised and non-exercised leg muscle. The classic study by Baar and Esser (1999) has shown a strong correlation (r=0.998) between S6K1 phosphorylation measured following electrical stimulation and subsequent muscle mass gain in rodents (1). Their data have been used to suggest that the phosphorylation status of key signaling proteins following resistance-type exercise might be applicable as biomarkers for the long-term increase in skeletal muscle mass. In humans, studies examining the effect of exercise and nutrition on both FSR and molecular

signaling are quite limited. Recently, it has been demonstrated that increases in muscle protein synthesis rate following either resistance exercise (12,13) or amino acid administration (10, 16) are accompanied by the activation of the mTOR signaling pathway. Interestingly, we did not observe any significant correlations between FSR and the (changes in) the phosphorylation state of the different key proteins in the mTOR signaling cascade (data not shown). From a physiological standpoint, lack of association between elevated FSR and anabolic signaling should not necessarily be considered surprising. Biopsies were taken immediately after cessation of exercise and following 6h of recovery, allowing the assessment of FSR during the entire post-exercise recovery period and the determination of anabolic signaling events at these two time-points. However, in the present study we did not aim to assess potential changes in the mTOR signaling cascade that could have occurred during early (2h) post-exercise recovery (12). In addition, it could be speculated that there is a temporal disconnect between phosphorylation of certain signaling proteins and maximal rates of muscle protein synthesis. Furthermore, we acknowledge the potential role of alternate signaling pathways such as MAPK in modulating the synthesis of muscle proteins. Clearly, more *in vivo* human research is warranted to assess the time dependent changes in both skeletal muscle signaling and FSR. So far, the latter has been restricted by the number of muscle biopsy samples that are being collected, which generally precludes a more detailed insight into the time-course of changes in skeletal muscle signaling.

2.6 Conclusion

In conclusion, resistance exercise in the fed state substantially increases mixed muscle protein synthesis rate. The greater increase in mixed muscle protein synthesis following muscle contraction is accompanied by an elevated 4E-BP1 and S6 phosphorylation status during post-exercise recovery.

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CHAPTER 3 - Intensified training attenuates the exercise-induced minimal mobilisation of effector memory CD8+ T lymphocytes.

Contributions to study: Sarah Jackman assisted with data collection and blood analysis. Dr Jos Bosch and Natalie Riddell provided flow cytometry training. Dr Jos Bosch, Dr Kevin Tipton, Professor Asker Jeukendrup and Dr Juul Achten designed the study. Lucy Walton, Rachel Turner and Sarah Jackman assisted with dietary control.

3.1 Abstract

Introduction: The cause of the increased incidence of upper respiratory tract infections (URTI) experienced by endurance athletes has yet to be elucidated. The purpose of this study was to compare lymphocyte counts during and after exercise following a period of intensified training (INT) compared to normal training (NOR). **Methods:** Trained cyclists (Wmax: 372±21W) completed a trial lasting two weeks, equally divided into NOR and INT. Extensive lymphocyte sub-population phenotyping was performed using multicolour flow cytometry on blood collected at baseline (BL), following submaximal exercise (SM), at immediate cessation of intense exercise (TT) and one hour post exercise (1h-POST). Specifically, flow cytometry was used to identify CD8+T lymphocyte (CD8+TL), CD4+T lymphocyte, Natural Killer cells and B lymphocytes. Additionally, CD8+TL were classified into functionally distinct subsets: Naïve, Central Memory, Effector Memory and CD45RA+ Effector Memory (RA+EM). **Results:** Over the entire exercise period, the minimal mobilisation of RA+EM CD8+TL was blunted following INT (3144±924 cells·µL blood^{-1}·165 min) compared to NOR (6417±2143 cells·µL blood^{-1}·165 min) ($P<0.05$). Subsequently, a tendency ($P=0.06$) for an attenuated post exercise minimal efflux of RA+EM CD8+TL was observed following INT (71±32 cells·µL blood^{-1}) compared to NOR (130±46 cells·µL blood^{-1}). INT blunted plasma cortisol concentrations at BL, TT and 1h-POST ($P<0.05$), as well as peak adrenaline concentrations (NOR=3.88±1.38ng/mL vs. INT=2.42±0.91ng/mL, $P<0.05$). **Conclusion:** Intensified training blunted the minimal mobilisation and eggression of highly cytotoxic CD8+TL in response to intense exercise. The reduced ability of these cytotoxic anti-viral lymphocytes to migrate in and out of the blood may render athletes less capable to respond to infection.

Keywords: anti-viral lymphocytes, lymphocyte trafficking, exercise training, stress hormones.

3.2 Introduction

Epidemiological evidence supports the suggestion that athletes experience an increased susceptibility to upper respiratory tract infections (URTI) (i.e. common colds, sore throat) following major competition or during intensified periods of training (14). A series of cross-sectional studies demonstrated a 50-70% greater incidence of URTI reported by endurance athletes during a two week period following a marathon (36) or an ultramarathon (42,43,44) compared to non-athletes. In addition, longitudinal studies reveal a >40% incidence of URTI in endurance athletes undertaking a period of increased training volume (7,19,32). Thus, recent research has examined the relationship between chronic exercise training and changes in immune parameters (31).

Chronic periods of intense training alter several aspects of immune function. Immunological perturbations include declines in neutrophil priming (45), natural killer (NK) cell cytotoxicity (52) and lymphocyte activation in response to antigen (27). In addition, salivary immunoglobulin A (S-IgA) concentrations measured at rest and after exercise have been shown to decline progressively as training intensity increased during the course of a season in trained swimmers (15,16). However, with the exception of S-IgA concentrations (13), changes in the functional capacity of immune parameters do not appear to correlate with increased incidence of infection in athletes (31). In an attempt to fully elucidate the causal development of URTI during intense training, alternative immune parameters should be considered.

Immune cells (leukocytes) continuously traffic around the body, traveling from the blood into various peripheral tissues and back into the blood. This trafficking pattern of immune cells is essential to maintaining an effective immune defence network (53) and is known to respond sensitively to exercise. Indeed, the rapid deployment of lymphocyte sub-

populations into the blood, known as lymphocytosis, is the best documented response of the immune system to exercise (1,48). This response is considered to reflect an adaptive mechanism, priming the immune system to fight pending infection (6). The lymphocytosis during strenuous exercise is followed by a state of 'lymphocytopenia' in the post exercise period, whereby lymphocyte counts reach a nadir. The open window for infection hypothesis predicts that this lymphocytopenic response following prolonged intense exercise is representative of a transient state of immune suppression (38). However, this idea is controversial (46). Animal studies (24,53) indicate that immediately following exercise, lymphocytes are transiently redistributed from the blood to the peripheral tissues. Thus, rather than a state of immune suppression, this phenomenon reflects an important functional response of the immune defence network, whereby sufficient cells numbers are redeployed to locations such as the skin and mucosa where antigens are most likely to be encountered first. Characterising how intense exercise affects this continuous redeployment of leukocytes may thus clarify how exercise influences the capacity of the immune system to protect its host (2).

The aim of the current study is to characterise how intensified training alters the redeployment of circulating lymphocytes. Recent methodological advances in immunology identify functionally distinct subsets of NK cell and T lymphocyte sub-populations. Two main subsets of NK cells can be distinguished phenotypically on the basis of surface expression on CD56. A minority of NK cells in the blood (~10%) express high levels of CD56 (CD56hi) (5). Functionally, these CD56hi NK cells exhibit mainly regulatory properties and are not directly involved in the killing of infected cells (5). The majority of NK cells in the blood (~90%) express relatively low levels of CD56 (CD56lo). Aided by the expression of CD11a, CD56lo NK cells have the propensity to migrate into inflamed peripheral tissues, where they may utilise their high cytolytic potential (30).

Four broad subsets of CD8+ T lymphocytes (CD8+TL) may be discerned: Naïve (NA); Central Memory (CM); Effector Memory (EM); and Effector Memory CD8+ TL that have re-expressed the naïve marker CD45RA (RA+EM) (47). Both NA CD8+TL, which have yet to encounter their cognate antigen, and CM CD8+TL continuously recirculate between the blood and secondary lymphoid organs. Memory or antigen experienced effector T cells are more diverse with respect to their migratory potential. Viral or tumour antigens may be located anywhere in the body, thus EM and RA+EM T lymphocytes have the propensity to migrate to the peripheral tissues, such as the skin, lungs and bone marrow. These EM, and in particular RA+EM subsets are highly cytotoxic, i.e., exhibit the functional capacity to eliminate antigen by killing off infected cells by direct cell-to-cell contact (18,29,47). Monitoring the trafficking pattern of functionally distinct lymphocyte subsets rather than sub-populations in response to exercise may be considered more representative of the capacity of the immune system to protect its host when challenged by repeated bouts of intense exercise.

Recently, it has been established that the recruitment of the highly cytotoxic CD56lo cells are largely responsible for the preferential exercise-induced mobilisation into the bloodstream of NK cells compared to other lymphocyte sub-populations (3,52). Interestingly, a recent study by Suzui *et al.* (52) demonstrated that intensified training increased the resting proportion of the non-cytotoxic CD56hi NK cells, concomitantly decreasing the proportion of highly cytotoxic NK cells. These data (52) provide promising support for the suggestion that athletes involved in periods of intensified training may compromise the functional capacity of NK cells. Additionally, Campbell *et al.* (3) recently demonstrated that both EM and RA+EM CD8+TL subsets were mobilised into the bloodstream to a greater extent than NA and CM CD8+TL during brief bouts of exercise in humans. However, the trafficking pattern of

functionally distinct T lymphocyte subsets which exhibit distinct migratory patterns during and following exercise has yet to be systematically investigated following intensified training.

The purpose of the present study was two-fold. First, we set out to monitor cell counts of functionally distinct CD8+TL and NK cell subsets during and following an acute bout of intense exercise. Second, our study was designed to compare the pattern of minimal mobilisation and minimal egression of lymphocytes in response to exercise following intensified, compared to normal training. A blunted minimal mobilisation and minimal egression of lymphocytes is taken to mean that the immune system is less capable of optimally redistributing lymphocytes in response to exercise, possibly rendering the athlete less protected when exposed to an infectious antigen.

3.3 Methods

3.3.1 Participant characteristics

Eight endurance-trained cyclists (age: 27±8yr; weight: 73.0±7.1kg; maximal oxygen uptake ($\dot{V}O_2$): 64.2±6.5ml·kg^{-1}·min^{-1} and Wmax: 372±21W) were recruited to participate in this study. All cyclists had a training history of at least five years. Prior to participation, the health status of each participant was assessed using a General Health Questionnaire (Appendix A). Participants were non-smokers, not taking any medication and had remained free of respiratory infection for four weeks prior to participation in the study. Written informed consent (Appendix B) was preceded by a detailed explanation of practical details, associated risks and required commitment applicable to the present study (Appendix C). Participants were reminded of their right to withdraw from the study at anytime, without provision of reason. The protocol was approved by the Research Ethics Committee of the School of Sport and Exercise Sciences, University of Birmingham, UK.

3.3.2 Experimental design

The aim of the present study was to examine the impact of intensified cycle training on the mobilisation of lymphocyte subsets into the bloodstream. Fig. 3.1 illustrates the experimental model. Each participant undertook a two week period of quantified training which consisted of one week of normal training (NOR) and one week of intensified training (INT). On day seven of both training weeks, a pre-loaded time trial was performed. Cell counts of a series of lymphocyte sub-populations were measured at rest, following exercise and after 1 h recovery (Fig. 3.1).

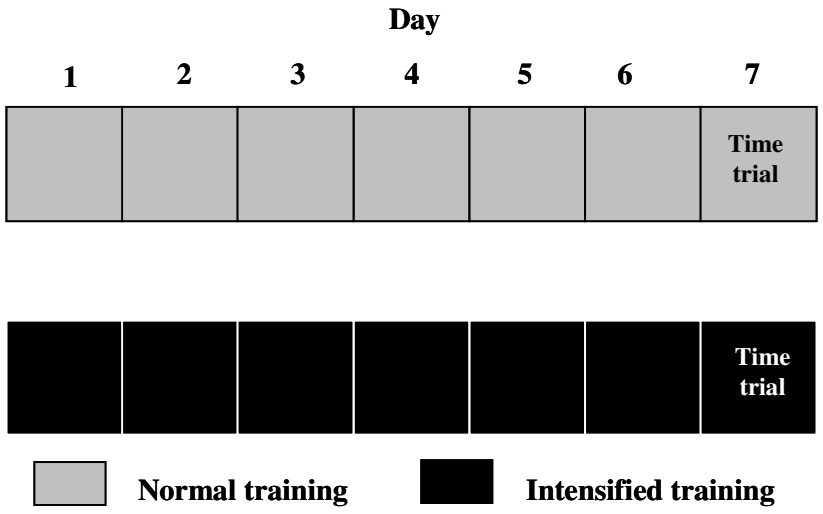

Figure 3.1: Schematic representation of experimental model.
Time trial, pre-loaded (120 min) time trial (45 min).

3.3.3 Preliminary assessments

3.3.3.1 Incremental test to exhaustion

Each participant completed a preliminary incremental test to exhaustion ($\dot{V}$ O_2max test) prior to the three week training period to determine maximal power outputs (Wmax) and lactate threshold (LT). Subjects reported to the laboratory after an overnight fast (≥10 h). A Teflon catheter connected to a three-way stopcock was inserted into a forearm vein and a resting blood sample (~1 ml) was drawn. The catheter was maintained patent with isotonic saline. Subjects then performed an incremental test to exhaustion using an electrically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). Subjects began cycling at 95W and workload was increased by 35W at three min intervals until volitional exhaustion (i.e. when the athlete could no longer maintain a cadence >60 rpm). Wmax was calculated according to the formula:

$$\mathbf{Wmax = Wfinal + (t/T)*W}$$

where Wfinal (W) is the final stage completed, t (s) is the amount of time reached in the final uncompleted stage, T (s) is the duration of each stage and W (W) is the workload increment (21).

In the final 30 s of each exercise intensity and at immediate cessation of the test, a blood sample was collected (~2 ml) into 20mM K3 ethylenediamine tetraacetic acid (EDTA) vacutainers for plasma lactate analysis. LT was determined using the maximal distance (Dmax) method as previously described (4). Five training zones were calculated from the $\dot{V}O_2$ max test in accordance to British cycling guidelines and expressed as target heart rate values. Test duration ranged from 21-29 min.

3.3.3.2 Dietary Analysis

In the week preceding study commencement, a three-day weighed food record was completed. Participants were asked to record accurately all food items they consumed during two weekdays and one weekend day. To increase the accuracy of the food analysis, subjects were provided with electronic scales to weigh all food products and were carefully instructed on how to document food intake. Diets were analysed using an internet-based nutrition analysis software (www.weightlossresources.co.uk). The habitual energy intake of the subjects was 2882±172 kcal/day and the diet consisted of 1.6±0.1g protein·kg body mass(BM)$^{-1}$·day^{-1}, 5.4±0.3g carbohydrate·kgBM^{-1}·day^{-1} and 1.3±0.1 g fat·kgBM^{-1}·day^{-1}.

3.3.4 Experimental trial periods

3.3.4.1 Dietary control

Diet was controlled for the duration of the study with all foods prepared by investigators in the Metabolic Research Kitchen of the School of Sport and Exercise Sciences, University of Birmingham. Prior to study commencement, participants completed a food questionnaire to determine food likes, dislikes and preferred items consumed during training rides (Appendix E). Participants were fed with the aim to maintain energy balance. Total energy intake was adjusted on a daily basis to ensure weight stability. Participants consumed

an energy-balanced diet based on a macronutrient intake equivalent to 1.5g protein·kg body mass $(BM)^{-1}\cdot day^{-1}$ and 6g carbohydrate·kg $BM^{-1}\cdot day^{-1}$, with the remainder of energy derived from fat. During INT, energy intake was increased to match energy expenditure, however diet composition was not manipulated. Carbohydrate-rich fruit smoothies were consumed daily on immediate completion of training sessions during INT. Five set menus were carefully composed using the internet-based nutrition analysis software (www.weightlossresources.co.uk). Food and drinks were provided as three main meals together with a collection of snacks. Participants were asked to refrain from consuming alcohol for the duration of the study.

3.3.4.2 Training quantification

Training was controlled and quantified over the two week training period. Each athlete was equipped with a downloadable heart rate monitor (Polar Electro, Kempele, Finland) for the duration of the study to monitor individual training sessions. During NOR, participants completed their usual volume and type of training. Individualised programs, consisting of training goals and recommended local routes were prescribed to cyclists during INT. During INT, athletes were required to train on a daily basis, which typically consisted of a combination of high-intensity interval sessions above LT, lasting between two and three hours, and long-continuous rides, usually between four and five hours in duration. These sessions were designed to increase total training hours and training intensity, as determined by time spent in training zones three, four and five. Individual training sessions were not supervised; however athletes were given the option to train in the laboratory. In an attempt to monitor training as accurately as possible and ultimately standardise training impulse between trials, each participant received an athlete handbook to record intensity and duration of each training

session. All participants were contacted on a daily basis to check adherence to training regimens.

3.3.4.3 Time Trial

On day seven of each training week, immunological and hormonal measurements were collected simultaneously with a previously validated (CV=3.34%) pre-loaded time trial (21) (Fig. 3.2). Participants reported to the laboratory in a fasted state and a Teflon catheter was inserted into a forearm vein. Following 10 min rest in a supine position, a baseline (BL) blood sample was collected. Thereafter, cyclists completed 120 min of submaximal exercise (pre-load) at a relative intensity of 50%Wmax. The electromagnetically braked ergometer was set in the hyperbolic mode (cadence-independent mode) so that the work rate (50%Wmax) was independent of pedalling rate. Upon immediate completion of submaximal exercise, participants typically toilleted, before commencing the simulated time trial. The ergometer was set in the linear mode so that by increasing pedaling rate, the work rate increased, in accordance with the following formula:

$$W = L * (RPM)^2$$

In which RPM is the pedalling rate and L is a (constant) linear factor. The linear factor is based on individual Wmax and was calculated so that 70%Wmax will be produced at a pedalling rate of 90rpm (21).

Participants were asked to complete a set amount of work in as short a time as possible, consequently eliciting a maximal effort on the part of each cyclist. Total work to be completed was individualised, equivalent to working at 70%Wmax for 45 min and was calculated according to the equation adapted from Jeukendrup *et al.* (1996):

$$\text{Total amount of work (kJ)} = 0.7 * \text{Wmax (W)} * 2700\text{(s)}$$

Where 0.7 refers to a factor equivalent to 70%, Wmax (W) is the maximum wattage attained during the incremental test in watts and 2700 refers to the number of seconds that make up 45 min.

All trials were performed under normal and standard environmental conditions (20-23°C dry bulb temperature and 50%-60% relative humidity) as immune cell counts have been observed to be effected by temperature (22). To minimise thermal stress, a floor-standing fan was positioned in front of cyclists and water was provided *ad libitum*. Blood samples (~25ml) were collected into K3 ethylenediamine tetraacetic acid (EDTA) vacutainers at BL, following 120 min submaximal pre-load (SM), at immediate cessation of time trial (TT) and after 1h of passive recovery (1h-POST). Blood volumes were divided equally for immunological and stress hormone analyses. For immunological analyses, EDTA vacutainers containing whole blood were positioned horizontally on the laboratory worktop at room temperature (~23˚C) to avoid undue agitation. Samples were analysed on the day of collection (within ~10h) for a range of lymphocyte sub-populations and individual lymphocyte subsets. Analyses of stress hormone concentrations were performed using blood that was kept chilled on ice during the trial, before centrifugation at 3000rpm at 4˚C for 10 min. Plasma was frozen at -80˚C until further analysis. Catecholamine responses could not be detected at 1h-POST.

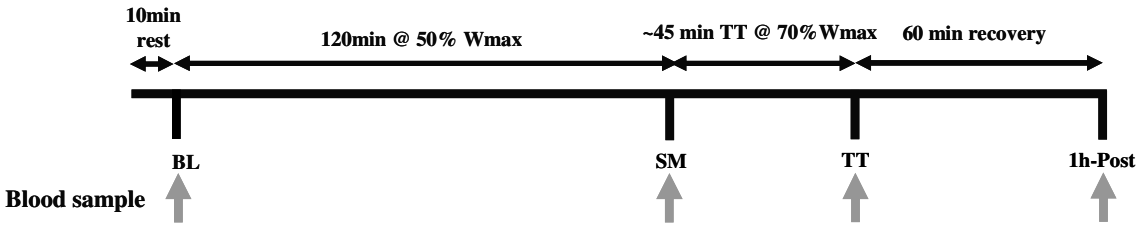

Figure 3.2: Schematic representation of time trial protocol.
BL, baseline; SM, immediate cessation of submaximal exercise, TT, time trial and 1h-POST, 1h after time trial.

3.3.4 Haematological analysis

Complete blood counts were determined using a Coulter GEN-S haematology analyzer (Beckman-Coulter, Miami, USA) at all time-points. Total white blood cell (WBC) counts were determined in addition to differential leukocyte sub-population counts, which

included neutrophil, lymphocyte and monocyte counts. Neutrophil:Lymphocyte (N:L) was also calculated.

3.3.5 Flow Cytometry

3.3.5.1 Immuno-fluorescent antibody staining of whole blood

Extensive leukocyte and lymphocyte sub-population phenotyping was performed using multicolour flow cytometry. Aliquots (50μL) of whole blood and immuno-fluorescent antibodies (4μL) were added to polystyrene tubes before being incubated in the dark for 25 min at room temperature with conjugated antibody. Erythrocytes were then lysed with 1.5 ml FACS lysing solution (10% concentrated hypotoxic buffer) (Becton Dickinson, San Jose, USA). Following a further 10 min incubation, cell suspensions were washed by centrifugation at 500 x g for 6 min at 4˚C, and then re-suspended in 300 μL of phosphate buffered saline (PBS) containing 1% formaldehyde, and stored in the dark at 4˚C before being analysed using flow cytometry. Analysis was based on whole blood rather than peripheral blood mononuclear cell preparations (PBMC's) to minimise the potential selective losses of subsets during preparation of cell suspensions.

Combinations of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- and allophycocyanin (APC)-conjugated (Pharmingen, San Diego, USA) and peridinin-chlorophyll-protein complex (PerCP)-conjugated (Becton Dickinson, San Jose, USA) monoclonal antibodies were used to stain peripheral blood for surface antigens common to CD8+TL, NK cells, CD4+TL and B lymphocyte cells and subsets within each sub-populations (Table 3.1). CD8+TL sub-populations were distinguished by positive cell surface marker expression of CD3 (PerCP) and CD8 (APC). Positive cell surface marker expression of CD3 (PerCP) and CD4 (APC) distinguished CD4+TL. Expression of CD45RA (FITC) in

combination with CD27 (PE) was used to further differentiate between naïve (CD27+CD45RA+), central memory (CM) (CD27+CD45RA-), effector-memory (CD27-CD45RA-) (EM), and terminally-differentiated effector-memory (CD27-CD45RA+) (RA+EM) T lymphocyte subsets. NK cells were distinguished by positive cell surface marker expression of CD56 (PE) and CD3 (PerCP) (CD3-CD56+). CD56hi and CD56lo cells were identified by combining CD56+ and CD16- antibodies and isolating the two fluorescently distinct sub-populations of CD56+ cells using appropriate electronic gates. B lymphocytes were distinguished by the positive cell surface marker expression of CD19 PerCP.

Table 3.1: Antibody combinations used to stain for surface antigens common to selected lymphocyte sub-populations.

Lymphocyte sub-population	Colour			
	FITC	**PE**	**PerCP**	**APC**
CD8+ TL	CD45RA	CD27	CD3	CD8
NK cells	CD16	CD56	CD3	CD14
CD4+ TL	CD25	CD69	CD3	CD4
B Lymphocytes	IgD	CD27	CD19	CD5
Isotype control I	IgG2b	IgG2b	IgG1	IgG1
Isotype control II	IgG1	IgG1	IgG2b	IgG2b

CD8+TL=CD8+ T Lymphocytes; NK=natural killer; CD4+TL=CD4+ T lymphocytes. Monoclonal antibody colours included, FITC=fluorescein isothiocyanate, PE=phycoerythrin, PerCP=peridinin-chlorophyll-protein complex and APC=allophycocyanin-conjugated.

3.3.5.2 Flow cytometric analyses

A fluorescence-activated cell sorter (Becton Dickinson FACS Calibur) enabled subsequent analysis of labelled cells by flow cytometry. Using the lymphocyte gate, 20,000 gated events were acquired from each sample. Cells were selected on the basis of their forward vs. side light scatter. Absolute numbers of lymphocyte sub-populations or sub-sets

were calculated by multiplying corresponding percentages of sub-population or subset by total lymphocyte count or sub-population count, respectively.

Absolute sub-population numbers = (%Sub-population/100) * Lymphocyte #) *1000
Absolute subset numbers = (%Subset/100) * Sub-population #

Where %Sub-population = lymphocyte sub-population expressed as a percentage of total lymphocyte population; Lymphocyte # = total lymphocyte number derived from whole blood count; %Subset = lymphocyte sub-set as a percentage of lymphocyte sub-population and, Sub-population # = total corresponding sub-population number as previously calculated.

Matched antibody isotype controls were used to identify positive and negative staining criteria. Lymphocyte sub-populations were analysed using FlowJo software v5.2 (Tree Star Inc., Aston, Oregon). Preparations were read within 24h using Cell Quest-pro software (Becton-Dickinson, San Jose, USA).

3.3.6 Stress hormones

Commercially available sandwich ELISA kits were used to determine plasma cortisol (Cortisol, IDS, Tyne and Wear, UK) and catecholamine (CAT-COMBO, IDS, Tyne and Wear, UK) concentrations. In each case, plates were read in duplicate on a Labsystems Original Multiskan MS at selected wavelengths (450nm=cortisol; 405nm=adrenaline and noradrenaline). Reported sensitivity of ELISA was 2.5ng/ml, 10pg/ml and 20pg/ml for cortisol, adrenaline and noradrenaline, respectively. Intra-assay variations were calculated for cortisol (7%), adrenaline (9%) and noradrenaline (13%), respectively.

3.3.7 Psychological Questionnaires

Incidence of URTI (throat, congestion and running nose) were determined by questionnaire. Symptoms of URTI were classified as ‘worse than normal’, ‘normal’ or ‘better than normal.’ The number of ‘worse than normal’ symptoms of URTI scores were summated on a weekly basis following NOR and INT. Athletes were asked to complete the questionnaire daily.

3.3.8 Statistical analyses

Statistical analyses were performed using SPSS 15.0 for Windows (SPSS Inc., Chicago, IL). All data are expressed as absolute change from BL, unless otherwise stated. Area under the curve (AUC) was calculated during the exercise period for selected immune parameters with zero set at BL, using PRISM software v4 (Graphpad software INC., San Diego, CA). The magnitude of the post exercise lymphocytopenic response of immune cell parameters were calculated as the difference in cell number between TT and 1h-POST. Students paired T-tests were used to compare total leukocyte/differential leukocyte sub-population counts, lymphocyte responses to exercise expressed as AUC, and post exercise lymphocytopenic responses between training weeks. Changes in lymphocyte count in response to exercise and stress hormone concentrations were analysed using a two way (training week and exercise time point) analysis of variance (ANOVA) with repeated (BL, SM, TT and 1h-POST) measures. Post hoc analysis was performed to determine time point differences between weeks using LSD adjustment. DALDA 'a' scores part B during INT, were compared between trials using a non parametric Two Related Samples Wilcoxon Signed Rank test. Data are expressed as means ± SE for eight subjects. Significance was set at $P \leq 0.05$.

3.4 Results

3.4.1 Quantification of intensified training

Mean total weekly training volume and time spent training in each training zone are displayed in Fig. 3.3. Training volume was increased by 58±7% following INT relative to NOR. Time spent in training zones 3-5 was increased during INT compared to NOR. Of the total training volume completed during INT, 64±8% of work was performed in training zones 3-5.

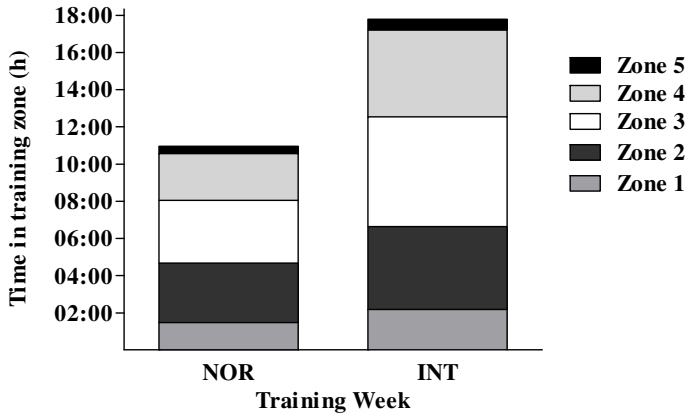

Figure 3.3: Time spent in each heart rate zone during normal (NOR) and intensified (INT) training.

Intensity of heart rate zone ranging from, zone 1=lowest intensity training zone - zone 5=highest intensity training zone; h=hour. Values are means.

3.4.2 Total Leukocyte and Lymphocyte counts

Total leukocyte and differential leukocyte sub-population counts are compared between NOR and INT at baseline (BL), following submaximal exercise (SM), at immediate cessation of time trial (TT) and 1h-POST in Table 3.2. Total leukocyte, monocyte and neutrophil counts increased during exercise, peaking at TT and remained elevated above BL

at 1h-POST. Exercise-induced elevations in lymphocyte counts were followed by a drop to below BL at 1h-POST.

INT blunted WBC, lymphocyte and neutrophil counts at TT ($P<0.05$). Training intensity had no effect on total leukocyte and differential leukocyte sub-population counts at all other time points.

Table 3.2: Total leukocyte and differential leukocyte sub-population counts to exercise and recovery following normal (NOR) and intensified (INT) training.

Cell type(cells $x10^9 \cdot L^{-1}$)	BL		SUBMAX		TT		1h-POST	
	NOR	INT	NOR	INT	NOR	INT	NOR	INT
WBC	4.8±0.3	4.7±0.3	8.2±0.7†	7.6±0.6†	12.8±1.3†	10.3±1.0†*	10.2±1.5†	8.4±0.6†
Lymph	2.0±0.1	1.9±0.1	2.8±0.2†	2.7±0.2†	4.0±0.2†	3.6±0.3†*	1.5±0.1†	1.6±0.1†
Neu	3.7±0.8	2.5±0.2	4.8±0.5	4.5±0.4†	8.5±1.1†	6.3±0.9†*	8.4±1.4	6.6±0.5
N:L	2.0±0.4	1.4±0.1	1.9±0.2	1.7±0.1	2.2±0.2	1.8±0.3	6.0±0.8†	4.3±0.3†

NOR=normal training, INT=intensified training. BL=baseline blood sample collected 10 min pre-exercise, SM=immediate cessation of preload, TT=immediate cessation of time trial, 1h-POST=1h following time trial, WBC=white blood cells, Lymph=lymphocytes, Neu=neutrophils, N:L=Neutrophil:Lymphocyte ratio. Values are means ± SE. †significantly different from BL in corresponding training week ($P<0.05$). *significantly different from NOR ($P<0.05$) at corresponding timepoint ($P<0.05$).

3.4.3 Peripheral blood CD8+TL counts during exercise

Fig. 3.4a presents CD8+TL responses to the time trial, expressed as absolute change from BL, following NOR and INT. A robust increase in CD8+TL counts during exercise (~112%) was followed by a decline in cell numbers below BL 1h-POST (~27%). INT tended ($P=0.092$) to blunt CD8+TL counts during and following the time trial. Expressed as area under the curve (AUC), INT tended ($P=0.07$) to ameliorate the total CD8+TL numbers during exercise compared to NOR (Fig. 3.4b).

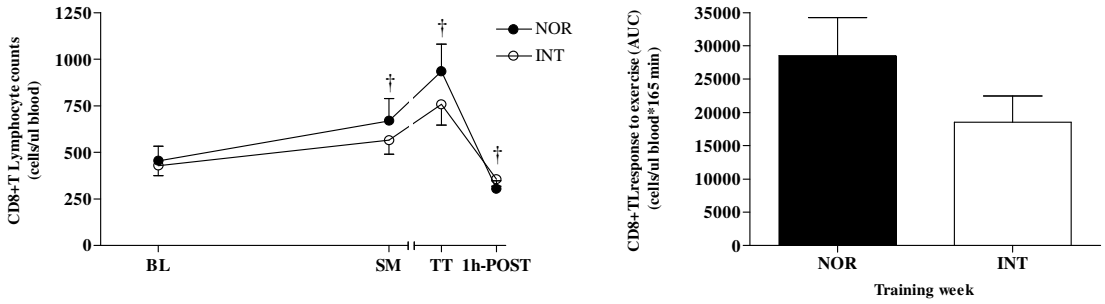

Fig. 3.4: CD8+TL counts expressed as a) absolute change from baseline (BL), following normal (NOR) and intensified (INT) training and b) AUC in response to exercise.

SM=immediate cessation of preload, TT=immediate cessation of time trial, 1h-POST=1h following time trial. Values are means ± SE (n=8). † significantly different from BL in corresponding training week (P<0.05).

3.4.4 Peripheral blood CD8+TL subset counts during exercise

During NOR, an acute bout of exercise induced a differential increase in CD8+TL subset counts. At TT, EM and RA+EM CD8+TL counts increased by ~150% and ~385%, respectively, compared to 85-110% increases in NA and CM CD8+TL counts following NOR (data not shown).

Fig. 3.5 display the absolute changes in cell number from BL for NA, CM, EM and RA+EM CD8+TL, respectively following NOR and INT. RA+EM CD8+TL counts were attenuated following INT compared to NOR (P<0.05), however no significant interaction was detected. Expressed as AUC, RA+EM CD8+TL responses over the exercise period were blunted following INT compared to NOR (P<0.05) (Fig. 3.6). No main effect of training week on EM, CM or NA CD8+TL counts was observed.

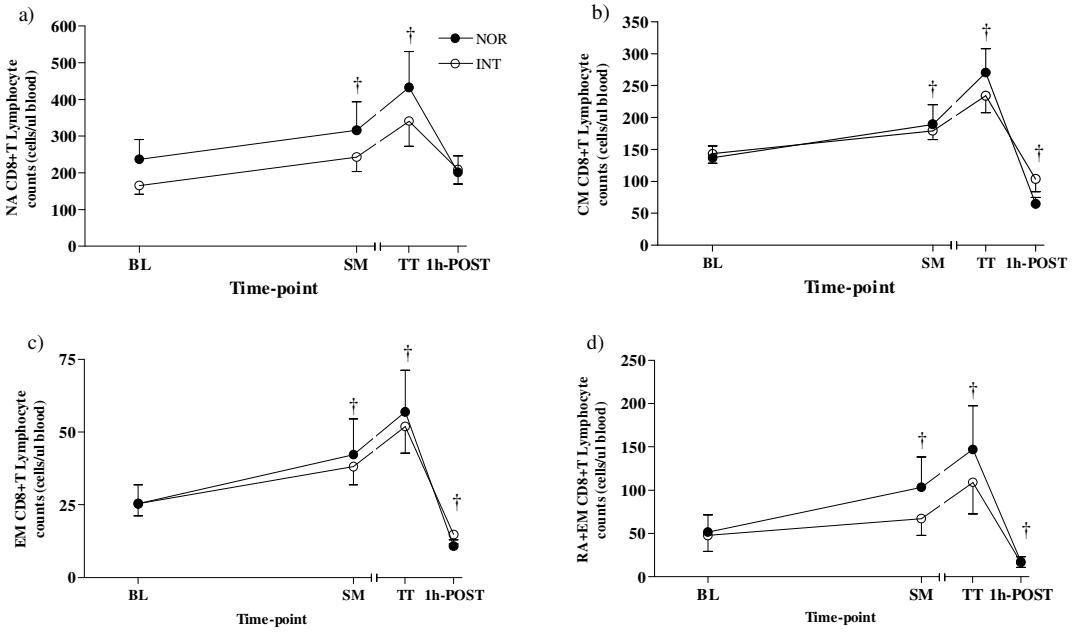

Fig. 3.5a-d: Cell counts (a) naïve (NA) CD8+TL, (b) central memory (CM) CD8+TL, (c) effector memory (EM) CD8+TL and (d) RA+EM CD8+TL, expressed as absolute change from baseline (BL) (cells/µL blood) following normal (NOR) and intensified (INT) training.

SM=immediate cessation of preload, TT=immediate cessation of time trial, 1h-POST=1h following time trial. Values are means ± SE (n=8). † significantly different from BL in corresponding training week ($P<0.05$).

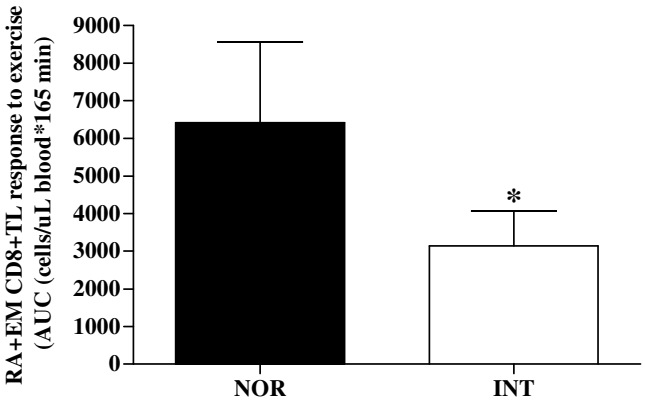

Fig. 3.6: RA+EM CD8+TL counts, expressed as area under the curve (AUC) over the exercise period, following normal (NOR) and intensified (INT) training.

Values are means ± SE (n=8). AUC for blood RA+EM CD8+TL counts calculated during the exercise period with zero set at baseline. *significantly different from NOR (P<0.05).

3.4.5 Post exercise efflux of CD8+TL

Fig. 3.7 displays the magnitude of the lymphocytopenic response (difference in cell count between TT and 1h-POST) of CD8+TL, RA+EM CD8+TL, EM CD8+TL, CM CD8+TL and NA CD8+TL following NOR and INT. The post exercise egression of CD8+TL and NA CD8+TL was attenuated following INT compared to NOR (P<0.05). The post exercise egression of CM CD8+TL and EM CD8+TL was similar between NOR and INT. However, a tendency (P=0.06) for an attenuated egression of RA+EM CD8+TL was observed following intensified training.

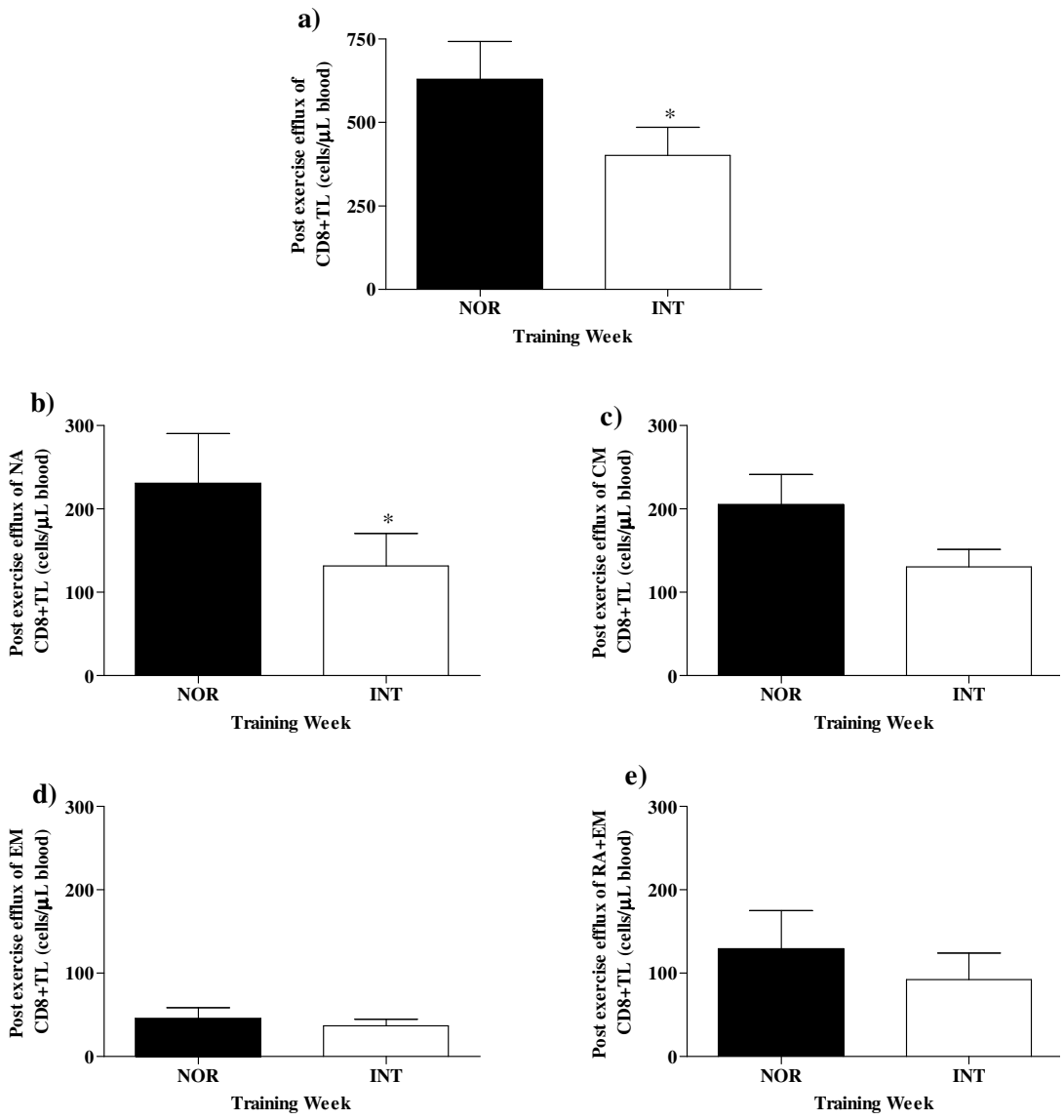

Fig. 3.7a-e: Post exercise cell counts for CD8+TL (a), naïve (NA) CD8+TL (b), central memory (CM) CD8+TL, effector memory (EM) and RA+EM CD8+TL out of the bloodstream 1h post exercise following normal (NOR) and intensified (INT) training.

Values are means ± SE (n=8). *significantly different from NOR ($P<0.05$).

3.4.6 Peripheral blood NK cell counts

Fig. 3.8 displays NK cell responses to the time trial, expressed as absolute change from BL following NOR and INT. A robust increase in NK cell counts during exercise (~420%) was followed by a decline in cell numbers below BL 1h-POST (~36%). No differences in the exercise-induced increase in NK cell count was observed between training weeks at any time point.

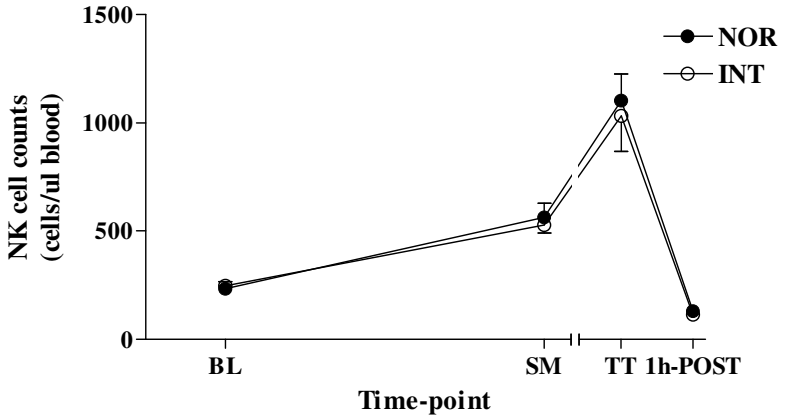

Fig. 3.8: NK cell counts expressed as absolute change from baseline (BL), following normal (NOR) and intensified (INT) training.

SM=immediate cessation of preload, TT= immediate cessation of time trial, 1h-POST=1h following time trial. Values are means ± SE (n=8).

3.4.7 NK cell subset counts

An acute bout of exercise elicited a differential increase in NK cell subset counts. A greater exercise-induced mobilisation of CD56lo (~433%) compared to CD56hi (~242%) cells was observed following NOR. Training intensity had no effect on CD56lo or CD56hi cell counts (data not shown).

The post exercise egression of NK cells, CD56hi NK cells and CD56lo NK cells were similar between NOR and INT (data not shown).

3.4.8 CD4+TL and B lymphocytes.

Exercise induced increases in CD4+TL (~45% compared to BL) and B lymphocytes (~56% compared to BL) were modest in comparison to NK cells and CD8+TL. Following one h of passive recovery, both CD4+TL and B lymphocyte counts returned to BL. No main effect of training week was observed on the exercise-induced increase in CD4+TL or B lymphocyte counts and their corresponding subset counts (data not shown). The post exercise decline in CD4+TL and B lymphocyte counts was similar between NOR and INT (data not shown).

3.4.9 Stress hormone responses

The plasma cortisol response to exercise and recovery following NOR and INT is illustrated in Fig. 3.9. Plasma cortisol concentrations typically increased by 55-85% above BL at TT, remaining elevated 1h-POST (15-50% above BL). INT attenuated plasma cortisol concentration compared to NOR ($P<0.05$). Post hoc analyses revealed blunted plasma cortisol concentrations at BL ($P<0.05$), TT ($P<0.05$) and 1h-POST ($P<0.05$) following INT compared to NOR.

Peak plasma adrenaline concentrations (typically detected at TT) were blunted following INT (2.42±0.91ng/ml) compared to NOR (3.88±1.38ng/ml) ($P<0.05$). Statistically, no significant difference in peak noradrenaline concentrations (typically detected at TT) were observed between NOR (13.9±2.9ng/ml) and INT (10.8±1.3ng/ml).

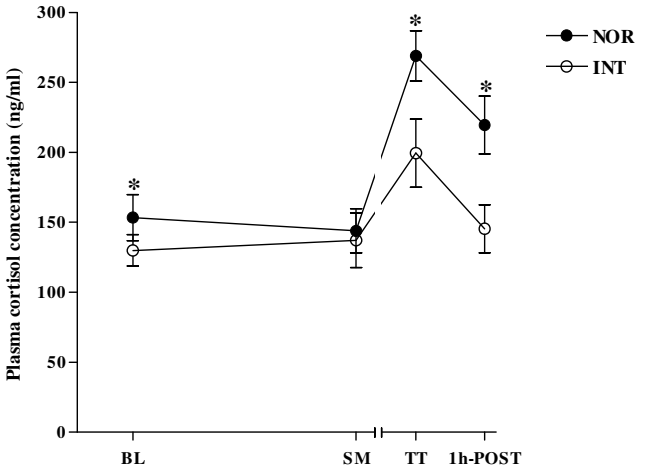

Fig. 3.9: Plasma cortisol concentration (ng/ml) following normal (NOR) and intensified (INT) training.

BL=baseline, SM=immediate cessation of preload, TT= immediate cessation of time trial, 1h-POST=1h following time trial. Values are means ± SE (n=8). *significantly different from NOR at corresponding timepoint (P<0.05).

3.4.10 Symptoms of URTI

Mean number of symptoms of URTI rated as 'worse than normal' were higher in INT (2.9±1.0 'a' scores part B, DALDA) compared to NOR (0.1±0.8 'a' scores part B, DALDA) following INT (P<0.05).

3.5 Discussion

This study provided a comprehensive phenotypical analysis of minimal lymphocyte mobilisation and egression of lymphocytes during and after a prolonged bout of strenuous exercise following normal and intensified training conditions. Our focus was on the response of distinct CD8+TL and NK cell subsets, which differ in functional characteristics (i.e. cytotoxicity) and migratory potential. Similar to previous research (3), a robust minimal mobilisation of cytotoxic lymphocytes which exhibit a strong effector and tissue-migratory potential into the blood was observed following intense aerobic exercise. Moreover, in the present study it was found that this cytotoxic-driven response was attenuated following intensified training, which was observed in conjunction with a blunted stress hormone response. Interestingly, a decline in the minimal egression of CD8+TL from the venous blood during exercise recovery was observed following INT compared to NOR. These results suggest that exposure to repeated bouts of high intensity exercise may partially impede the capacity of the immune system to mobilise antiviral cells into the bloodstream and presumably inhibit the migratory potential of highly cytotoxic lymphocytes to the 'injured' peripheral tissues. Such a response during intensified training may render athletes more susceptible to the risk of infection.

Acute bouts of prolonged intense exercise challenge, rather than suppress, the immune system. In accordance with previous studies, strenuous aerobic-based exercise elicited a preferential deployment of NK cells and CD8+TL from their donor marginal pools and peripheral lymphoid organs into the peripheral blood (10,11,37,49,50). Recent evidence suggests that within lymphocyte sub-populations, specific subsets which share distinct functional and phenotypic characteristics are preferentially mobilised during exercise (3). We demonstrated a subset-specific selective minimal mobilisation of EM and RA+EM CD8+TL

into the bloodstream during the pre-loaded time trial. Furthermore, a greater minimal mobilisation of cytotoxic CD56lo NK cells compared with the more immunomodulatory subset, i.e. CD56hi NK cells was observed. These findings replicate a recent study by Campbell *et al.* (2009) in which a preferential mobilisation of EM, and to a greater extent RA+EM CD8+TL, during brief bouts (16 min) of either low (35%VO_2max) or high (85%VO_2max) intensity cycling exercise was observed. Likewise, the recruitment of the highly cytolytic and tissue-migratory CD56lo NK cells in preference to the more regulatory CD56hi cells has been previously reported in response to both psychological stress (2) and exercise (3,52). Elevating ventilation rates during exercise may increase the exposure of the lungs to numerous potentially harmful agents. Thus, our data support the notion that modulating the proportion of circulating effector CD8+TL and NK cells during intense prolonged exercise may be considered an important adaptive response of the immune system, whereby the functional potential of the blood is modified when increasingly exposed to air-borne pathogens.

The post exercise minimal egression of CD8+TL and NK cell subpopulations/subsets also appears to be specific. The exercise stimulus implemented in the present study was of sufficient duration to induce a state of lymphocytopenia (25% decline below BL in total lymphocyte number 1 h post exercise). In accordance with previous studies (10,12,49), a non-parallel post exercise minimal efflux of cytotoxic lymphocyte sub-populations from was observed. The magnitude of the lymphocytopenic responses of CD8+TL (~27% below BL) and NK cells (~36% below BL) were greater compared to CD4+TL (15% below BL) and B lymphocytes (4% below BL). Previous studies (9,26) demonstrated a preferential removal from the bloodstream of circulating lymphocytes which express high surface levels of CD11a and CD18 and low levels of CD62; features characteristic of effector-memory CD8+TL. In

support of these findings, our data demonstrated a post exercise subset-specific minimal egression of CD8+TL and NK cells which express these characteristic adhesion molecules on their cell surfaces. RA+EM CD8+TL (~36% drop to below BL) and EM CD8+TL (~47% drop to below BL) were largely accountable for the minimal efflux of CD8+TL 1 h post exercise. In addition, the minimal efflux of NK cells following exercise was almost exclusively attributed to CD56lo cells. Collectively, these data demonstrate that the highly cytotoxic effector subsets, rather than naïve/immunoregulatory subsets of CD8+TL and NK cells, are preferentially deployed out of the bloodstream in the post exercise period.

The implications of reduced post exercise peripheral blood lymphocyte cell counts may be considered to be a contentious issue. Post exercise declines in peripheral blood lymphocyte subpopulation counts have previously been interpreted as representing an 'open window' for infection (38). Transiently low blood lymphocyte counts following exercise were thought to represent a period of vulnerability, whereby invading pathogens could potentially gain a foothold (40). However, this traditional viewpoint is based only on changes in lymphocyte sub-population counts in venous blood, and thus fails to consider the destination of lymphocytes removed from the blood. Recent data collected from an animal-based study demonstrated that an acute exercise stress induced a redeployment of T lymphocytes to the lungs post exercise (24). We demonstrated a preferential post exercise minimal egression of CD8+TL and NK cell subsets which not only exhibit highly cytotoxic effector properties, but also exhibit a strong tissue-migratory potential. The definitive destination of these highly cytotoxic cells post exercise could not be determined with our methodological techniques. Nevertheless, this selective post exercise redeployment of highly cytotoxic lymphocytes out of the bloodstream, presumably to the infected peripheral tissues, may be considered representative of an adaptive, rather than suppressive response of the immune system.

The second aim of the present study was to determine the impact of intensified training on the trafficking pattern of highly cytotoxic lymphocytes in response to an acute bout of exercise. The minimal mobilisation of NK cells, CD4+TL and B lymphocytes during strenuous exercise was similar between normal and intensified training conditions. However, one week of increased training selectively impeded the minimal mobilisation pattern of highly cytotoxic CD8+TL into the blood during exercise and subsequent post exercise minimal egression of tissue-migratory CD8+TL out of the bloodstream post exercise. Expressed as AUC over the entire exercise period (~165 min), intensified training attenuated the minimal mobilisation of RA+EM CD8+TL. Furthermore, the magnitude of the post exercise minimal efflux of this antigen-experienced tissue migratory CD8+TL subset tended to be blunted following an intensified period of training. Thus, we may speculate that the potential homing of RA+EM CD8+TL, presumably into peripheral tissues such as the skin, gut, intestine, lungs, joints or the central nervous system that may contain pathogens (53), may be ameliorated by increased training stress. We propose that the accumulation of intense bouts of intense exercise, as typically performed by endurance athletes, may partially compromise the anti-viral immune defence network by selectively blunting the normal trafficking pattern of highly cytotoxic CD8+TL in response to exercise. If so, regular profiling of CD8+TL subset counts may provide a useful additional diagnostic marker indicating training stress experienced by the athlete (17).

A mechanistic explanation underpinning the training induced blunted minimal mobilisation of RA+EM CD8+TL during exercise remains to be fully elucidated. Exercise intensity provides the most obvious explanation. In our experimental design, trained cyclists completed the same exercise model on two occasions; following one week of normal and intensified training. The exercise model consisted of 120 min of steady state exercise

performed at a set relative intensity (50%Wmax), immediately followed by a time trial, whereby cyclists completed a set amount of work in as short a time as possible. Calculated using Hopkins (1999) equation (20), average exercise intensity elicited during the time trial was predicted to be ~76%VO_2max and ~70%VO_2max following normal and intensified training, respectively. Such a relatively minor difference in exercise intensity is unlikely to confound the magnitude of the decrement in percentage mobilisation of effector lymphocytes during strenuous exercise following intensified training compared to normal training (23). Potential mechanisms responsible for this blunted effect are of paramount importance to establishing the aetiology of immune-suppression following intensified training.

A limitation of many studies which examined immunological responses to intensified training is the failure to concurrently measure endocrine responses. In addition to increasing haemo-dynamic shear stress (8), exercise elevates catecholamine levels (1), thus initiating the demargination of lymphocytes from the marginal pools into the peripheral blood (11). Increased cyclic adenosine monophosphate concentrations and resulting elevated catecholamine concentrations is proposed to provide a key stimulus for the exercise-induced mobilisation of lymphocytes which express the greatest surface density of ß2 adrenergic receptors (28). Hence, the high density of ß2 adrenergic receptors expressed on the cell surfaces of both CD8+TL and NK cells likely explains the preferential mobilisation of these highly cytotoxic lymphocyte sub-populations during exercise (28). In accordance with this notion, previous observations have implicated the release of hormones, most notably adrenaline, as facilitative for the mobilisation of lymphocytes into the blood (1,24,25). In the present study, the blunted minimal mobilisation of RA+EM CD8+TL into the bloodstream following intensified training was accompanied by attenuated peak plasma concentrations of adrenaline. These data suggest that an amelioration of the peak adrenaline response, following

intensified training, blunted the minimal mobilisation of highly cytotoxic CD8+TL into the bloodstream.

In contrast to lymphocytosis, a paucity of data exists examining the mechanisms responsible for the state of lymphocytopenia which typically follows strenuous exercise. Programmed cell death provides an unlikely explanation for the decrease in blood lymphocytes post exercise, since previous studies report insignificant levels of apoptosis in the peripheral circulation following exercise (1,33,34,35,51). Elevated plasma concentrations of cortisol during exercise are known to exert a time-delayed effect on the distribution of lymphocyte counts post exercise (41). In accordance with previous findings (37,39), lymphocytopenic responses of CD8+TL and NK cells were observed following both training weeks. Interestingly, the blunted peak cortisol response to exercise following intensified training was accompanied by a strong tendency (P=0.06) for an ameliorated post exercise efflux of highly cytotoxic tissue-migratory CD8+TL. Collectively, these data support the role of cortisol as a possible mediator for the blunted post exercise minimal egression of highly cytotoxic CD8+TL following intensified training.

3.6 Conclusions

In conclusion, intensified training blunted the pattern of minimal mobilisation and egression of highly cytotoxic CD8+TL in response to intense exercise, which was likely mediated by an attenuated stress hormone concentration in the blood. Future studies should be conducted with the aim to investigate whether this partially blunted antiviral response explains the increased incidence of viral infections experienced by athletes. Furthermore, more work in humans needs to be carried out witgh the aim to fully elucidate the post exercise destination of cytolytic subsets of CD8+TL preferentially mobilised during exercise.

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CHAPTER 4 - Increased protein intake restores the minimal mobilisation of cytotoxic CD8+ T lymphocytes during intensified training

Contributions to study: Sarah Jackman assisted with data collection and blood analysis. Dr Jos Bosch and Natalie Riddell provided flow cytometry training. Dr Jos Bosch, Dr Kevin Tipton, Professor Asker Jeukendrup and Dr Juul Achten designed the study. Lucy Walton, Rachel Turner and Sarah Jackman assisted with dietary control.

4.1 Abstract

Introduction: Nutrition has recently been touted as a beneficial strategy to counter the immuno-suppressive effects of intensified periods of training; however most interest has focused on carbohydrate, rather than protein, feeding. Therefore, the purpose of the present study was to examine the impact of increasing dietary protein intake on immunological perturbations induced by a period of intensified training. **Methods:** Trained cyclists (VO_2max: 64.2±6.5ml·kg^{-1}·min^{-1}) completed two separate trials, both equally divided into a single week of normal (NOR) and intensified (INT) training. In a randomised, single-blinded, crossover experimental design, cyclists received either a high protein (PRO) (3g protein·kg body mass (BM)$^{-1}$·day^{-1}) or a normal diet (CON) (1.5g protein·kgBM^{-1}·day^{-1}) during INT. Dietary carbohydrate content remained constant at 6g·kgBM^{-1}·day^{-1}. Cell counts of CD8+ T lymphocytes (CD8+TL) was measured using flow cytometry in the fasted state at baseline, following 120 min submaximal exercise, at immediate cessation of a time trial and following 1 h of passive recovery (1h-POST). CD8+TL were classified into their functionally distinct subsets: Naïve, Central Memory, Effector Memory and CD45RA+ Effector Memory (RA+EM). Cyclists completed a questionnaire on a daily basis to determine the number of 'worse than normal scores' for symptoms related to upper respiratory tract infections (URTI). **Results:** Mean number of 'worse than normal' scores for symptoms related to URTI were higher in CON (3.3±1.0) compared to PRO (1.4±0.8) following INT ($P<0.05$). The blunted minimal mobilisation of RA+EM CD8+TL during exercise following INT in CON was prevented with PRO ($P<0.05$). Despite observing an ~61% greater post exercise minimal efflux of RA+EM CD8+TL following INT in PRO compared with CON, no statistically significant difference was detected. Additional protein intake did not modulate the blunted stress hormone concentrations observed following INT. However, following INT, plasma urea

concentrations were higher in PRO (367±65 mmol/L*165 min) compared to CON (183±30 mmol/L*165 min) ($P<0.05$). **Conclusions:** Additional dietary protein intake during a period of intensified training restored the minimal mobilisation pattern of highly cytotoxic CD8+TL into the bloodstream during exercise. Further investigations are needed before protein nutrition may be touted as an effective nutritional countermeasure to the immunosuppressive effects of intensified training.

Key words: Protein feeding, CD45RA+ effector memory CD8+ T lymphocytes, adrenaline concentration.

4.2 Introduction

Heavy training schedules, commonly practiced by endurance athletes, compromise the functional response of the immune defence network to protect its host. Antiviral CD8+ T lymphocytes (CD8+TL) are highly influential to immune function (35). Specifically, the highly cytotoxic tissue-migratory CD45RA+ Effector Memory (RA+EM) CD8+TL subset eliminates antigens by killing off virally infected cells by direct cell-to-cell contact (15,20,29). Hence, this CD8+TL subset may play an important role in reducing the incidence of upper respiratory tract infections (URTI) experienced by athletes. The minimal mobilisation of RA+EM CD8+TL into the blood during exercise and subsequent post exercise minimal egression presumably to potentially infected peripheral tissues, appears to be impeded following an increased training stress (Chapter 3). Thus, strategies aimed at restoring the trafficking pattern of highly cytotoxic CD8+TL in response to exercise should be endorsed in an attempt to minimise the detrimental impact of intensified training on the functional capacity of the immune defence network.

The interrelationship between nutrition and immunity during and following exercise, particularly in endurance-trained individuals, has received recent attention (14,27). Nutrient intake has the potential to affect virtually all aspects of the immune system (6). In particular, carbohydrate (CHO) feeding has been touted as a potentially beneficial nutritional strategy to attenuate the immunosuppressive effects of intense exercise (27). This notion is supported by an acute-based study which demonstrated that consuming CHO (30-60 g/h) during 2.5 h of strenuous cycling precluded the post exercise suppression of interferon (IFN)-γ production and attenuated the release of inflammatory cytokines (interleukin (IL) 6, IL-10 and IL-1 receptor agonist) from T lymphocytes during exercise (5). CHO ingestion during exercise has also been associated with fewer perturbations in lymphocyte counts during exercise (26).

Alternative nutritional strategies which modulate the mobilisation of antiviral T lymphocytes may be advantageous to athletes undertaking intensified periods of training, whom appear more susceptible to minor cases of viral infection.

Perhaps surprising is the lack of studies that have been conducted to examine the impact of manipulating dietary protein intake in an attempt to counter the immunosuppressive effects of heavy exertion. The dual role of amino acids in supplying energy substrate for lymphocyte functioning (7) and immune cell replication (34) support the suggestion that additional dietary protein intake may play a beneficial role in attenuating the compromised anti-viral immune defence following intensified training (Chapter 3). Two field-based studies demonstrated that supplementing the habitual diet with branched chain amino acids (BCAA) over a thirty day period attenuated the exercise-induced decline in plasma glutamine concentration and restored the ability of lymphocytes to proliferate in response to mitogens following an Olympic distance triathlon (2,3) or 30 km run (3). In addition, a recent study conducted by the late Paul Flakoll and colleagues (12) reported improvements in informative, albeit crude markers of immune status, such as 33% fewer medical visits, a reduced incidence of bacterial/viral infection and 37% fewer medical visits due to muscle/joint problems when United States marine recruits consumed nutritional supplements which included protein immediately following each training session of a basic 54 day training period. However, to date the impact of manipulating dietary protein intake on direct measures of immune status following exercise training is yet to be examined. Taken together, these data (2,3,12) suggest that chronic supplementation of the diet with protein sources may play a pivotal role in maintaining general health during intensified periods of training.

Hence, the purpose of the present study was to examine the effect of increasing the protein content of the diet on the minimal mobilisation and egression of highly cytotoxic

RA+EM CD8+TL following a period of intensified training. We hypothesised that additional dietary protein intake would attenuate the blunted pattern of CD8+TL minimal mobilisation and egression previously observed following intensified training.

4.3 Methods

4.3.1 Participant characteristics

Eight endurance-trained cyclists (age: 27±8 y; weight: 73.0±7.1 kg; maximal oxygen uptake ($\dot{V}O_2$max): 64.2±6.5 ml·kg^{-1}·min^{-1} and Wmax: 372±21 W) were recruited to participate in this study. All cyclists had a training history of at least five years. Prior to participation, the health status of each participant was assessed using a General Health Questionnaire (Appendix A). Participants were non-smokers and were not taking any medication immediately prior to participation in the study. Written informed consent (Appendix B) was preceded by a detailed explanation of practical details, associated risks and required commitment applicable to the present study (Appendix C). Participants were reminded of their right to withdraw from the study at anytime without provision of reason. The protocol was approved by the research ethics committee of the School of Sport and Exercise Sciences, University of Birmingham, UK.

4.3.2 Experimental design

This study was designed to determine the impact of increasing dietary protein intake on the mobilisation of lymphocyte sub-populations during a period of intensified training. Each participant completed two separate trials, both consisting of a two week period of quantified training. Training periods were divided into one week of normal training (NOR) and one week of intensified training (INT). On day seven of both training weeks, a pre-loaded time trial was performed. Cell counts of a series of immune parameters were measured at rest, following exercise and after 1 h recovery. In a randomised crossover experimental design, participants received either a high protein diet (PRO) or an energy and carbohydrate matched

normal protein diet (CON) during INT. Trial order was counter-balanced and separated by at least a two week washout period in which participants resumed their normal training program (Fig. 4.1).

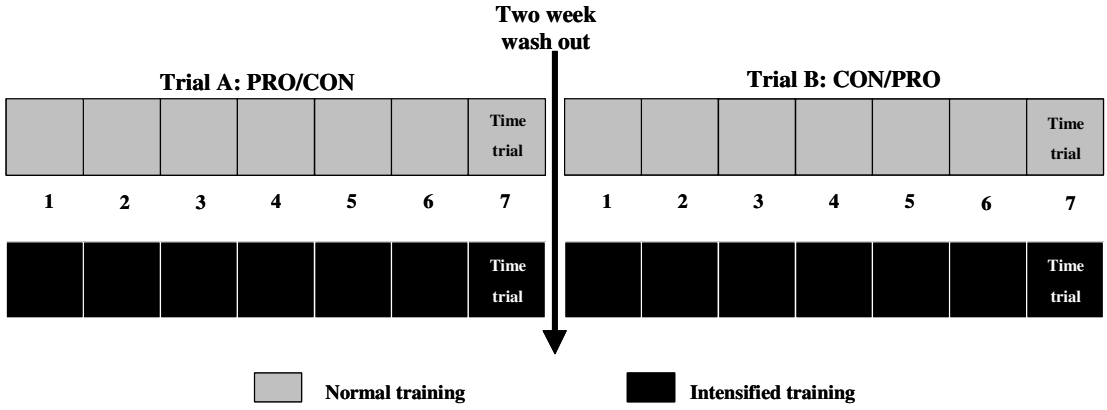

Figure 4.1: Schematic representation of experimental model.
Time Trial, pre-loaded (120 min) time trial (45 min).

4.3.3 Preliminary assessments

Each participant completed a preliminary incremental test to exhaustion, a training diary and a three day diet diary prior to both experimental trials.

4.3.3.1 Incremental test to exhaustion

Maximal power outputs (Wmax) and lactate threshold (LT) were determined within seven days of commencement of each experimental trial. Subjects reported to the laboratory after an overnight fast (≥10 h). A Teflon catheter connected to a Lectrocath with a three-way stopcock was inserted into a forearm vein and a resting blood sample (~1 ml) was drawn. The catheter was maintained patent with isotonic (0.9%) saline. Subjects then performed an incremental test to exhaustion using an electrically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). Subjects began cycling at 95 W for three min. Workload was increased by 35 W at three min intervals until volitional exhaustion (i.e., when the athlete

could no longer maintain a cadence >60 rpm). Wmax was calculated according to the formula (17):

$$\mathbf{Wmax = Wfinal + Wfinal + (t/T)*W}$$

where Wfinal (W) is the final stage completed, t (s) is the amount of time reached in the final uncompleted stage, T (s) is the duration of each stage and W (W) is the workload increment.

In the final 30 s of each interval and at immediate cessation of the test, a blood sample was collected (~2 mL) into K3 ethylenediamine tetraacetic acid (EDTA) tubes for plasma lactate analysis. LT was determined using the maximal distance (Dmax) method as previously described (9). Five training zones were calculated from the preliminary MT in accordance to British cycling guidelines (1) and were expressed as target heart rate values. Test duration ranged from 21-29min.

4.3.3.2 Training diary

Cyclists completed a training diary prior to participation in the study, to quantify habitual training. Information included daily training volume (min), objective rating of daily training intensity (1-10), type of training session (i.e., continuous vs. interval), location of training session.

4.3.3.3 Dietary analysis

In the week preceding study commencement, a three-day weighed food record was completed. Participants were asked to record accurately all food items they consumed during two weekdays and one weekend day. To increase the accuracy of the food analysis, subjects were provided with electronic scales to weigh all food products and were carefully instructed on how to document food intake. Diets were analysed using an internet-based nutrition analysis software (www.weightlossresources.co.uk). The habitual energy intake of the

subjects was 2882±172 kcal/day and the diet consisted of 1.6±0.1g protein·kg body mass(BM)$^{-1}$·day^{-1}, 5.4±0.3g carbohydrate·kgBM^{-1}·day^{-1} and 1.3±0.1 g fat·kgBM^{-1}·day^{-1}.

4.3.4 Experimental trial periods

4.3.4.1 Dietary manipulation

Diet was controlled for the duration of the study with all foods prepared by investigators in the Metabolic Research Kitchen of the School of Sport and Exercise Sciences, University of Birmingham. For each dietary period, participants were fed with the aim to maintain energy balance. Total energy intake was adjusted on a daily basis to ensure weight stability. Basal metabolic rate (BMR) (kcal/d) (ambulating status), predicted using the Harris Benedict equation, was added to estimated energy expenditure for cycling activity (23,32) to determine total energy expenditure on a daily basis. Energy intake during both weeks of training was matched between trials. During NOR in both trials, participants consumed an energy-balanced diet based on a macronutrient intake equivalent to 1.5g protein·kgBM^{-1}·day^{-1} and 6g carbohydrate·kgBM^{-1}·day^{-1} with the remainder of energy derived from fat. During INT, energy intake was increased to match energy expenditure in both trials. Diet composition was not manipulated during INT in CON. In PRO, protein intake was doubled to 3g protein·kgBM^{-1}·day^{-1} during INT. The carbohydrate content of the diet remained constant at 6g carbohydrate·kgBM^{-1}·day^{-1} with the remainder of energy derived from fat. In both dietary conditions, carbohydrate-rich fruit smoothies were consumed daily during INT. In PRO, 60 g (3x20 g) of casein protein (DSM Foods, The Netherlands) was disguised in each fruit smoothie to assist the attainment of 3g protein·kgBM^{-1}·day^{-1}. Smoothie's were ingested on immediate completion of training sessions where possible. The order of the diets was counterbalanced. Prior to study commencement, participants completed a food questionnaire

to determine food likes, dislikes and preferred items consumed during training rides (Appendix D). For each diet period, five set menus were carefully composed using the internet-based nutrition analysis software (www.weightlossresources.co.uk). Food and drinks were provided as three main meals together with a collection of snacks. Participants were asked to refrain from consuming alcohol for the duration of the study. The last meal and evening snack before the laboratory trials were standardised within individual participants, because it has been shown that these can still affect metabolism >8 h after ingestion (24,32).

4.3.4.2 Training quantification

Training was controlled and quantified over a two week training period, which was divided equally into normal (NOR), and intensified (INT) training (Fig. 6.1). Each athlete was equipped with a downloadable heart rate monitor (Polar Electro, Kempele, Finland) for the duration of the study to monitor individual training sessions. During NOR, participants completed their usual volume and type of training. Individualised programs, consisting of training goals and recommended local routes were prescribed by the research team, designed to increase the training impulse during INT. Athletes were required to train on a daily basis, which typically consisted of a combination of high-intensity interval sessions above LT, lasting between two and three hours, and long-continuous rides, usually between four and five hours in duration. These sessions were designed to increase total training hours and training intensity, as determined by time spent in training zones three, four and five.

Individual training sessions were not supervised, however athletes were given the option to train in the laboratory. In an attempt to monitor training as accurately as possible and ultimately standardise training impulse between trials, each participant received an athlete handbook, completing both a training diary and an assessment of well-being. Subjects

mirrored the exact type, intensity, duration and location of training session (training impulse) in the second trial. All participants were contacted on a daily basis.

4.3.4.3 Time Trial

On Day 7 of respective training weeks, participants reported to the Human Performance Laboratory in a fasted state to perform a previously validated (3.34%) pre-loaded TT (17) (Fig. 4.2). Following 10 min rest in a supine position, a Teflon catheter was inserted into a forearm vein and a baseline (BL) blood sample was collected. Thereafter, cyclists completed 120 min of submaximal exercise at a relative intensity of 50% Wmax. The electromagnetically braked ergometer was set in the hyperbolic mode (cadence-independent mode) so that the work rate (50% Wmax) was independent of pedalling rate. Upon immediate completion of submaximal exercise, participants typically toileted, before commencing the simulated TT. The ergometer was set in the linear mode so that by increasing pedaling rate, the work rate increased, in accordance with the following formula:

$$W = L * (RPM)^2$$

In which RPM is the pedalling rate and L is a (constant) linear factor. The linear factor is based on individual Wmax and was calculated so that 70% Wmax will be produced at a pedalling rate of 90 rpm.

Participants were asked to complete a set amount of work in as short a time as possible, consequently eliciting a maximal effort on the part of each cyclist. Total work to be completed was individualised, equivalent to working at 70%Wmax for 45 min and was calculated according to the equation adapted from Jeukendrup *et al.* (1996):

$$\text{Total amount of work (kJ)} = 0.7 * \text{Wmax (W)} * 2700\text{(s)}$$

Where 0.7 refers to a factor equivalent to 70%, Wmax (W) is the maximum wattage attained during the incremental test in watts and 2700 refers to the number of seconds that make up 45 min.

All trials were performed under normal and standard environmental conditions (20-23°C dry bulb temperature and 50%-60% relative humidity). To minimise thermal stress, a

floor-standing fan was positioned infront of cyclists and water was provided *ad libitum.* Blood samples (~25ml) were collected into K_3EDTA vacutainers at BL, following 120 min submaximal pre-load (SM), at immediate cessation of the time trial (TT) and after 1h of passive recovery (1h-POST). Vacutainers containing whole blood were positioned horizontally on the laboratory worktop at room temperature (~23˚C) to avoid undue agitation and was analysed on the day of collection (within ~10 h) for a range of lymphocyte sub-populations and individual lymphocyte subsets Plasma cortisol concentrations were measured at all time-points. Plasma adrenaline responses could not be detected at 1h-POST. In addition**,** at regular time points (20, 40, 60, 80, 100 min), blood samples (~15 ml) were drawn during the preload of TT, collected into Lithium Heparin vacutainers. Enzymatic analysis for plasma urea concentration (Urea, ABX diagnostics, UK) were determined in duplicate using a semi-automated analyser (COBAS MIRA S-plus, ABX, UK) in a basal state, at each time point during the preload and at immediate cessation of the time trial.

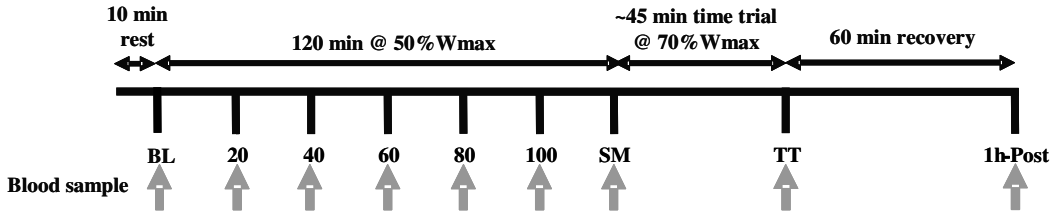

Figure 4.2: Schematic representation of time trial protocol.

BL=baseline; SM=immediate cessation of submaximal exercise, TT=time trial and 1h-POST=1h after time trial.

4.3.5 Haematological analysis

Complete blood counts were determined using a Coulter GEN-S haematology analyzer (Beckman-Coulter, Miami, USA) or a Coulter ACT^{diff} (Beckman Coulter, High Wycombe, UK). Total leukocyte counts were determined in addition to differential leukocyte

counts, which included neutrophil and lymphocyte counts. Blood indices of mean cell volume (MCV) and platelet counts were also determined.

4.3.6 Flow Cytometry

4.3.6.1 Immuno-fluorescent antibody staining of whole blood

Extensive leukocyte and lymphocyte sub-population phenotyping was performed using multicolour flow cytometry. Aliquots (50μL) of whole blood and immuno-fluorescent antibodies (4μL) were added to polystyrene tubes before being incubated in the dark for 25 min at room temperature with conjugated antibody. Erythrocytes were then lysed with 1.5 ml FACS lysing solution (10% concentrated hypotoxic buffer) (Becton Dickinson, San Jose, USA). Following a further 10 min incubation, cell suspensions were washed by centrifugation at 500 x g for 6 min at 4˚C, and then re-suspended in 300 μL of phosphate buffered saline (PBS) containing 1% formaldehyde, and stored in the dark at 4˚C before being analysed using flow cytometry. Analysis was based on whole blood rather than peripheral blood mononuclear cell preparations (PBMC's) to minimise the potential selective losses of subsets during preparation of cell suspensions.

Combinations of fluorescein isothiocyanate (FITC)-, phycoerythrin (PE)- and allophycocyanin (APC)-conjugated (Pharmingen, San Diego, USA) and peridinin-chlorophyll-protein complex (PerCP)-conjugated (Becton Dickinson, San Jose, USA) monoclonal antibodies were used to stain peripheral blood for surface antigens common to CD8+TL subsets. CD8+TL sub-populations were distinguished by positive cell surface marker expression of CD3 (PerCP) and CD8 (APC). Expression of CD45RA (FITC) in combination with CD27 (PE) was used to further differentiate between naïve (NA) (CD27+CD45RA+), central memory (CM) (CD27+CD45RA-), effector-memory (EM)

(CD27-CD45RA-), and CD45RA+ Effector Memory (RA+EM) (CD27-CD45RA+) CD8+TL subsets.

4.3.7 Flow Cytometric Analyses

A fluorescence-activated cell sorter (Becton Dickinson FACS Calibur) enabled subsequent analysis of labelled cells by flow cytometry. Using the lymphocyte gate, 20,000 gated events were acquired from each sample. Cells were selected on the basis of their forward vs. side light scatter. Absolute numbers of lymphocyte sub-populations or sub-sets were calculated by multiplying corresponding percentages of sub-population or subset by total lymphocyte count or sub-population count, respectively.

Absolute sub-population numbers = (%Sub-population/100) * Lymphocyte #) *1000
Absolute subset numbers = (%Subset/100) * Sub-population #

Where %Sub-population = lymphocyte sub-population expressed as a percentage of total lymphocyte population; Lymphocyte # = total lymphocyte number derived from whole blood count; %Subset = lymphocyte sub-set as a percentage of lymphocyte sub-population and, Sub-population # = total corresponding sub-population number as previously calculated.

Matched antibody isotype controls were used to identify positive and negative staining criteria. Lymphocyte sub-populations were analysed using FlowJo software v5.2 (Tree Star Inc., Aston, Oregon). Preparations were read within 24 h using Cell Quest-pro software (Becton-Dickinson, San Jose, USA).

4.3.8 Stress Hormones

Commercially available sandwich enzyme-linked immunosorbent assay's (ELISA's) were used to determine plasma cortisol (Cortisol, IDS, Tyne and Wear, UK) and adrenaline (CAT-COMBO, IDS, Tyne and Wear, UK) concentrations. In each case, plates were read in duplicate on a Labsystems Original Multiskan MS at selected wavelengths (450nm = cortisol; 405nm = adrenaline). Reported sensitivity of ELISA's were 2.5ng/ml, and 10pg/ml for

cortisol and adrenaline, respectively. Intra-assay variations (CV%) were calculated for cortisol (7%) and adrenaline (9%), respectively.

4.3.9 Incidence of URTI

Incidence of URTI (throat, congestion and running nose) were determined by questionnaire. Symptoms of URTI were classified as 'worse than normal', 'normal' or 'better than normal.' The number of 'worse than normal' symptoms of URTI scores were summated on a weekly basis following NOR and INT in both PRO and CON. Athletes were asked to complete the questionnaire daily.

4.3.10 Data presentation and statistical analysis

All data are presented as means ± SE, unless otherwise stated. The minimal mobilisation of CD8+TL is expressed as absolute change in cell count (cells/μl blood) from NOR to INT at each corresponding timepoint (i.e. BL, SM, TT). The post exercise minimal efflux following INT was calculated as the difference between CD8+TL numbers measured at TT and 1h-POST. Due to difficulties with analysis, CD8+TL data are shown and analysed for n=7. Hormonal data are expressed as percentage change in peak plasma cortisol and adrenaline concentrations (typically measured at TT) following INT relative to NOR. The profile of urea concentration over time was presented as raw values following NOR and INT in both dietary condition. The response of plasma urea concentration to maximal exercise was calculated as area under the curve (AUC) following INT (baseline set to resting values measured during NOR of corresponding dietary condition) using PRISM software v4 (Graphpad Software INC., San Diego, CA).

Statistical analyses were performed using SPSS 15.0 for windows (SPSS Inc., Chicago, IL). Two-way analysis of variance (ANOVA) with two between factors, diet (PRO and CON)

and time (SM, TT) was used to detect differences in the change of cell counts during exercise and also the urea profile during exercise. Lymphocytopenic responses, i.e. the decline in lymphocyte counts to below baseline, and percentage change in peak hormone concentrations were compared between conditions using Students paired T tests. DALDA 'a' scores part B during INT, were compared between dietary conditions using a non parametric Two Related Samples Wilcoxon Signed Rank test. Significance was set at $P \leq 0.05$.

4.4 Results

4.4.1 Training Quantification

No difference in training intensity or duration between trials was observed during NOR and INT. Time spent in training zones three, four and five were increased during INT compared to NOR in both trials.

4.4.2 Dietary regimen

Dietary energy intake and macronutrient composition during each week of both trials is shown in Table 4.1. Energy intake, expressed as average daily kilocalorie intake per day was not significantly different between dietary periods. Energy intake was increased by ~23% following INT compared to NOR in both trials. As intended, dietary protein intake was significantly greater in PRO compared to CON following INT ($P<0.001$). No difference in CHO intake was observed between PRO and CON. Intake of fat was greater in CON compared to PRO during INT ($P<0.05$).

Table 4.1: Dietary intake of cyclists during normal (NOR) and intensified (INT) training under two dietary conditions.

	NOR	INT	
	PRO/CON	**PRO**	**CON**
Energy intake (kcal/day)	3511±161	4273±153*	4300±155*
Protein intake (g/day)	110±3	218±6*†	110±3
CHO intake (g/day)	436±14	446±18	440±12
Fat intake (g/day)	148±15	185±9*†	240±11*

PRO=high protein diet, CON=normal protein diet. Data are means ± SD. *Significantly greater than NOR (either dietary condition) ($P<0.05$). †Significantly different from CON during INT ($P<0.05$).

4.4.3 CD8+TL subset counts

Change in total CD8+TL and CD8+TL subset counts following INT relative to NOR are shown in Fig. 4.3. A tendency (P=0.08) for an augmented exercise-induced increase in total CD8+TL into the blood following INT was observed in PRO compared to CON. Additionally, PRO augmented RA+EM CD8+TL counts measured at TT following INT compared to CON (P<0.05). However, no statistically significant protein intake-by-timepoint interaction was observed. Tendencies for a main effect of protein intake (P=0.097) and a protein intake-by-timepoint interaction (P=0.057) were detected for NA CD8+TL. No main effect of protein intake on the change in EM and CM CD8+TL counts was observed.

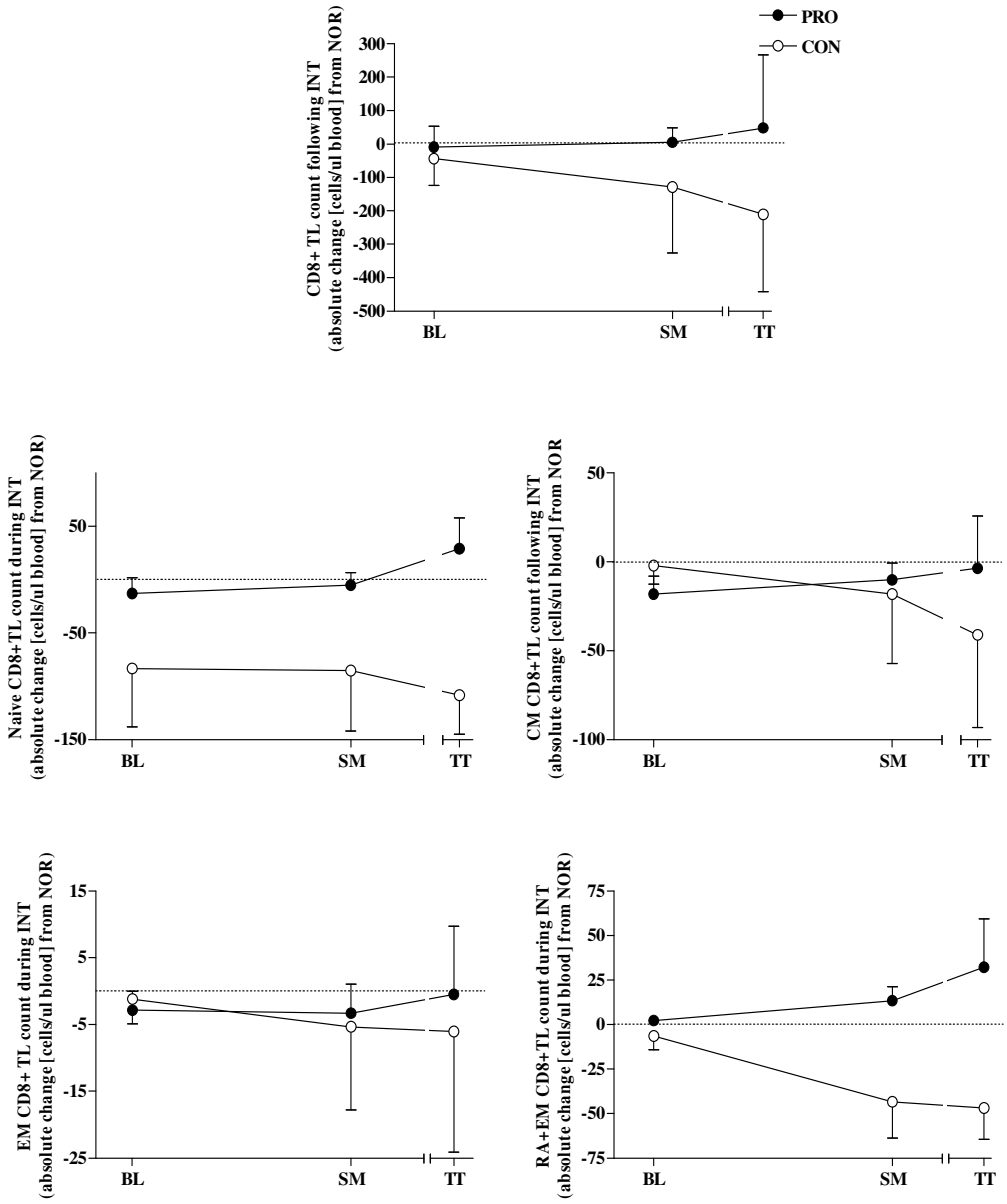

Figure 4.3: Change in a) CD8+T lymphocyte (CD8+TL), b) Naïve CD8+TL, c) central memory (CM) CD8+TL, d) effector memory (EM) CD8+TL, and e) CD45RA+Effector Memory (RA+EM) CD8+TL counts following intensified (INT) compared to normal (NOR) training in high dietary protein (PRO) and normal dietary protein (CON) conditions.

BL=baseline, SM=following 120 min submaximal exercise, TT=immediate cessation of time trial. Values are means ± SE (n=7).

4.4.4 Post exercise CD8+TL subset counts

The magnitude of the post exercise (measured over 60 min) lymphocytopenic response of CD8+TL and their functionally distinct subsets following INT for PRO and CON are displayed in Fig. 4.4. The higher the value, the greater the lymphocytopenia of CD8+TL. No differences in the post exercise decline in total CD8+TL, or NA, CM and EM CD8+TL subsets were detected between dietary conditions. Despite observing an ~61% greater post exercise decline in RA+EM CD8+TL counts following INT in PRO compared with CON, no statistically significant difference was detected.

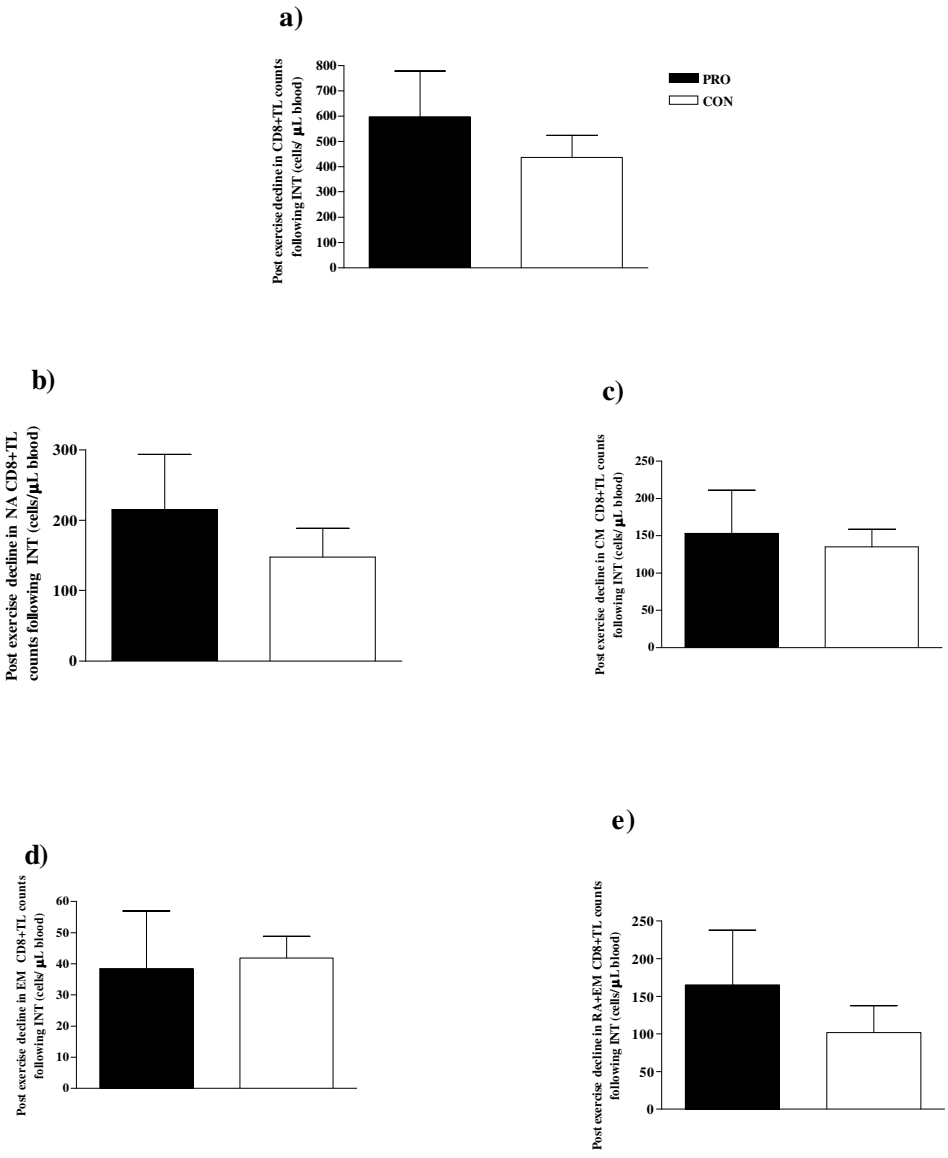

Figure 4.4: Post exercise counts of a) CD8+T Lymphocytes (CD8+TL), b) RA+EM CD8+TL, c) effector memory (EM) CD8+TL, d) central memory (CM) CD8+TL and e) NA CD8+TL following INT in both high dietary protein (PRO) and normal dietary protein (CON) conditions.

Post exercise declines in CD8+TL counts were calaculated as the difference between CD8+TL numbers measured at TT and 1-POST. Values are means ± SE (n=7).

4.4.5 Blood metabolite responses

Changes in peak plasma cortisol and adrenaline concentrations (typically peaking at TT) following INT compared to NOR were compared between PRO and CON in Fig. 4.5.

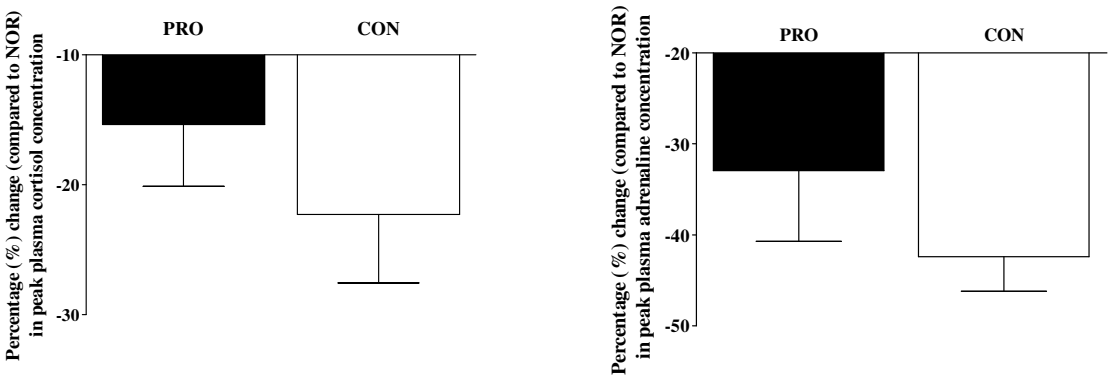

Figure 4.5: Percentage change in a) peak plasma cortisol and b) peak adrenaline concentration following intensified training (INT) compared to normal training (NOR) in high dietary protein (PRO) and normal dietary protein (CON) conditions.

Values are means ± SE (n=7).

No main effect of diet on the decrement in peak plasma cortisol and adrenaline concentrations following INT, expressed as percentage change from NOR, were observed, this despite smaller mean decrements in peak plasma cortisol and adrenaline concentration in PRO compared to CON.

The profile of urea concentration over time during NOR and INT is displayed for both PRO and CON in Fig. 4.6. The blood urea response, expressed as AUC over the exercise period following INT, was higher in PRO (367±65 mmol/L*165 min) compared to CON (183±30 mmol/L*165 min) ($P<0.05$).

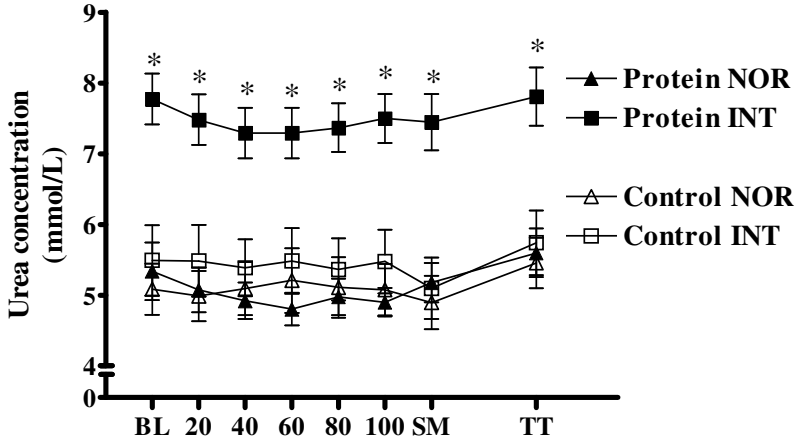

Figure 4.6: Profile of urea concentration during exercise following normal and intensified training in both high dietary protein (PRO) and normal dietary protein (CON) conditions.

Values are means ± SE. *significantly different from all other conditions at corresponding time point.

4.4.6 Symptoms of URTI

Mean number of 'worse than normal' scores for symptoms related to URTI during INT were higher in CON (3.3±1.0 'worse than normal' scores) compared to PRO (1.4±0.8 'worse than normal' scores) following INT ($P<0.05$).

4.5 Discussion

The present study is novel in examining the impact of increasing dietary protein intake on the exercise-induced minimal mobilisation and subsequent post exercise minimal egression of functionally distinct CD8+TL subsets. We previously demonstrated that a short-term period of intensified training selectively blunted the minimal mobilisation and egression of CD8+TL in response to exercise, in particular RA+EM CD8+TL, thus potentially reduced the functional capacity of the immune system to protect its host (Chapter 3). Therefore, in this study we focused on the response of these highly cytotoxic antiviral cells to additional protein intake during intensified training. The present findings indicate that additional dietary protein intake prevents the blunted exercise-induced minimal mobilisation of RA+EM CD8+TL into the blood following overload training.

Facilitating the mobilisation of highly cytotoxic antiviral lymphocytes into the bloodstream during exercise may indirectly be considered an advantageous functional response of the immune system (11). This notion is based on the premise that acute bouts of prolonged intense exercise challenge, rather than suppress, the immune system (33). Brief (~15 min) (8) and prolonged (>2.5 h) bouts of acute exercise (Chapter 3) elicit the preferential minimal mobilisation of effector memory subsets of CD8+TL (i.e. EM and RA+EM CD8+TL) into the bloodstream. Highly cytotoxic RA+EM CD8+TL subsets contribute only a small proportion of total CD8+TL (~5-10%), however their distinct cytolytic capacities mean these cells are highly influential to immune function. In the present study, doubling the protein content of the diet restored the minimal mobilisation of highly cytotoxic CD8+TL into the blood during exercise following intensified training. Intuitively, augmenting the minimal mobilisation of RA+EM CD8+TL into the blood during exercise enhances the functional capacity of the immune system to redeploy these highly cytotoxic tissue-migratory CD8+TL

into peripheral tissues such as the skin and lungs that may contain pathogens (10) post exercise. Thus, these data provide promising support for the notion that additional protein intake may be an effective nutritional strategy to 'prime' the lymphocyte system to fight impending infections when challenged by intense training.

The restored exercise-induced minimal mobilisation of highly cytotoxic CD8+TL into the blood following intensified training with increased dietary protein intake did not seem to be endocrine-mediated. Acute CHO feeding strategies have been demonstrated to attenuate the immuno-suppressive effects of intense exercise in association with a modulated endocrine response (4,16). Furthermore, the release of adrenaline is well documented to provide a key stimulus driving the mobilisation of lymphocytes into the bloodstream during exercise (18). Thus, the restored minimal mobilisation pattern of CD8+TL with an increased dietary protein intake may be expected to be linked with higher plasma adrenaline concentrations. However, in the present study the decline in peak adrenaline concentration induced by overload training was not attenuated with additional dietary protein intake. Hence, perhaps surprisingly, the restored minimal mobilisation of RA+EM CD8+TL into the blood following intensified training with increased protein intake was not modulated by plasma adrenaline concentrations.

A second possibility to explain the modulated CD8+TL response with additional protein intake may be substrate availability. The direct functional capacity of T lymphocytes is highly dependent on glucose (31) and glutamine (21,25) as energy substrates. Several authors (16,26) attributed the amelioration of the decline in lymphocyte proliferation following an acute bout of intense exercise with CHO supplementation to the maintenance of blood glucose concentrations. Analogous with this potentially beneficial effect of CHO feeding on direct measures of T lymphocyte function within an acute setting, increasing dietary protein intake during intensified training restored the minimal mobilisation of highly cytotoxic CD8+TL

into the blood during exercise. A recent study demonstrated that the rate of gluconeogenesis was elevated by ~40% with a high protein diet, consequently accounting for a large proportion of endogenous glucose availability (22). Urea concentrations measured in the basal and exercised state were greater with increased protein intake following intensified training. Hence, it is possible that more amino acids were made available for deamination and ultimately the rate at which carbon skeletons could be used for gluconeogenesis or oxidation for energy was enhanced in the high protein condition. In the present study, no direct measure of CHO utilisation by lymphocytes was made. However, increasing the availability of glucose does not seem to be the primary mechanism driving the mobilisation of CD8+TL into the blood during exercise (19). Thus, enhanced substrate availability due to increased protein intake is not the mechanism responsible for the augmented minimal mobilisation of CD8+TL following intensified training with additional protein intake. The exact mechanism responsible for the modulated mobilisation of RA+EM CD8+TL cannot be deduced with our study design.

The impact of protein feeding on the post exercise minimal efflux of highly cytotoxic CD8+TL remains unclear. Statistically, no difference in the magnitude of change in RA+EM CD8+TL cell counts from TT to 1h-POST was detected between PRO and CON. However, the mean decline in circulating RA+EM CD8+TL counts one hour following exercise was ~65% greater with additional protein intake. This may be indicative of a type II statistical error. Further support for this suggestion comes from previous studies which reported the nadir in lymphocyte counts to be typically reached between 3-72h following exercise cessation (13,30). Thus, it seems logical to speculate that a significant difference in the post exercise minimal egression of RA+EM CD8+TL may have been detected between dietary conditions if continuous blood samples had been collected post exercise. Practically, it was not possible to collect fasted blood samples >1h following a ~3h bout of exercise. Therefore,

it remains unknown whether additional protein intake would facilitate the minimal egression of highly cytotoxic tissue-migratory CD8+TL during exercise recovery.

The post exercise minimal efflux of highly cytotoxic CD8+TL is thought to be indicative of a redeployment of cells into the peripheral tissues (20). Elevated ventilation rates during exercise expose the upper respiratory tract or lungs to infectious agents (35). However, a comprehensive examination of the destination of migrated lymphocytes post exercise in humans is hindered by ethical constraints. In healthy human beings, it is not possible to collect tissue samples from sites of impending infection, such as the upper respiratory tract or lungs. However, flow cytometric analyses employed in a recent rodent-based study (18) revealed declined T lymphocyte counts in the spleen and liver, with a concomitant increase in T lymphocyte numbers located in the lungs, bone marrow and peyers patches following intense exercise. Over the course of the recovery period, if indeed these cells were destined for infected peripheral tissues, in particular the lungs, upper respiratory tract or damaged muscle, our data may suggest that protein feeding could enhance immune vigilance during recovery. On the other hand, the post exercise decrease in cell counts has traditionally been interpreted as immuno-suppression (28). Hence, augmenting the lymphocytopenic response has previously been deemed to be a negative immune response. Clearly, further research is needed to fully elucidate the interpretation of lymphocytopenic responses post exercise.

The practical application of administering and maintaining a diet consisting of 3g protein·kgBM^{-1}·day^{-1} may be questioned. This high level of protein intake may not be tolerated well by athletes in their natural training setting, and would likely have to be administered at the expense of CHO intake. Therefore, the promotion of protein feeding as an effective counter-measure to the compromised anti-viral defence induced by intensified training should be considered with caution. Whereas we intended to maximise the effect of

protein feeding, future studies should endeavour to determine whether more practical levels of protein intake (e.g. 2 g·kg BM^{-1}·day^{-1}) during intensified training impacts not only on the distribution of CD8+TL, but also the direct functioning of CD8+TL before and after a period of intensified training.

4.6 Conclusion

These data suggest that very high protein intakes restore the minimal mobilisation of highly cytotoxic CD8+TL into the bloodstream during intensified periods of training. The impact of administering more practical protein intakes must be examined before protein feeding may be considered as an effective strategy to reduce episodes of infection commonly experienced by athletes.

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CHAPTER 5 - Increasing dietary protein intake can help maintain endurance performance during intensified periods of training in cyclists

Contributions to study: Sarah Jackman assisted with data collection and blood analysis. Dr Kevin Tipton, Professor Asker Jeukendrup and Dr Juul Achten designed the study. Lucy Walton, Rachel Turner and Sarah Jackman assisted with dietary control.

5.1 Abstract

Introduction: Nutrition is commonly regarded as a useful strategy to improve tolerance to increased training volume; however despite promising rationale, little attention has been given to increasing protein intake. Therefore, the purpose of the present study was to examine, in the context of a period of intensified training, the impact of additional dietary protein intake on short-term decrements in endurance performance, exercise-induced alterations in endocrine and biochemical parameters, and increased mood disturbance. **Methods:** Trained male cyclists ($\dot{V}O_2$max: 64.2±6.5ml·kg^{-1}·min^{-1}) completed two three-week trials, both divided equally into normal (NOR), intensified (INT) and recovery (REC) training. In a randomised, single blinded, crossover experimental design, cyclists received either a high protein (PRO) (3 g protein·kg body mass (BM)$^{-1}$·day^{-1}) or a normal diet (CON) (1.5 g protein·kg BM^{-1}·day^{-1}) during INT and REC. Dietary carbohydrate content remained constant at 6 g·kg BM^{-1}·day^{-1}. Endurance performance was assessed with a pre-loaded time trial. Alterations in blood metabolite and hormonal responses to exercise were measured at rest, following 120 min submaximal exercise, at immediate cessation of the time trial and following 1 h of passive recovery. Cyclists completed the Daily Analysis of Life Demands for Athletes (DALDA) questionnaire on a daily basis. **Results:** Time trial completion increased (performance decreased) following INT (2893±160s) compared to NOR (2555±111s) in CON ($P<0.05$). A main effect of PRO on time trial performance during INT and REC was observed ($P<0.05$). Inferential statistics detected a possibly beneficial (35%) effect of PRO for amelioration of impaired endurance performance following INT. No discernible changes in peak plasma lactate or cortisol concentration were observed in PRO following an intensified period of training. The number of psychological symptoms of stress reported following intensified training was greater in CON (58±8 'a' scores part B, DALDA) compared to PRO

(41±7 'a' scores part B, DALDA) ($P<0.05$). **Conclusion:** Better maintenance of endurance performance following a period of increased training stress with elevated intake of dietary protein was likely mediated by an improved psychological status during training.

Key words: overload training, protein feeding, time trial completion, endocrine responses, psychological status.

5.2 Introduction

In an attempt to improve exercise performance, athletes often incorporate periods of high training volume, combined with limited recovery time, into carefully planned training regimens (13). A disproportionate balance between training impulse and recovery is likely to result in an accumulation of fatigue (27), together with perturbations in biological functioning and psychological status (46). Such an imbalance in homeostasis with intensified training ultimately results in an acute impairment in exercise capacity (12) and performance (2,14,16,22).

Nutritional strategies are commonly recommended for endurance athletes interested in enhancing recovery from each intense bout of exercise, thus potentially maximising the adaptive response to endurance training (17). Carbohydrate (CHO) feeding has been demonstrated to attenuate short-term decrements in endurance performance following intensified periods of training (2,16). Recent studies reported an attenuated impairment in endurance performance when endurance athletes supplemented their habitual diet for eight days with carbohydrate before and after each intense training session (16) or strictly adhered to a high (8.5 g·kg body mass $(BM)^{-1}\cdot day^{-1}$ = ~65% total energy intake) compared to a moderate (5.4 g·kg $BM^{-1}\cdot day^{-1}$ = ~41% total energy intake) CHO diet (2) before and during periods of increased training volume. Better maintenance of endurance performance and mood state with CHO feeding during intensified training was associated with attenuated impairments in endocrine responsiveness to exercise (16) and maintained rates of muscle glycogenolysis (2). In both studies (2,16), protein content of the diet remained constant. Whereas CHO has received considerable attention, despite promising rationale, the effect of protein feeding on exercise recovery from an intensified period of training is yet to be investigated.

The importance of protein intake with reference to recovery from endurance exercise has recently received more attention, almost entirely in an acute setting (38). The addition of protein to a CHO-rich drink, administered during and following exercise, was reported to improve the endurance capacity of subsequent exercise compared to a CHO-matched but non-isoenergetic drink (31,36,39). Mechanisms proposed to explain the improved recovery with additional protein intake are three-fold, including the remodelling of previously damaged proteins (31,36,39), the modulation of post exercise protein balance (25,29) and initiation of insulin-mediated accelerated rates of glycogen repletion following exercise (47). Taken together, these findings support the beneficial role of protein nutrition for optimising recovery from a strenuous acute bout of endurance exercise (38).

Despite promising findings from acute protein feeding studies, no direct scientific evidence exist that chronic feeding of protein impacts exercise recovery during intensified periods of training. In a recent field-based study (11), U.S Marine recruits reported reduced muscle soreness (i.e., 37% fewer medical visits due to muscle/joint problems), improved recovery from damaging exercise (33% fewer medical visits) and improvements in markers of immune function (i.e., a reduced incidence of bacterial/viral infection) when protein was added to nutritional supplements consumed immediately following each training session of a basic 54 day training period compared to those without protein. These data have led to the suggestion that chronic supplementation of the diet with protein may play a pivotal role in maintaining general health and minimising local muscular disruptions during intensified periods of training (11). Moreover, a recent laboratory-based study provides support for amino acid supplementation pre- and post- exercise as an effective strategy to attenuate impairments in maximal strength experienced in the initial period of high volume resistance training (26). In addition to the protective effect of protein nutrition for the amelioration of

postexercise markers of muscle damage, a recent study which supplemented with amino acids rather than protein appeared to preclude the blunted testosterone response to exercise associated with intensified training, indicating the maintenance of an anabolic environment despite an increased training volume (26). Taken together, these data provide preliminary evidence that the beneficial role of protein supplementation in exercise recovery observed in acute-based studies may be extrapolated to the chronic effect of protein feeding on recovery from a period of intensified training. However, to date no study has examined the effect of protein feeding on exercise recovery in the chronic setting of an intensified training period.

Therefore the main purpose of the present study was to examine the impact of manipulating dietary protein intake during a short term period of intensified training on subsequent endurance performance. In a crossover experimental design, trained cyclists consumed a diet composed of either a typical habitual protein intake for endurance athletes (1.5 g·kg BM^{-1}·day^{-1}) (6), or a high protein diet (3 g·kg BM^{-1}·day^{-1}) during and following a week of intensified training. We hypothesised that trained cyclists would experience an improved tolerance to the stress of increased training volume when consuming a high protein diet during and following intensified training, ultimately resulting in the better maintenance of endurance performance.

5.3 Methods

5.3.1 Participants

Eight endurance-trained cyclists (age: 27±8yr; weight: 73±7kg; maximal oxygen uptake ($\dot{V}$ O_2max): 64.2±6.5ml·kg^{-1}·min^{-1}; Wmax: 372±21W) were recruited to participate in this study. All cyclists had a training history of at least five years. Prior to participation, the health status of each participant was assessed using a General Health Questionnaire (Appendix A). A detailed explanation of practical details, associated risks and required commitment applicable to the present study (Appendix B) preceded the attainment of written informed consent (Appendix C). Participants were reminded of their right to withdraw from the study at anytime without provision of reason. The protocol was approved by the Research Ethics Committee of the School of Sport and Exercise Sciences, University of Birmingham, UK.

5.3.2 Experimental design

This study was designed to test the hypothesis that increasing dietary protein intake attenuates the short-term decrement in endurance performance, elevated mood disturbance and perturbations in biological functioning induced by a period of intensified training. Each participant completed two trials, both consisting of a three week period of quantified training. Training periods were divided into one week of normal training (NOR), one week of intensified training (INT) and one week of recovery training (REC). Endurance performance was assessed by a $\dot{V}$ O_2max test (MT) on day six and a pre-loaded time trial on day seven of each training week. Identical diets were administered during NOR in both conditions. In a randomised, crossover experimental design, participants received either a diet consisting of a high protein intake (PRO) or a normal protein intake (CON) during INT and REC. Trial order

was counter-balanced and separated by at least a two week washout period in which participants resumed their normal training volume and intensity (Fig. 5.1).

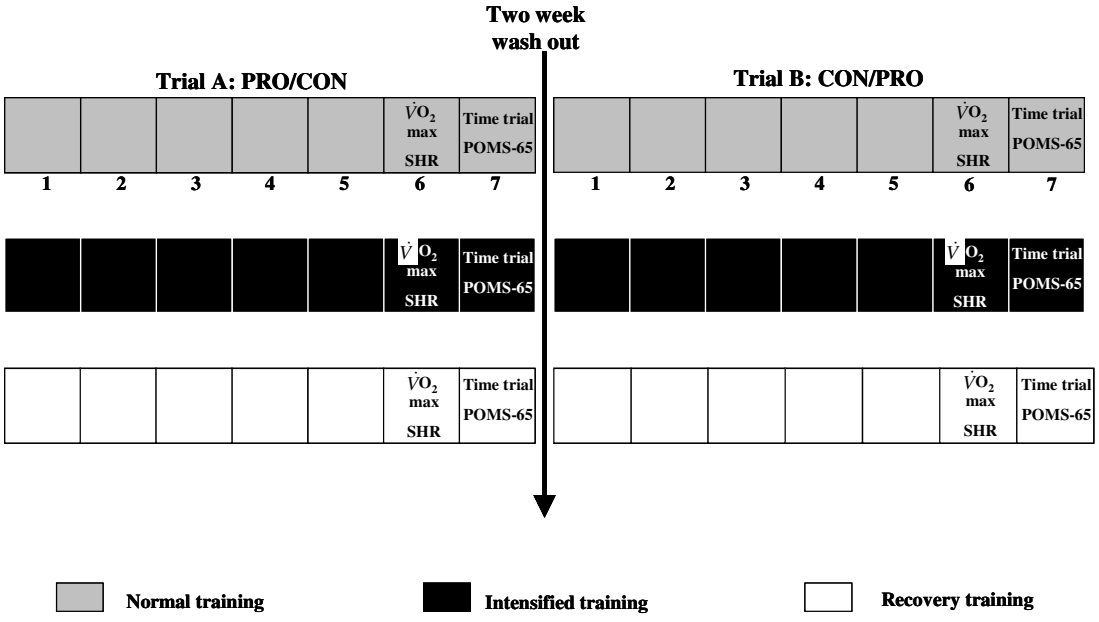

Fig. 5.1: Schematic representation of experimental model.

DALDA=Daily Analysis of Life Demands for Athletes questionnaire (not shown in figure) was completed daily. PRO=high protein trial, CON=normal protein trial, $\dot{V}$ O_2max=maximal $\dot{V}$ O_2 uptake, SHR=sleeping HR, Time trial=pre-loaded (120min) time trial (45min), POMS-65=Profile of Mood State-65 questionnaire.

5.3.3 Preliminary testing

Each participant completed a preliminary MT, familiarisation time trial and a three day diet diary prior to each experimental trial.

5.3.3.1 Preliminary Max Test

Maximal power output (Wmax), maximal aerobic capacity ($\dot{V}$ O_2max) and lactate threshold (LT) were determined within the seven days prior to commencing each experimental trial. Participants reported to the laboratory after an overnight fast (≥10 h), and measurements of body mass (kg) and height (cm) were determined. A catheter was inserted

into a forearm vein and connected with a three-way stopcock before a resting blood sample (~1 ml) was then drawn. The catheter was maintained patent with isotonic saline. Resting data were collected prior to subjects performing an incremental test to exhaustion using an electrically braked cycle ergometer (Lode Excalibur Sport, Groningen, The Netherlands). Subjects began cycling at 95W. Workload was increased by 35W at 3 min intervals until volitional exhaustion. Wmax was calculated according to the formula (21):

Wmax = Wfinal + (t/T)*W

where Wfinal (W) is the final stage completed; t (s) is the amount of time reached in the final uncompleted stage; T (s) is the duration of each stage and W (W) is the workload increment.

To determine submaximal and maximal $\dot{V}$ O_2, expired gas samples were collected into previously evacuated Douglas bags. The final expired gas collection to determine maximal $\dot{V}$ O_2 was collected when participants signalled 1 min until volitional exhaustion. Expired air samples were analysed for fractions of O_2 and CO_2 using a Servomex analyser. The analyser was calibrated prior to each test using certified reference gases (100% Nitrogen, 15.1%O_2/5.0%CO_2 and 17.1%O_2/2.0%CO_2). The volume of expired air was measured using a Harvard dry-gas meter. Heart rate was recorded continuously during the test using radio telemetry (Polar Vantage NV, Polar Electro Oy, Kempele, Finland). $\dot{V}$ O_2 was considered to be maximal when at least two of the following three criteria were met: 1) $\dot{V}$ O_2 did not increase with an increase in intensity (increase of no more than 0.2 L/min), 2) a heart rate within 10 beats/min of the predicted maximum (220 beats/min minus age), and 3) a respiratory exchange ratio >1.05. In the final 30 s of each exercise intensity and at immediate cessation of the test, a blood sample was collected (~2 ml) into K3 ethylenediamine tetraacetic acid (EDTA) vacutainers for plasma lactate analysis. LT was determined using the maximal distance method as previously described (7). Training zones, expressed as target HR values, were calculated from the preliminary MT in accordance with British Cycling

guidelines (1). Five training zones were defined as: Zone 1 (Z1) = <70% HRmax; Zone 2 (Z2) = 70-80% HRmax; Zone 3 (Z3) = 80-90% HRmax; Zone 4 (Z4) = 90-95% HRmax; Zone 5 (Z5) = >95% HRmax. Test duration ranged from 21-29 min.

5.3.3.2 Familiarisation time trial

All participants completed a habituation time trial within seven days prior to commencement of the study. Those participants who were unable to complete the familiarisation time trial were excluded from the study.

5.3.4 Dietary analysis

In the week preceding the first trial, a three-day weighed food record was completed. Participants were asked to record accurately all food items they consumed during two weekdays and one weekend day (Appendix D). To increase the accuracy of the food analysis, subjects were provided with electronic scales to weigh all food products and were carefully instructed on how to document food intake. Diets were analysed using internet-based nutrition analysis software (www.weightlossresources.co.uk). The habitual daily energy intake of the subjects was 2882±172 kcal·kg BM^{-1}·day^{-1} and the habitual diet consisted of the following macronutrient intake (relative to body mass): Protein: 1.6±0.1 g·kg BM^{-1}·day^{-1}, CHO: 5.4±0.3 g·kg BM^{-1}·day^{-1} and fat: 1.3±0.1 g·kg BM^{-1}·day^{-1}.

5.3.5 Experimental trial periods

5.3.5.1 Training quantification

Each three week training period was divided equally into NOR, INT and REC training (Fig. 5.1). Athletes were equipped with a downloadable heart rate monitor (Polar Electro, Kempele, Finland) for the duration of each trial to monitor individual training sessions.

During NOR, participants completed their usual volume and type of training. Individualised programs, consisting of training goals and recommended local routes, were designed to increase the training impulse during INT. Athletes were required to train on a daily basis during INT, which typically consisted of a combination of high-intensity interval sessions above LT, lasting between two and three hours, and long-continuous rides, usually between four and five hours in duration. These training sessions were implemented to increase total training hours and training intensity, as determined by time spent in Z3, Z4 and Z5. In REC, training volume was reduced to 60% of NOR, consisting primarily of low-intensity rides set relative to training zones one and two. Individual training sessions were not supervised, however athletes were given the option to train in the laboratory. In an attempt to monitor training as accurately as possible and ultimately standardise training impulse between trials, each participant received an athlete handbook. On a daily basis, athletes completed a training diary (time of day training session was started, duration of training (min), average and maximal HR (beats/min) for training sessions, location of training, weather conditions and distance covered (km)) and an assessment of well-being (morning resting HR (beats/min) (RHR), sleep rating (likert scale:1-10), stress rating (likert scale:1-10), fatigue rating (likert scale:1-10), muscle soreness (likert scale:1-10) (43), total sleep (h) and morning weight (kg)). Upon waking, RHR was recorded as the average HR over a 4 min period. On day six of each training week, participants wore their HR monitor throughout the night (on going to bed at night to getting up in the morning) to record an average sleeping heart rate (SHR). Cyclists mirrored the exact type, intensity, duration and location of each training session in the second trial. All participants were contacted on a daily basis.

5.3.5.2 Dietary control

Diet was controlled for the duration of the study with all food prepared by investigators in the Metabolic Research Kitchen of the School of Sport and Exercise Sciences, University of Birmingham. Prior to study commencement, participants completed a food questionnaire to determine food likes, dislikes and preferred items consumed during training rides (Appendix E). Five set menus were carefully composed using the internet-based nutrition analysis software (www.weightlossresources.co.uk). Food and drinks were provided as three main meals together with a collection of snacks. Participants were asked to refrain from consuming alcohol for the duration of the study. The last meal and evening snack before the laboratory trials were standardised within individual participants.

For each dietary period, participants were fed with the aim to maintain energy balance. Total energy intake was adjusted on a daily basis to ensure weight stability. The summation of basal metabolic rate (kcal/day) (ambulating status), predicted using the Harris Benedict equation and estimated energy expenditure in cycling activity (32) was used to estimate daily energy requirements of cyclists. Energy intake during each week of training was matched between trials. During NOR in both trials, participants consumed an energy-balanced diet based on a macronutrient intake equivalent to 1.5g protein·kg BM^{-1}·day^{-1}, 6g CHO·kg BM^{-1}·day^{-1} with the remainder of energy derived from fat. In CON, diet composition remained the same as NOR in both INT and REC, with energy intake modified to match daily changes in energy expenditure. In PRO, during both INT and REC, protein intake was doubled to 3g·kg BM^{-1}·day^{-1}. The CHO content of the diet was held to 6g·kg BM^{-1}·day^{-1} with the remainder of energy derived from fat.

In both dietary conditions, carbohydrate-rich fruit smoothies were consumed daily during INT and REC. In PRO, 60g (3x20g) of casein protein isolate (DSM Foods, The

Netherlands) was disguised in each fruit smoothie to assist the attainment of increased dietary protein intake. In CON, 60g (3x20 g) of cream (Sainsbury's, UK) was added to ensure the desired fat intake could be attained. Where possible, smoothie's were ingested on immediate completion of training sessions.

5.3.6 Performance tests

Excluding preliminary testing, subjects completed a total of six MT's and six time trials to assess maximal power output and endurance performance, respectively. MT and time trials were always performed in the morning (start of exercise between 06:30 – 08:00 am), under similar environmental conditions (~19°C and ~55% relative humidity) and in a fasted state (>10h). Within subjects, every attempt was made to schedule performance tests at the same time of day throughout the study period, to limit the impact of changes in circadian rhythms from influencing results.

5.3.6.1 $\dot{V}O_2$ max test

The same MT protocol was performed during experimental trial periods as was previously detailed in 'Preliminary testing.'

5.3.6.2 Time Trial

To determine endurance performance, a pre-loaded (120 min) 45 min simulated time trial was performed in which cyclists attempted to complete a target amount of work (kJ) in as short a time as possible (21). Fig. 5.2 summarises the time trial protocol and accompanying measurements. Participants were fitted with a HR monitor, and a Teflon catheter was inserted into a forearm vein. Following a 10 min rest period, a baseline (BL) blood sample was drawn. Participants then cycled for 120 min at 50% Wmax with the electromagnetically braked ergometer set in the hyperbolic mode (cadence-independent mode) so that work rate was

independent of pedalling rate. Thereafter, at regular time points (20, 40, 60, 80 and 100 min), blood samples (~15 ml) were drawn, HR was recorded and ratings of perceived exertion (RPE), using the modified Borg scale (5), were obtained. These measurements were repeated immediately following completion of 120 min of the submaximal exercise preload (SM). Upon immediate completion of the 120 min submaximal exercise preload, participants typically voided before commencing the simulated time trial. Further blood samples were collected at immediate cessation of time trial (TT) and following 1h passive recovery (1h-POST). The ergometer was set in the linear mode so that with increasing pedaling rate, work rate increased, in accordance with the following formula:

$$W = L * (RPM)^2$$

In which RPM is the pedalling rate and L is a (constant) linear factor. The linear factor is based on each subjects Wmax and was calculated so that ~70%Wmax will be produced at a pedalling rate of 90rpm.

Participants were asked to complete a set amount of work in as short a time as possible. Total work to be completed was individualised, equivalent to working at 70%Wmax for 45 min and was calculated based on Wmax using the following equation (adapted from Jeukendrup et al., 1996).

Total amount of work (kJ) = 0.7 * Wmax (W) * 2700(s)

where 0.7 refers to a factor equivalent to 70%, Wmax (W) is the maximum wattage attained during the incremental test in watts and 2700 refers to the number of seconds that make up 45 min.

During the time trial, the ergometer was connected to a computer which recorded power output, cadence and total work completed at 20s intervals (21). Participants did not receive any verbal encouragement or feedback regarding power output, cadence or HR. All timekeeping devices (e.g. clocks, stopwatches) were not visible to the athlete during the time trial. Every attempt was made to ensure participants were not disturbed for the duration of the time trial. The only information available to the participants was the amount of work performed, target work and percentage of work completed relative to the target amount (i.e.

0% at start and 100% at completion of the trial). To minimise thermal stress, a floor-standing fan was positioned infront of cyclists and water was provided *ad libitum* throughout both pre-load and time trial aspects of the protocol. In healthy endurance-trained cyclists, the coefficient of variation for this type of performance measurement is 3.34% (21). An energy target equivalent to 70%Wmax was preferred to more demanding targets (i.e. 75%Wmax) to increase the likelihood of time trial completion immediately following INT.

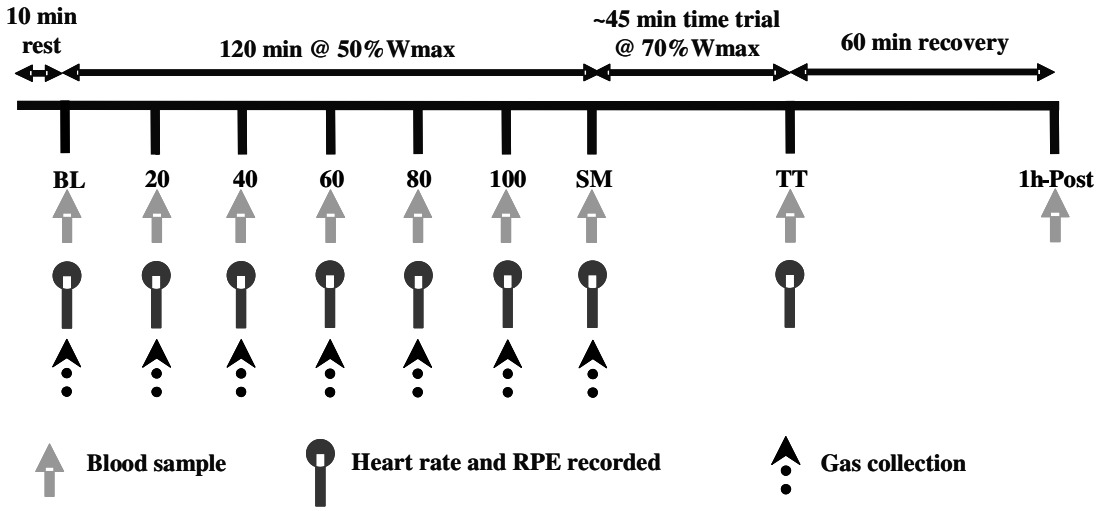

Figure 5.2: Schematic representation of time trial protocol.

120 min @ 50%Wmax=120 minutes of submaximal exercise at a relative intensity of 50% of maximum wattage, ~45 min time trial @ 70% Wmax=simulated time trial set at an energy target whereby cyclists will complete a set energy target in 45 min if an average relative exercise intensity of 70%Wmax was maintained, BL=baseline, SM=immediate cessation of preload, TT = immediate cessation of time trial and 1h-POST=60 min passive recovery following time trial, RPE=rating of perceived exertion (5). Expired air collection performed at 20 min intervals during pre-load of time trial.

5.3.7 Blood Analyses

All blood samples were collected into pre-chilled EDTA or Lithium Heparin vacutainers. Whole blood was immediately placed on ice until centrifugation at 3000 rpm for 10 min at 4 °C, within 2 h of collection. Aliquoted volumes of plasma were dispensed equally into 0.5 mL Eppendorf tubes before storage at -80°C until further analysis. Enzymatic analysis for blood lactate (Lactic Acid, ABX diagnostics, UK) and plasma urea concentration

(Urea, ABX diagnostics, UK) were determined in duplicate using a semi-automated analyser (COBAS MIRA S-plus, ABX, UK). Commercially available sandwich enzyme-linked immunoabsorbent assay's (ELISA's) were used to determine plasma cortisol (Cortisol, IDS, Tyne and Wear, UK), testosterone (Testosterone, IDS, Tyne and Wear, UK) and catecholamine (CAT-COMBO, IDS, Tyne and Wear, UK) concentrations. In each case, plates were read in duplicate on a Labsystems Original Multiskan MS at selected wavelengths (450nm = cortisol and testosterone; 405nm = adrenaline and noradrenaline). Reported sensitivity of ELISA's were 2.5ng/ml, 0.083ng/ml and 10pg/ml and 20pg/ml for cortisol, testosterone, adrenaline, and noradrenaline, respectively. Intra-assay variations were calculated for cortisol (7%), testosterone (8%), adrenaline (9%), noradrenaline (13%) and dopamine (12%), respectively. Plasma urea concentrations were determined at all timepoints during time trial. Hormone concentrations were measured at BL, SM, TT. Additionally, plasma cortisol was also measured at 1h-POST. Blood samples collected during each stage of the MT were analysed for blood lactate concentrations.

5.3.8 Questionnaires

5.3.8.1 Daily Analysis of Life Demands for Athletes

Participants completed the Daily Analysis of Life Demands for Athletes (DALDA) questionnaire (37) (Appendix F) on a daily basis to monitor psychological status. The DALDA is divided into parts A and B, which represent the sources of- and manifestation of stress in the form of signs and symptoms, respectively. For both parts, the number of items marked as 'worse than normal' ('a' scores) were reported.

5.3.8.2 Profile of Mood State-65

On day seven of each training week, participants completed a 65-item version of the Profile of Mood State-65 questionnaire (POMS-65) (33) (Appendix G). POMS-65 items are divided into six categories; tension, depression, anger, vigour, fatigue, and confusion. GMS-65 was calculated as the sum of all negative categories minus the score for vigour, plus 100. Athletes were asked to complete all questionnaires (including those previously mentioned in section 'Training Quantification') on waking.

5.3.9 Data Presentation and Statistical Analysis

All data are expressed as mean ± SE for eight subjects, unless otherwise stated. Statistical analysis was performed using Statistical Package for Social Sciences (SPSS) 15.0 for Windows (SPSS Inc., Chicago, IL). Differences were considered significant when the probability of chance occurrence (alpha) was equal to or less than 0.05. To establish the effect of intensified training, a one-way (week) analysis of variance (ANOVA) was used to examine the effect of training on measured variables in CON, only. To determine differences between dietary conditions, all variables were analysed using a two way (dietary condition and week/day) analysis of variance (ANOVA) with repeated measuresm, unless otherwise stated. If there was a significant treatment effect, pairwise comparisons with least significant difference (LSD) adjustments were performed to determine differences between trials for each week (between-trial comparison). Plasma urea concentration measured during the time trial in each week of training was expressed as area under the curve (AUC) (baseline set as resting values measured during NOR of corresponding dietary condition). DALDA 'a' scores part B during INT, were compared between trials using a non parametric Two Related Samples Wilcoxon Signed Rank test.

Data also are expressed in terms of inferential statistics using 90% confidence limits to produce magnitude-based values about the probabilities of the outcome (20). For time trial performance, a substantial effect was assumed to be a reduction or increase of more than 1.7%, and for Wmax this value was 2.5%. These values were derived from 0.5 multiplied (19) by published coefficients of variation of respective performance tests (9,28). The likelihood of the outcome being of beneficial or detrimental nature was also determined using a published spreadsheet (20). The chance of observing a detrimental/beneficial effect was divided into the following categories: <1%=no chance, 1-5%=very unlikely, 5-25%=unlikely, 25-75%=possible, 75%-95%=likely, 95-99%=very likely and >99%=almost certain.

5.4 Results

5.4.1 Training quantification

Mean total weekly training volume and time spent training in each training zone are displayed in Fig. 5.3 for both PRO and CON. No difference in training intensity or duration between trials was observed during any training week (P>0.05). Time spent in training zones three, four and five were increased during INT compared to NOR in both trials. During REC, training volume was reduced to ~60% relative to NOR in both dietary conditions.

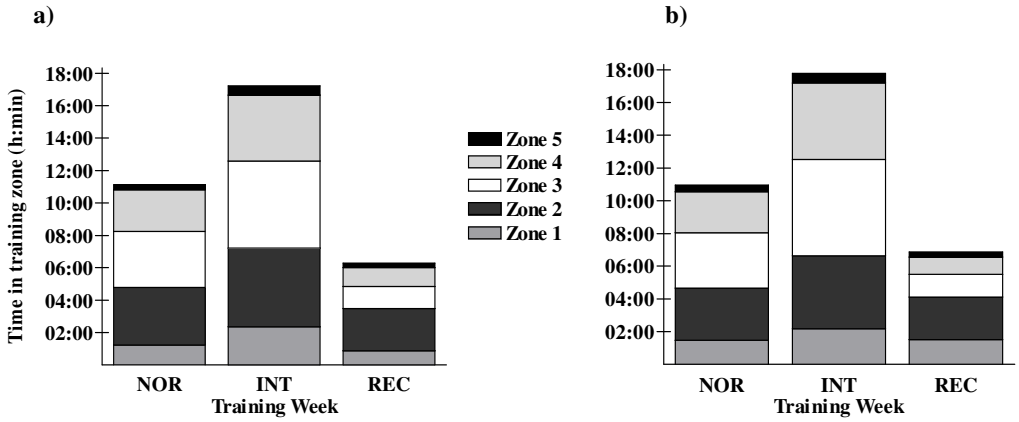

Figure 5.3: Time spent in each heart rate zone during normal (NOR), intensified (INT) and recovery (REC) training in, a) high protein (PRO) and b) normal protein (CON) trials.

Zones 1-5 represent low to high training zones, respectively.

5.4.2 Dietary regimen

Dietary energy intake and macronutrient composition during each training week of both trials are given in Table 5.1. Energy intake, expressed as average daily calorie intake over the course of a week, was matched between dietary periods in each week of training. Participants remained weight stable during both dietary periods. As intended, dietary protein intake was significantly greater in PRO compared to CON during INT (P<0.05) and REC

(P<0.05). There was no difference in CHO intake between PRO and CON. Intake of fat was greater in CON compared to PRO during both INT (P<0.05) and REC (P<0.05).

Table 5.1: Dietary intake of cyclists during normal (NOR), intensified (INT) and recovery (REC) training weeks in high protein (PRO) and control (CON) conditions.

		NOR	INT		REC	
		PRO/ CON	PRO	CON	PRO	CON
Protein	(g/day)	106±3	210±6*†	106±3	210±6*†	105±4
intake	(g/kg)	1.5±≤0.1	3.0±≤0.1*†	1.5±≤0.1	3.0±≤0.1*†	1.5±≤0.1
CHO intake	(g/day)	421±14	420±18	422±12	420±18	420±13
	(g/kg)	6.0±≤0.1	6.0±0.1	6.0±≤0.1	6.0±0.1	6.0±≤0.1
Fat	(g/day)	178±15	216±9*†	221±11	71±19†	118±18
intake	(g/kg)	2.1±0.2	2.3±0.1*†	3.7±0.1	1.4±0.3†	1.7±0.3
Energy intake	(kcal/day)	3711±161	4409±153*	4410±155	3096±178	3096±153

PRO=high protein diet, CON=normal protein diet. *Significantly greater than NOR (either dietary condition) (P<0.05). †Significantly different from CON during corresponding training week (P<0.05). Data are means and SD.

5.4.3 Performance

5.4.3.1 Time Trial

Fig. 5.4 demonstrates the decrement in time trial performance following INT and REC in relation to NOR. Mean time trial completion time was increased (performance decreased) following INT in CON (NOR: 2555±111s; INT: 2893±160s, P<0.05). No effect of diet intervention on time trial performance during INT and REC was detected by two-way ANOVA, nor was any statistically significant week-by-diet interaction observed.

The impact of training week on time trial performance for each cyclist is illustrated in Fig. 5.5. In PRO, seven out of eight cyclists demonstrated a decrement in time trial performance following INT compared with NOR. Following REC, time trial performance remained depressed in four cyclists, but three cyclists exhibited a super-compensation effect. Time trial performance was improved following both INT and REC compared to NOR in one cyclist. Seven out of eight cyclists in CON demonstrated a decrement in time trial performance following INT compared with NOR.

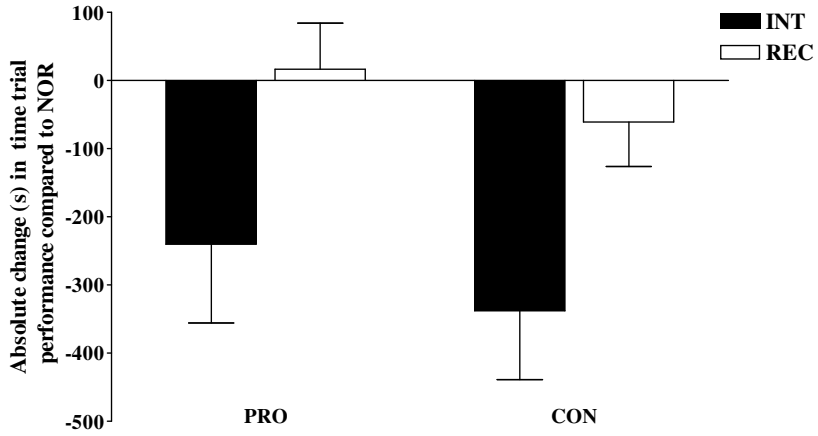

Figure 5.4: Absolute change in time-trial performance following intensified training (INT) and recovery (REC) compared to normal training in high protein (PRO) and habitual protein (CON) trials.

INT=absolute change in time trial performance following INT compared to NOR; REC=absolute change in time trial performance following REC compared to NOR. Values expressed as mean absolute change from NOR ± SE

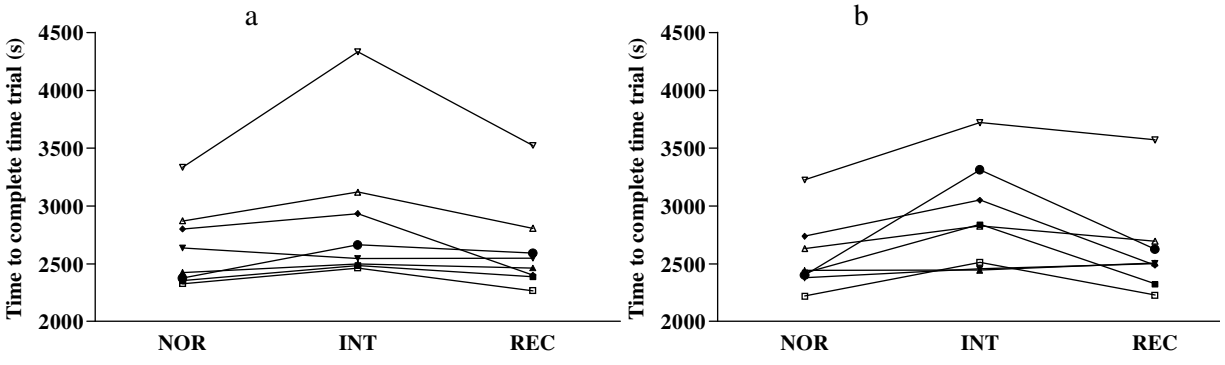

Figure 5.5: Time trial completion times for individual cyclists following normal (NOR), intensified (INT) and recovery (REC) training in, a) high protein (PRO) and b) habitual protein (CON) trials.

Inferential statistics revealed that additional high protein intake elicited an ~35% chance of being beneficial and only an ~4% chance of being detrimental to time trial performance.

5.4.3.2 $\dot{V}O_2$ max test

Six out of eight cyclists in PRO and seven out of eight cyclists in CON exhibited decrements in Wmax following INT compared to NOR. Mean Wmax decreased following INT compared to NOR in CON (NOR: 357±10W; INT: 335±11W, P<0.01). Fig. 5.6 displays the decrement in Wmax following INT and REC in relation to NOR between PRO and CON. No effect of diet was observed on Wmax using either traditional hypothesis (p value) testing or inferential statistical analysis.

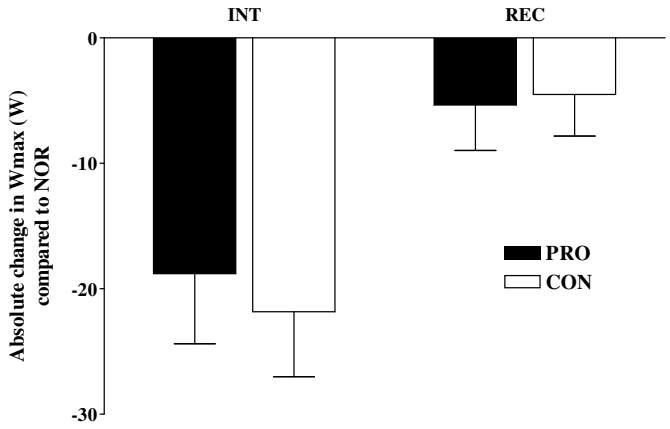

Figure 5.6: Absolute change in maximum power output (Wmax) following intensified training (INT) and recovery (REC) compared to normal training (NOR) in high protein (PRO) and habitual protein (CON) conditions.

INT=absolute change in Wmax following INT compared to NOR; REC,=absolute change in Wmax following REC compared to NOR. Values expressed as mean absolute change from NOR ± SE.

5.4.4 Physiological, metabolic and biochemical responses

5.4.4.1 Maximal exercise responses

Table 5.2 summarises physiological, metabolic and biochemical responses to maximal exercise following intensified training in PRO and CON. $\dot{V}$ O_2max tended (P=0.103) to decrease following INT in CON. $\dot{V}$ O_2max was unchanged by training week or diet. Maximal HR (HRmax) and lactate (LACmax) responses to exercise were impaired by 6±1 beats/min and 1.88±0.59 mmol/L, respectively following INT (compared to NOR) in CON (P<0.05). No effect of diet on HRmax, LACmax or RERpeak was observed.

Table 5.2: Selected changes in maximal test variables over the course of the study period

Maximal responses	PRO			CON		
	NOR	INT	REC	NOR	INT	REC
VO_2 (L/min)	4.24±0.20	4.36±0.04	4.49±0.07	4.49±0.23	4.19±0.27	4.43±0.07
VO_2 (mL·kg^{-1}·min^{-1})	58.8±3.6	59.9±2.8	61.2±2.1	60.9±1.4	55.9±2.5	60.7±2.0
Maximal HR (beats/min)	178±5	170±5*	179±3	181±3	171±5*	180±3
Maximal RER	1.10±0.03	1.02±0.03*	1.07±0.02	1.09±0.02	1.05±0.01	1.05±0.01
Maximal lactate (mmol/L)	10.44±1.33	7.21±1.04**	10.67±1.42	9.94±0.78	8.07±0.48*	8.82±0.83

VO_2, volume of oxygen consumed; L, litres; min, minute; mL, millilitres; kg, kilogram; mmol, millimoles, HR, heart rate; RER, respiratory exchange ratio. Significantly different from NOR ** (P<0.01); * (P<0.05). Data expressed as means ± SE.

5.4.4.2 Submaximal exercise and resting responses

No effect of diet on mean HR and RPE values recorded during 120 min preload of time trial, RHR or SHR was observed (data not shown).

AUC for urea over the course of the exercise period was calculated following NOR, INT and REC (Fig. 5.7). The plasma urea response was higher (~46%) in PRO compared to CON following INT (P<0.05).

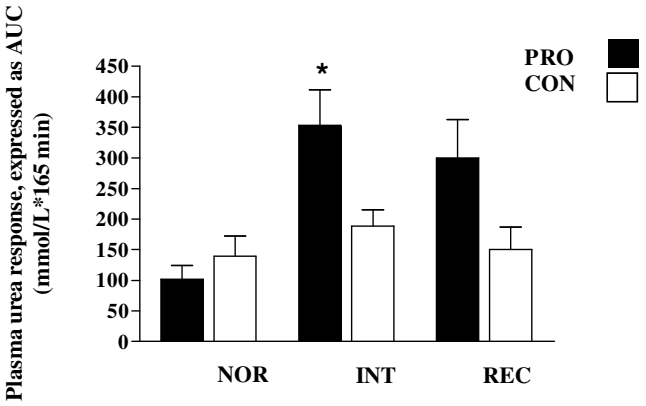

Figure 5.7: Plasma urea concentration responses, expressed as area under the curve (AUC) (mmol·L^{-1}·165 min^{-1}) following normal (NOR), intensified training (INT) and recovery (REC) in high protein (PRO) and habitual protein (CON) conditions.

AUC calculated with baseline set as resting values measured during NOR of corresponding dietary condition. *Significant difference compared to CON (P<0.05).

5.4.4.3 Hormonal responses

Percentage decrement in peak plasma cortisol and adrenaline concentration following INT and REC compared to NOR in both PRO and CON are displayed in Figs. 5.8 and 5.9, respectively. No effect of diet was observed on peak plasma cortisol concentration. A tendency (P=0.095) for an amelioration of the decrease in peak adrenaline concentration due to higher protein intake was noted. Decrements in peak plasma noradrenaline or testosterone concentrations observed following INT, expressed as percentage change relative to NOR, were not statistically different between trials.

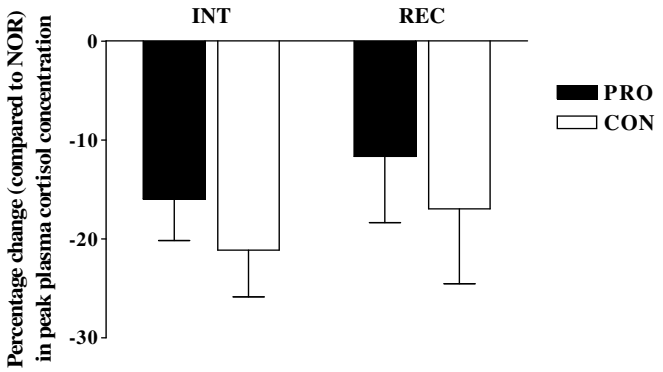

Figure 5.8: Percentage change in peak plasma cortisol concentration following intensified training (INT) and recovery (REC) compared to normal training (NOR) in high protein (PRO) and habitual protein (CON) conditions.

Peak cortisol concentrations typically measured at TT. Values described for INT reflect absolute change in peak cortisol concentration compared to NOR; Values described for REC reflect absolute change in peak cortisol concentration compared to NOR. Values expressed as mean absolute change from NOR ± SE.

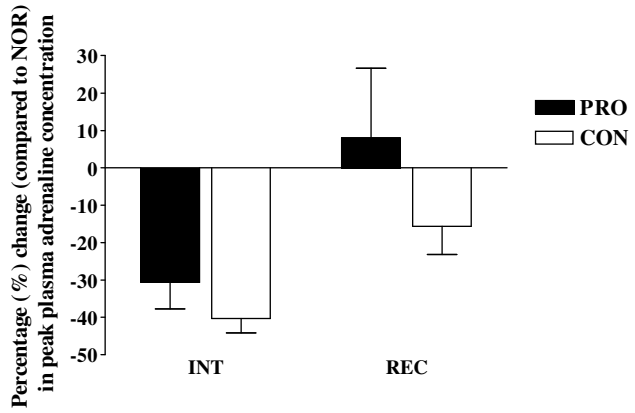

Figure 5.9: Percentage change in peak plasma adrenaline concentration following intensified training (INT) and recovery (REC) compared to normal training (NOR) in high protein (PRO) and habitual protein (CON) conditions.

Peak adrenaline concentrations typically measured at TT. Values described for INT reflect absolute change in peak adrenaline concentration compared to NOR; Values described for REC reflect absolute change in peak adrenaline concentration compared to NOR. Values expressed as mean absolute change from NOR ± SE.

Peak testosterone concentrations during each training week and dietary condition are displayed in Fig. 5.10. Peak testosterone concentrations tended (P=0.066) to be elevated following INT and were significantly higher following REC (~32%) (P<0.05) compared to

NOR in CON. A main effect of diet and diet by week interaction on peak testosterone concentration was observed (P<0.05). Peak testosterone concentrations were significantly lower following INT and REC in PRO compared to CON (P<0.05). No main effect of diet on testosterone:cortisol ratio was observed.

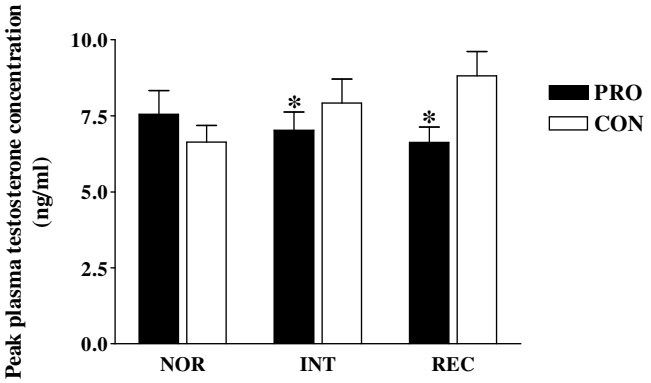

Figure 5.10: Plasma testosterone concentration following normal (NOR), intensified training (INT) and recovery (REC) in high dietary protein (PRO) and habitual dietary protein (CON) conditions.
*Significant difference compared to CON (P<0.05).

5.4.5 Questionnaires

5.4.5.1 Daily Analysis of Life Demands for Athletes

Fig. 5.11 displays the total number of 'a', i.e. 'worse than normal' scores on part B of the DALDA questionnaire for both PRO and CON. Notable increases in the number of fatigue-related symptoms including, 'need for a rest', 'muscle pains', 'between session recovery', 'general weakness', 'sleep' and 'irritability' were prevalent following INT compared to NOR in both trials. The number of psychological symptoms of stress reported during INT was significantly greater in CON compared to PRO (P<0.05).

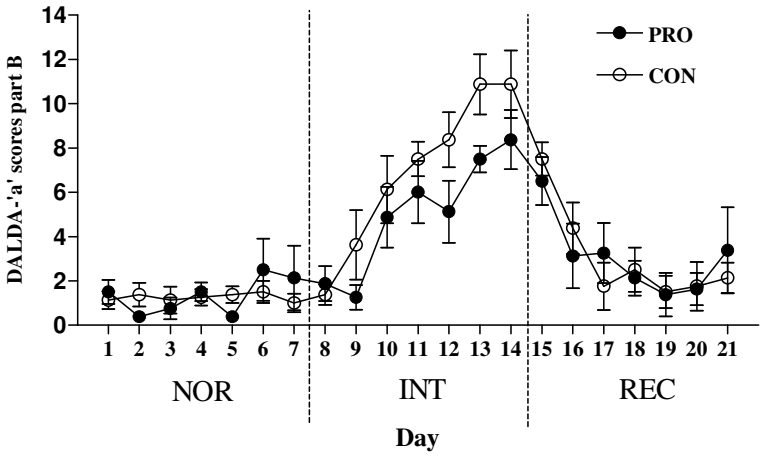

Figure 5.11: Daily reported number of, a) "worse-than-normal" 'a' scores on part B (symptoms of stress) on the Daily Analysis of Life Demands of Athletes (DALDA) questionnaire during normal (NOR), intensified trining (INT) and recovery (REC). Cyclists completed the DALDA questionnaire upon waking in a rested state on a daily basis.

5.4.5.2 Global Mood State scores: Profile of Mood State-65 questionnaire

Fig. 5.12 compares the change in mood state following INT and REC in relation to NOR between PRO and CON. No main effect of diet on GMS was observed.

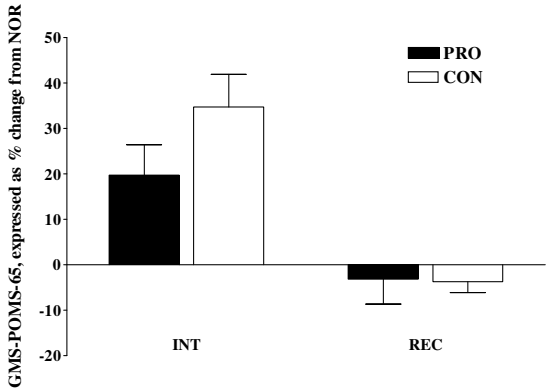

Figure 5.12: Percentage change in Global Mood State (GMS) (derived from Profile of Mood State (POMS)-65) following intensified training (INT) and recovery (REC) compared to normal training (NOR) in high protein (PRO) and habitual protein (CON) conditions.
GMS=Global Mood State scores, POMS-65=Profile of Mood State Inventory-65. Values expressed as mean percent change from NOR ± SE. Cyclists completed the POMS-65 questionnaire upon waking in a rested state on a daily basis.

The subscales of the POMS-65 are presented in Figs. 5.13a and 5.13b for PRO and CON, respectively. Iceberg profiles were 'flattened', indicating increased stress, following INT in both PRO and CON. Most notable changes were observed in 'fatigue' and 'vigor.' An increase in number of reported feelings of 'fatigue' were reported following INT compared to NOR in both PRO (NOR: 7±2 vs. INT: 15±1, P<0.05) and CON (NOR: 5±1 vs. INT: 17±1, P<0.001). In contrast, feelings of 'vigor' declined following INT (8±2) compared to NOR (16±3) in CON (P<0.05). No effect of diet on 'fatigue' or 'vigor' was observed. Feelings of depression, anger and confusion increased to a similar extent following INT in both trials.

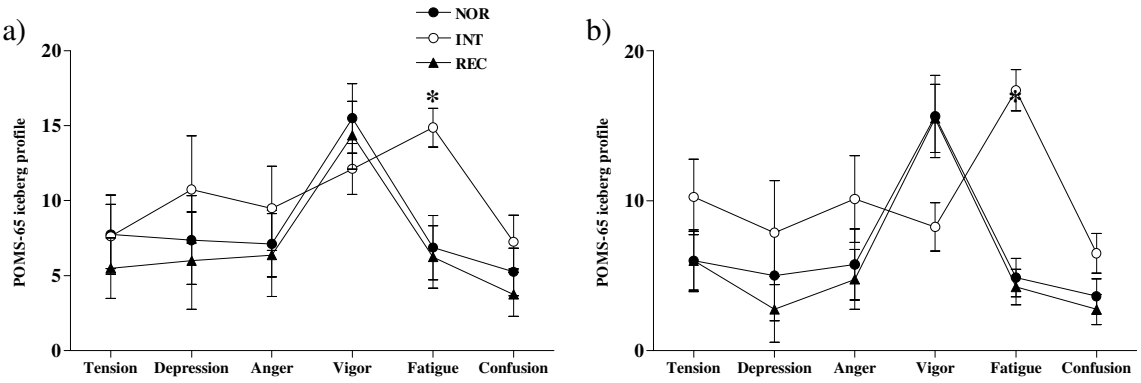

Figure 5.13: Iceberg profile (POMS-65) questionnaire following normal training (NOR), intensified training (INT) and after recovery (REC) in, a) high protein (PRO) and b) habitual protein (CON) conditions. POMS-65=Profile of Mood State Inventory-65. Cyclists completed the DALDA questionnaire upon waking in a rested state on a daily basis.

5.4.5.3 Psychometric ratings/ Subjective complaints

Average weekly fatigue ratings (likert scale) were significantly higher following INT (5.4±0.5) (P<0.0001) and REC (3.7±0.4) (P<0.05) compared to NOR (2.5±0.3) in CON. No effect of diet on fatigue rating or RPE reported during the pre-load was observed. Stress ratings increased following INT (3.6±0.5) compared to NOR (2.5±0.3) in CON (P<0.05). No effect of diet on stress or sleep ratings was detected. Muscle soreness scores, expressed as AUC during INT and REC, were similar between PRO (19±3 mm) and CON (22±5 mm).

5.5 Discussion

The purpose of the present investigation was to examine the impact of a high protein diet on the change in endurance performance and exercise recovery following an intensified period of training. Our data are consistent with earlier studies (14,22), whereby impaired endurance performance and elevated mood disturbances were accompanied by biochemical abnormalities such as blunted maximal plasma lactate concentrations and attenuated stress hormone concentrations following intensified training. Our findings are novel in providing evidence that increasing dietary protein intake to 3 g·kg BM^{-1}·day^{-1} may attenuate mood disturbances and decrements in endurance performance following an intensified period of training.

Protein nutrition may play a beneficial role in better maintaining endurance performance during intensified periods of training. A substantial decrement (13±4%) in time trial performance was observed immediately following one week of overload training when protein intake was similar to both the habitual intake of recruited cyclists (calculated as ~1.6 g protein·kg BM^{-1}·day^{-1}) and recently published recommended dietary protein requirements for endurance athletes (42). Doubling the protein intake of an energy-matched, iso-CHO diet resulted in better maintenance of endurance performance in the period following intensified training. P value hypothesis testing failed to detect a main effect of protein on time trial performance for INT and REC combined; this despite observing a super-compensation effect in four out of eight cyclists following REC in PRO. Support for the notion that protein enhanced the efficacy of cyclists to maintain endurance performance during intensified training was emphasised by magnitude-based inferential statistics. Increased dietary protein intake was determined to provide a 35% chance of a 'beneficial' effect and only 4% chance of a 'harmful' effect on time trial performance following one week of intensified training. Thus,

our data are the first to suggest that additional dietary protein intake may attenuate decrements in time trial performance following intensified training.

Previous research has focused on the impact of CHO rather than protein nutrition for reducing the negative impact of intense training. Depleted muscle glycogen concentrations following excessive training (8,24,40) are thought to provide a potential explanation for impaired endurance performance typically experienced following intensified training (23). The better maintenance of endurance performance following intensified training, previously observed with carbohydrate supplementation (16), was attributed to a maintained rate of carbohydrate oxidation. In the present study, cyclists were fed a moderate CHO diet (6 g·kg BM^{-1}·day^{-1}) which is lower than levels recommended (8-10 g·kg BM^{-1}·day^{-1}) for intense endurance training (6). Increased dietary protein intake could indirectly increase CHO availability or muscle glycogen stores via gluconeogenic pathways. Support for this notion is exemplified by a study which demonstrated that the rate of gluconeogenesis was elevated by ~40% with a high protein diet, consequently accounting for a large proportion of endogenous glucose availability (30). Our urea data may provide evidence that gluconeogenesis was increased during PRO. Urea concentration was greater with increased protein intake following intensified training, suggesting more amino acids were deaminated, possibly so that the carbon skeletons could be used for gluconeogenesis. However, increased performance due to gluconeogenesis would likely be related to increased CHO oxidation, and consequently the maintenance of peak lactate concentrations. Contrary to the prevented decrement in maximum lactate production during maximal exercise with CHO supplementation (16), peak lactate concentrations measured during maximal exercise declined following intensified training despite additional dietary protein intake. Furthermore, no differences in RER were observed between dietary conditions in the present study. Thus, the better maintenance of endurance

performance following intensified training observed in the high protein condition is unlikely to be attributed to a restored rate of CHO oxidation.

The beneficial effect of CHO nutrition in attenuating decrements in performance associated with intensified periods of training has also previously been attributed to a modulated endocrine response (2,16). The cumulative effect of releasing high levels of stress hormones during consecutive bouts of intense exercise has been suggested to desensitise the hypothalamic pituitary axis (HPA) (18), and ultimately reduce availability of selected hormones (4,44). This notion is supported by an attenuated peak cortisol response to maximal exercise that we and others (16,41,45) observed following intensified training. CHO supplementation before, during, and after training was suggested to attenuate the desensitisation of the HPA following intensified training, and was thus deemed responsible for restoring the blunted peak cortisol response (16). However, increased dietary protein intake during a period of intensified training had no impact on peak cortisol concentration, nor were any differences in plasma adrenaline concentrations observed between dietary conditions. Thus, whereas it is clear that CHO impacts the endocrine response to intensified training, the mechanism responsible for the better maintenance endurance performance with protein feeding does not appear to be related to a modulated stress hormone response.

One endocrine response which was clearly affected by additional dietary protein intake was testosterone. Blunted resting plasma testosterone concentrations have been previously reported following a period of heavy training to coincide with a decline in performance (12). Increasing amino acid availability has previously been demonstrated to prevent the decline in blood testosterone concentrations following overload training (26). In the present study, diet modulated plasma testosterone concentrations during maximal exercise to a greater extent than training. Peak plasma testosterone concentrations were augmented by

~25% following intensified training in the control condition. In contrast, increasing the protein content of the diet failed to modulate peak testosterone concentration. Testosterone levels in healthy males have been reported to range from ~3-10ng/mL (3). Clearly, peak testosterone concentrations remained within the 'normal' physiological range in both dietary conditions, increasing from ~7ng/mL (NOR) – ~9ng/mL (REC). Thus, despite a statistically significant change in testosterone concentration (and resultant elevation in T:C) following intensified training, this increase may not actually be physiologically relevant to biological functioning. Future investigations which not only monitor changes in testosterone concentrations with intensified training, but examine the physiological significance of these changes, may be beneficial.

In addition to endocrine and biochemical parameters, protein nutrition failed to impact physiological and subjective markers of increased training stress. In accordance with previous findings, the number and severity of post-exercise markers of fatigue, such as increased resting and sleeping heart rate (10) and muscle soreness ('heavy legs') (46) increased following consecutive days of excessive training (15). Increasing dietary protein intake during and following intensified training had no impact on these physiological perturbations. These data suggest that the apparently beneficial effect of increasing dietary protein intake on endurance performance during excessive periods of training observed in the present study was not mediated by a reduction in physiological markers of fatigue, and therefore is more likely explained by other mechanisms.

We propose that indirect centrally-driven mechanisms may be responsible for the better maintenance of endurance performance during intensified periods of training with additional protein intake. In agreement with previous findings (2,16,35), psychological symptoms of stress, in particular the number of fatigue-related symptoms of stress (i.e., 'need

for a rest,' and 'general weakness'), deteriorated following overload training. CHO feeding has previously been reported (2,16) to attenuate the negative psychological symptoms of stress associated with intensified training. In the present study, this athlete-specific DALDA questionnaire revealed that the number of reported psychological symptoms of stress reported by cyclists during and following a period of intensified training was attenuated when dietary protein intake was increased. On the otherhand, the POMS-65 questionnaire, which was developed for psychiatric patients, detected no differences in mood state between dietary conditions, questioning the utility of this clinically-based questionnaire to monitor mood states of athletes (35). The apparent improved psychological status of athletes during and following a short-term period of intensified training in the high protein trial potentially contributed to the better maintenance of endurance performance following overload training. It is suggested that tyrosine supplementation attenuates decrements in stress related mood (34), thus it is possible that an increased availability of amino acids, in particular tyrosine, mediated an improved mood state in the protein condition. Further research should be conducted to fully elucidate the mechanism(s) responsible for this beneficial effect of protein feeding on psychological status.

Informing athletes that by increasing dietary protein intake, they have an ~35% chance of better maintaining endurance performance during intensified periods of training may appear attractive. However, a recommendation for such high protein intakes may not be warranted. It should be emphasised that the cyclists in this study ingested 3 g protein·kg BM^{-1}·day^{-1}. This high level of protein intake may not be tolerated well by athletes in their natural training setting, and would likely have to be administered at the expense of CHO intake. Hence, whereas we intended to maximise the effect of protein feeding, future studies should

endeavor to determine whether more practical levels of protein intake (e.g. 2 g·kg BM^{-1}·day^{-1}) during intensified training results in better maintenance of endurance performance.

5.6 Conclusion

Our data suggest that increased dietary protein intake may have a beneficial role in exercise recovery by attenuating impairments in endurance performance which accompany intensified periods of training. Likely mediators of this beneficial effect of protein feeding on exercise recovery include attenuated perturbations in psychological symptoms of stress.

5.7 References

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CHAPTER 6 - General Discussion

Exercise-induced changes in protein metabolism may be further modulated by protein feeding. A plethora of studies demonstrate that administration of different types (14,44) and dosages (31) of protein sources, ingested alone or co-ingested with carbohydrate (CHO) (7,29,46), further stimulate muscle protein synthesis (MPS) when consumed following resistance exercise. Our study was novel in examining the influence of resistance-based exercise on MPS following meal ingestion. Another area of interest is the role of protein ingestion for recovery from endurance exercise training. CHO is the most important substrate for skeletal muscle metabolism during prolonged moderate to high-intensity dynamic exercise, thus most research has focused on manipulating CHO intake in an attempt to enhance recovery from endurance exercise. Only recently have investigations explored the role played by protein feeding for enhancing recovery from endurance exercise. Thus, the purpose of this thesis was twofold:

1) To determine the influence of resistance exercise on MPS in practical setting whereby resistance exercise is performed following ingestion of a meal.

2) To determine the role of dietary protein intake on recovery from an intense period of endurance training.

The general discussion that follows is sub-divided into two sections. Section A summarises the major findings from **Chapter 2**, which examined the additive effect of resistance exercise to nutrition on MPS in a practical situation whereby muscle protein synthesis is already stimulated by food intake. In section B, **Chapters 3-5** utilised an experimental model whereby the training volume of experienced cyclists was intensified for a period of a week. The detrimental effect of a period of intensified training on the immune status of trained cyclists was determined with the measurement of peripheral blood counts of functionally distinct lymphocytes (**Chapter 3**). Dietary protein intake was manipulated in an

attempt to modulate the pattern of lymphocyte trafficking following a period of intensified training **(Chapter 4)**. Finally, we examined the effect of increasing dietary protein intake during a period of intensified training and a subsequent period of reduced training on endurance performance, indices of exercise recovery and mood state **(Chapter 5)**.

Section A – Muscle protein synthesis, exercise and nutrition

Resistance exercise augments the protein synthetic rate of muscle already stimulated by food intake. Exercise, at least of sufficient intensity (26), is known to stimulate the rate of post exercise mixed (all proteins in the muscle) MPS in healthy young people. Indeed, an acute bout of resistance exercise, performed in the fasted state, can elicit a profound increase in mixed muscle fractional synthetic rate (FSR) after exercise compared with the basal (rested and fasted) condition (9,25,26,40), which can persist for ~48 h in untrained individuals (40). However, recreational activities are generally performed in a fed condition, rather than in the fasted state. Our study **(Chapter 2)** examined the impact of resistance-based exercise on MPS following meal ingestion. We reported FSR values of ~0.10%/h during recovery from an acute bout of resistance exercise performed in the postprandial state, which corresponded with an 80-150% increase in MPS compared with previously reported basal muscle protein synthetic rates (0.04-0.06%/h) (4,25,40,49). Taken together, these data suggest that an acute bout of exercise stimulates MPS during recovery whether performed in the fed or fasted state. Post-exercise feeding has been well investigated in the fasted state (44,45). Thus, in an attempt to further replicate the training habits of athletes, the logical extension to these findings would be to examine the muscle protein synthetic response to exercise preceded by food intake and followed by single/repeated ingestion of protein.

The synergistic effect of exercise followed by nutrition on mixed MPS is well established (5,39,43). A recent study by Phillips et al. (39) demonstrated that repeated

administration of small boluses of mixed macronutrient drinks (10 g casein protein + 75 g glucose) during exercise recovery accelerated muscle protein synthetic rates by ~20% compared with nutrient intake alone in untrained males. The novel finding of the present study was that an acute bout of resistance exercise, performed in the postprandial state 75 min following meal ingestion, further accelerated mixed muscle protein synthetic rates by ~20% compared to a fed state alone. Thus, it seems clear that the additive effect of exercise and intake of mixed macronutrients on MPS rates are similar whether mixed macronutrients are consumed via more practical means as a meal prior to activity, or as small repeated drink boluses post exercise. Thus, the proposed 'metabolic window of opportunity' notion that protein must be ingested within a short period after exercise in order to create an anabolic response after exercise (23) is not supported by these data.

Increased MPS following resistance exercise performed in the fed state may be partially mediated through the upregulation of the mammalian target of rapamycin (mTOR) signalling cascade. To our knowledge, the present study was the first to compare the phosphorylation status of mammalian target of rapamycin (mTOR)-associated signaling proteins between the additive effect of exercise + pre-exercise meal ingestion versus meal ingestion alone, simultaneously with MPS. Accelerated muscle protein synthetic rates when food intake preceded exercise were accompanied by an elevated phosphorylation status of eukaryotic initiation factor (eIF) 4E-Binding Protein 1 (4E-BP1) and ribosomal S6 (rpS6) during exercise recovery, however no changes in the phosphorylation status of mTOR or S6K1 at T^{389} were observed. Thus, in agreement with previous studies (13), an apparent disconnect between the phosphorylation of mTOR and some of its downstream signalling proteins, 4E-BP1 and S6 were reported. The reasons for this apparent disconnect are unclear, but may be related to differences in the time course of the response of the various signaling

proteins. In an attempt to explain this apparent disconnect within the mTOR signalling cascade, future studies investigating the regulation of translation initiation by both exercise and nutrition should examine alternate signalling pathways involved in translation initiation, such as the mitogen activated protein-dependant kinase (MAPK) pathway, as well as the time course of the response to exercise and nutrition.

Section B – Protein and intensified endurance training

Section B of this general discussion attempts to tie together the major findings from a research project which investigated the impact of dietary protein manipulation on exercise recovery during and following a short-term period of intensified endurance training. Changes in exercise performance (**Chapters 5**), endocrinology (**Chapter 3-5**), blood metabolite parameters (**Chapter 5**), physiological indices (**Chapter 5**), psychological status (**Chapter 3-5**) and immune function were included in our analyses of exercise recovery following a period of intensified training. This study allowed us to determine alterations in the functional capacity of the immune system in response to an acute bout of exercise following both normal and intensified training (**Chapter 3**). The impact of additional protein intake on the mobilisation of antiviral lymphocytes (**Chapter 4)**, endurance performance **(Chapter 5)** and mood state **(Chapter 5)** was investigated. In summary, our data suggest that doubling dietary protein intake may be beneficial for maintaining exercise performance, mood state and immune status within the context of an intensified period of training.

Regular profiling of the minimal mobilisation and minimal egression of functionally distinct antiviral CD8+ T lymphocytes (CD8+TL) may provide a useful diagnostic tool to determine the underlying cause of the increased incidence of infections experienced by athletes. Our assessment of immune status was comprehensive, incorporating the minimal mobilisation and minimal egression of functionally and phenotypically distinct subsets of

each lymphocyte sub-population (CD8+TL, CD4+ T lymphocytes (CD4+TL), Natural Killer (NK) cells and B lymphocytes). Specifically, the minimal mobilisation and minimal egression of a highly cytotoxic subset of anti-viral CD8+TL responded to intensified training, potentially rendering cyclists more susceptible to viral infection **(Chapter 3)**. The minimal mobilisation of CD45RA+ Effector Memory (RA+EM) CD8+TL into the bloodstream during exercise was reduced, subsequently translating into an attenuated post exercise minimal egression of these antiviral cells. In accordance with previous studies (41), we observed an increase in the frequency of 'worse than normal' symptoms of minor viral infections (i.e. sore throat, congestion and running nose) reported by trained cyclists during one week of intensified training. However, reported illnesses and infections were not clinically confirmed in the present study, thus no attempt to directly associate the apparently increased incidence of infection following intensified training with the modulated pattern of CD8+TL minimal mobilisation and egression could be made. To fully elucidate the immunological significance of blunted CD8+TL trafficking, future studies should simultaneously monitor changes in the patterns of antiviral lymphocyte trafficking and incidence of clinically diagnosed episodes of upper respiratory tract infections (URTI).

Our data **(Chapter 4)** offer the opportunity for an interesting argument challenging the open window hypothesis. Intense bouts of prolonged exercise are well documented to increase circulating CD8+TL counts during exercise, followed by a decline to below baseline during recovery (33,35). This post exercise lymphocytopenic response is often referred to as the open window, perceived to be indicative of a transient suppression of the immune system (36). Traditionally, nutritional strategies which attenuate perturbations in T lymphocyte sub-population counts following exercise are considered effective counter-measures to the immuno-suppressive effects of intense exercise training (36). Accordingly, CHO

supplementation strategies which attenuate this postexercise egression of total lymphocyte subpopulations have previously been deemed effective counter-measures to the immuno-suppressive effects of intense exercise (6,16,30).

Alternatively, facilitating the continuous trafficking of functionally distinct CD8+TL subsets into the bloodstream, through various tissues, and back into the peripheral blood may be considered an effective strategy to increase the capacity of the immune system to maintain an effective immune defence network (12). Highly cytotoxic RA+EM CD8+TL contribute only a small proportion of total CD8+TL (~5-10%), however their distinct cytolytic capacity mean these cells are highly influential for immune function. Our findings indicate that increasing the protein content of the diet during intensified training augmented the minimal mobilisation of highly cytotoxic RA+EM CD8+TL into the blood during exercise. Increasing the availability of these tissue-migratory CD8+TL cells in the blood may lead to a greater post -exercise deployment of these highly cytotoxic CD8+TL to the peripheral tissues that may contain pathogens. Thus, restoring the minimal mobilisation of highly cytotoxic CD8+TL into the blood during exercise with additional protein possibly represents an adaptive cell-mediated response of the immune system to fight impending infection when challenged by intense training. We acknowledge that this interpretation of lymphocyte trafficking is not universally accepted within the field of exercise immunology. However, we propose that the differentiation status of CD8+TL subsets should be considered when interpreting the effectiveness of a nutritional strategy to counter the blunted response of the immune system typically experienced following an intensified period of training.

Substrate availability has been suggested as the possible explanation underpinning the capacity for nutritional strategies to counter the immuno-suppressive effects of intense exercise. T lymphocytes depend on blood glucose (42) and glutamine (28,32) as energy

substrates. The maintenance of blood glucose concentrations during acute bouts of intense exercise with CHO supplementation has previously been shown to attenuate the post-exercise decline in lymphocyte proliferation (20,34). In accordance with this potentially beneficial effect of CHO feeding on direct measures of T lymphocyte function, increasing dietary protein intake during intensified training restored the indirect functional capacity of CD8+TL, i.e. the capacity of highly cytotoxic CD8+TL to traffic between the blood and locations such as the peripheral tissues where antigens may be impending. Urea concentrations, measured in the basal and exercised state, were greater with increased protein intake following intensified training. Hence, it may be argued that more amino acids were deaminated and ultimately the rate at which carbon skeletons could be used for gluconeogenesis and/or oxidation was enhanced in the high protein condition. Directly comparing the immune response to feeding CHO in relation to an acute bout of exercise (17,20) with the present study whereby dietary protein intake was manipulated during a sustained period of intensified training appears non-sensical. Furthermore, enhancing the availability of glucose with increased dietary protein intake is not supported by current pertinent literature (27) as a potential mechanism driving the mobilisation pattern of CD8+TL in response to exercise. Thus, substrate availability provides an unlikely explanation underpinning the apparent restored indirect functional capacity of CD8+TL following intensified training with additional protein intake.

An alternative mechanism suggested to be related to the modulated pattern of minimal mobilisation and egression of lymphocytes intensified training with additional dietary protein intake is the endocrine system. The cumulative effect of releasing high levels of stress hormones during consecutive bouts of intense exercise has been suggested to desensitise the hypothalamic pituitary axis (HPA) (19), and ultimately reduce hormone availability (2,47). We demonstrated that blunted maximal plasma adrenaline and cortisol concentration

following intensified training were accompanied by an attenuated minimal mobilisation and egression pattern of highly cytotoxic antiviral CD8+TL in response to exercise **(Chapter 3)**. Adrenaline initiates the demargination of lymphocytes from the marginal pools into the peripheral blood (3), hence RA+EM CD8+TL which express relatively high densities of ß2 adrenergic receptors on their cell surface membranes (21) are preferentially mobilised during exercise **(Chapter 3).** Thus, blunted adrenaline concentrations induced by intensified training likely inhibit the preferential minimal mobilisation of these highly cytotoxic CD8+TL into the bloodstream during exercise. Moreover, reduced plasma cortisol concentrations during prolonged exercise following intensified training have previously been shown to limit the post-exercise minimal egression of lymphocytes (38). Additional protein intake during a period of intensified training had no impact on measured peak plasma concentrations of cortisol or adrenaline during exercise. It may be considered surprising that the restored minimal mobilisation pattern of highly cytotoxic CD8+TL during exercise did not coincide with a modulated stress hormone (adrenaline or cortisol) response. The exact mechanism responsible for the modulated minimal mobilisation of RA+EM CD8+TL cannot be determined with our study design.

Alongside health status and well-being, performance is of ultimate importance to endurance athletes during intensified periods of training. A substantial decrement (13±4%) in time trial performance was observed immediately following one week of overload training when protein intake was similar to both the habitual intake of recruited cyclists (calculated as ~1.6 g protein·kg body mass(BM)$^{-1}$·day^{-1}) and recently published recommended dietary protein requirements for endurance athletes (10). In **Chapter 5**, we reported impaired time trial performances following one week of intensified training was attenuated by ~5% when cyclists ingested a daily dietary protein intake of 3 g·kgBM^{-1}·day^{-1}. Magnitude-based

inference analysis (22) revealed a 35% chance of a 'beneficial' effect and only 4% chance of a 'harmful' effect of additional protein intake on endurance performance following a period of intensified training. Thus, our data are the first to suggest that additional dietary protein intake may attenuate decrements in endurance performance following intensified training.

It remains unclear whether the beneficial effect of additional dietary protein intake on exercise performance may be explained by a modulated metabolic response. The recently reported better maintenance of endurance performance during intensified training when dietary CHO intake was increased from 5.4 g CHO·kg body mass $(BM)^{-1}\cdot day^{-1}$ to 8.5 g CHO·kg $BM^{-1}\cdot day^{-1}$ was attributed to an enhanced restoration of muscle glycogen stores between exercise bouts, and hence more stable CHO and fat oxidation rates during exercise (1,18). Similar to the lower CHO intake administered by (1), athletes in the present study were fed a moderate CHO diet (6 g·kg body mass $(BM)^{-1}\cdot day^{-1}$) in both conditions, which is less than levels recommended (8-10 g·kg$BM^{-1}\cdot day^{-1}$) for intense endurance training (10). Thus, glycogen stores may have been depleted by the relatively low CHO intake during such intense training (11). If depleted glycogen stores were responsible for the decrement in endurance performance following intensified training in the present study, it is possible that an increased dietary protein intake could have indirectly increased CHO availability or muscle glycogen stores via gluconeogenic pathways, ultimately maintaining endurance performance. An interesting future study would be to compare the impact of supplementing the habitual diet with CHO + protein vs. CHO alone during an intensified period of training on the maintenance of muscle glycogen stores and exercise performance.

In accordance with previous studies (1,18), the determination of muscle glycogen content was not be made in the present study. However, we observed no effect of dietary protein manipulation on maximal plasma lactate concentrations, nor the respiratory exchange

ratio calculated during submaximal exercise. Thus, the better maintenance of performance observed with increased dietary protein intake does not seem to be explained by changes in blood metabolite parameters that we measured in association with time trial performance.

Alternatively, we propose that indirect centrally-driven mechanisms may be responsible for the better maintenance of endurance performance during intensified periods of training with additional protein intake. In accordance with CHO feeding studies (1,18), we demonstrated that the negative psychological effects of one week of intensified training, as determined by both the Daily Analysis of Life Demands for Athletes and Profile of Mood State-65 questionnaires, were attenuated with dietary protein manipulation **(Chapter 5)**. The increased availability of amino acids, in particular tyrosine, has previously been implicated to improve stress-related mood state (37). Furthermore, recent findings advocate the role of increasing the protein content of the diet in enhancing satiety (48), and thus potentially mood state (24). Altered mood states are associated with immune status and subsequent risk of illness in athletes (8). Therefore, our data may also suggest a role for protein feeding for maintaining the well-being and health status of athletes during intensified training (**Chapter 4**), Thus, central mechanisms, rather than modulated blood metabolite concentrations or endocrine responses to intensified training, are likely responsible for the beneficial affect of additional dietary protein intake on endurance performance.

Our intention was to maximise the chance of detecting an effect of protein feeding on recovery from an intensified period of training. Informing athletes that by increasing dietary protein intake, they have an ~35% chance of better maintaining endurance performance during intensified periods of training may appear attractive. However, a recommendation for such high protein intakes may not be warranted. It should be emphasised that the cyclists in this study ingested 3 g protein·kg BM^{-1}·day^{-1}. We acknowledge that maintaining a diet of 3

g·kg BM^{-1}·day^{-1} over an extended period of time is not practical for endurance athletes. This high level of protein intake may not be tolerated well by athletes in their natural training setting, and would likely have to be administered at the expense of CHO intake. Thus, future studies should endeavour to examine the inter-relationship between more realistic levels of dietary protein intake (e.g. 2 g·kg BM^{-1}·day^{-1}), exercise performance and immune status.

To summarise, collectively these data suggest that protein feeding may provide an effective nutritional strategy to, at least partially, attenuate the compromised immune status and impairment in exercise performance typically experienced following chronic, high volume, high intensity training. Additional protein intake during a period of increased training stress restored the exercise-induced minimal mobilisation pattern of highly cytotoxic CD8+TL **(Chapter 4)**, which may be deemed indicative of restored anti-viral defence and subsequent improved health status. Interestingly, the number of 'worse than normal' scores for symptoms related to URTI following intensified training was reduced when the dietary protein intake of trained cyclists was doubled. These data possibly extend previous findings which reported reduced incidence of viral infection with protein feeding during extended periods of intense physical exertion (15). The exact mechanism by which protein feeding restored CD8+TL trafficking cannot be deduced with the measurements collected in our study design. A modulated psychological status provides the most plausible mechanism underpinning the better maintenance of endurance performance following intensified training with protein feeding **(Chapter 5)**.

Conclusions

The study described in **Chapter 2,** which examined the additive effect of resistance exercise when preceded by food intake on MPS and intracellular signalling, demonstrated that:

1) An acute bout of resistance exercise, preceded by a meal, stimulates MPS to a greater extent than a meal alone.

2) Accelerated rates of MPS when exercise follows a meal may be partially mediated via an upregulation in the mTOR signalling pathway.

Studies described in **Chapters 3 and 5** which investigated the impact of dietary protein manipulation on exercise recovery during and following a short-term period of intensified training show that:

3) Intensified training partially blunts the minimal mobilisation and egression response of highly cytotoxic antiviral lymphocytes to exercise.

4) Additional dietary protein intake restores the minimal mobilisation and egression pattern of CD8+TL following intensified training in response to exercise.

5) Additional dietary protein intake induces a better maintenance of endurance performance following a period of intensified training.

6) The impact of increased dietary protein intake on endurance performance may be associated with an improved psychological status during intensified training.

Future research questions

This thesis examined the role of protein feeding on recovery from, i) an acute bout of resistance exercise and ii) an intense period of endurance training. **Chapter 2** provides a further contribution to our current understanding of the response of MPS to resistance exercise and nutrition. **Chapters 3-5** add to promising recent evidence that protein feeding benefits endurance athletes who commonly undertake intense period of training. However, several follow up questions should be addressed. These include:

1) What is the response of MPS to protein ingestion following exercise preceded by a meal?

2) What are the roles of alternative anabolic signalling pathways, such as MAPK in regulating the response of MPS to exercise and nutrition?

3) Is MPS upregulated in the non-exercised leg following a unilateral bout of resistance exercise?

4) Which specific tissues are highly cytotoxic effector CD8+TL and NK cells destined for following exercise in humans?

5) Does the blunted trafficking pattern of antiviral lymphocytes following intensified training translate into an increased incidence of clinically confirmed upper respiratory tract infections?

6) What is the impact of a lower, more practical dietary protein intake that can be better tolerated on the maintenance of endurance performance?

7) What is the impact of supplementing the habitual diet with CHO + protein vs. CHO alone during an intensified period of training on the maintenance of muscle glycogen stores and exercise performance?

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Appendices

Appendix A

The University of Birmingham

School of Sport and Exercise Sciences

General Health Questionnaire

Name: .....

Address: .....

.....

.....

Phone: .....

Name of the responsible investigator for the study:

.....

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

1.	You are.....	Male	Female
2.	What is your exact date of birth? Day..... Month.....Year..19..... So your age is..... Years		
3.	When did you last see your doctor? In the: Last week..... Last month..... Last six months..... Year..... More than a year.....		
4.	Are you currently taking any medication?	YES	NO
5.	Has your doctor ever advised you not to take vigorous exercise?	YES	NO
6.	Has your doctor ever said you have “heart trouble”?	YES	NO
7.	Has your doctor ever said you have high blood pressure?	YES	NO

8.	Have you ever taken medication for blood pressure or your heart?	YES	NO
9.	Do you feel pain in your chest when you undertake physical activity?	YES	NO
10.	In the last month have you had pains in your chest when not doing any physical activity?	YES	NO
11.	Has your doctor (or anyone else) said that you have a raised blood cholesterol?	YES	NO
12.	Have you had a cold or feverish illness in the last month?	YES	NO
13.	Do you ever lose balance because of dizziness, or do you ever lose consciousness?	YES	NO
14.	a) Do you suffer from back pain b) if so, does it ever prevent you from exercising?	YES YES	NO NO
15.	Do you suffer from asthma?	YES	NO
16.	Do you have any joint or bone problems which may be made worse by exercise?	YES	NO
17.	Has your doctor ever said you have diabetes?	YES	NO
18.	Have you ever had viral hepatitis?	YES	NO
19.	If you are female, to your knowledge, are you pregnant?	YES	NO
20.	Do you know of any reason, not mentioned above, why you should not exercise?	YES	NO
21.	Are you accustomed to vigorous exercise (an hour or so a week)?	YES	NO

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

Signed: .....

Date: .....

Appendix B

Human Performance Laboratory

Participant Information Sheet

THE UNIVERSITY OF BIRMINGHAM

Study title: Intake of protein hydrolysate during intensified training in athletes.'

Location: Human Performance Laboratory, University of Birmingham

Investigators: Professor Asker Jeukendrup

Mr Oliver Witard

Supervisor: Dr Kevin Tipton

Background:

The elite level endurance athlete completes many long hours of training in a given week, often at a very high intensity, with little time for recovery. If intensity and volume of training become too great over an extended period of time, short-term declines in performance may result. Recovery may take from several days to several weeks. This phenomenon has been termed 'overreaching.' If overreaching is not quickly recognised and recovery is insufficient, the athlete may experience long term overtraining. Overtraining has longer-term ill effects on performance and, as such is far more detrimental to an athlete.

Overreaching and overtraining result in physiological and biochemical changes in addition to some psychological changes. It has been shown on a number of occasions that correct nutrition can help combat the effects of overreaching, in particular increased intake of carbohydrate. However, the impact of post exercise protein ingestion on symptoms resulting from a greatly intensified period of exercise is unknown.

Study Design:

If you agree to take part in this study, you will be required to complete two weeks of low to moderate intensity training, separated by one week of high intensity training on two separate occasions.

All laboratory tests will be performed in the laboratory of the School of Sport and Exercise Sciences, University of Birmingham under the supervision of study personnel. In addition, dietary control is extremely important for this study. All food will be provided for you for the entire three-week study period on two separate occasions. You must consume all food provided and not consume anything, including alcohol, other than the food provided for you.

The quality of the data collected and the success of this research project will depend on you adhering to all the training, testing and dietary instructions. Therefore, we will provide you with an instruction booklet that will spell out everything that you will need to do to help us carry out this important research project.

Procedures:

Details of the three week intensified training schedule are presented to you diagrammatically below.

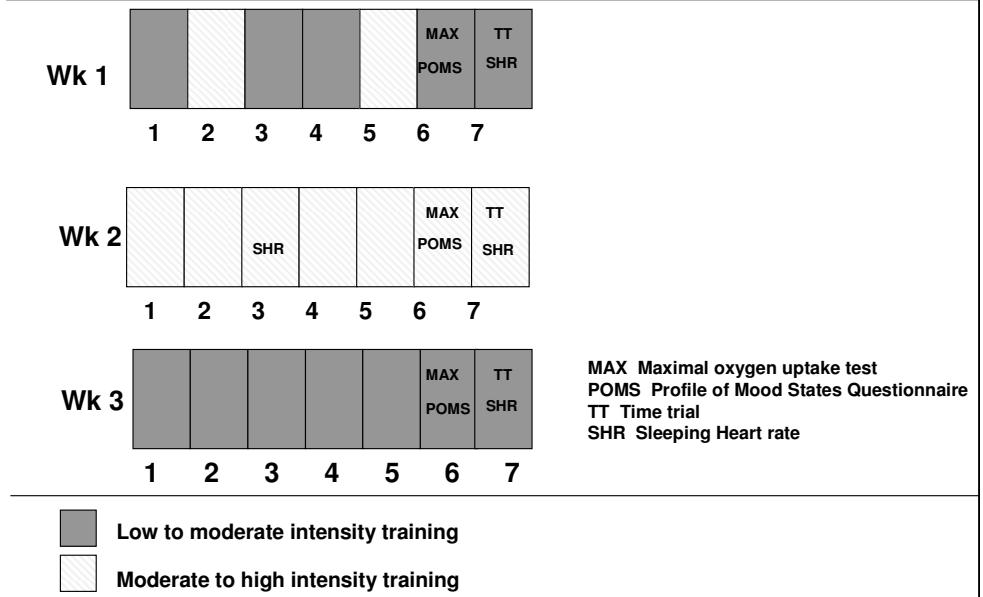

Diagram of the three weeks of intensified training schedule with the

Prior to the start of the experimental periods, you will complete two preliminary testing sessions consisting of a time trial and a VO_2 max test. The VO_2 max test will be completed on a cycle ergometer in the Human Performance Laboratory. You will be weighed prior to commencement of the test and fitted with a heart rate monitor. The test will be comprised of an initial three minute period at 95 watts, with 35 Watt increases every three minutes until you have reached volitional fatigue. Throughout the test, you will breathe into a mouthpiece, enabling us to measure oxygen uptake and CO_2 release from the body.

Blood samples will be taken in order to measure your lactate threshold and other metabolic parameters. We will take up to 12 blood samples during each VO_2max test (one at rest, one on completion of the max test and approximately eight samples during the incremental test) from each participant. The volume of blood taken will be 1.5 mL). In addition, blood (10 ml) will be drawn both pre and post each TT for measurement of immune parameters. The total amount of blood drawn will be 304 mL. This amount of blood will be taken over no less than 8 wks, so the total is well below the maximum amount of blood that can safely be taken (480 mLs/ 8 wks), nor is it enough to influence performance or add duress to you as a volunteer.

Finally, you will have two cannulations per week for a total of 16 throughout the study period (at least 8 wks). On completion of these tests, providing you show no signs of overreaching, the trials will begin.

Weeks one and three: In the first and last week of each three week block of testing, you will complete a week of low-moderate intensity training, representative of your normal amount and type of training. Rest days will be taken at your discretion. On days 6 and 7, you will complete a max test and time trial, respectively. Each time trial involves a steady state cruise for 2 hours at 50% Wmax followed by a race against the clock in which the challenge is to complete a target amount of work as fast as possible. The third week will be the recovery period. Intensity of training will be controlled by means of pre-determined calculated training zones.

Week two (high intensity): The second week will consist of 7 days of moderate to high intensity training. Duration of time spent training will be twice that of week one. Again, on days 6 and 7, you will complete a max test and time trial respectively.

You will be provided with detailed instructions on your training during each week. On four days of each week (including the two testing days), you will complete all training in the laboratory of the School of Sport and Exercise Sciences. On the other three days, you will complete training on your own. All training will be recorded. You will be provided with a heart rate monitor and a power crank to record your training.

Other measurements

On each day during all three weeks of the study, you will complete two questionnaires to assess your mood state and determine any symptoms of stress. In order to maintain energy balance, you will report to the researchers on each morning of the 21 days to be weighed and to receive your food allowance for the following 24 hours, which will be prepared to match your dietary requirements.

Study logistics

You will complete two three week testing periods in random order, which will be scheduled at least 14 days apart. The testing periods will be identical except for the amount of protein consumed in your diet during the final two weeks of each period. In one testing period, you will consume an average level of dietary protein (1.5 g/kg BW/day), and in the other period, you will consume a high level of dietary protein (3.0 g/kg BW/day) during the final two weeks of each. The additional protein will be provided in the form of a protein hydrolysate supplement. A placebo containing a similar amount of carbohydrate will be provided in the normal protein diet period, so that you will receive the same amount of calories and carbohydrates in each testing period. In the normal protein period, you will be given extra fat to ensure that calorie intake is equal in both trials. You will be required to finish a prepared drink in the hour immediately after completion of exercise, which will contain either the additional protein, or a placebo.

Dietary and Activity controls

You will be asked to record your food intake for 3 days prior to testing. All food for two periods of 21 days will be provided throughout the testing. Guidelines on how to record food intake will be provided. You are to consume only the food provided for you, as well as any supplements provided, for both of the two three week testing periods.

Risks:

As a result of the intensified training, you may experience some of the following effects: decreased performance, work capacity and muscular strength, chronic fatigue, prolonged recovery, loss of appetite, feelings of depression, apathy, difficulty in concentrating and irritability. Furthermore, you may become more susceptible to flu like symptoms and minor infection. If you do experience any of these symptoms, they will disappear on completion of the study. There are minor risks of drawing blood, including slight pain, bruising and infection. Trained personnel will draw the blood in order to minimize these risks.

Confidentiality:

All data obtained will be dealt with in a confidential manner. Your name will not be used in any publications resulting from this study, making it impossible to identify you in the report. No additional measurements to those described above will be made and all samples will be discarded after use.

Rights:

It is your choice whether or not you wish to take part in this study. If you wish to take part in this study, you will be given this information sheet to read and be asked to sign a consent form. You are reminded that if you decide to take part in the study, you are still free to withdraw from the study at anytime without provision of reason. Requests for a copy of the results attained will be honoured following study completion and publication in a peer-reviewed scientific journal.

Incentives:

You will be reimbursed for lost time, travel, parking, etc. in the amount of £1000 on completion of the study. In addition, if for any reason you decide to pull out of the study, you will be reimbursed for your time spent in the study based on a pre determined pro-rata scale of £8 or £10/h. However**,** you are reminded that this payment should not be used as a motivation to carry out the study. You will also receive food for each of two three week training periods, worth approximately £380.

Benefits:

The results of the VO_2 max test will give you an indication of your performance capacity and training status. You will also get information about sport nutrition in general and there is an opportunity to ask the experiment leaders anything about sport nutrition and training.

Contact:

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Appendix C

School of Sport and Exercise Sciences

Consent Form

Investigation: 'Can the intake of a protein hydrolysate during and after exercise reduce the symptoms of overtraining during intensified training in athletes.'

Investigators: Dr Kevin Tipton (Principal Investigator)
Professor Asker Jeukendrup (co-investigator)
Mr Oliver Witard, MSc.

Subject:

Name:

Address:

DOB:

I have read the attached Information sheet and discussed the investigation with..... who has explained the procedures to my satisfaction. I understand that participation in this protocol will involve a significant time commitment and I have considered the implications of participation. I am willing to undergo the investigation but understand that I am free to withdraw at any time without having to give an explanation and that doing so will not affect any treatment or care I may receive.

Signed

Witnessed

Date

Appendix D

Keeping a food diary

1. Please try and be as exact as possible, we have supplied scales to try and help you with this. The more information you provide the more accurate the feedback we give to you can be. It also ensures that we match your energy intake and expenditure when we provide your food.

2. Please do not alter your diet because you know someone is going to be looking at it. This needs to be a reflection of your normal diet.

3. Please keep your form with you all day, and write down everything you eat or drink as and when you eat and drink it. Do not rely on your memory at the end of the day. Record your eating as you go.

How to record your food and drink

1. Start a new sheet for each new day, putting the date and the day at the top of the page.

2. In the first column record the time that you are eating and/or drinking, please try and be as exact as possible so that this can be matched with your training log.

3. In the second column describe the food/drink you are consuming trying to give as much detail as possible, giving the brand name, the food and how it was cooked, for example:
    - One slice, wholemeal bread, Hovis, medium sliced, toasted
    - Asda, chicken breast, grilled
    - Walkers salt and vinegar crisps,
    - Robinsons no added sugar orange squash
    - McDonald's Big Mac sandwich

4. In the third column record how much of each food item you ate. Where possible try and weigh the food, making sure you indicate whether it was cooked or not when you weighed it. For solid foods, the food should be placed on the scale on a plate or container. The plate or container must be weighed empty first and the scales can then be zeroed. Each item of food can then be added to the plate and weighted individually, returning the scales to zero between each item.

5. If there is any food left over, try and estimate how much you did not eat and record it in column 4

6. Remember to record any extras, such as mayonnaise, gravy, sugar etc, any snacks/drinks you ate/drank throughout the day. If you ate in a restaurant, please indicate this as well as recording what you ate and drank.

   If you have any questions please ask the investigators.

Appendix E

Dietary Information/Questionnaire

Name: _____

It is important that we provide you with food sources that appeal to you. This questionnaire aims to gain an insight into the foods you like and dislike. Please tick the appropriate box for each item.

FOOD SOURCE	Dislike	Dislike but will eat if required	Don't mind it	like	Additional information (e.g. brand/type preference)
Breakfast items					
Bagels					
Chocolate spread					
Corn Flakes					
Coco Pops					
Milk (semi skimmed)					
Milk (whole)					
Museli (fruit)					
Oats					
Peanut butter					
Strawberry jam					
Weetabix					
Apple juice					
Orange juice					
Fruit yoghurt					
Plain yoghurt					
Lunch Items					
Babybel cheese					
Brown bread					
Butter (anchor)					

Caesar salad dressing					
Cucumber					
Honey roast ham					
Houmous					
Iceberg lettuce					
Margarine					
Mayonnaise					
Medium cheddar cheese					
Pickle					
Pork pie					
Prawn cocktail					
Salad cream					
Sausage roll					
Tuna					
Turkey breast					
White bread					
Dinner Items					
Avocado					
Baked beans					
Carrots (tinned)					
Chicken chow mein stir fry sauce					
Chicken curry with rice					
Chicken fillets					
Cod steaks					
Double cream					
Extra thick double cream					
Fruit cocktail (tinned)					
Honey and Mustard sauce					
Mixed vegetables					
Mushroom soup					
New potatoes					

Noodles					
Olive oil					
Parsley sauce					
Pasta (penne)					
Pasta sauce (tomato & herb)					
Peas					
Quiche Lorraine					
Spaghetti					
Stir fry vegetables					
Sweet and Sour chicken					
Sweet and sour stir fry sauce					
Sweetcorn					
Szeuchen tomato stir fry sauce					
Tinned pears					
Whole wheat pasta					
Snacks					
Apples					
Bananas					
Brazil nuts					
Cashew nuts					
Crisps					
Fruit gums					
Jelly Babies					
Lucozade					
Macadamia nuts					
Maltesers					
Mars bar					
Marshmallows					
Milkway bars					
Nutrigrain elevenses bars- choc chip					
Nutrigrain elevenses bars-raisin					

Olives					
Powerade					
Roasted almonds					
Smoothie- mango & passion fruit					
Smoothie- pineapple, banana & coconut					
Snickers bar					
Strawberries					
Walnuts					
Wine gums					

Additional information/ diet recommendations, (eg: allergies, additional foods that you really could not contemplate eating, additional foods/drinks you could not contemplate being without, ie: coffee). Please include Information detailing your favourite foods when training on the bike.

Appendix F

DALDA

Initials _____

Date _____

Please circle the correct response for this moment

a= worse then normal
b= normal
c= better than normal

Part A

1	a	b	c	Diet
2	a	b	c	Home-Life
3	a	b	c	School/College/Work
4	a	b	c	Friends
5	a	b	c	Sport Training
6	a	b	c	Climate
7	a	b	c	Sleep
8	a	b	c	Recreation
9	a	b	c	Health

Part B

1	a	b	c	Muscle Pains
2	a	b	c	Techniques
3	a	b	c	Tiredness
4	a	b	c	Need for a Rest
5	a	b	c	Supplementary Work
6	a	b	c	Boredom
7	a	b	c	Recovery Time
8	a	b	c	Irritability
9	a	b	c	Weight
10	a	b	c	Throat
11	a	b	c	Internal
12	a	b	c	Unexplained Aches
13	a	b	c	Technique Strength
14	a	b	c	Enough Sleep
15	a	b	c	Between Session Recovery
16	a	b	c	General Weakness
17	a	b	c	Interest
18	a	b	c	Arguments
19	a	b	c	Skin Rashes
20	a	b	c	Congestion
21	a	b	c	Training Effort
22	a	b	c	Temper
23	a	b	c	Swellings
24	a	b	c	Likeability
25	a	b	c	Running Nose

APPENDIX G **POMS-65** Initials: ____

Below is a list of words that describe feelings people have. Please read each one carefully. Then circle the number that best describes how you have been feeling during the past 10-14 days.

		Not at all	A little	Moderately	Quit a bit	Extremely
1	Friendly	0	1	2	3	4
2	Tense	0	1	2	3	4
3	Angry	0	1	2	3	4
4	Worn out	0	1	2	3	4
5	Unhappy	0	1	2	3	4
6	Clear-headed	0	1	2	3	4
7	Lively	0	1	2	3	4
8	Confused	0	1	2	3	4
9	Sorry for things done	0	1	2	3	4
10	Shaky	0	1	2	3	4
11	Listless	0	1	2	3	4
12	Peeved	0	1	2	3	4
13	Considerate	0	1	2	3	4
14	Sad	0	1	2	3	4
15	Active	0	1	2	3	4
16	On edge	0	1	2	3	4
17	Grouchy	0	1	2	3	4
18	Blue	0	1	2	3	4
19	Energetic	0	1	2	3	4
20	Panicky	0	1	2	3	4
21	Hopeless	0	1	2	3	4
22	Relaxed	0	1	2	3	4
23	Unworthy	0	1	2	3	4
24	Spiteful	0	1	2	3	4
25	Sympathetic	0	1	2	3	4
26	Uneasy	0	1	2	3	4
27	Restless	0	1	2	3	4
28	Unable to concentrate	0	1	2	3	4
29	Fatigued	0	1	2	3	4
30	Helpful	0	1	2	3	4
31	Annoyed	0	1	2	3	4
32	Discouraged	0	1	2	3	4
33	Resentful	0	1	2	3	4
34	Nervous	0	1	2	3	4
35	Lonely	0	1	2	3	4
36	Miserable	0	1	2	3	4
37	Muddled	0	1	2	3	4
38	Cheerful	0	1	2	3	4
39	Bitter	0	1	2	3	4
40	Exhausted	0	1	2	3	4
41	Anxious	0	1	2	3	4
42	Ready to fight	0	1	2	3	4
43	Good natured	0	1	2	3	4
44	Gloomy	0	1	2	3	4
45	Desperate	0	1	2	3	4
46	Sluggish	0	1	2	3	4
47	Rebellious	0	1	2	3	4
48	Helpless	0	1	2	3	4
49	Weary	0	1	2	3	4
50	Bewildered	0	1	2	3	4
51	Alert	0	1	2	3	4
52	Deceived	0	1	2	3	4
53	Furious	0	1	2	3	4
54	Efficient	0	1	2	3	4
55	Trusting	0	1	2	3	4
56	Full of pep	0	1	2	3	4
57	Bad-tempered	0	1	2	3	4
58	Worthless	0	1	2	3	4
59	Forgetful	0	1	2	3	4
60	Carefree	0	1	2	3	4
61	Terrified	0	1	2	3	4
62	Guilty	0	1	2	3	4
63	Vigorous	0	1	2	3	4
64	Uncertain about things	0	1	2	3	4
65	Bushed	0	1	2	3	4