The Effect of Endurance Exercise on Energy and Protein Balance

Anna K. Rolleston

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy, The University of Auckland, 2009

Abstract

Background

The energy cost of endurance exercise is significant and the ability to consume enough food to maintain energy balance is critical for success. In addition maintenance of body protein stores is also critical and there is a well known relationship between energy and protein balance. The establishment of any relationship between energy and protein balance and markers of overtraining in endurance athletes is also beneficial for endurance performance.

Purpose and Aims

The purpose of this thesis was to investigate the effects of endurance exercise on energy balance and protein balance. The aims were to establish accurate values for EI, EE and protein balance in two endurance sports, Road Cycling and Ironman. The rationale was to determine whether endurance athletes are able to maintain energy and protein balance over periods of increased EE and whether parameters of energy and protein balance related to markers of overtraining

Methods

Two research projects were conducted, the first at the Tour of Southland cycle event (5 high performance male cyclists) and the second at an Ironman training camp (7 male Ironman athletes). In both studies EE was determined by the DLW technique and EI by supervised weighed diet records. Protein balance was determined by an 18-hour primed continuous infusion of ^{13}C-leucine at the Tour of Southland and via the traditional 24-hour nitrogen balance technique at the Ironman camp. Both techniques were performed prior to and immediately after the given event. Nude body weight, blood overtraining parameters, urine samples and a mood questionnaire were collected in the mornings for analysis of daily parameters.

Results

The Tour of Southland: Subjects maintained energy balance with a mean EI of 27.3±3.8 MJ/day and a mean EE of 27.4 ± 2.0 MJ/day. There was no significant change in protein breakdown or parameters of overtraining. Ironman Training Camp: Athletes maintained energy balance with a mean EI of 20.27±2.91 MJ/day and a mean EE of 20.5±3.4 MJ/day and nitrogen balance was positive. There were indications of a relationship between energy and protein balance and markers of overtraining in both studies.

Conclusions

Endurance athletes are able to maintain energy balance when EE is high. When nutrients are sufficient to meet energy demands, protein stores are maintained.

Dedication

In memory of my grandparents

EDWARD RATIMA ROLLESTON

17 March 1912 – 23 August 1987

WILENA PARETIAKI ROLLESTON

18 June 1924 – 29 August 1993

ELIZABETH MARY HILDRETH

19 September 1920 – 28 October 2001

Acknowledgements

I would like to warmly thank the following people who made significant contributions to the research presented here:

Dr Lindsay Plank, Department of Surgery, University of Auckland, New Zealand

Dr Benjamin Miller, Department of Health and Exercise Science, Colorado State University, USA

Dr Nancy Rehrer, School of Physical Education, University of Otago, New Zealand

Ien Hellemens, Department of Human Nutrition, University of Otago, New Zealand

Dr Elaine Rush, School of Sport and Recreation, Auckland University of Technology, New Zealand

Kerry McIllroy, Nutrition Services, Auckland and Starship Hospitals, New Zealand

Eduardo Ferriolli, School of Medicine of Ribeirao Preto, University of Sao Paulo, Brazil

I would also like to thank the following people for their input into my doctorate: The athletes who participated as subjects, Dr Allen Lim, Cheryl Murphy, Darren Ellis, Aimee Burns, Meredith Miller, Amy Taylor, Dr Norbert Baume, Rachael Gardiner, Dr Carmel Nottle, Yvonne Wood, Carl Bradford, Briar Rudsits

And to my family and friends; Thank-you for your love, support and encouragement throughout my journey.

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List of Abbreviations

%ΔPV	percent change in plasma volume	IRMS	isotope ratio mass spectrometry
αKIC	α-ketoisocaproate	kcal	kilocalorie
AA	amino acid	kJ	kilojoule
AGF	Atwater general factor	LBM	lean body mass
APE	atom percent excess	MJ	megajoule
AV balance	arteriovenous balance	MS	mass spectrometer
BCAA	branched chain amino acid	POMS	profile of mood state
BD	body density	R_a	rate of appearance
BF%	body fat percentage	R_d	rate of disappearance
CO_2	carbon dioxide	RMR	resting metabolic rate
DLW	doubly labelled water	RQ	respiratory quotient
DXA	dual energy x-ray absorptiometry	TBW	total body water
EE	energy expenditure	TDEE	total daily energy expenditure
EI	energy intake	TEF	thermic effect of food
FFM	fat free mass	ToS	Tour of Southland
FQ	food quotient	TUN	total urinary nitrogen
FSR	fractional synthesis rate	UUN	urine urea nitrogen
GC	gas chromatography	VO_2max	maximal oxygen consumption
Hct	haematocrit	VO_2peak	peak oxygen consumption
Hgb	haemoglobin	WBPT	whole body protein turnover

Chapter 1 Introduction

1.1 Background

All athletes, regardless of their sport, aim to improve their performance. Many professional and elite level athletes have access to the latest technology and information to help them in the pursuit of excellence and additionally many have highly qualified and experienced coaches, trainers and sport scientists working with them toward their goal. It has become more common in recent years for non-elite athletes to access leading edge technology and specialized knowledge in an effort to improve. In particular, athletes who participate in endurance sports seek the latest nutrition recommendations to ensure that they are adequately fuelled for their event. An optimal amount and quality of nutrients means an optimal ability to expend energy and therefore reach the finish line as quickly and as efficiently as possible. Road cycling and Ironman are examples of two endurance sports that have an extensive professional and elite level of competitors and a large contingent of recreational, non-elite participants all seeking 'the edge' and striving for optimal performance.

In road cycling, competitions can last for as little as 1 hour or can be as long as 100 hours in touring events like the Tour de France. Road cyclists have been studied extensively in laboratory based projects in an effort to determine the optimal physiological, metabolic, anthropometric and psychological factors that lead to success. In fact it has been suggested that physiological factors determined under laboratory conditions, when expressed relative to anthropometric data, can accurately predict cycling performance in the field (Padilla et al., 1996). This type of evidence is helpful for professional, elite and non-elite cyclists in determining their abilities and also in tailoring where their training needs to focus if a weakness

or deficiency is identified. Laboratory based tests used to determine physical characteristics of a rider can be collated to give a picture of the optimal characteristics needed to be a top level cyclist. **Table 1.1** shows that male professional endurance cyclists have a wide range of characteristics due to the fact that road cyclists can be specialist time trialers, climbers, flat terrain riders or all terrain riders. However, knowing these physical characteristics means very little to cycling performance if other practical factors related to success are not optimal (e.g. adequate energy intake (EI) for the energy expenditure (EE) that occurs).

Table 1.1: Characteristics of male professional road cyclists

	Mean	Range	
		Min	Max
Age (yr)	26	20	33
Height (cm)	180	160	190
Body weight (kg)	69	53	80
Body surface area (m^2)	1.87	1.54	2.08
Frontal area (m^2)	0.35	0.28	0.38
PO_{max} (W)	439	349	525
PO_{max} (W/kg)	6.4	5.7	6.8
VO_{2max} (L/min)	5.4	4.4	6.4
VO_{2max} (ml/kg/min)	78.8	69.7	84.8
HR_{max} (bpm)	194	187	204
$[La]_{peak}$ (mmol/L)	9.9	6.9	13.7
PO_{LT} (W)	334	202	417
VO_{2LT} (% VO_{2max})	77	74	83
HR_{LT} (bpm)	163	146	174
PO_{OBLA} (W)	386	275	478
VO_{2OBLA} (%VO_{2max})	86	81	91
HR_{OBLA} (bpm)	178	168	191

Table modified from (Mujika and Padilla, 2001). PO = power output, VO_{2max} = maximal oxygen uptake, HR_{max} = maximal heart rate, $[La]_{peak}$ = peak blood lactate concentration, $_{LT}$ = lactate threshold, $_{OBLA}$ = onset of blood lactate accumulation.

Laboratory based assessments of an athlete are limited by the controlled environment and the lack of free-living conditions. Practical recommendations for cyclists are largely based on laboratory findings but the ability to monitor riders during an actual event gives worthwhile and 'real' information about what is required to be successful. Nevertheless, performing

measurements on athletes who are training or competing in an event can be difficult because of the potential interference that the measurement techniques might have on performance and success. There have been many laboratory based studies performed on road cyclists that have provided vital information for training and competition, but when athletes are constantly looking for the best way to improve, field based research is needed to consolidate and validate laboratory findings.

The Ironman is a relatively young sport and there is little in the way of research of any description with Ironman competitors especially in comparison to studies performed in cycling, running and swimming. The Ironman triathlon is a grueling multi-discipline event in which competitors swim (3.8km), cycle (190km) and run (42.2km) in a consecutive fashion with no rest between disciplines. The inaugural running of the event in Hawaii in 1978 saw 15 male competitors start the race of which 12 completed and the winning time was 11hr 46min. Today the Ironman format remains unchanged, and the Hawaiian Ironman is still regarded as the most prestigious triathlon event to win in the world, deemed to be the World Championship. To be successful in an Ironman, competitors must be able to sustain a high level of EE for an extended length of time and also maintain an energy supply that meets the demands of the exercise.

The main cause of fatigue in triathlon events, especially the ultra-distance events like the Ironman, is energy depletion and dehydration and these factors also apply when training (O'Toole and Douglas, 1995). Ironman competitors train 6-7 days each week with more than one training session scheduled on most days. It is important that daily EI is adequate during training phases to ensure that adaptations occur in response to that training. There is a body of evidence that describes the carbohydrate (Hawley et al., 1997, Coyle et al., 2001), sodium (Rehrer, 2001, Laursen and Rhodes, 2001, Armstrong et al., 1993) and fluid requirements (Cheuvront et al.,

2003, Convertino et al., 1996, Rogers et al., 1997) for training and competition for triathletes but there is a lack of evidence to describe dietary protein requirements, protein kinetics in response to training and competition and also total EI and expenditure available to compete or train at an optimal level. Review articles that discuss dietary recommendations for triathletes use evidence from generic endurance exercise studies to produce recommendations when there are no specific findings in triathlon subjects. This method of providing recommendations has some validity but in one triathlon nutrition review it was noted that, with respect to protein, "there is some evidence that amino acid ingestion in combination with and without CHO [carbohydrate] may increase post-exercise protein synthesis and net muscle protein balance" (Jeukendrup et al., 2005). The referenced papers for this statement used resistance training as the exercise mode (Rasmussen et al., 2000, Tipton et al., 1999) and although these findings may well cross-over to endurance-based exercise it is not entirely satisfactory to relate them to Ironman or even shorter distance triathlons.

In cycling there are a number of methodologically sound studies in both laboratory settings and under free-living conditions which have improved the understanding of the effect of cycling exercise on the body. However, there is no evidence for the effect of extremely high EE on protein kinetics. With respect to Ironman there is a general lack of evidence all round for the effect of training and competing in such an event on physiological, metabolic and other parameters. Both sports are likely different in their energy requirements but are similar in their endurance based nature and the significant volume of training required to compete consistently at even an age group level.

1.2 Energy intake and expenditure

Information about the EE required for an endurance event and consequently how much food is necessary to sustain that EE, is vital for performance. If food intake is less than EE the athlete will be in energy deficit and will not have adequate substrates available to participate at an optimal level. Accurate determination of EE in the field requires the use of the doubly labeled water (DLW) technique which is the current gold standard. A landmark field based study at the Tour de France used multiple doses of DLW to estimate EE (Westerterp et al., 1986). It was found that riders expended between 29 MJ/day and 35 MJ/day over the 3-weeks of the tour. However, the use of multiple doses of DLW and the length of the study (3 weeks) may have introduced some error. In a follow-up study, Saris et. al. (1989) estimated that EE was approximately 25 MJ/day but DLW was not used and instead EE was estimated from an assumed resting metabolic rate, assumed non-racing EE, and a calculation of EE while racing from speed, time, and altitude change (Saris et al., 1989). Regardless, both studies found that the energy requirements of the Tour de France are exceptional and therefore maintaining energy balance can be a challenge.

There is little evidence which describes the EE of triathletes. Kimber et. al. (2002) describe EE during an actual Ironman event. They reported that mean EE was 42 MJ ± 10 MJ which seems excessive but the method used to collect the data was less than rigorous with EE predicted from heart rate readings throughout the event (Kimber et al., 2002). The findings by Kimber and colleagues illustrate a significant energy cost of participation in an Ironman but more accurate findings are needed and further research into the energy cost of training is also required to provide an understanding of energy balance for these athletes.

An inability to maintain adequate EI, especially in cycling tour events which require repeated bouts of exercise over a number of days, means that an athlete will be unable to restore depleted substrates like muscle glycogen (Burke et al., 2001). They may also be unable to maintain nitrogen stores and therefore protein mass may decline (Butterfield and Calloway, 1983, Todd et al., 1984). It may be that there is a limit to an athlete's ability to expend energy and that the limit is set by how much food they are able to consume. There is evidence that a decrease in EI can result in a partially compensatory reduction in physical effort (Gorsky and Calloway, 1983). Some researchers also suggest that it may be difficult to ingest and digest enough food to maintain energy balance when prolonged exercise leads to EE in excess of 20 MJ/day (Brouns et al., 1989a, Brouns et al., 1989b).

Determining EI during field based studies then becomes an important consideration for researchers and accurate methods need to be utilised. Many studies in cyclists have used the relatively inaccurate method of food diaries to estimate EI when the weighed food record is the more exact method (Gibson, 2005). Saris et. al. (1989) estimated mean EI at the Tour de France to be 24.7 MJ/day with a range that had a maximum of 32.4 MJ/day (Saris et al., 1989). The use of simple diet records by these researchers means that the EI data is fairly inaccurate. However the findings of Saris et al relate well to those presented by Garcia-Roves et. al. (1998) who used the more accurate method of weighed diet records and found a mean EI of 23.5 MJ/day in Tour de France riders (Garcia-Roves et al., 1998). Knowing mean values for EI is helpful for cyclists as it gives them an estimate of how much food needs to be consumed each day to maintain energy balance. However, as **Table 1** shows, professional cyclists display a wide range of physiological parameters and as such probably also have a wide range of EI requirements. In addition, it is likely that findings in Tour de France riders are in excess to the requirements for

elite and sub-elite riders due to the difference in level of competition and research with elite, sub-elite and development level riders is warranted.

The day-to-day EI patterns of triathletes are not well documented but as with other endurance sports it may be difficult for a triathlete to maintain energy balance over a training week, especially in high volume phases when EE increases. Elite Australian male triathletes were found to consume only 17 MJ/day with a weekly training regime that involved 13km of swimming, 323km of cycling and 75km of running (Burke and Read, 1987). These findings compare well to a subsequent study by Burke et al (1991) who reported a mean EI of 248 kJ/kg/day (or approximately 17 MJ for a 70kg athlete) for triathletes training on average 19 hours per week. In addition, male triathletes participating in the Hawaii Ironman consumed a mere 15 MJ/day during the week prior to the Ironman event (Khoo et al., 1987) and although there is no EE data from either of these studies the intake values do seem very low in comparison to those reported in cycling (Westerterp et al., 1986, Saris et al., 1989) and is considerably less than the 42 MJ of EE estimated by Kimber and colleagues (Kimber et al., 2002). Again, further research into the level of EE and subsequent patterns of EI during triathlon training and competition is warranted to formulate definitive nutrition recommendations.

1.3 Protein kinetics

In addition to absolute EI, the macronutrient make-up of an athlete's diet is also very important. Managing EE with adequate EI is important for replenishment of carbohydrate stores (Burke et al., 2004) and to maintain nitrogen balance (Butterfield and Calloway, 1983, Todd et al., 1984), especially during the course of repeated activity. Indeed, it has been demonstrated that even short-term deficiencies in EI during a period of intense training can lead to symptoms of "overreaching" (Halson et al., 2004). Therefore, it is important to quantify the energy requirements of prolonged, repeated, endurance exercise and determine whether athletes normally consume an adequate amount of macronutrients to meet these needs.

It is known that protein requirements are dependent on energy balance, but it is yet to be established whether a very high EE, independent of energy balance, changes protein kinetics. A substantial understanding of protein kinetics in relation to resistance exercise exists in the literature. The evidence indicates that resistance exercise increases rates of protein metabolism when a person consumes sufficient calories in their diet (Phillips et al., 1997, Tipton et al., 1999, Balagopal et al., 2001), but the effect of endurance exercise on whole body protein turnover is not as clearly understood. The relative contribution of protein metabolism to energy provision is small despite changes in protein synthesis and breakdown, commonly termed turnover, that occur during an acute bout of endurance exercise (Tarnopolsky, 2004, Rennie et al., 2006). However, over the course of a multiple day event, requiring a large daily EE, it is possible that the relatively small contribution of protein to energy metabolism could result in a meaningful absolute contribution of protein to energy metabolism or structural repair. Several studies have reported increases in whole body protein degradation during exercise (Rennie et al., 1981, Wolfe et al., 1982, Wolfe et al., 1984) while others have failed to observe any change (Carraro et al.,

1990a, Stein et al., 1989). A concrete understanding of whole body protein turnover in endurance athletes has been hampered by methods that, although sound, lack free-living considerations. For example many studies have been performed after an overnight fast, which may not be able to be extrapolated to an actual 24-hr period of training or competition because athletes ingest large amounts of carbohydrate before, during and after exercise.

Human protein requirements have been determined from nitrogen balance studies. The rationale is that nitrogen is a major constituent of protein and therefore measurement of nitrogenous metabolites in the urine, and other wastes, reflects protein turnover in the body. The calculation of nitrogen balance (the amount of nitrogen consumed minus the amount of nitrogen excreted) is useful as an index of nutritional status. Maintenance of protein stores in the body requires a balance between the nitrogen that is consumed, with the nitrogen that is lost through tissue turnover and exchange between tissues. It is acknowledged that nitrogen balance studies may underestimate the protein and amino acid requirements of a population (Young et al., 1989, Fuller and Garlick, 1994, Zello et al., 1995) but it remains a valid measure of protein use in the body.

The use of stable isotopes to determine protein turnover is becoming more common in metabolic research. Accurate determination of protein kinetics by the stable isotope method is a sophisticated and expensive technique. In a laboratory setting muscle biopsy protocols are useful and accurate in determining mixed muscle protein kinetics in response to acute bouts of exercise or training. Their use in field studies is however limited, if not impossible, because of the invasive nature of the procedure. Whole body protein turnover methodologies that do not use muscle biopsies suit the field environment better but are still relatively invasive and also costly to run. Research that focuses on the response of protein turnover to repeated bouts of endurance

exercise is necessary to gain a clear understanding of what an athlete's protein intake requirements really are, and what effect high energy endurance exercise has on protein kinetics.

Cyclists tend to have protein consumption levels well in excess of the recommended protein intake for endurance athletes which currently stand at between 1.2g protein/kg and 1.7g protein/kg (Tarnopolsky, 2004). However, it is likely that the absolute increase in protein intake that occurs when cyclists compete in a multi-day event relates to the overall increase in total EI that occurs. The evidence suggests that the relative contribution of protein to total energy is generally in the vicinity of 14% to 18% for cyclists regardless of the total EI (Saris et al., 1989, Westerterp et al., 1986). Reports regarding protein intake by ultra endurance triathletes have determined that the level of intake is equivalent to 1.5 - 2.0g protein/kg/day (Burke and Read, 1987, Khoo et al., 1987) which is in line with the recommendations for endurance athletes. However, there is no specific research that has determined protein or nitrogen balance in Ironman athletes. Knowing how protein synthesis and breakdown respond to increased EE will mean that athletes have a better understanding about whether the amount of protein that they are consuming is sufficient for the protein metabolism that occurs. Additionally, concurrent measurement of energy balance and protein or nitrogen balance could provide a clear picture of how one affects the other and whether increased energy demands alter protein kinetics.

1.3 Over-reaching and Overtraining

Training for an endurance event requires many hours of disciplined exertion. Adequate rest and recovery is required to allow for the physiological adaptations to occur in response to that training that will ultimately lead to improved performance. The principle of overload states that the body will not adapt unless it experiences more stress than that to which it is accustomed (Hellebrandt and Houtz, 1956). However, the training stimulus, while inducing overload, must not overstress the athlete and cause performance decrements. High volume and/or high intensity training without adequate recovery can limit an athlete's ability to perform at an optimal level (Fry et al., 1991). The overtraining syndrome results in stagnation or decrement in athletic performance following a state of prolonged fatigue and requires significant rest for recovery (Fry et al., 1991). A range of physiological measures have been investigated as possible markers of the overtraining syndrome and although training stress and altered homeostasis are accepted as being precipitating factors, the underlying mechanism is still unknown. A number of hypotheses have been proposed and adequate nutrition is one that has been investigated. The effects of 10 days of increased training volume on performance and muscle glycogen levels showed that athletes who did not respond positively to the increased training consumed about 1,000 kcal per day less than their estimated energy requirement (Costill et al., 1988). There is currently no research that establishes a relationship between energy, protein and/or nitrogen balance and markers of overtraining and therefore some investigation into the effect of high EE on the risk and/or development of overtraining is required.

Regardless of the mechanism, an alteration in mood and elevated plasma cortisol concentrations are two markers widely accepted as reliable in identifying over-reaching and subsequent overtraining. These markers, when found in conjunction with the classical symptoms

of profound fatigue, history of frequent illness and deterioration in performance have been used in a number of overtraining studies (O'Connor et al., 1989, Hooper et al., 1995, Hooper et al., 1993, Morgan et al., 1987a). Decreased serum ferritin has been indicated as a possible marker of over-reaching and overtraining but is not widely accepted as a true indicator. However, decreased ferritin and iron levels are of consequence in fatigue because low levels will alter oxygen carrying capacity and subsequently have an effect on performance. The rationale for the monitoring of ferritin comes from an investigation that demonstrated that a significant reduction in serum ferritin concentration was associated with a significant decline in performance in response to a specific overload training protocol (Fry et al., 1992). In addition, another study found that maintenance of low serum ferritin resulted in no improvement in VO_2max performance in ferritin depleted male and female athletes compared to ferritin depleted athletes who received iron supplementation (Freidmann et al., 2001).

Overtraining is a complex issue that plagues endurance athletes and there are no certain markers of the syndrome. Prevention involves minimising factors that are known to cause fatigue and reduce performance. High EE, inadequate EI and subsequent negative energy balance have not been implicated in overtraining and nor has negative nitrogen balance or altered protein kinetics. However, these factors do have a detrimental effect on performance and the relationship between them and overtraining deserves consideration.

1.4 Statement of the Problem

The overall goal of this thesis was to collect and interpret energy, protein and nitrogen balance evidence in free-living endurance athletes with a view to building a better understanding of the energy and protein requirements of ultra-endurance sport. In addition the goal was to determine whether energy balance and protein balance parameters have any relationship to factors related to over-reaching and the risk of overtraining. This relationship was developed in an attempt to provide recommendations that not only ensure optimal performance but which help in the prevention of under-performance that occurs in an overtrained athlete. The specific aims of the thesis were to determine:

1. whether high energy expenditure, independent of energy balance, affects whole body protein turnover during a 6-day cycling tour
2. whether high energy expenditure, independent of energy balance, affects nitrogen balance during a 6-day Ironman camp
3. whether athletes are able to maintain energy balance over a period of increased energy expenditure
4. energy requirements of a 6-day cycling tour and a 6-day Ironman camp and exactly what athletes eat during this time, and
5. whether energy balance, protein turnover or nitrogen balance relate to measured parameters of fatigue and overreaching.

The following experimental hypotheses were tested:

1. High energy expenditure during a 6-day cycling tour event will not have any effect on whole body protein turnover
2. High energy expenditure during a 6-day Ironman training camp will not have any effect on nitrogen balance
3. Athletes will be able to maintain energy balance during their 6-day event
4. Energy requirements will be in excess of 20MJ/day and both cyclists and Ironman athletes will consume protein in excess of the recommendations for endurance athletes
5. Negative energy, nitrogen and protein balance will be associated with increased presence of overtraining markers

1.5 Thesis Overview

A comprehensive review of literature (Chapter 2) begins the body of this thesis and commences by explaining the measurement techniques used in the research component to determine energy expenditure, energy intake and protein balance. The literature review also examines the energy expenditure and energy intake evidence in endurance athletes in an effort to understand the energy demands placed on endurance competitors and their ability to accommodate those demands. The endurance-based protein turnover research is sparse and therefore evidence from protein studies in resistance trained individuals is canvassed to illustrate the affect of exercise on protein kinetics. Interactive relationships between energy, protein and exercise are also examined. The final part of the literature review is dedicated to overtraining and determining any relationship between markers of overtraining and parameters of energy and protein balance.

Chapter 3 and 4 are the research components of the thesis. Chapter 3 describes a study performed at the 6-day Tour of Southland cycle race that aimed to determine the effect of a cycling tour event on protein turnover and energy balance. Chapter 4 describes a study that used slightly different methods to answer the same aim in athletes participating in a 6-day Ironman training camp. Both chapters include a full methodological description of procedures, comprehensive results and discussion with conclusions and recommendations to close.

Chapter 5 completes the body of the thesis and presents an overall discussion, conclusions and recommendations for future research.

Chapter 2 Review of Literature

2.1 Introduction

The purpose of this thesis is to investigate the effects of endurance exercise on energy balance and protein balance. Energy balance is the difference between energy intake (EI), in the form of dietary nutrients, and energy expenditure (EE). Protein balance is the difference between dietary protein intake and the appearance of protein metabolites in the body. The research aims to establish values for EI and EE in two endurance sports, Road Cycling and Ironman. The rationale for doing so is to determine whether endurance athletes are able to maintain energy balance over periods of increased EE. In addition, this thesis examines the relationship between energy and protein in the body, asking the questions; what effect does high EE have on protein metabolism; what effect does energy balance have on protein balance and conversely what effect does protein balance have on energy balance?

To determine these things, sophisticated, reliable and valid techniques have been utilised to produce meaningful findings that are valid both from an academic viewpoint and importantly, in a practical sense, for athletes, their coaches, trainers and advisors. Specifically, the following methodological approaches were used:

- Stable isotopes to determine EE, via the doubly labeled water technique, and whole body protein turnover using a primed continuous infusion of ^{13}C-leucine;
- Traditional nitrogen balance method, ascertaining nitrogen intake and excretion and subsequent protein balance;
- Supervised, microgram accurate, weighed diet records

Additionally, the purpose of this thesis is also to establish the presence or absence of a relationship between energy and protein balance and markers of overtraining in athletes. The inclusion of overtraining as a thread throughout the research is in an effort to identify if energy and/or protein kinetics play any part in the decline in performance that is seen in athletes progressing into an overtrained state. It does not attempt to explain the complexities of the overtraining syndrome.

The literature review that follows describes in detail the major methodological techniques and the rationale for their use. It also examines the energy and protein balance research to discuss the effects of exercise on energy and protein kinetics with particular reference to endurance exercise. Furthermore, the literature review presents evidence that links endurance sport and the overtraining syndrome.

2.2 The Use of Stable Isotopes in Metabolic Research

An isotope is a variation of an element. It has the same number of protons but a differing number of neutrons which gives it a different atomic mass (**Figure 2.1**). The biological environment is comprised of the common stable isotopes ^{1}H, ^{12}C, ^{14}N and ^{16}O but there are rarer, uncommon types of each element that also occur in a stable form (^{2}H, ^{13}C, ^{15}N, ^{18}O) at between 0.02 - 1.1% in nature (Rennie, 1999). Radioactive analogues (^{3}H, $^{11,14,15}C$, $^{13,16}N$, $^{11,15}O$) also exist but are even rarer than the uncommon stable isotopes. Radioactive isotopes differ from their stable counterparts in that their masses are not constant but change with radioactive decay that results in the emission of sub-atomic particles. An increased awareness of the health hazards associated with radioactive emissions has led to the predominant use of stable isotopes in human metabolic research.

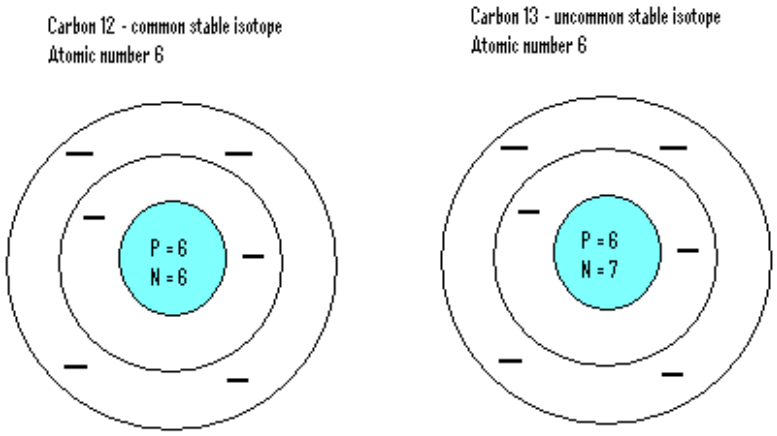


Figure 2.1: Carbon Isotopes. The common stable isotope of carbon (^{12}C) has an atomic number of 6, 6 protons and 6 neutrons. The uncommon stable isotope of carbon (^{13}C) has an atomic number of 6, 6 protons but 7 neutrons. ^{13}C is often called a 'heavy' isotope of carbon.

When incorporated into a substrate, an isotope can be used as a 'tracer', in that it follows the fate of that substrate (tracee) in the body. For example, ^{13}C-leucine (an amino acid with one carbon 12 replaced with a carbon 13) can be used to trace the metabolic fate of the more common ^{12}C-leucine in the body. Stable isotopes have been used in metabolic research since the 1930's when Schoenheimer and colleagues used deuterium (^{2}H) to study the metabolic fate of fat in mice (Schoenheimer R and D, 1935) and ^{15}N-glycine to assess the energetics of the body's protein pools (Schoenheimer R et al., 1939).

The risks associated with the use of stable isotopes are negligible when given in trace amounts making them ideal for research in humans. A study by Gregg et. al. (1973) showed no perceptible effect on mice that were fed ^{13}C enriched food pellets over several months which resulted in 60% of the animal's body carbon being replaced with ^{13}C (Gregg et al., 1973). Uphaus et. al. (1967) suggest that excess amounts of ^{13}C, ^{18}O and ^{15}N can affect some cellular

functions however the enrichment levels used would never be attained in human in-vivo research (Uphaus et al., 1967). There have been no reports of harm published in relation to the use of stable isotopes in human metabolic research.

2.2.1 Analysis of Stable Isotopes in Biological Samples

Biological studies using stable isotopes require a method of detecting the isotope within the biological sample. Stable isotopes are detected on the basis of their molecular mass by means of a mass spectrometer (MS). Ions are introduced into the MS and sorted according to their mass-to-charge ratio (m/z). An ion detector converts their rate of flow in to an electrical signal that is transformed into a mass spectrum. The relative abundance of an ion can then be determined from the spectrum (**Figure 2.2**).

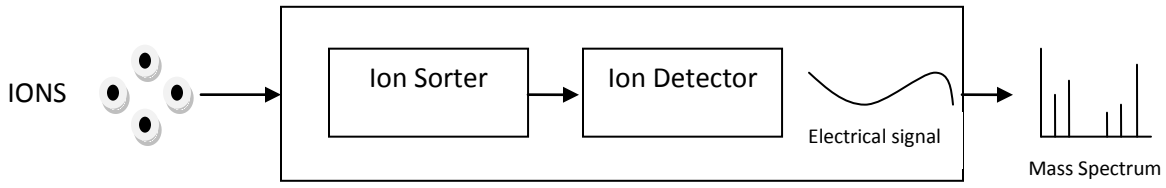


Figure 2.2: Schematic diagram of a mass spectrometer. Ions are sorted, detected and then converted into an electrical signal. The end result is a mass spectrum that is used to determine the abundance of various ions.

The gas chromatograph (GC), another instrument for separating compounds and determining isotopic abundance or enrichment, was proposed in the 1940's (Martin and Synge, 1941) but it was not until many years later that it became the predominant apparatus for the separating of compounds in organic mixtures (Cirillo, 1973). The GC separates chemicals based on their volatility. A small amount of a liquid mixture is injected into the GC through a port that is heated to very high temperatures (approximately 300ºC). The liquid becomes a gas that is then

carried by an inert gas through the apparatus. The GC has a specialised oven and within the oven is a column, comprised of a thin tube with an inner polymer coating. Chemicals travel through the column at different speeds and separate depending on their volatility. Highly volatile substances travel more quickly than their less volatile counterparts and a detector device identifies chemicals as they elute. The identified substances are then represented on a chromatogram (Meier-Augenstein, 1999a, Matthews and Bier, 1983) (**Figure 2.3**).

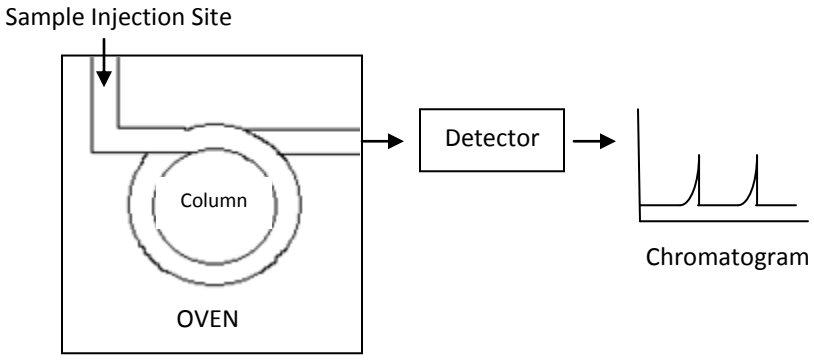


Figure 2.3: Schematic diagram of a gas chromatograph. A sample liquid mixture is injected at high temperature rendering the mixture a gas. The gas sample is carried through the column and ions elute depending on their volatility. Eluted ions are identified by a detector and are then represented on a chromatogram.

Soon after its introduction it was found that the GC on its own did not provide sufficient specific identification of complex organic mixtures, and the use of a manual transfer of the GC eluates to the MS began (Gates and Sweeley, 1978). Coupling of the GC to the MS was perfected in the 1960's (Matthews and Bier, 1983) and in doing so provided an instrument that allowed analysis of a wide range of organic substances.

Samples to be analysed by GC-MS need to be derivatised, making them volatile and more easily able to convert into a gas that can pass through the GC (Rennie, 1999). The derivatisation of molecules is carefully considered, as a wide range of derivatives can be prepared and the derivative chosen can affect the selectivity of the GC-MS assay (Matthews and

Bier, 1983). Tracer-to-tracee ratios greater than 0.5 atom % excess (APE) are required for accurate and reliable enrichment measurements with the GC-MS and is the most common enrichment in metabolic studies that use plasma as the sampling pool (Patterson et al., 1997). The reliability of the GC-MS for determination of enrichment in tissue samples is however, questionable because tissue enrichment is between 0.001 APE and 0.05APE (Wolfe and Chinks, 1992) and thus below the reliable range.

Isotope ratio mass spectrometry (IRMS) accurately measures tracer-to-tracee ratios in the order of 0.001APE to 0.1APE but requires a larger sample (>10nmol compared to <1nmol for GC-MS) and can only analyse substances in purified gases (e.g. CO_2 and N_2) (Patterson et al., 1997). In comparison to GC-MS which gives structural information by scanning a range of masses for characteristic ions, IRMS gives quantitative information on a given compound (Meier-Augenstein, 1999b). The standard IRMS has a duel inlet gas system which allows the measurement of the isotope ratio of the sample gas against the isotope ratio of a reference gas with a known isotopic content (Mook and Grootes, 1973). The sample and reference gases are introduced into two separate 'bellows' and a constrictor valve is used to switch between each, in an effort to maintain continuous gas flow (Meier-Augenstein, 1999a). Gas flows through the ion source where it is ionised, accelerated and directed at a collector magnet which deflects the ions depending on their mass (Capriole, 1972). The resulting ion currents are analysed and isotopomer peaks provide information for the calculation of isotope ratios (**Figure 2.4**).

In protein and amino acid tracer studies, enrichment can be determined from within the muscle, in plasma and/or in breath samples depending on the aims of the study. Biological studies with protein and energy most commonly utilise IRMS to find skeletal muscle intracellular enrichments (Wolfe and Chinks, 1992) and the presence of infused label in urine

and CO_2 from breath samples (Halliday and Read, 1981). The GC-MS is used mostly to determine plasma isotope enrichments.

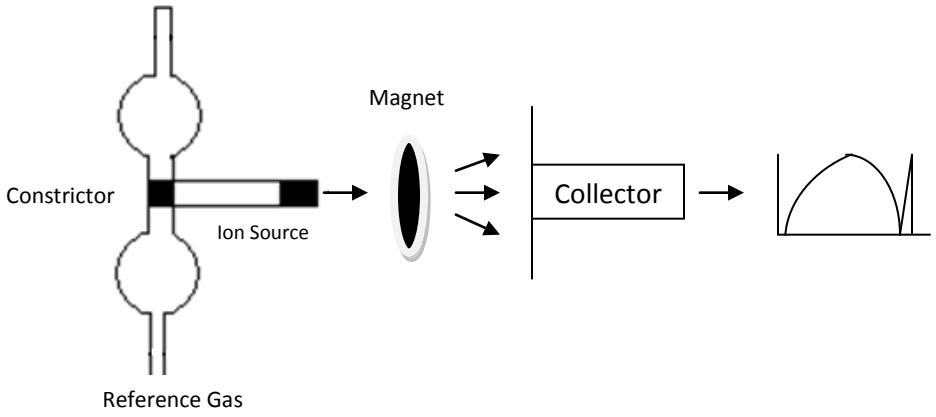


Figure 2.4: Schematic diagram of an isotope ratio mass spectrometer. The sample and reference gases are introduced and passed though the ion source where they are ionised, accelerated and collected by a magnet. The enrichment of the sample is determined against the known isotope value of the reference gas.

2.2.2 Isotopes and the Single Pool Model

The Law of Conservation of Mass states that matter can neither be created nor destroyed. With respect to a biological system, if a stable isotope tracer is added to the system then the total mass added (including that which is metabolised, exhaled and excreted) must remain constant. Therefore measurement of the tracer dilution in various pools within the system will enable measurement of the size of those pools. The dilution principle is therefore one model for the application of stable isotopes in metabolic research.

The single pool model of substrate kinetics is the most common approach for estimating substrate rate of appearance via the dilution principle. The model was first proposed by Steele et. al. in 1956 and assumes that substrates are confined to a single homogenous pool and that mixing

within the pool is rapid (Altszuler et al., 1956). Calculations of isotopic enrichment are used to determine the kinetics of a biological substrate within the single pool. Enrichment describes the amount of labelled substance present and is often expressed as APE which is the difference between the natural abundance of the labelled substance compared to that found in a sample (Buckley et al., 1985). For example, the natural abundance of ^{13}C is 1.084 APE (Capriole, 1972), if a sample contained 5% ^{13}C then the enrichment would be 5 – 1.084 = 3.916 APE. Calculation of enrichment using the APE is valid if the amount of tracer added to the biological pool is less than 10% of the pool size (Buckley et al., 1985). If the tracer constitutes more than 10% of the pool, calculation of isotopic enrichment using the ratio of the mass of the tracer to the mass of the tracee (tracer/tracee ratio) is a more valid method (Colby and McCaman, 1979). Most metabolic studies in humans using isotope methodologies use a tracer infusion that is less than 10% of the pool size and therefore the calculation of enrichment using the APE is the accepted method.

The rate of appearance (R_a) of a substrate into a given single pool is equal to its rate of disappearance (R_d) from the pool in steady state conditions (**Figure 2.5**). The steady state enrichment is dependent on the rate of infusion of the isotope, which is in turn dependent on the method of administration. Administration of a stable isotope to a pool can be via a continuous infusion over a set period of time, as a bolus amount (e.g. injection or ingestion) or as a primed continuous infusion which combines a single bolus with a continuous infusion.

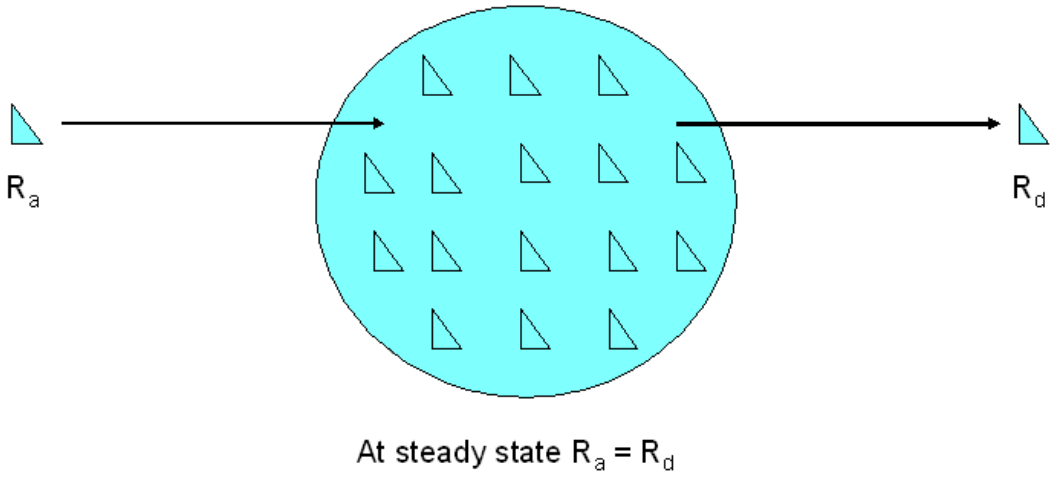


Figure 2.5: The single pool model of substrate kinetics. During steady state conditions the rate of appearance (Ra) of a substrate into the pool is equal to its rate of disappearance (Rd) from the pool (Figure adapted from (Wolfe and Chinks, 1992)). = substrate

Isotopic equilibrium is required in metabolic studies and **Figure 2.6** illustrates the achievement of isotopic equilibrium by a continuous tracer infusion. **Figure 2.7** shows the enrichment over time during a continuous infusion of a tracer and the equilibrium that is achieved. On reaching isotopic equilibrium sampling from the biological pool (e.g. plasma) can begin and the fate of the tracee can be determined.

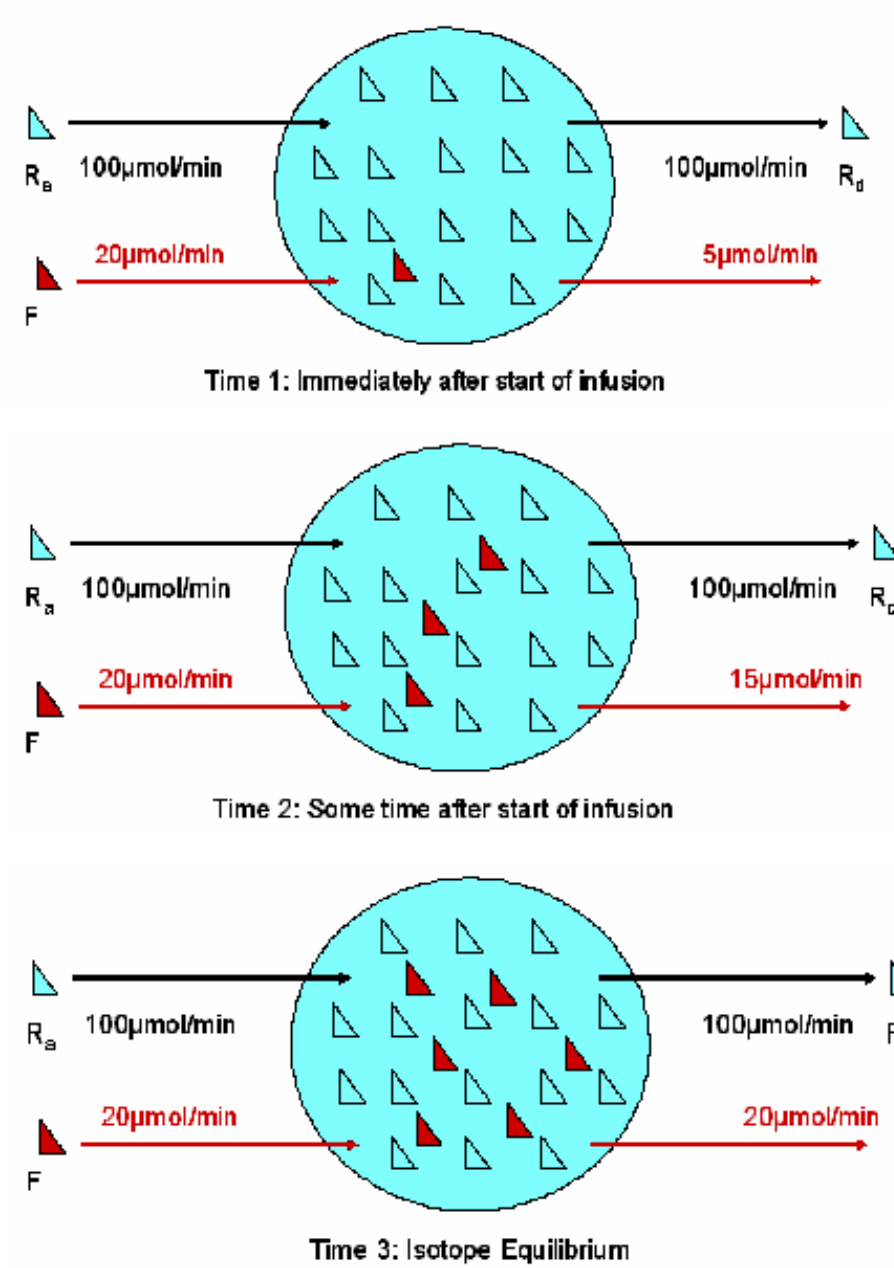


Figure 2.6: Continuous infusion of tracer to a pool through three time periods. At Time 1 there is only a minimal amount of tracer that has entered into the pool. The rate of flow (F) into the pool is not yet equal to the rate of disappearance from the pool (Rd). At Time 2, isotopic equilibrium has still not occurred but by Time 3, the F into the pool is equal to the rate of disappearance from the pool and equilibrium has been achieved. = tracee = tracer (Figure adapted from (Wolfe and Chinks, 1992)).

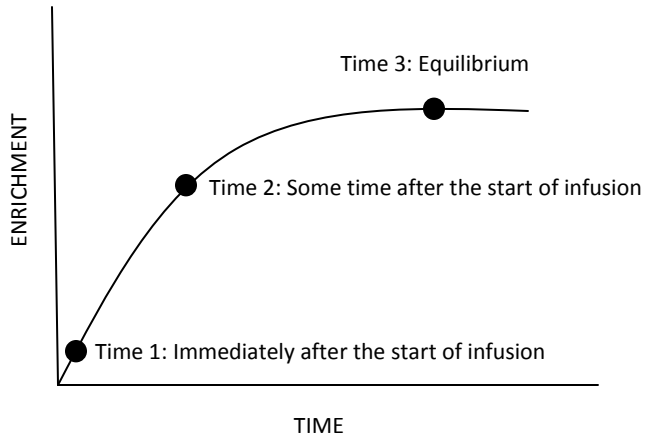


Figure 2.7: Achievement of isotopic equilibrium from a continuous infusion of tracer. (Figure adapted from (Wolfe and Chinks, 1992))

A bolus administration of tracer will result in an assumed instantaneous increase in the ratio of tracer to tracee with that ratio declining over time as entry of tracee into the pool dilutes the tracer present. Additionally, the tracer may leave the pool (i.e. to be incorporated into body tissues) which further dilutes the pool. Movement of the tracee can thus be calculated by the rate of dilution of the tracer within the pool. For example, the enrichment from a bolus injection of a stable isotope incorporated within an amino acid will decline as more of the common form – tracee – enters into the pool and as the stable isotope leaves the pool to be incorporated into body proteins. Over time the stable isotope becomes more and more dilute within the pool and this is represented in **Figure 2.8**.

If the rate of substrate turnover in a pool is relatively slow it can take several hours to achieve isotopic equilibrium via the continuous infusion method. This can cause practical difficulties when performing studies, especially on human subjects where time spent awaiting isotopic equilibrium might alter substrate kinetics. The use of a priming dose (bolus injection) with a continuous infusion was first described in the 1950's when the method was used to assess

glucose metabolism in dogs (Searle et al., 1954). Priming the substrate pool with a bolus injection, in an effort to shorten the time to isotopic equilibrium has since been proven to be beneficial in kinetic measurements of many substrates, including amino acids (Waterlow et al., 1978). Calculation of isotopic equilibrium from a primed continuous infusion is made from the rate of decline in enrichment from the bolus injection plus the rise in enrichment from the continuous infusion (Matthews et al., 1980). A perfect priming dose would bring about equilibrium instantaneously (a biological impossibility) but in reality it simply shortens the time that it takes to reach that point (**Figure 2.9**).

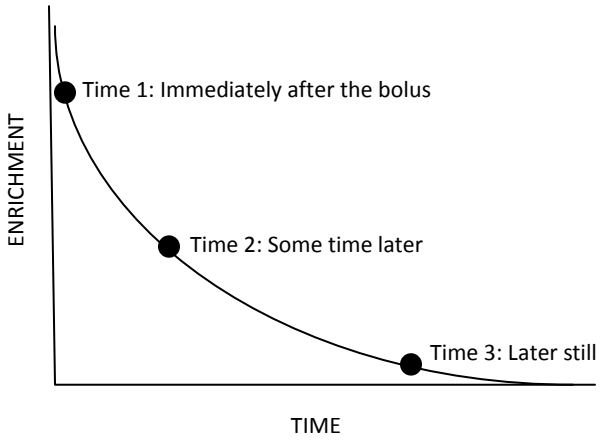


Figure 2.8: The decline in enrichment with bolus administration of tracer. (Figure adapted from (Wolfe and Chinks, 1992))

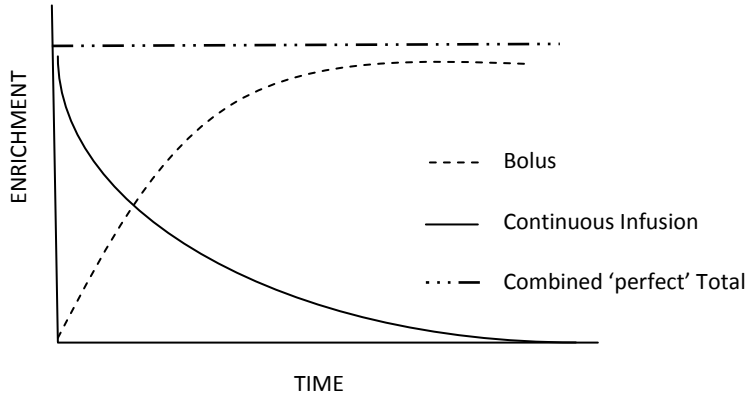


Figure 2.9: Isotopic equilibrium achieved via a 'perfect' primed, continuous infusion of tracer. (Figure adapted from (Wolfe and Chinks, 1992))

Another method used to speed up the time it takes to reach isotopic equilibrium is the flooding dose. Generally applied to the multiple pool model, the flooding dose combines a dose of unlabelled and labelled substrate in an effort to reduce the plasma enrichment of the unlabelled substance so that it is closer to the intracellular enrichment (Garlick et al., 1989). Similar enrichments means that equilibrium between compartments is rapid and that sampling from any pool can occur and enrichment within one is representative of enrichment in the others (Rennie et al., 1994). The flooding dose does much to improve the problem of an assumed instantaneous mixing of isotope within a pool but has caused an overestimation of protein synthesis rates (Garlick et al., 1989). Smith et. al. (1998) gave twenty three subjects a primed continuous infusion of ^{13}C leucine over a 7.5-hour period and after the 6th hour subjects were given a flooding dose of labelled or unlabelled amino acid (phenylalanine, threonine, arginine, ^{13}C-glycine or ^{13}C serine) (Smith et al., 1998). Findings were that during the flooding period, calculated rates of muscle protein synthesis almost doubled in the subjects given phenylalanine and threonine whereas in the groups who received non essential amino acids there was no significant change due to the flooding dose. Thus the administration of a flooding dose resulted in an enhanced uptake of essential amino acids into skeletal muscle and overestimation of the rate of protein synthesis. The flooding dose essentially violates one of the major assumptions associated with the use of labelled isotopes in metabolic research, that administration of the tracer does not affect normal metabolic processes (see **section 2.2.2.1**). Additionally, there seems to be greater variability in the values gained from flooding dose experiments compared to those using a continuous infusion method (Rennie et al., 1994). Evidence gained by flooding methods has not been included in this review.

2.2.2.1 Assumptions: As with most methods which endeavour to understand processes within the human body, a number of assumptions are implicit when using stable isotopes in metabolic research. The assumptions are that:

- Addition of a tracer to a pool will not affect the metabolism of the tracee
- Mixing of the tracer in the pool is instantaneous
- The pool size remains constant
- Recycling of the tracer within the pool does not occur

The first assumption is based on the knowledge that isotopes of the same element have the same chemical and functional properties and thus different isotopes should not have any differing effect on normal metabolic processes (Voet and Voet, 1990). In protein turnover research the assumption of no metabolic effect is of particular importance because in most cases the tracer is an amino acid and amino acids are the building blocks of protein. If the amino acid tracer is given in more than 'trace' amounts then protein synthesis can be stimulated by the increase in circulating amino acids (see **Section 2.6.1.1**). The assumption of no isotopic effect on metabolism is acceptable when using carbon, nitrogen and oxygen isotopes (Wolfe and Chinks, 1992) but not when using hydrogen's isotope deuterium. Argoud et. al. (1987) demonstrated that deuterium labelled glucose was metabolised more slowly than unlabelled glucose leading to inaccurate calculations due to high enrichment values (Argoud et al., 1987). This metabolic property of ^{2}H has been used to advantage in the development of the doubly labelled water technique for the measurement of EE (Westerterp, 1999) which is explained fully in **Section 2.4** of this review.

The assumptions that mixing of the tracer in the pool is instantaneous and that the size of the pool remains constant may not be the case with amino acid tracers. When exercise or diet are used as an intervention both could stimulate amino acid uptake into body protein and therefore the pool sizes will change (Buckley et al., 1985). In such a case food intake that includes amino acids will also dilute the tracer because of the addition of unlabelled amino acid into the pool. Accounting for exercise and/or food intake is therefore essential when performing exercise or nutrition research that uses stable isotopes incorporated into amino acids. Lastly, it is assumed that recycling of the tracer within the pool does not occur within the time course of a normal metabolic study. Considering the relatively slow turnover of skeletal muscle recycling is unlikely (Rennie et al., 1994). In protein turnover research this means that if an amino acid tracer disappears from the pool to be incorporated into protein, it will not reappear back into the pool as a result of protein breakdown. Despite these assumptions causing possible inaccuracies in results they are an accepted part of stable isotope use in metabolic studies.

2.3 Measurement of Protein Metabolism

Protein exists within pools in the body and these pools are dynamic with constant synthesis, breakdown and recycling that, in a normal healthy person, results in a relatively stable lean body mass (LBM) (Schoenheimer R et al., 1939). Protein synthesis exceeds breakdown during periods of growth throughout the lifespan. The progression through childhood and adolescence is a period of increased protein synthesis compared to breakdown as a person moves toward their genetically determined adult size. In adulthood when synthesis and breakdown are fairly evenly matched the body becomes anabolic in order to perform successful restorative processes or in response to an appropriate stimulus like resistance exercise (Biolo et al., 1999, Biolo et al., 1995b). Age brings about a gradual decline in LBM, mainly from skeletal muscle,

and this is seen in synthesis rates of protein that are lower in the elderly (>65yr) than in the young (<30yr) (Yarasheski et al., 1993, Welle et al., 1993). LBM also declines in pathological conditions for example muscular dystrophies, malnutrition and metabolic disorders such as uncontrolled diabetes mellitus when protein breakdown exceeds the rate of protein synthesis.

Human protein requirements have traditionally been determined from nitrogen balance studies. The rationale is that nitrogen is a major constituent of protein and therefore measurement of nitrogenous metabolites in the urine, and other wastes, reflects protein turnover in the body. The calculation of nitrogen balance (the amount of nitrogen consumed minus the amount of nitrogen excreted) is useful as an index of nutritional status. However, nitrogen balance studies are widely recognised as underestimating the protein and amino acid requirements of a population (Young et al., 1989, Zello et al., 1995, Fuller and Garlick, 1994). Maintenance of protein stores in the body requires a balance of nitrogen and amino acids that are consumed with nitrogen and amino acids that are lost through tissue turnover and exchange between tissues. Therefore stable isotope methodologies have been used over the past 10-15 years to study protein dynamics in both animals and humans and are considered to be the method of choice for metabolic studies.

2.3.1 Measurement of Protein Turnover by Stable Isotopes

Schoenheimer et. al. (1939) established the use of stable isotopes to show the active nature of proteins within the body (Schoenheimer R et al., 1939). Their methodology used ^{15}N tyrosine and paved the way for numerous metabolic studies using stable isotope methodology (Schoenheimer R and D, 1938). The single pool model of substrate kinetics is used for the determination of whole body protein turnover (WBPT) and assumes that amino acids, which are the building blocks of protein, are found within a single pool in the body and that the intra- and

extracellular compartments of protein rich skeletal muscle are uniform. The size of the free amino acid pool changes with alterations in the balance between protein synthesis and breakdown and amino acid catabolism in the post absorptive state (**Figure 2.10**).

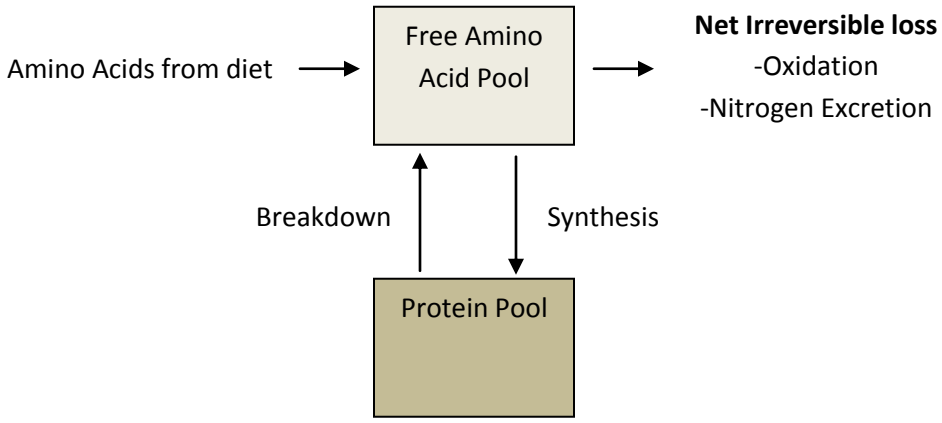


Figure 2.10: Model of WBPT dynamics. Amino acids are introduced into the free amino acid pool from the diet or as a result of protein breakdown. Amino acids disappear from the pool to be incorporated into protein or they enter into the catabolic pathway and are lost by oxidation or excretion (Figure adapted from (Wagenmakers, 1999)).

When considering a single amino acid, the term 'flux' is often used to describe the movement of that amino acid into and out of the free amino acid pool. Outward flux indicates the R_a of that amino acid into the free amino acid pool from the protein pool in the post-absorptive state. Inward flux indicates the R_d of the amino acid from the free amino acid pool into either the protein pool or the catabolic pathway where irreversible conversion occurs (Rennie, 1999). It is important to note that R_a describes all points of entry into any given pool, i.e. glucose enters into the blood from a single point of entry which is the liver so the R_a of glucose is relatively easy to quantify. However in some cases there are multiple points of entry of a substrate into a pool

especially if consideration is being made about the metabolism of a substrate in the postprandial state. Entry of substrates via dietary sources influences the R_a by introducing unlabelled substrate into the sampling pool which will dilute the tracer being infused. Correction of the amount of unlabelled substrate introduced can be performed to ensure accuracy, or the dietary source itself can be labelled to prevent dilution.

The overall sum of the rates of turnover of individual body proteins, determines whether an individual gains, loses or maintains lean body mass (LBM). Rates of turnover of specific body proteins can be inferred by the WBPT method. For example, in the transition from rest to exercise, any alterations in WBPT will almost certainly indicate skeletal muscle protein alterations. Quantification of rates of synthesis and breakdown of individual body proteins is important. The drawback of the WBPT method is that rates of turnover of body proteins differ depending on their function and the method does not distinguish between proteins. For example, the turnover rate of myocardial proteins is approximately three times greater than the turnover rate of skeletal muscle proteins (Revkin et al., 1990). Changes in slow-turning-over proteins may be masked by changes in faster-turning-over proteins when the WBPT method is used. However, this situation does not cause significant problems under normal conditions and is most common in pathological conditions such as cardiomyopathies when the myocardium hypertrophies at the same time that skeletal muscle atrophies due to the decreased cardiac output (Drexler, 1992).

The use of muscle biopsy techniques to determine protein turnover in skeletal muscle can define more accurately the kinetics within the skeletal muscle compartment compared to the WBPT method. However the invasiveness of the biopsy technique prevents its use in many human subject situations (e.g. athletes who are training or competing). The method used in the primary research of this thesis is WBPT. Despite the greater accuracy of the biopsy method the

subjects are high performance athletes within competition phase and the use of biopsy in that population is not practical. The WBPT technique allows for determination of protein turnover without obstructing the athletes ability to compete at an optimal level.

2.3.1.1 ^{13}C-Leucine Technique for Determination of Whole Body Protein Turnover: There are twenty-one amino acids in the body (**Table 2.1**). Eight are considered to be essential in that they must be consumed in the diet because they cannot be synthesised in vivo. Leucine is an essential amino acid that is catabolised primarily in skeletal muscle (Odessey and Goldberg, 1972) and therefore is useful in the determination of WBPT.

Table 2.1: Essential and nonessential amino acids

Essential Amino Acids	Nonessential Amino Acids
Isoleucine	Alanine
Leucine	Arginine*
Lysine	Asparagine
Methionine	Aspartate
Phenylalanine	Cystine^
Threonine	Glutamate
Tryptophan	Glycine
Valine	Histidine#
	Proline
	Serine
	Tyrosine+

*Children have a reduced ability to synthesis arginine, ^cytosine is synthesised in the body from methionine, #Infants cannot synthesis histidine, +tyrosine is synthesised in the body from phenylalanine

Protein breakdown can be determined in the post-absorptive state if the rate of leucine appearance from protein and the quantity of leucine per gram of protein within the tissue is known. The R_a of leucine can be calculated from the tracer infusion rate and the intracellular enrichment using α-ketoisocaproate (αKIC). αKIC is produced intracellularly solely from transamination of leucine (Motil et al., 1981a) and plasma αKIC enrichment better reflects intracellular leucine enrichment than does plasma leucine (Schwenk et al., 1985).

Bauman et al. (1994) provide evidence that plasma leucine enrichment is actually higher than intracellular leucine enrichment (Baumann et al., 1994) which illustrates that using plasma leucine as a reflection of intracellular leucine is not sound. It is generally accepted that plasma ^{13}C-αKIC enrichment gives adequate results in WBPT studies when ^{13}C-leucine is used as a tracer (Motil et al., 1981a, Wolfe et al., 1982, Schwenk et al., 1985). However it is important to note that the intracellular compartment is not entirely homogenous and thus plasma αKIC, although a better indicator than plasma leucine, is not in equilibrium with all intracellular leucine (Carraro et al., 1990b). **Figure 2.11** explains the rationale for the use of plasma αKIC as an estimate of intracellular leucine. The metabolic fate of leucine determines its R_d. Leucine can be incorporated into muscle or transformed to αKIC and irreversibly converted to carbon dioxide (CO_2). The label then either appears in exhaled CO_2 or is retained within the bicarbonate pool of the body.

To determine rates of protein breakdown and synthesis via the WBPT method the amount that the amino acid (used as the tracer) contributes to whole body protein stores must be known. Leucine contributes approximately 590μmol/g protein to whole body protein stores (Wolfe and Chinks, 1992) and as such the equation for the determination of protein breakdown via the ^{13}C-leucine technique is:

$$Breakdown\ (g\ protein/kg/hr)\ = \frac{F}{\mathrm{E_{KIC}} \times 590} \qquad \text{(Equation 2.1)}$$

where F is the infusion rate (µmol/kg/hr) and $\mathrm{E_{KIC}}$ is the tracer to tracee ratio of αKIC.

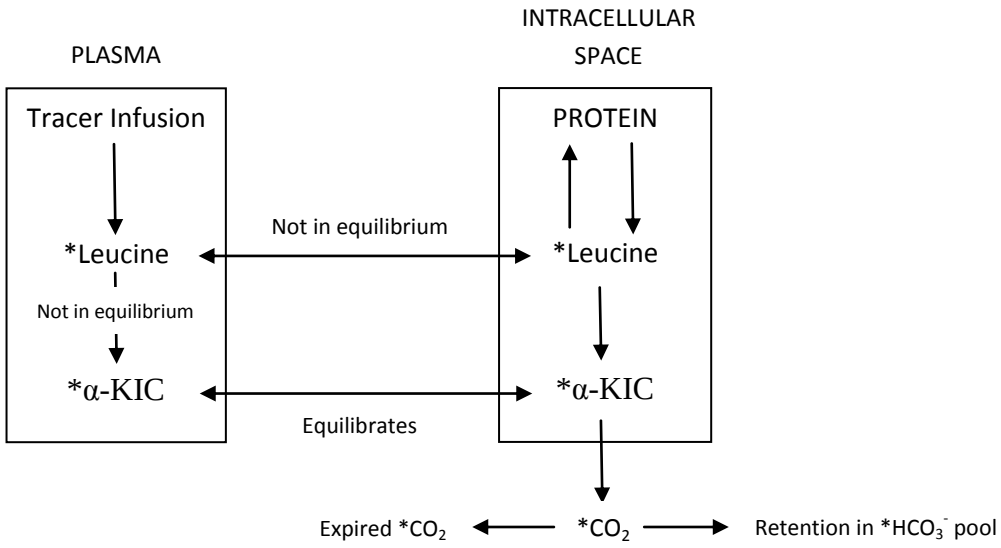


Figure 2.11: Rationale for the use of ^{13}C-αKIC for the determination of WBPT when using ^{13}C-leucine as a tracer. Leucine can be incorporated into protein or transformed into αKIC and irreversibly converted to CO_2 or retained in the bicarbonate pool. * indicates appearance of the label (Figure adapted from (Wolfe and Chinks, 1992)).

Oxidation and reincorporation into protein are the two fates of leucine released from protein breakdown (van Hall, 1999). During oxidation the first carbon of the leucine molecule which is often the labelled carbon, is oxidised to CO_2. The amount of label lost through oxidation can thus be determined by collection of expired CO_2, an irreversible step. If protein breakdown (via **Equation 2.1**) and CO_2 excretion is known, then protein synthesis in the post absorptive state can be determined by **Equation 2.2**:

$$Synthesis\ (g\ protein/kg/hr)\ = Breakdown\ - \left(\frac{CO_2\ excretion}{\mathrm{E_{KIC}} \times 590}\right) \qquad \text{(Equation 2.2)}$$

where, CO_2 excretion is the total rate of CO_2 production (µmol/kg/hr) multiplied by the tracer/tracee ratio and corrected for CO_2 retention in the bicarbonate pool (explained in **Section 2.3.2.2**). The ability to calculate protein breakdown as well as protein synthesis makes the ^{13}C-leucine technique widely acceptable for WBPT research.

2.3.1.2 CO_2 retention: Calculation of leucine kinetics (particularly oxidation) assumes an amount of CO_2 fixation where some of the labelled CO_2 produced is not excreted via the breath and thus needs to be accounted for in an experiment. CO_2 passes through the bicarbonate pool before being expired and therefore can be removed prior to reaching the lung. For example CO_2 can be incorporated into urea and excreted as urine (Wiklund, 1996) or it can be integrated into a glucose molecule via the Krebs cycle (Peronnet et al., 1990). These alternate fates of CO_2 mean that the collection of the expired label cannot fully account for total leucine oxidation.

CO_2 fixation is determined by the recovery of labelled CO_2 after administration of labelled $NaHCO_3$. Many researchers use a recovery value of 81% as a correction factor instead of directly determining labelled CO_2 recovery (Motil et al., 1981a). The correction factor is used despite research which has found that values for recovery of labelled CO_2 range from 50-100% (Irving et al., 1983, Allsop et al., 1978, Clugston and Garlick, 1983). The variability could be due to physiological inconsistencies and technical problems, such as loss of the tracer prior to infusion due to equilibration with ambient air (Wolfe and Chinks, 1992). To ensure accurate determination of the percent of the label that has undergone oxidation experimental data is required. In most cases a separate experimental study using the same or similar subjects determines CO_2 fixation (Irving et al., 1983).

It is also important to note that CO_2 recovery values determined by continuous infusion can be affected by the duration of infusion whereby there is a higher recovery rate when infusion time is longer. Wolfe and Chinks thus recommend performing recovery infusion, with labelled $NaHCO_3$, for the same time period as the tracer experiment infusion (Wolfe and Chinks, 1992). Recovery experiments in specific populations also need to consider that metabolic rate affects recovery. A study by Shaw and Wolfe (1987) showed a general relationship between metabolic rate and recovery values in cancer patients with varying degrees of hypermetabolism (Shaw and Wolfe, 1987). Patients with higher metabolic rates had higher rates of CO_2 recovery. An increase in recovery with increased metabolism has also been described in infants (Van Aerde et al., 1985) and in normal volunteers (Hoerr et al., 1989). Therefore results from recovery studies that do not accurately match subjects should be interpreted with caution.

2.3.2 Nitrogen Balance Method

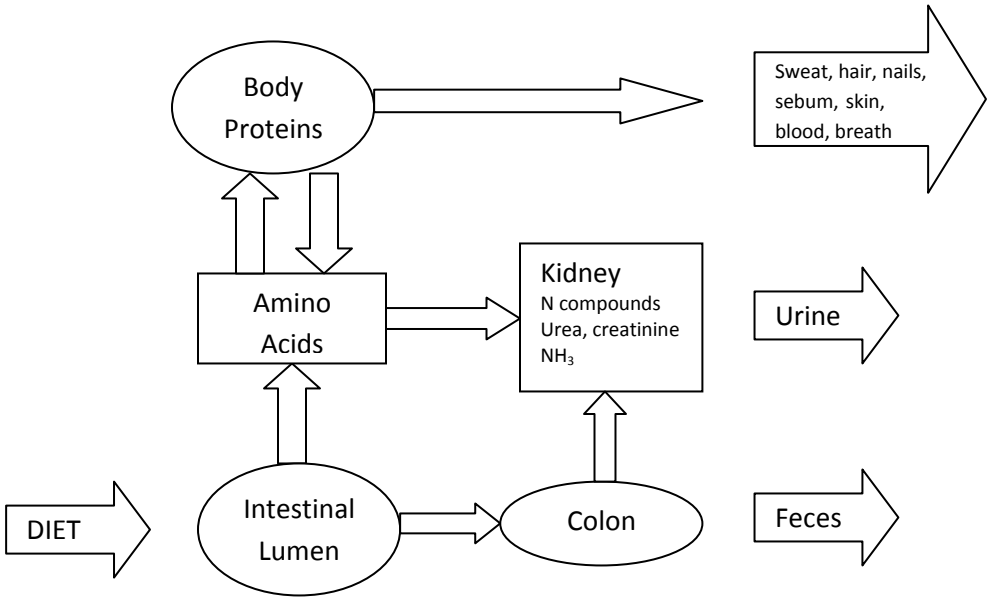
The traditional method for determining protein requirements has been the nitrogen balance method. Nitrogen is a major component of all protein molecules and the net balance of nitrogen content has been used as an index of protein balance. The fact that the percentage of nitrogen in human proteins is relatively constant, at about 16%, means that net nitrogen balance can be easily converted to net protein balance by multiplying by the nitrogen constant 6.25 and from there dietary recommendations can be formulated (Kopple, 1987). The recommended daily allowances (RDA) for amino acids and protein are largely still based on nitrogen balance findings despite the advent of stable isotope methodologies for determining protein turnover. The method requires the measurement of nitrogen consumed in the diet and nitrogen lost from the body and has been a useful index of nutritional status especially in surgery and trauma patients (Elwyn, 1980, Blackburn et al., 1977). A person who is in negative nitrogen balance is using

their protein stores, predominantly skeletal muscle, to meet the metabolic energy requirements of the body and is therefore in a catabolic state.

Nitrogen intake from the diet is relatively easy to establish if good recording of food consumed is performed. However it is acknowledged in the literature that nitrogen intake is usually over-estimated because of loss of nitrogen from food on utensils, crockery and other food storage and consumption items (Kopple, 1987). Determining nitrogen loss from the body is not a straight forward task and in many cases nitrogen loss is underestimated. Nitrogen loss from the body mainly occurs from urinary losses in the form of urea, creatinine and ammonia but also from faeces and miscellaneous losses (e.g. skin, hair etc) (Calloway and Margen, 1971) (**Figure 2.12**). The measurement of total urinary nitrogen (TUN) by the Kjeldahl and micro-Kjeldahl methods are the accepted protocols for determining both intake and output nitrogen but are not used as frequently these days because of their expense, technical procedures and the advent of automated assays (Jacobs, 1968). Some researchers use measured urinary urea nitrogen (UUN) instead because UUN can be used to calculate TUN as 80% - 90% of TUN is in the form of urea (Skogerboe et al., 1990).

In most studies of nitrogen balance, unmeasured nitrogen losses are accounted for by using fixed values that were established in complex metabolic studies. Unmeasured losses include nitrogen loss from integument (desquamated skin, sweat, hair, nails), breath in the form of ammonia, blood drawing, menstrual fluids, sputum, saliva, emesis, and semen (Kopple, 1987). It is generally accepted that these unmeasured losses work together to falsely increase the positivity of any balance calculations. Carroway et. al. (1971) estimated that the average daily unmeasured loss of nitrogen was about 400mg of nitrogen per day with any blood drawing additional to that and estimated at 32 ± 2mg N/ml of blood drawn (Calloway et al., 1971). Of particular

importance to nitrogen balance studies is the fact that unmeasured losses are not constant and can change with alterations in metabolic status (e.g. disease or sweating) and also can increase with increasing intake of dietary nitrogen (Calloway et al., 1971, Fisher et al., 1967).



Nitrogen In = Nitrogen Out

Figure 2.12: Metabolic pathways determining nitrogen balance. Nitrogen balance is the result of dietary intake and nitrogen recovered from urine, faeces and miscellaneous losses. NH_3 = ammonia. (Adapted from (Tome and Bos, 2000))

Standard formulae are used for the calculation of nitrogen balance (NBal) (Gibson, 2005). When TUN data is available the equation is:

NBal = nitrogen input – (TUN + 2) (Equation 2.3)

where the constant 2 corrects for faecal, perspiration and other losses of nitrogen. However, if only UUN is known then the equation is:

NBal = nitrogen input – (UUN + 4) (Equation 2.4)

where the constant 4 is a correction for faecal, perspiration and other losses of nitrogen as well as non-urea forms of nitrogen in urine. Determination of nitrogen balance by **Equation 2.4** is often referred to as the crude nitrogen balance. These formulae can be altered to account for losses that have actually been measured and individual loss measurements have been reported in various publications so that researchers can be more accurate in their determination of nitrogen balance (Calloway and Margen, 1971, Calloway et al., 1971).

Although the nitrogen balance method is well established and has generated valuable information regarding nutrition and protein metabolism over the years, there are a number of limitations and sources of error associated with the method. The major limitations are (modified from (Kopple, 1987)):

- Losses of foods that adhere to dishes, cooking utensils and cutlery lead to an over-estimate of nitrogen intake
- Losses of faecal matter and urine on toilet tissue leads to an under-estimate of nitrogen loss
- Unmeasured losses from skin, breath, tooth brushing, blood draws, menstrual loss, semen and sweating can lead to an under-estimate of nitrogen loss
- The balance value is calculated from two large numbers, input and output, and produces a small number as a result. Small percentage changes or errors in the original input and output figures can lead to marked changes in the balance value
- Balance measurements taken over a long time period e.g. days, are more likely to be erroneous due to the length of time that input and output have to be monitored

- Balance studies do not describe the fate of ingested nitrogen they simply give a value for input and output with no explanation for what is happening in the body

Given the limitations of the nitrogen balance technique and the accessibility of the stable isotope method, the present day value of the method is often questioned. However, it does provide a relatively accurate means of determining protein balance if researchers acknowledge and account for unmeasured nitrogen losses and it is also comparatively inexpensive.

2.4 Measurement of Energy Expenditure

The importance of respiration and ventilation for the energy requirements of animals has been well documented over the past 200 years. In 1660 Robert Boyle noticed that mice confined to sealed jars died at the same time that a flame became extinguished and thus he established two important principles: that fire and life were both combustion processes and that there is a requirement that air be present to support both processes. Over a century later the discovery of CO_2 in 1757 by Joseph Black and the discovery of oxygen in 1774 by Joseph Priestly bought about methods similar to those used by Boyle to determine oxygen consumption and CO_2 production of animals and humans in confined spaces. These methods led to three important discoveries about oxygen consumption by French chemists, Lavoisier and Seguin; 1) that larger people consumed more oxygen than smaller people; 2) that people standing up or moving around consumed more oxygen then people sitting and resting and; 3) that oxygen consumption was increased after eating a meal even in people who were sitting. In addition and perhaps the most important contribution made by Lavoisier and Seguin, was the development of the method of indirect calorimetry for the quantification of animal and human EE, a method that is still in use today (Speakman, 1998).

Metabolism is an energetic process that describes all the chemical reactions that take place in the body and is described in terms of EE. Total daily energy expenditure (TEE) is the amount of energy in kilojoules (or kilocalories) that a person uses each day to function. TEE is made up of three parts:

- Resting metabolic rate (RMR)
- Thermogenic effect of food (TEF)
- Activity EE

RMR, or resting EE, accounts for between 60-75% of TEE and includes basal and resting metabolism. The body has a minimum requirement to sustain the function of vital organs and this basal level of activity (basal metabolic rate (BMR)) is dependent on such factors as fat free mass, gender, thyroid hormone function and rate of protein turnover. BMR is measured on waking after an extended period of fasting, while a person is still lying in bed. In comparison, RMR is the energy required for an awake person to function in a resting state and is measured typically 3-4 hours after a meal. The TEF accounts for approximately 10% of TEE and as Lavoisier and Seguin discovered, describes the increase in energy metabolism that occurs with food consumption. Physical activity is the third component of TEE and has the most influence on human EE. Under normal conditions, for a healthy adult, physical activity accounts for between 15-30% of TEE. High level athletes can double their TEE with just 3-4 hours of hard training each day and therefore the contribution of physical activity to their TEE is increased.

TEE can be accurately measured by direct calorimetry. In the 1890's Atwater and Rosa used the first human calorimeter to determine EE in a person (Webb, 1985). In doing so they verified the 1st law of thermodynamics: Energy can neither be created nor destroyed, it can only

change forms. All metabolic functions produce heat and the calorimeter was designed to measure that heat production. It consisted of a chamber in which a person lived for a period of time. They ate, slept and did exercise on a cycle ergometer and their heat production was measured by the change in temperature of water moving through a series of pipes in the chamber. Over the years a number of heat measuring devices have been developed but it is widely acknowledged that direct measurement of heat production in humans is not practical. Indirect methods of calorimetry are used for research nowadays and depend on the premise that all energy-releasing reactions in the body depend on oxygen use. Measuring oxygen consumption therefore gives an accurate method for EE determination.

2.4.1 The Double Labelled Water Technique

In the absence of calorimetry analysis TEE can be estimated by taking accurate records of all food intake over a period of time and measuring the change in body composition that occurs during that time. EI ± any change in body composition then equates to EE. However, this method although easy to implement has obvious limitations and when the doubly labelled water method was first suggested by Lifson et. al. in the 1950's it was no surprise that it soon became the 'gold standard' for determination of free-living EE (Lifson et al., 1955). Lifson et. al. observed that oxygen atoms in exhaled CO_2 and in body water were in isotopic equilibrium (Lifson et al., 1949). The earlier discovery of the rare heavy stable isotope of oxygen (^{18}O versus the more common ^{16}O) and hydrogen (^{2}H versus the more common ^{1}H) in the 1920's and 1930's respectively meant that the heavier atoms could be used as tracers for oxygen and water and that they would label both the CO_2 and water pools. When using doubly labelled water ($^{2}H^{1}H^{18}O$) the ^{18}O is excreted both as CO_2 and as water and thus is a measure of water plus CO_2 flux. The ^{2}H is eliminated only from the body as water and thus is a measure of water flux. Therefore, the

difference between the two flux measures is equal to the CO_2 flux. After administration of an appropriate dose of $^{2}H^{1}H^{18}O$, collection of urine, plasma and/or saliva over a period of time is required. Measurement of isotopic concentrations in the body water are used to determine both the dilution spaces for the isotopes, and thus total body water (TBW), and the rate of CO_2 elimination from the body. TBW is determined from the ^{18}O dilution space by the equation (Schoeller et al., 1980):

$$TBW = (\text{dilution space} - \text{dose}) / 1.039 \qquad \text{(Equation 2.5)}$$

where 1.039 is the fractionation factor (described below). Fat-free mass (FFM) can be determined from TBW by the relationship:

$$FFM = TBW / 0.73 \qquad \text{(Equation 2.6)}$$

where 0.73 is the fraction of water in FFM (Prentice et al., 1952). Fat mass (which by definition contains no water) can then be determined from the difference in body mass and FFM. The rate of CO_2 production (r_{CO2}) is represented by the mathematical equation:

$$r_{CO2} = N/2\ (k_{18} - k_2) \qquad \text{(Equation 2.7)}$$

where r_{CO2} is measured in mol/day, N is the size of the total body water pool (mol) and k_{18} and k_2 are the ^{18}O and ^{2}H elimination rates per day (Lifson et al., 1955).

Practically, to prepare a dose of DLW, ^{2}H and ^{18}O are combined to form a stock mixture from which individual doses are apportioned. The solutions available of ^{2}H usually contain 99.9 atom % and for ^{18}O contain 10 atom % and stock solutions are mixed using tap water to make a solution of between 5-10 atom %. Individual doses are calculated based on the expected dilution spaces of subjects which are estimated from body weight and estimated body fat percentage. In

general, the dose provides an initial enrichment that is about 600 times the random analytical error (Schoeller, 1988). Stock mixture preparation and dose storage should be in glass bottles that have screw-caps and in many cases are secondary sealed with a product such as Parafilm™ to ensure that there is no loss of isotope to the environment. Plastic bottles are not suitable for mixing or storing as they have been shown to be permeable to water and therefore isotope exchange can occur (Westerterp, 1999). Prior to administration of the dose a baseline sample is collected from the subject to determine the background enrichment of ^{2}H and ^{18}O in the body. Background samples differ between subjects and over time periods and in some studies more than one baseline sample is collected. Background values for ^{2}H ranging from 138 ± 0.2 to 155.6 ± 0.4 10^{-4} atom % and for ^{18}O ranging from 1979.7 ± 0.4 to 2006.7 ± 1.0 10^{-4} atom % have been described and illustrate the individual variation in background levels (Westerterp et al., 1995).

Five major assumptions are inherent in the calculation of the rate of CO_2 production by the DLW technique: (1) the labels enter into and are lost from total body water only; (2) the ^{2}H label is lost as water only; (3) ^{18}O is lost to both water and CO_2 and the exchange between water and CO_2 is rapid due to the action of the enzyme carbonic anhydrase; (4) the fractional output rate of water and CO_2 are constant; and (5) background isotope intake rates are constant (Coward, 1988). It is generally agreed that fractionation occurs, a process whereby when a molecule undergoes a change of state (e.g. liquid to gas) the heavier molecules tend to remain in the lower energy state (Wolfe and Chinks, 1992). The occurrence of fractionation therefore must be accounted for when determining rates of water and CO_2 flux. There are three fractionation factors to consider in the DLW method and **Equation 2.7** can thus be altered to account for fractionation factors:

$$r_{CO2} = N/2f_3 (k_{18} - k_2) - ((f_2 - f_1)/2f_3) r_G \qquad \text{(Equation 2.8)}$$

where f_1 and f_2 represent the fractionation of ^{2}H and ^{18}O respectively between liquid and gas states, f_3 is the fractionation factor for the exchange of ^{18}O between CO_2 and water, and r_G is the assumed rate of water loss as vapour (evaporation) (Schoeller, 1983). The correction factor of 2.1 is often used for r_G however the correction is based on the assumption that water losses are related to the average 24-hour ventilatory volume for healthy adults and also that transcutaneous losses are related to average rates of water loss per square metre of body surface. In subjects who deviate from the normal population (e.g. those with significant metabolic disease or athletes who expend a large amount of energy) the correction factor may account for up to 4% of the error in the determination of TEE (Ruby et al., 2002).

The calculated rate of CO_2 production can be used to determine EE if the respiratory quotient (RQ) is known or can be estimated. In experimental studies it is unlikely that a measured RQ is available over the entire sampling period when doubly labelled water is used. Some studies use measured resting values of RQ but in reality this value will change over the course of a day due to physical activity, exercise and food consumption. The measured food quotient (FQ) can be used as an estimate of RQ because RQ is equal to FQ when a person is in energy balance (Black et al., 1986). FQ can be determined from diet records where a person's intake of protein, fat, carbohydrate and alcohol are expressed as a percentage of total EI (Southgate and Durnin, 1970). Southgate and Durnin developed an equation for the calculation of FQ:

$$FQ = 0.81P + 0.71F + 1C + 0.67A \qquad \text{(Equation 2.9)}$$

where P, F, C and A are protein, fat carbohydrate and alcohol respectively with the numbers preceding each being constant values (Southgate and Durnin, 1970). The formula can be adjusted

in situations where a person either gains or losses mass, if the amount of lean mass/fat lost or gained is known. TEE can then be calculated by the equation:

$$\text{TEE (kcal/day)} = \frac{3.941\text{rCO2}}{FQ} + 1.106\text{rCO2} - 2.17\text{UN} \qquad \text{(Equation 2.10)}$$

where 3.941 is the calorie value for oxygen consumed at non-protein RQ, 1.106 is the calorie value of 1-litre of oxygen metabolising carbohydrate and fat and UN is the urinary nitrogen excretion in g/day and is the protein correction (de Weir, 1949). The protein correction is equal to a deduction of 1% when 12.3% of the total calories come from protein metabolism, and therefore using this estimation as a constant **Equation 2.6** becomes:

$$\text{TEE (kcal/day)} = 99\% \left(\frac{3.941\text{rCO2}}{FQ} + 1.106\text{rCO2}\right) \qquad \text{(Equation 2.11)}$$

There are many assumptions involved in the determination of EE via the double labelled water technique and with these assumptions there is a margin of error. Potential sources of error include 1) incorrect estimation of fractionation, 2) errors in the calculation of water and CO_2 elimination rates, 3) changes in water and/or CO_2 flux within the total body water pool during the experimental period, 4) errors in the calculation of the dose required or poor oral administration of the dose, and 5) error associated with the individual equations for r_{CO2} and TEE and also the two equations together. Seale et. al. (1989) tested the impact of these variables on the final determination of EE and found that a 5% error in dose, water production rate and fractionation produced an approximate +25%, -20% and -5% error respectively in the calculation of CO_2 production (Seale et al., 1989).

A variety of participants and metabolic conditions have been used to validate the doubly labelled water technique, including active and sedentary adults, and those in energy balance and imbalance (Schoeller et al., 1986b, Coward and Prentice, 1985, DeLany et al., 1989, Westerterp et al., 1988, Schoeller and van Santen, 1982). Validation usually occurs with the concurrent measurement of EE by doubly labelled water and respiratory gas exchange. Schoeller et. al. (1996) found that from 14 field studies, with repeated doubly labelled water measurements, the analytical variation in the method was approximately 4% and the within subject variability was approximately 7% (Schoeller and Hnilicka, 1996).

The doubly labelled water technique for the analysis of TEE has been rigorously validated and is used universally as the most accurate measure of TEE. As with any method, limitations need to be restricted and can be controlled for through good study design and accurate calculation and interpretation of results.

2.5 Measurement of Energy Intake

Foods are described energetically by kilocalories (kcal). 1 kcal is the amount of heat required to increase the temperature of 1 litre of water by 1°C. Therefore, if a food contains 300 kcal it is able to increase the temperature of 300L of water by 1°C when its potential energy is released. The kilojoule (kJ) is the standard international unit for expressing the energy content of food. Kcal can be converted kJ by multiplying by 4.186 (McArdle et al., 2001).

Bomb calorimeters measure the heat or energy liberated from a food as it burns. Food enclosed within a sealed container, surrounded by water, is ignited and the heat produced is absorbed by the water causing the temperature to increase. The degree of temperature increase in the water is then used to determine the energy content of that food. Energy liberated from the

various macronutrients differs because of their different chemical structures. The energy liberated from lipid depends on the structural composition of the fatty acids and averages 9.4 kcal/gm. Carbohydrates also vary in their ability to liberate heat depending on the organisation of the atoms within the molecule. Generally, a value of 4.2 kcal/gm represents the energy liberated from carbohydrates. The release of energy from protein depends on two factors; 1) the type of protein in the food and 2) the nitrogen content of the protein in the food. On average protein liberates 5.65 kcal/gm (Brooks et al., 2005).

There is a difference between the energy liberated from the three macronutrients in the bomb calorimeter and the energy available for use in the body. Nitrogen that is released as part of the breakdown of protein is not able to be oxidised by the body and instead is eliminated as urea in the urine. The nitrogen excreted does not take part in energy reactions and accounts for approximately 19% of the protein molecule. The energy liberation from protein is therefore corrected to 4.6 kcal/gm in the body. In comparison, the energy liberated from carbohydrate and fat, neither of which have nitrogen atoms, is the same in the body as it is in the bomb calorimeter. For practical reasons most nutritionists round the average kcal values to whole numbers (carbohydrate 4 kcal/gm, lipid 9 kcal/gm, protein 4 kcal/gm) that are referred to as Atwater General Factors (AGF) (Atwater, 1910). In addition, alcohol has an AGF of 7 kcal/gm.

The ability to accurately determine food intake of a person or group is essential to ensure nutritional status conclusions and subsequent nutritional recommendations are precise. Standard methods for collecting food intake information include; self-report diet records, 24-hour recalls, or food frequency questionnaires. These methods are inexpensive and relatively effective in determining EI but their obvious limitation is the lack of validation against an objective reference in an effort to eliminate reporting bias (Mertz, 1992). The main problem with these dietary

assessment tools is the under-reporting of intakes that commonly occurs (Hill and Davies, 2001, Schoeller, 1995). A number of factors influence the reporting of dietary intakes including; educational level, gender, race, obesity status and age (Johnson et al., 1994, Sawaya et al., 1996, Kristal et al., 1997, Heitmann and Lissner, 1995). Self-report diet records are particularly prone to under-reporting and inaccuracies; however the 24-hour recall interview is not much better because it depends on the subject remembering what they have eaten and also their ability to accurately recount portion sizes. In addition, the degree of motivation of the subject is an important factor in the accuracy of the 24-hour recall (Acheson et al., 1980). The semi-quantitative food frequency questionnaire is used widely in epidemiological enquiries to assess the frequency that foods are consumed over a specific time period. However, caution is required in the use of food frequency questionnaires, as with other methods, because its validity is questionable (Briefel et al., 1992). For example, Schaefer et. al. (2000) found that the food frequency questionnaire did not provide reliable estimates of the quantity of fat and cholesterol consumed by middle aged healthy adults (Schaefer et al., 2000). However, with the availability of the DLW technique it is possible to validate diet records in an effort to quantify any misreporting. If a subject is weight stable then they should be in energy balance as measured by DLW and diet recording. If they are not in energy balance then it can be assumed that some misreporting of dietary intake has occurred and the extent of the misreporting can be quantified. A review of studies that have used DLW to validate dietary records in athletes revealed that misreporting accounts for 10-45% of TEE and is mainly due to under-reporting rather than under-eating (Hill and Davies, 2001).

Weighed diet records are considered to be more accurate than self reported diet recording in determining EI (Magkos and Yannakoulia, 2003). The weighing of all food and beverages

consumed is a valid method of collecting EI data because it gives an actual amount of food and removes the need to estimate portion size. In addition, individual food constituents are weighed separately meaning that the nutrient content of mixed foods is more accurate. If weighed food records are to be used in research however the subject(s) need to be motivated, literate and numerate. In fact, experimental studies that require participants to weigh their own food are hindered by lack of time, patience and, in athletic populations where dietary intake is dependent on TEE and may not have a set pattern, the lack of transportability of the method becomes a major limitation (Benardot and Thompson, 1999). A better approach is for researchers to prepare and weigh the food to be consumed. The supervised weighed diet records are accurate, but require substantial time from the research team. It requires researchers to be present at all feeding times which may become a problem if the experimental period is longer than a few days. However, reproducibility is high for weighed recording and when supervised weighing occurs, under-reporting of food intake is less of a limiting factor. In fact, when food intake is weighed and recorded by trained researchers or nutritionists the EI closely matches the DLW EE regardless of the subject population (Sjodin et al., 1994, Branth et al., 1996).

The determination of protein intake is part of the dietary intake recording. The ideal method of determining nitrogen and thus protein intake in foods is not by diet records but by analysis of a duplicate of an actual meal and homogenising the meal as described by Southgate and Durin (Southgate and Durnin, 1970). The Southgate and Durin method is rigorous but it adds to the expense of the research, the time required for analysis, and really is impractical for free-living assessments, especially when determining protein intake over a number of days. The commonly used method in nutrition research is input of dietary intake data into a commercially available software programme which calculates the nutrient values of the food.

The measurement of *habitual* food intake is said to be one of the hardest tasks of nutritional research (Acheson et al., 1980). The basic problems are; 1) as discussed above, the method of data collection needs to be accurate and 2) the subject must maintain their habitual intake despite the research protocol. Any technique used must be accurate but must not be applied so rigorously that it alters habitual eating patterns. Studies that collect EI data probably influence habitual intakes solely because it makes people more aware of what they are eating. The longer the reporting period the greater the risk of deliberate alteration of eating behaviour to simplify the recording process (Magkos and Yannakoulia, 2003). Reducing the bias in this respect is an essential part of the planning process for nutritional studies.

Metabolic studies that require accurate EI information will always be hindered by the limitations of the various recording methods. It is therefore essential that these limitations are understood by researchers and controlled for as best as possible to ensure the accuracy of research findings.

2.6 Energy and Protein Balance in Athletes

An individual who neither gains nor looses body mass is in energy balance. When in energy balance, EI from dietary sources is equal to the amount of energy expended by the body. Energy is expended during resting conditions when the body produces heat during its various metabolic activities and expenditure increases above resting levels with exertion. Individuals who have a TEE that is less than their daily EI are in positive energy balance and as a result their body mass will increase. The type of tissue that will be amassed in the body (e.g. adipose tissue, muscle protein) depends on the composition of the diet and the type of exercise, if any, the individual undertakes. Conversely, an individual with a TEE greater than their daily intake will be in negative energy balance and body mass will decline. Energy balance status has a dramatic

effect on the utilisation and turnover of protein in the body (Calloway and Spector, 1954) and therefore is an important consideration for athletes in endurance sports who may need to call on protein stores for energy.

When an individual's daily intake of nitrogen from protein equals their daily excretion of nitrogen they are said to be in nitrogen balance. Anabolic stimulants that contribute to a positive nitrogen balance in adults include resistance training, a large dietary protein intake and ergogenic steroid drugs (McArdle et al., 2001). Individuals who are ill or have a poor protein intake or total EI may be in negative nitrogen balance where lean tissue is being degraded to supply energy. Nitrogen balance can be maintained with a dietary intake of protein of between 0.8 and 1.2g protein/kg/day in normal healthy individuals (Jeukendrup and Gleeson, 2004). However, Pellet and Young (1991) have established a relationship between energy balance and protein balance. They evaluated the nitrogen balance literature and found that for EIs between 2100 kcal and 4200 kcal and protein intakes between 18 g protein/day and 90 g protein/day, both the increase in EI and the increase in nitrogen intake improved nitrogen balance separately (Pellett and Young, 1991). However, the effects of changes in protein intake on energy metabolism were considered to be less significant in the context of estimating energy requirements than were the effects of energy on protein metabolism and requirements. There remains a lack of consensus about the quantity of protein that needs to be consumed by athletes to maintain nitrogen balance especially during periods of high EE when EI may not be sufficient to maintain energy balance.

2.6.1 Protein Balance

It is important to understand the factors that contribute to the relationship between energy and protein balance. Energy balance is dependent on EI and EE and is a fairly straight forward concept. If an individual’s EI is greater than their EE then their body mass increases. Conversely

if an individual's EE is greater than their EI then their body mass declines. A normal healthy person should be in energy balance. Protein balance is however a more complex process and is dependent on a number of factors which have an effect on protein turnover and therefore indirectly on energy balance and body mass.

2.6.1.1 Factors controlling protein turnover: There are a multitude of different proteins in the body that are distributed amongst all body cells and fluids. These proteins include: structural proteins, contractile proteins and transport proteins. To maintain the integrity of an organ or organism there is continuous turnover of protein. Each day 5-6g of protein / kg body mass are metabolised (De Feo et al., 1994) and that turnover process requires significant energy for both protein synthesis and breakdown (Welle and Nair, 1990). A normal healthy adult who consumes an adequate amount of protein in their diet should be in protein balance where synthesis and breakdown are matched and whole body protein mass remains constant (Wagenmakers, 1999). Under resting conditions there are two main controllers of protein turnover, 1) the availability of amino acids and 2) the presence of insulin. Hyperaminoacidemia, at rest, stimulates muscle protein synthesis (Bennet et al., 1989, Bennet and Rennie, 1991) and the provision of amino acids, either orally (Rasmussen et al., 2000) or via intravenous infusion (Bennet et al., 1990), can stimulate muscle protein synthesis due to the increased amino acid concentration of the blood. Bohe et. al. (2003) found that elevations in plasma essential amino acids (EAA) to 41% and 82% of basal levels resulted in an almost linear increase in muscle protein synthesis (20% and 40% respectively). However, increases above 82% in plasma EAA levels caused little further increase in rates of protein synthesis (Bohe et al., 2003). Hence individuals with an adequate intake of protein and the ability to metabolise that protein to amino acids are better able to amass protein and maintain protein balance.

Insulin's role in the regulation of protein metabolism was established by early investigations using animal, in vitro, models that demonstrated that insulin has a stimulatory effect on muscle protein synthesis (Manchester and Young, 1958, Jefferson et al., 1972, Pain and Garlick, 1974). Manchester and Young concluded that "insulin stimulates the transfer of the carbon of amino acids from extracellular fluid into protein" (Manchester and Young, 1958). These findings from the 1950's are supported by Jeffereson et. al. who in the 1970's, found that insulin induced a 2-fold increase in the rate of labelled phenylalanine incorporation into isolated rat psoas and gastrocnemius muscles (Jefferson et al., 1972). Support for the role of insulin in the stimulation of muscle protein synthesis has also been provided in more recent papers, with Kimball and colleagues (1998) suggesting that the mechanism of action is in part through insulin's regulation of the complex process of mRNA translation, where mRNA binds to the ribosomal subunit 40S, the beginning of the protein synthesis pathway (see (O'Connor et al., 2003, Kimball et al., 1998) for explanation of the pathways). These findings in an in vitro animal model concur with those by O'Connor et. al. (2003) who found similar results in a study which used the skeletal muscle of neonatal pigs.

Initial investigations into insulin's anabolic effect in humans were confounded by methodological dilemmas. Studies that infused insulin into subjects induced hypoaminoacidemia which counteracted the hypothesised anabolic effect of insulin (Tessari et al., 1991, Fukagawa et al., 1986, Tessari et al., 1986). Improvements in methods resulted in studies which maintained plasma amino acid levels while insulin infusion occurred and these studies provided the evidence that the effect seen in animals was also apparent in humans (Biolo et al., 1999, Biolo et al., 1995a, Bell et al., 2005). These studies are however potentially confounded by the infusion of amino acids because the increased availability may have stimulated muscle protein synthesis

directly. Regardless, in free-living humans or athletes, it is unlikely that insulin will ever be present in the absence of amino acids as normal feeding will stimulate both hyperinsulinemia and hyperaminoacidemia. Muscle protein synthesis that results from feeding is therefore most likely due to increased amino acid availability rather than increased insulin.

It is more likely that insulin's role is in the suppression of protein breakdown rather than the stimulation of protein synthesis. Insulin is a known inhibitor of lysosomal proteolysis (Moritmore et al., 1978, Furuno and Goldberg, 1986) which is the major pathway of membrane and glycoprotein breakdown. In 1970 Mortimore and colleagues showed that insulin blunts the proteolytic activity of lysosomes in the isolated rat liver (Moritmore et al., 1978). This effect concurs with findings by Furono et. al. (1986) who found that proteolysis in skeletal and cardiac muscle of rats is high in the absence of insulin and inhibited by adding a weak base which stops the action of the lysosomal proteases (Furuno and Goldberg, 1986). However, from a skeletal muscle turnover point of view there is some suggestion that insulin does not suppress myofibrillar proteolysis because it occurs via a pathway other than the lysosomal (Lowell 1986b). Incubation of rat skeletal muscle in the absence of amino acids or insulin caused a large increase in overall proteolysis as indicated by increased tyrosine production but did not cause any increase in the major marker of myofibrillar proteolysis, 3-methylhistadine (3MH) (Furuno et al., 1990). Therefore proteolysis was unchanged in myofibrillar proteins under those conditions indicating little or no effect of insulin on inhibition of skeletal muscle protein breakdown. Additionally, an earlier study by the same group found that the release of 3MH from rat skeletal and cardiac muscle was not changed by the infusion of insulin, although overall protein breakdown was suppressed (Lowell et al., 1986).

Studies in human subjects better support the role of insulin in the suppression of skeletal muscle proteolysis. WBPT studies in patients with diabetes provide evidence that the absence of insulin increases muscle protein breakdown. Nair and colleagues used ^{13}C-leucine to determine WBPT in post-absorptive insulin treated diabetic subjects and found that leucine flux increased when insulin was withdrawn indicating an increase in protein breakdown (Nair et al., 1983, Nair et al., 1987). Additionally, when insulin was replaced in insulin deficient subjects with diabetes, the leucine R_a decreased demonstrating an insulin induced restraint on proteolysis. Findings by Pacy et. al. (1989) also support this notion with plasma levels of isoleucine, leucine and valine, which make up a third of the content of skeletal muscle, significantly increased in subjects with diabetes when insulin was withdrawn (Pacy et al., 1989). The appearance of these amino acids is an indication of muscle protein degradation that would normally be suppressed by the action of insulin.

In healthy subjects infusion of insulin also causes a decrease in leucine R_a. Fukagawa et. al. (1998) found that plasma leucine levels were significantly reduced below basal levels in five, non-obese healthy, young men at four different infusion rates of insulin (Fukagawa et al., 1985). A 12% reduction in the R_a of leucine in response to insulin infusion in seven healthy male subjects supports the findings of the Fukugawa study (Bennet et al., 1989). One group of researchers have reported that whole body proteolysis is resistant to suppression by insulin but subjects were individuals with Type 1 diabetes who probably have an altered response to insulin's action (Tessari et al., 1991).

Evidence of regional skeletal muscle responses to insulin has been provided using the arteriovenous (AV) balance method (**Figure 2.13**). Briefly, the AV balance method involves blood sampling from catheters inserted into an artery and its corresponding vein (e.g. femoral

artery and vein). A tracer infusion occurs and the difference in amino acid levels between the artery and vein indicates net amino acid balance across the muscle in the local area. Muscle accounts for the majority of protein turnover that occurs across a limb because it makes up 60-70% of total limb volume. The other tissue structures (i.e. bone, skin and adipose) do not contribute a large amount to protein metabolism (Biolo and Wolfe, 1993) and thus determining the appearance of a tracer into the venous circulation gives an indication of local muscle protein breakdown.

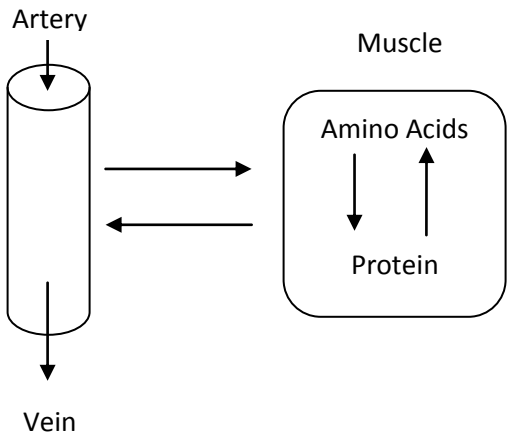


Figure 2.13: Three compartment model of muscle amino acid kinetics when using the AV balance technique. Free amino acid pools in the artery, vein and muscle are connected by arrows which indicate unidirectional amino acid flow between the compartments. Pictured adapted from (Wolfe and Chinks, 1992).

Gelfand et. al. (1987) found that infusion of insulin into the brachial artery of seven healthy subjects suppressed the release of phenylalanine and leucine from the forearm (Gelfand and Barrett, 1987). Fryburg et. al. (1995) also used phenylalanine across the forearm and found that there was a sustained decline in breakdown over a 6-hour study protocol when insulin and amino acids were infused (Fryburg et al., 1995). These findings are supported by a larger study of thirty-six subjects which found that insulin suppressed protein breakdown across the leg as

indicated by decreased appearance of leucine, phenylalanine and tyrosine (Nygren and Nair, 2003).

It is apparent that the two main controllers of protein turnover are the availability of amino acids and the presence of insulin. Hyperaminoacidemia seems to stimulate muscle protein synthesis while insulin inhibits proteolysis. The combined action occurs with feeding and is in part responsible for the anabolic condition that arises in the postprandial state.

2.6.1.2 Nutrition and protein turnover: Over a typical day, a person who consumes a weight maintaining diet will be in net protein balance. Daily net protein balance is accomplished despite periods of negative balance throughout the day in the post-absorptive state. These negative balance periods are counterbalanced by the positive balance that occurs after eating. Following the consumption of a protein containing meal, protein accretion occurs and the extent of accretion depends on the amount of protein and also the total energy content of the meal. To a certain extent, the more protein and energy in a meal the greater the protein gain.

Evidence that feeding increases protein synthesis is seen in work by Rennie et. al. (1982) that was amongst the first of its kind (Rennie et al., 1982). Rennie and colleagues used ^{13}C leucine to determine rates of whole body protein synthesis, amino acid catabolism and skeletal muscle protein synthesis in seven healthy men. They found markedly increased rates of whole body protein synthesis and muscle protein synthesis in the fed compared to the fasted state. Interestingly, they also showed that protein breakdown was not significantly different between fed and fasted conditions. Subsequent research by others have confirmed the original findings by Rennie and colleagues in a variety of subjects and using various stable isotope methodologies. Hoffer et. al. (1985) demonstrated that feeding was associated with an increase in protein

synthesis and a suppression of proteolysis after they used ^{13}C leucine and ^{2}H alanine and took blood samples after a period of fasting and then hourly after the consumption of 6 small meals over a 6-hour time period (Hoffer et al., 1985). Welle et. al. (1994) concluded that whole body protein synthesis is greater in both young and old subjects in the fed state compared to after an overnight fast (Welle et al., 1994). Interestingly, the findings of Welle et. al. also showed that even though ageing slows the rate of protein synthesis it does not impair the stimulatory effect of feeding. Tessari et. al. (1996), using the AV balance technique, found that meal ingestion stimulates forearm muscle protein deposition through enhanced protein synthesis and an inhibition of proteolysis (Tessari et al., 1996) and many muscle biopsy studies have also found that feeding leads to an increase in FSR (Rennie et al., 1982, Halliday et al., 1988, Welle et al., 1994, Cheng et al., 1987). Motil et. al. (1981) explored the effect of the amount of protein consumed on protein turnover. Subjects were fed surplus, marginal or inadequate protein and it was found that an increase in protein intake from marginal (06.g/kg) to surplus (1.5g/kg) was associated with increased incorporation of leucine into body protein (Motil et al., 1981b). In addition when protein intake was reduced from marginal to inadequate (0.1g/kg) there was a reduction in leucine flux and protein synthesis. These findings indicate that feeding stimulates protein synthesis and suppresses proteolysis. They also show that the quantity of protein within a meal has an effect on these processes.

After a meal the R_a of an amino acid reflects both proteolysis and the appearance of amino acids from the meal. In tracer studies where feeding occurs, proteolysis could be determined by simply subtracting the amount of consumed amino acid from the total R_a. The result would be the actual appearance of amino acid from proteolysis (Forslund et al., 1998, Boirie et al., 1997, Gibson et al., 1996). However, the suppression of proteolysis that occurs with

feeding can be overestimated by a calculation as such because it assumes that all the amino acids within the meal are absorbed through the gut as free amino acids and that each amino acid elicits the same insulin response, which they do not. The reality is that not all dietary proteins have the same fate. Additionally there is a splanchnic first-pass extraction of amino acids from the digestive tract and to this extent the dietary contribution to R_a is probably less than the total amino acid content of the food (van Goudoever et al., 2000). Overestimating the contribution of the meal results in an overestimation of how much that meal is able to inhibit proteolysis. The R_a in the postprandial state therefore needs to be corrected, not only for the amount of protein fed but for splanchnic extraction and for incomplete absorption that may occur (**Figure 2.14**).

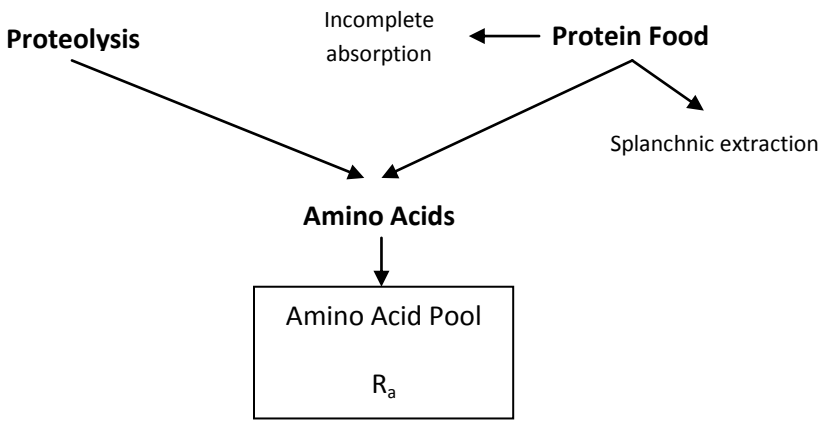


Figure 2.14: Appearance of amino acid into the free amino acid pool. The R_a of any essential amino acid in the postprandial state is due to proteolysis and supply of amino acids from food. Any calculation of proteolysis in the postprandial state must take into account splanchnic extraction and incomplete absorption as free amino acid

The appearance of an amino acid into the free amino acid pool, from protein breakdown, occurs less when food has been consumed. However, if that food is low in protein or protein free then the meal does not suppress R_a to the same extent (Gibson et al., 1996, Cayol et al., 1997).

This makes it appear that protein is required in a meal to cause the greatest effect in suppressing proteolysis. However, when estimations of splanchnic turnover are made, adding protein to a meal does not suppress proteolysis any more than a protein free diet (Cayol et al., 1997). Obviously it is the insulin response to the meal and not the protein content that has the inhibitory effect. In fact, a protein meal that increases the amino acid availability but does little to stimulate insulin release produces only a slight suppression of proteolysis (Boirie et al., 1996). These findings suggest that a meal has the ability to suppress proteolysis but the extent of that suppression is dependent on the protein and caloric composition and the extent to which hyperinsulinemia is stimulated.

There is no doubt that feeding contributes to protein kinetics and there is significant backing for the role of feeding in the stimulation of protein synthesis. The body of evidence using both whole body and regional skeletal muscle methodologies encourages the notion that feeding aids protein synthesis and thus emphasises the role of adequate nutrition in muscle protein turnover. For athletes, optimal nutrition is key to successful performance and despite the obvious benefits associated with increasing nutrient supply, more research into the amount, quality and timing of nutrition and the effect of these things on protein kinetics is required.

2.6.1.3 Effect of exercise on protein turnover: the effect of exercise on protein turnover has been widely studied because skeletal muscle makes up about approximately 40% of whole body protein mass (Rennie and Tipton, 2000). Resistance exercise, in the fed state, causes increased rates of muscle protein synthesis and breakdown, with the increased synthesis exceeding that of breakdown, resulting in an improved net balance (Phillips et al., 1997, Phillips et al., 1999, Biolo et al., 1995a, Chesley et al., 1992). The anabolic response is seen in both young and old (Balagopal et al., 2001, Yarasheski et al., 1993), male and female populations (Phillips et al.,

1997) and also following a single acute bout of resistance exercise (Yarasheski et al., 1993). Resistance exercise training also produces an anabolic response, although the effect is less pronounced with training (Phillips et al., 1999).

Aerobic exercise causes different physiological adaptations compared to resistance exercise, including an increase in mitochondrial volume, increased capillary density and improved activity of aerobic enzymes (Holloszy, 1967). These adaptive differences mean that aerobic exercise probably affects protein turnover differently as well. There is very little evidence about the effects of aerobic exercise on protein turnover but studies in rats show a decrease in protein synthesis after a single bout. The incorporation of ^{3}H-tyrosine into muscle was, on average, 18% lower in animals following a 1-hour swimming bout compared to rest (Dohm et al., 1980). Later findings by the same group concur, they found a 70% reduction in the incorporation of ^{13}H-tyrosine into muscle following a run to exhaustion also when compared to rest (Dohm et al., 1982a).

In human subjects several studies have found that there is an increase in protein degradation with aerobic exercise (Wolfe et al., 1984, Wolfe et al., 1982, Rennie et al., 1981, Phillips et al., 1993, Knapik et al., 1991) while others have found no protein degradation at all after exercise (Carraro et al., 1990b). Lamont and colleagues described the WBPT response to 1-hour of cycle exercise in 14 healthy, trained and untrained subjects (Lamont et al., 1999). They found that leucine oxidation increased during exercise in both groups and that during recovery leucine oxidation returned to baseline in untrained but dropped below baseline in trained subjects. Another study by the same group also established that leucine oxidation increased after a 1-hour bout of cycle exercise in untrained male and female participants (Lamont et al., 2003). Others have also found an increase in leucine oxidation with exercise using similar protocols to

the Lamont group which supports the notion that aerobic exercise is catabolic (McKenzie et al., 2000, Phillips et al., 1993, Rooyackers et al., 1996). One study however has produced contrary findings. Devlin et. al. (1990) found that leucine oxidation was decreased in comparison to rest after cycling cycling at 75% VO_2max (Devlin et al., 1990). However, the study does not report exercise duration but instead state that subjects exercised at the required intensity until exhaustion or until they were unable to maintain a cadence of 50rpm. Authors affirm that their exercise protocol was set to induce glycogen depletion but the duration is unlikely to have been longer than 1-hour as in the other studies because 75% VO_2max is above lactate threshold in most people, especially the untrained.

Protein breakdown can be measured by the rate of urea production because urea production indicates net protein degradation and oxidation. Several studies using ^{15}N-urea indicate that rates of urea production are unchanged and therefore there is no change in whole body protein synthesis or breakdown with aerobic exercise (Carraro et al., 1990b, Wolfe et al., 1982). These findings are contrary to those in a field-based nitrogen balance study that found increased urea production with prolonged aerobic exercise (Decombaz et al., 1979). The conflicting results may be due to vastly different exercise durations. Subjects in the latter study competed in a 100km running event and although exercise duration was not reported it would be reasonable to expect that subjects spent more than 8 hours exercising. 8-hours of outdoor running is very different to 4-hours and 105-minutes respectively of laboratory based exercise for the subjects in the studies which showed no change in urea production (Carraro et al., 1990b, Wolfe et al., 1982). Although, it is interesting to note that Wolfe et. al. (1982) found that leucine oxidation increased with 105-minutes of cycling exercise but urea concentration and production did not change (Wolfe et al., 1982). During exercise, urea production on its own may not

accurately reflect amino acid metabolism and thus should be used cautiously in such situations or in conjunction with a nitrogen balance type methodology. Amino acid oxidation has also been found to increase in proportion to the intensity of aerobic exercise (Rennie et al., 1981). The increase is thought to be caused by increased amino acid utilisation as fuel to support that the more intense exercise. Glycogen depletion that limits ATP production by glycolysis during prolonged and/or intense exercise is the likely reason for the use of protein as a fuel to support work. Wagenmakers et. al. (1989) found an increase in the activity of the enzyme, branched chain α-keto acid dehydrogenase complex, (responsible for leucine oxidation) when glycogen stores were depleted. (Wagenmakers et al., 1989, Wagenmakers et al., 1991). Glycogen depletion was not measured in the 100km run study mentioned previously but it can be assumed that significant glycogen depletion would have occurred during such strenuous exercise. As such the glycogen depletion may explain why urea production increased in the 100km run study but did not change in the others. It could however be argued that all three exercise protocols had the potential to be glycogen depleting. Perhaps instead of either exercise duration or exercise intensity being the stimulus for increased protein oxidation, it may be that aerobic exercise that induces depletion of glycogen stores or an inappropriate EI that does not replenish those stores may be the cause of the response.

It is important to note that many of these studies are laboratory based and do not give ‘free living’ results. It is likely that athletes, during their normal training, would consume some form of nutrition either before, during and/or after training. Studies assessing the effect of aerobic exercise on protein turnover, for the benefit of athletic populations, need to consider this point when designing methods. In fact, a study by Koopman et al. (2004) examined eight endurance athletes in an environment that matched their normal training environment which

meant supplying nutrients throughout the rest and exercise protocols (Koopman et al., 2004). Subjects were studied at rest and after 6-hours of exercise with a carbohydrate or carbohydrate and protein supplement provided at half hourly intervals. Findings showed that 6-hours of aerobic exercise caused an increase in leucine oxidation suggesting the exercise is catabolic despite the provision of nutrients.

Skeletal muscle methodologies more clearly show the effect of aerobic exercise on protein turnover than do whole body techniques. These methodologies use stable isotope protocols and sample, by needle biopsy, skeletal muscle to determine the fractional synthetic rate (FSR) (Bergstrom, 1975). FSR is usually determined by the rise in tracer enrichment in the muscle bound amino acid over a set time period, divided by the tracer enrichment in the precursor pool. Therefore, FSR is the percentage of the existing pool that has been synthesised into muscle or protein over that time period (Wagenmakers, 1999). The acute effect of aerobic exercise on skeletal muscle protein turnover has been studied under fasting conditions (Carraro et al., 1990b, Sheffield-Moore et al., 2004, Tipton et al., 1996). Carraro et. al. (1990) found an increase in mixed muscle FSR in young subjects during a 4-hour recovery period which followed 4-hours of treadmill walking at 40% VO_2max (Carraro et al., 1990b). Sheffield-Moore et. al. (2004) also found that treadmill walking increased FSR (Sheffield-Moore et al., 2004). 45-minutes of treadmill walking at 40% VO_2peak increased FSR in both older and younger men and the response was evident 10-minutes post-exercise, and remained elevated until 60-minutes post-exercise in young subjects.

A study by Miller et. al. (2005) utilised a one-legged kicking model to determine muscle and collagen synthetic rates (Miller et al., 2005). Findings concur with those previously described. Rates of protein synthesis, specifically myofibrillar and sarcoplasmic fractions,

increased in the exercised leg by 6-hours post exercise. The synthesis rates peaked by 24-hours and by 48-hours post exercise had fallen slightly but still remained above the resting level. One point of difference with the one-legged kicking study is that subjects were provided with energy in the form of a nutrient drink given intermittently throughout the protocol. Knowing that caloric intake and amino acid supply play a role in protein synthesis it could be argued that the increased FSR was in fact due to the EI as opposed to the exercise bout. However, Miller et. al. (2005) believe that "any observed alterations were not the result of variation in nutritional state" because of steady plasma leucine and insulin levels at the 24-hour and 48-hour time periods of the study. Regardless, athletes are unlikely to perform exercise in the absence of nutrients and thus Miller et. al. (2005) have provided findings that are more applicable to athletic populations than the other studies mentioned.

Contrary to the findings already described, Tipton et. al. (1996) found that a 1.5 hour, 4.6km intermittent training session did not change FSR in well trained swimmers (Tipton et al., 1996). However, these findings in swimmers are difficult to compare because the study looked at an acute bout of exercise in already well-trained athletes who perform similar sessions on a daily, if not twice daily, basis. The stimulus for change may have been absent in this group of athletes because the exercise could be considered normal and thus no changes would ensue. Investigations using trained swimmers need to be weary of the possibility that these athletes may have more efficient protein turnover rates simply due to the volume of exercise they perform and thus changes may be difficult to see if normal training loads are used.

On the whole it appears that research from muscle biopsy studies indicate that an acute bout of aerobic exercise induces an increase in FSR. These findings are in contrast to those using WBPT methodologies which suggest aerobic exercise is in fact catabolic. However, comparison

of the two groups of findings are difficult due differing exercise modalities and durations. Additionally, the determination of FBR from muscle biopsy studies would be beneficial to understand the entire process of protein turnover. Further evidence is required before conclusions can be made regarding protein turnover under acute aerobic conditions.

The effect of an aerobic training programme on skeletal muscle protein turnover is even less well known than the response to acute aerobic exercise. Short et. al. (2003) found that 4-months of aerobic cycling training resulted in an increase in FSR at rest but no change in WBPT (Short et al., 2003a). The study was performed in seventy eight, previously untrained men and women who exercised 3-4 times each week for up to 45-minutes at 80% peak heart rate. A more recent investigation in previously untrained subjects, who participated in 4-weeks of aerobic exercise training demonstrated that training induced an increase in FSR at rest, a decreased net protein balance and a trend toward an increase in fractional breakdown rate (FBR) ($p = 0.06$) (Pikosky et al., 2006). These two studies had similar increases in FSR (22% and 17% respectively) despite differing training periods and similar improvements in measured VO_2peak. The increase in synthesis of mixed muscle proteins that seems to occur with aerobic training may be a result of an increase in proteins that are responsible for the adaptations associated with this type of training. For example, aerobic training increases mitochondrial density and enzyme activities as well as increasing capillary density (Gollnick and Saltin, 1982, Hoppeler, 1986). Neither Short et. al. (2003) nor Pikosky et. al. (2006) provided data to support this hypothesis, but a separate study by Short et. al. did find increased glucose transporters GLUT-4 mRNA along with mitochondrial biogenesis after training indicating an avenue for the increased protein synthesis (Short et al., 2003b).

The effect of training on nitrogen balance suggests that there may be a period of adaptation of protein balance to increasing exercise training. Gontzea et. al. (1975) showed that individuals on a diet containing a normal protein intake (1g protein/kg/day) moved into negative nitrogen balance when they began a training programme (Gontzea et al., 1975). The negative nitrogen balance resulted despite adequate energy being supplied to compensate for the increased EE. These findings suggest that increasing the protein content of the diet when initiating a training programme could improve the negative protein balance, if total energy requirements are being met. However, if extra protein is not included in the diet, physiological adaptation should occur within about 2 weeks and the negative protein balance will be rectified if energy balance is maintained (Brooks et al., 2005).

The need for further investigation into the effects of aerobic exercise on protein kinetics, both acutely and with training, is clear. Care needs to be taken when designing athletic studies to ensure that results are meaningful practically to athletes with more emphasis on free-living studies instead of tightly controlled laboratory investigations. Extreme endurance exercise stresses normal metabolic processes and is likely to have a dramatic effect on skeletal muscle and WBPT. Research that is designed to identify the mechanisms through which endurance exercise causes alterations in protein turnover, if at all, will help exercise scientists to formulate evidence based guidelines for endurance exercise and training.

2.6.1.4 Nutrition and exercise interaction: The reality of sport and optimal sports performance is that any effect of exercise cannot be considered in the absence of caloric intake. Adequate dietary intake is essential for adaptation to training and knowing the optimal amount of food, the optimal macronutrient make up and when it is best to eat that food allows optimisation of energy conditions. With respect to protein the question that is often asked is “Do athletes

require additional protein in their diet compared to non-athletes?" The evidence available to answer this question is scarce but it is clear that energy availability regulates protein kinetics during exercise. For example, the positive net protein balance that results from resistance exercise can be reversed in the absence of feeding. In such a case, protein breakdown can exceed protein synthesis, causing a net negative protein balance (Biolo et al., 1995b). Hence, resistance exercise needs to be coupled with an adequate EI to be anabolic. In this regard, the answer to the question would be 'no' because it is additional total calories that are important and not simply additional protein. In fact Butterfield and Calloway (1983) have suggested that athletes may need less protein than normal healthy individuals because training adaptations provide for more efficient protein utilisation (Butterfield and Calloway, 1983).

The current recommended daily allowance (RDA) for protein and amino acid intake varies worldwide between 0.8 and 1.2g protein/kg/day for the general population (Jeukendrup and Gleeson, 2004). The American Food and Nutrition Board (AFNB) have released dietary reference intakes (DRIs) for protein of between 10% and 35% of total daily energy. This means for a 70kg male the AFNB recommends anywhere between 0.89 to 3.1g protein/kg/day (Macronutrients, 2005). There remains the perception that athletes, both endurance and strength based, require a greater amount of daily protein than the general population due to evidence from WBPT studies that shows an increase in leucine oxidation with exercise (Phillips et al., 1993, Tarnopolsky et al., 1992, el-Khoury, 1997). As such, strength and speed athletes are recommended to consume 1.2-1.7g protein/kg/day and endurance athletes about 1.2-1.4g protein/kg/day (Tipton and Wolfe, 2004). However there is evidence which suggests that the development of protein intake recommendations from studies that focus on the kinetics of a single amino acid could be incorrect. Work by Wolfe and colleagues demonstrates that the fate

of leucine may not be the same as for other amino acids and therefore focussing on leucine metabolism as a descriptor of complete protein metabolism could be presumptuous (Wolfe et al., 1984). Wolfe et. al. (1984) found from the infusion of ^{15}N and ^{13}C lysine and ^{15}N and ^{13}C leucine that not only was the increase in lysine oxidation smaller with exercise compared to leucine, but the relationship between the labels (^{15}N and ^{13}C) was different for leucine compared to lysine. In support of the notion that athletes do not require higher levels of protein in their diet to sustain positive protein balance, Owen et. al. (1992) found that nitrogen retention increased as calorie content of a meal increased. These findings occurred with a stable protein content of 15% at each caloric intake (Owen et al., 1992).

The macronutrient composition of any nutrition is however an important consideration. It is known that addition of a carbohydrate supplement after resistance exercise does not have any effect on muscle protein synthesis. However, a decrease in 3MH and urea excretion is evident with a post-resistance exercise carbohydrate supplement suggesting a suppressive effect on proteolysis when carbohydrate is given (Borsheim et al., 2004, Roy et al., 1997). Ingestion or infusion of a combined carbohydrate and protein or amino acid source produces an increase in the rate of protein synthesis, a reduction in protein breakdown and a positive net protein balance after resistance exercise (Biolo et al., 1997, Borsheim et al., 2002, Rasmussen et al., 2000, Koopman et al., 2005). The improvement in net balance that is seen in these studies is the result of providing amino acids as precursors for protein synthesis, the combined ingestion of protein and carbohydrate which causes a hyperinsulinemic response and proteolytic inhibition, and the overall anabolic effect of resistance exercise.

Under aerobic exercise conditions Koopman et. al. (2004) demonstrated that net protein degradation does not increase during 6-hours of activity, performed at 45% of maximal

workload, when carbohydrate (0.7g/kg/hr) is ingested (Koopman et al., 2004). The same study also found that whole body protein synthesis at rest, during and after exercise, increased when a combined protein and carbohydrate supplement (0.7g CHO/kg/hr + 0.25g PRO/kg/hr) was given compared to when a carbohydrate only supplement was given. Additionally, a positive net protein balance was observed with the combined ingestion of carbohydrate and protein, but the net balance was negative with the carbohydrate only supplementation. Whether the positive net balance findings in the combined supplement were a result of the addition of the protein to the supplement or simply the effect of the added calories is unclear.

Of the 20 amino acids only the branched chain amino acids (BCAA'S) leucine, isoleucine, valine along with alanine, glutamate and aspartate are thought to be oxidised at a substantial rate in skeletal muscle (Goldberg and Odessey, 1972). BCAA's make an ideal supplement for athletes because they are taken up by skeletal muscle during exercise while the other amino acids are taken up by the liver (Ahlborg et al., 1974). Well-trained endurance athletes consumed a mixture of BCAA's (7.5g, consisting of 35% leucine, 50% valine and 15% isovaline) during a 30km cross country running event and during a marathon (12g, consisting of 35% leucine, 40% valine and 25% isovaline) (Blomstrand and Newsholme, 1992). Both plasma and vastas lateralis levels of BCAA's increased due to supplementation. Muscle levels of BCAA's decreased and plasma levels remained unchanged in the control group who did not receive the supplement. A negative net protein balance in the control group is assumed due to a 20% to 40% increase in tyrosine and phenylalanine levels in the muscle during both exercise bouts and an increase in plasma levels of both amino acids after the marathon. The increased level of these amino acids after exercise points to an increase in protein degradation when no BCAA's were given.

During training and competition endurance athletes exercise for many hours at high workloads and cause substantial depletion of glycogen stores and thus possible reliance on gluconeogenic amino acids for energy production. Gluconeogenesis must have an effect on protein turnover which means that understanding how to prevent or suppress it is an important goal for future research. Because protein supplies amino acids, anecdotally it might seem appropriate to recommend a high level of protein for endurance athletes in an effort to prevent the possible negative effects of gluconeogenesis. However, making appropriate recommendations about protein requirements for endurance athletes is currently haphazard as research is limited.

The limited evidence available on the effect of feeding on protein turnover, accompanied by the varying methodologies utilised by researchers in this area has led to protein intake guidelines for athletes that may not be appropriate. Further research into the timing of nutrition in relation to exercise, and the most suitable caloric value and macronutrient composition of that nutrition is required to minimise protein losses and optimise athletic performance, while maintaining a metabolically appropriate state.

The timing of nutrient ingestion may be slightly outside the scope of this review but it is possible that it is not the absolute amount of protein or calories that an athlete consumes that is important when considering gluconeogenesis but the timing of that intake around exercise. Replenishment of muscle glycogen following endurance exercise can be maximised by nutritional intake immediately post exercise (Costill et al., 1981, Ivy et al., 1988b, Ivy et al., 1988a). Additionally, the consumption of carbohydrate immediately after resistance exercise can increase whole body protein synthesis (Roy et al., 2000), potentially increase muscle protein synthesis (Roy et al., 1997) and decrease myofibrillar protein degradation (Roy et al., 1997). The

ingestion of protein or mixed carbohydrate and protein supplements immediately post resistance exercise will enhance the anabolic effect of that exercise. (Biolo et al., 1997, Tipton et al., 1999, Rasmussen et al., 2000).

The first study to investigate the effect of timing on protein metabolism after endurance exercise was performed in dogs (Okamura et al., 1997). Dogs were infused, either immediately or 2-hours post treadmill exercise, with an amino acid and glucose solution. A net protein breakdown was seen in the pre-exercise and exercise periods with a positive net balance achieved with nutrient infusion. Muscle amino acid uptake and protein synthesis was greater when the infusion occurred immediately as opposed to 2-hours post exercise.

In human subjects, Levenhagen and colleagues studied the effects of a carbohydrate and protein supplement on protein turnover after a 60-minute, moderate bout of exercise on a cycle ergometer (Levenhagen et al., 2001). The supplement was given either immediately or 3-hours after exercise and findings were that protein degradation was unaffected by timing but that protein synthesis increased when the supplement was given immediately post exercise. Additionally, there was no increase in protein synthesis when supplementation took place 3-hours post exercise. Roy et. al. (2002) performed a study which supports the findings of Levenhagen. They used a nitrogen balance method to determine the effect of timing of a supplement on endurance athletes exposed to an increased training volume (Roy et al., 2002). Subjects consumed a mixed nutrient supplement with additional carbohydrate or nil energy value placebo supplement which tasted the same as the experimental supplement immediately after a bout of exercise. Consumption of the mixed nutrient supplement resulted in positive nitrogen balance while those who consumed the placebo supplement were in negative nitrogen balance. The difference in nitrogen balance between the two groups was not statistically significant

despite a strong trend (p = 0.06). It could be argued that the results were due to the provision of nutrients alone and not the timing of that provision and therefore future research into the optimal timing of nutrients around exercise is warranted

2.6.2 Energy Balance

For most adults, body weight remains relatively stable over a number of years. The human body thus conforms to the 1st law of thermodynamics in that if food intake matches EE than there is no change in body mass and the person is said to be in energy balance. Energy balance can be impaired to cause weight loss if: 1) calorie intake is reduced with no change in activity status, 2) EE is increased with no increase in intake to compensate or 3) calorie intake is decreased and EE is increased. **Figure 2.15** depicts the relationship between energy nutrients into the body and EE by the body. The concept of energy balance is simple, when energy input equals energy output then a person is in energy balance.

The contribution of physical activity to energy output is significantly increased in endurance athletes and during an event such as the Tour de France cycle race, daily EE has been measured as among the highest recorded in human subjects. The extremely high energy demand required to undertake an event like a cycling tour can mean that maintaining energy balance is a challenge. Westerterp et. al. (1986) performed a landmark study with DLW to calculate EE in Tour de France cyclists (Westerterp et al., 1986). EE was found to be between 29.4 MJ/day and 36 MJ/day. There must exist a ceiling to TEE in humans and it is possible that this ceiling is set by maximal daily EI. Kirkwood et. al. (1983) reported EI scaled to body weight across a variety of animals where EI equated to 3 to 6 times RMR. Elite endurance athletes have a very large TEE and are at the extreme end of measured human EI, but are in line with the norm for various animals (Kirkwood, 1983). Indeed, the above findings in Tour de France cyclists indicated that

average daily EE was 3.6 – 5.3 times RMR (Westerterp et al., 1986) or within the range predicted by the study of Kirkwood. At such extreme levels of EE a significant amount of food is required to maintain energy balance and it has been speculated that it is difficult to ingest and digest enough food when prolonged exercise leads to EE in excess of 20 MJ/day (Brouns et al., 1989b, Brouns et al., 1989a). It has been suggested that food intake may be the limit to exercise performance in ultra endurance events because performance is dependent on the ability to maintain energy balance and muscle mass.

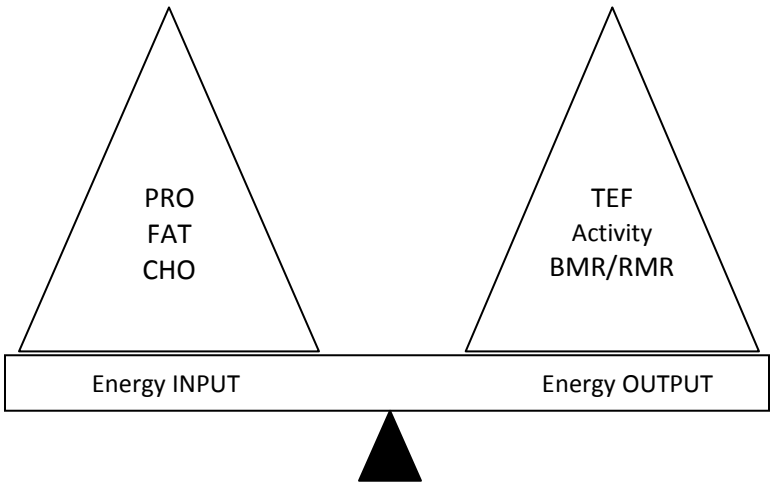


Figure 2.15: Energy Balance. Energy balance is a summation of energy input, in the form of protein (PRO), fat and carbohydrates (CHO) and energy output in the form of the thermic effect of food (TEF), activity EE and basal and resting metabolic rates (BMR/RMR).

Several researchers have measured energy balance during intense endurance exercise. Male cross-country skiers expend a similar amount of energy as Tour de France cyclists and are able to maintain energy balance despite the extreme energy cost (Sjodin et al., 1994). EE over a week of skiing training ranged between 25.4 MJ/day and 34.9 MJ/day with a corresponding EI of between 25.7 MJ/day and 36.0 MJ/day. It is interesting, and perhaps atypical, that energy

balance was maintained under these training conditions in light of such high EE. In fact, swimmers who expended on average 23.4 MJ/day ± 2.1 MJ/day over a week of increased training consumed a mere 13.1 MJ/day ± 1 MJ/day of energy leaving them in energy deficit by approximately 43% (Trappe et al., 1997). The cause of the discrepancy between EE and EI in swimmers was likely to be in part due to some under-reporting of food intake. Swimmers were all female, and it is common for under-reporting to occur in female populations. However, the sheer quantity of food that would need to be eaten to maintain an EE of greater than 20MJ/day is also likely to have been a cause of the negative balance.

It is not only at extremely high energy outputs that endurance athletes find it difficult to maintain energy balance. Hill and Davies (2002) found that female rowers expended on average 16.56 MJ/day ± 5.1 MJ/day for a concurrent EI of just 9.27 MJ/day ± 13 MJ/day (Hill and Davies, 2002). Hill and Davies (2002) also found that rowers were unable to maintain body weight over this time with a decrease of 1.2 kg on average. Under-reporting of food intake was indicated to be a confounding factor in the findings but the reduction in body mass indicates that actual EI was less than that required to maintain energy balance. In a more recent investigation, male endurance runners, during a period of high volume training, had a moderate EE of 14.6 MJ/day ± 1.0 MJ/day (Fudge et al., 2006). The runners were in slight energy deficit during the training period, consuming 13.2 MJ/day ± 1.3 MJ/day. There was no change in mean body weight but reported individual body weights show that only two subjects out of nine were able to maintain body mass. The findings by Fudge et. al. (2006) are consistent with those found in endurance runners in other parts of the world. Japanese endurance runners had a reduction in body weight after an 11-day training period where EE was estimated at 18.9 MJ/day ± 3.1

MJ/day and EI was well below maintenance levels at 15.8 MJ/day ± 0.3 MJ/day (Motonaga et al., 2006).

2.6.2.1 Energy balance and protein balance relationship: The physiological effect of negative energy balance on protein metabolism and therefore the ability to perform work has been an area of interest since the early 1900's. The initiative for such research came from the famines that occurred from war and other periods of upheaval that occurred during the early part of the 20th century. There is an established relationship between energy balance and the ability to maintain protein stores in the body (Calloway and Spector, 1954). Nitrogen and therefore protein balance cannot be maintained if EI is insufficient and feeding protein to extreme levels (6.9g protein/kg) is of little benefit to nitrogen balance when total calories are insufficient.

Butterfield and others have demonstrated that energy balance is a major factor in the maintenance of nitrogen balance and lean mass (Butterfield and Calloway, 1983, Todd et al., 1984). In pioneering work, Butterfield and Calloway emphasised that nitrogen balance depends on having both adequate protein and total energy in the diet (Butterfield and Calloway, 1983). Energy from non-protein sources can maintain nitrogen balance even when protein content is low, as long as the calories are sufficient to maintain energy balance. Findings from a study by Gater et. al. (1992) concur with this concept. Three dietary conditions were applied to resistance trained individuals over a 10 week period (Gater et al., 1992). One group trained with no dietary changes, the second group was given an amino acid supplement (66mg/kg fat free mass/day of both arginine and lysine) and the third group consumed one to one-and-a-half cans of a commercial dietary supplement daily in addition to their normal diet to increase their total EI. The absolute increase in fat free mass was significantly greater under the third dietary condition of positive energy balance compared to the increase in the other two groups. These findings

support the view that increased energy consumption sustains nitrogen balance, or in this case improves nitrogen balance, more so than an increase in amino acid availability. Butterfield et al also concluded that changing EI from deficient to weight maintaining, promoted nitrogen retention (Todd et al., 1984). This effect was more evident in active rather than sedentary men who were consuming a diet containing adequate amounts of protein (0.8g protein/kg).

Consumption of adequate protein with low total dietary calories can have a negative impact on protein balance. Synthesis of muscle is an energetic process requiring approximately 340kcal/day (or approximately 20% of resting metabolic rate) for the average adult male (Welle and Nair, 1990). Total energy in the diet is therefore a key factor in determining the rate of muscle protein synthesis. Giving a high calorie diet with no protein content causes a standard net negative balance of -7gm/day for diets ranging from 700kcal/day all the way up to 2800kcal/day (Calloway and Spector, 1954). Conversely, giving a high amount of protein with low total calories does not improve the negative balance. However, in cases where there is a caloric deficit, nitrogen balance is less negative when protein content of the diet is high (Hoffer et al., 1984, Calloway, 1975). In starvation, when both amino acids and energy are sparse, the gluconeogenic amino acids are released from muscle protein as precursors for glucose production (Ruderman, 1975). Subsequently, loss of protein mass occurs as protein stores within the body are degraded in order to maintain energy production. Amino acid levels in the blood and total EI are thus both important regulators of muscle protein turnover. These findings give weight to the idea that athletes possibly do not need to consume high amounts of protein to maintain body weight and lean mass if total calorie intake is adequate. In fact it has been was show that feeding higher energy meals at a constant protein level results in net protein retention in the fed state when energy was in excess to maintenance requirements (Motil et al., 1981a).

A comprehensive study by Friedlander et. al. (2005) examined the energy and protein balance relationship using nitrogen balance and WBPT methodologies (Friedlander et al., 2005). The aim of the research was to investigate the response of men to 21 days of negative energy balance which was defined as 40% less calories than their normal diet. The primary finding was that there was a reduction in leucine kinetics during exercise in response to caloric restriction but despite this, nitrogen balance did not improve over the 18-day study period. There was also a resultant loss of fat free mass that was equivalent to 50% of the total body weight lost and resulted in a reduction in arm curl endurance capacity. The reduction in body mass had little effect on aerobic exercise performance suggesting that the caloric restriction altered protein metabolism in a way that allowed for preservation of performance capacity.

The key to understanding energy balance is simply that if athletes are able to consume food in amounts equal to their EE then they will be in energy balance. In ultra endurance events or high volume training periods it may be that EI is the limiting factor in the achievement of energy balance. In addition is seems that the relationship between energy and protein is that if energy balance is achieved then protein retention is also achieved regardless of the level of protein in the diet. Negative nitrogen or protein balance is better tolerated when an athlete is in energy balance but feeding high levels of protein with inadequate total energy will not improve protein retention.

2.7 Overtraining

The overload principle of training states that; physiological adaptation will only occur when an athlete experiences stress that is greater than that to which they are accustomed. In

addition, in order for an athlete to adapt to training they must have adequate recovery from each training stimulus. Too much training in conjunction with inadequate recovery can yield less than optimal performance improvements and in severe cases a breakdown in the physiological adaptation processes (Budgett, 1998, Ketner and Mellion, 1995). The overtraining syndrome can occur and is characterised by stagnation or decrement in athletic performance following a state of prolonged fatigue and requires significant rest for recovery (Fry et al., 1991). Individual athletes have varied recovery capacities, training responses and stress tolerance and therefore a training regime that induces physiological adaptation and improvement in one athlete may be the cause of overtraining in another. The concept of varied susceptibility is key in understanding overtraining and in its prevention and is illustrated in swimmers and runners who traditionally train in squads where training is not necessarily individually tailored. Overtraining has been found in 10% of college swimmers and in 60% of endurance runners at some point in their career (Morgan et al., 1987a, Morgan et al., 1987b).

A range of physiological, biochemical and psychological measures have been investigated as possible markers of the overtraining syndrome but concrete recommendations are still not available mainly due to the ethical issue around inducing overtraining in an athletic population. In addition, different sports have different training requirements that make identification of general markers unlikely. Fry et. al. (1991) provide a comprehensive list of parameters that have been investigated as markers of overtraining but acknowledge that no marker alone can be predictive and in fact it is likely that it is a combination of a number of markers appearing at the same time that best predicts or diagnoses an overtrained athlete (Fry et al., 1991). Hooper and Mackinnon suggest methods for preventing the overtraining syndrome that include (Hooper et al., 1995):

- Identification of susceptible athletes
- Minimising known causes such as sudden increase in training load, lack of recovery between seasons and poor nutrition
- Individualising training
- Programming recovery as an integral component of training
- Monitoring athletes for early warning signs

The monitoring of athletes for early warning signs is possibly a difficult task due to the lack of consensus about what the early warning signs are and especially because athletes tend to ignore symptoms such as fatigue and heavy muscles until performance has already started to decline (Dyment, 1993).

The psychological stress that can occur in a person's life can lead to considerable fatigue and has been implemented in the overtraining syndrome. The Profile of Mood State (POMS) questionnaire has been used widely as a marker of overtraining and has had significant success in identifying at risk athletes (Morgan et al., 1987a). The POMS questionnaire provides a score for psychological mood state as well as scores for the individual mood states of fatigue, anger, tension, confusion, vigor and depression (Raglin and Morgan, 1994). A mood score is determined by adding the five negative mood states and subtracting the only positive mood state, vigour (Fatigue + Anger + Tension + Confusion + Depression – Vigour). Morgan's Mental Health Model proposes that an athlete who is performing well and is not at risk of overtraining will have a negative mood score and display a typical 'iceberg profile' (Morgan et al., 1987a). The iceberg profile, depicted in **Figure 2.16**, provides an easy method of interpreting the mood data available from the POMS and is also easy to understand and administer.

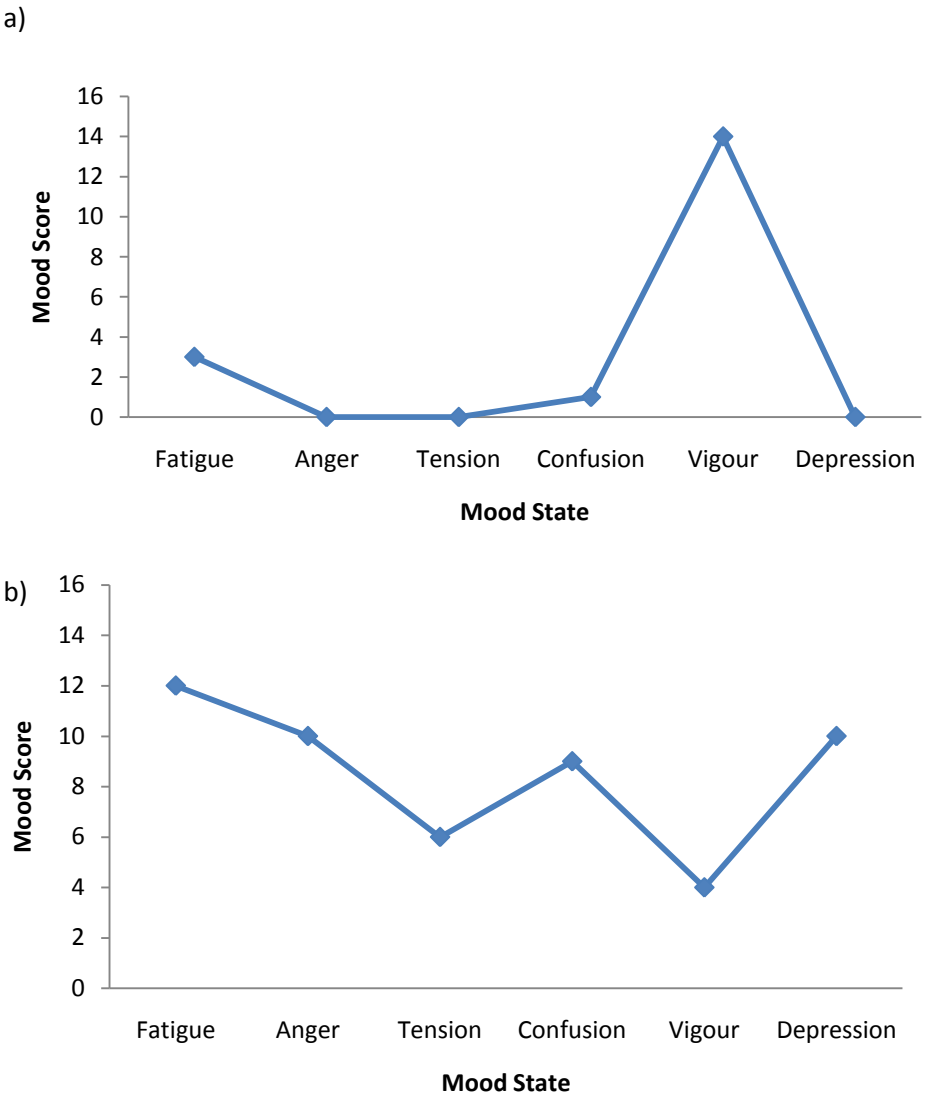


Figure 2.16: Morgan's Mental Health Model (the iceberg profile), An athlete who is performing well and is not at risk of overtraining will display an iceberg profile (a). The profile depicts low scores for the five negative mood states of fatigue, anger, tension, confusion and depression and a high score for the singular positive mood state, vigour. An athlete who is at risk or who is overtrained has high scores for the negative mood states and a low score for vigour which alters the profile and removes any distinguishable peak (b).

The POMS questionnaire provides a practical method for understanding how changes to training load affect an athlete and there is a general consensus that sudden increases in volume and/or intensity have a negative effect of mood state (Morgan et al., 1987a, O'Connor et al.,

1989, O'Connor et al., 1991). The POMS questionnaire was chosen as an indicator of overtraining in this thesis because of the volume of research that supports its application. Morgan et. al. performed studies on over 400 swimmers between 1975 and 1986 that showed that mood disturbance increased with step-wise increases in training volume (Morgan et al., 1987a). In addition, they found that following a two week period of taper, mood disturbance improved.

Elevated cortisol levels in the body have also been targeted as a marker of overtraining because the hormone is commonly released in response to stress. The theory that increased cortisol may be a marker for overtraining relates to its action on target tissues and also that increased levels are associated with some of the common symptoms of the syndrome including; weight loss, muscle soreness and weakness, insomnia, depressed mood and increased susceptibility to illness (**Figure 2.17**) (Axelrod and Reisine, 1984, Biglieri, 1989). Although there is no agreement in the overtraining literature as to the role of cortisol, the hormone has shown to be elevated in overtraining research (O'Connor et al., 1989, Kirwan et al., 1988). Irrespective of overtraining, chronic elevated levels in an athlete are indicative of stress and cannot be beneficial for performance. The inclusion of cortisol as a biological measurement in this thesis is to complement the POMS information and also to determine if any potential negative energy or protein balance changes are associated with physiological stress.

It is not the intent of the research contained within this thesis to gain a better understanding of the overtraining syndrome. The inclusion of overtraining as a thread throughout the research is in an effort to identify if energy and/or protein kinetics play any part in the decline in performance that is seen in athletes progressing into an overtrained state. The complexities of the overtraining syndrome are not described nor discussed in detail.

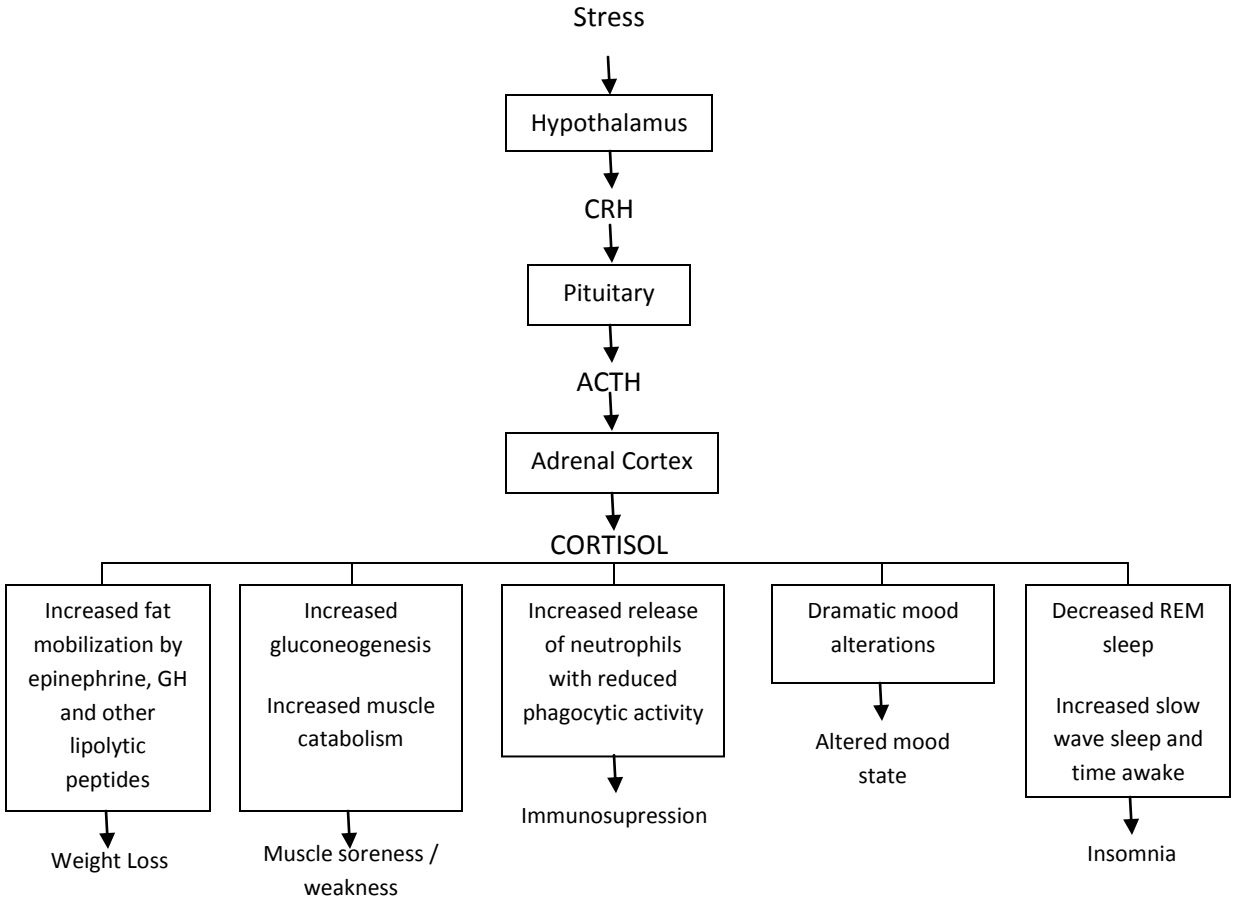


Figure 2.17: Cortisol release in response to stress. Stress induced cortisol release via the hypothalamic-pituitary-adrenal axis and the tissue specific responses leading to symptoms typical of the overtraining syndrome. CRH = corticotrophic hormone, ACTH = adrenocorticotrophic hormone, GH = growth hormone, REM = rapid eye movement.

2.8 Summary

Endurance athletes expend large amounts of energy both during training and while competing. The large EE needs to be matched with adequate food intake to ensure availability of substrates to fuel the metabolic machinery. Endurance athletes therefore need to maintain a balance between EE and food intake for optimal performance capacity. In addition, maintenance of a stable protein mass is essential and a balance between amino acids available for protein synthesis and amino acids released from protein breakdown is required for performance.

It is clear that the total number of calories in the diet is the important factor in maintenance of both energy and protein balance. If total calories are sufficient to maintain energy balance than an athlete is better able to retain protein stores even if protein intake is not optimal. The hyperinsulinemic response that occurs after eating inhibits proteolysis and is in part responsible for the retention of protein at adequate EIs and interestingly the inhibition of proteolysis occurs even when protein is absent from a meal. The addition of protein to a meal causes hyperaminoacidemia which stimulates protein synthesis and when coupled with the hyperinsulinemic response has a greater effect on retention of protein stores than either response on its own. If total energy is the important factor in the retention of protein mass then it is questionable whether endurance athletes require a protein intake higher than the general population. The catabolic effect of endurance exercise must be considered when it comes to making protein recommendations for athletes but it is still likely that the total EI is more important than the protein content of the diet in the maintenance of protein stores.

The ability to maintain energy balance is therefore an important consideration for endurance athletes. There is some evidence that suggests that it is difficult to consume enough calories when EE is in excess of 20MJ/day. In fact it may be that EI, not excessive EE, limits

maintenance of energy balance. However, in studies with cyclists at least, EE has been estimated at between 25MJ and 30^{+}MJ/day with equivalent EI values in most athletes. The accuracy of EE and intake data is of extreme import and therefore comes down to the methodological approach used.

The use of stable isotopes in metabolic research brings about an accuracy that may have been lacking in earlier investigations of EE and protein balance. The doubly labeled water technique is the gold standard for determination of EE and can also be used to validate dietary recording methods which are prone to inaccuracies. ^{13}C-leucine, as a tracer of the fate of amino acids, is an approach that has been used since the early 1980's to determine whole body and skeletal muscle protein kinetics and despite the assumptions associated with the method is an accepted protocol for protein turnover research. The traditional nitrogen balance technique is still an established method for estimation of protein balance. There are errors associated with its use but an understanding of those errors and knowledge of how to limit the bias associated with them means that the method is still valid despite the advent of stable isotopes. Nitrogen balance is also less invasive and therefore may be a sound option for metabolic research in athletic populations. To complement these techniques an accurate method for estimating EI is crucial. The supervised weighed diet record is the best method to ensure accuracy of food intake information as it eliminates under-reporting and takes the onus off the subject to remember and record their food intake. There is still the possibility that the supervised weighed recording of food may alter a subject's habitual food intake but the bias is associated with all dietary recording protocols and is probably impossible to eliminate.

Endurance athletes, with their significant training loads and frequent competition, need to train for optimal physiological adaptation but also ensure that they have adequate recovery to

prevent overtraining. There is limited evidence for the role that energy balance, or more correctly negative energy balance, plays in the development of overtraining. It is possible that poor nutrient intake functions in some way to progress an athlete from over-reaching to overtraining but this relationship is not clear and has not been extensively investigated. In addition, the relationship between negative protein balance and overtraining has also not been developed. There is unlikely to be a clear relationship between either energy or protein balance and overtraining because of the complexity of the condition. However, improving the information available may provide valuable insights to assist in the development of endurance training recommendations.

Chapter 3: The Tour of Southland

3.1 Introduction

The Tour of Southland (ToS) is a 6-day, 10 stage cycle tour that is based from Invercargill, New Zealand and travels through alpine terrain. The tour is the leading men's cycling tour event in New Zealand and attracts many top cyclists from New Zealand and around the globe. This study was conducted using a team of riders selected by the New Zealand national cycling body – Bike NZ. The team, named Pro4CycleOps, comprised five high performance male riders who were studied, before, during and after the ToS.

During multi-day cycling tour events daily energy expenditure (EE) is among the highest recorded in human subjects. Given the high energy demand, maintaining energy balance can prove to be a challenge. It has been observed that during such events, on a day-to-day basis, energy intake may not match EE, but over multiple days energy balance is maintained (Brouns et al., 1989a, Brouns et al., 1989b). In a laboratory environment energy intake (EI) and EE are relatively easy to measure however it is difficult to apply the rigid protocols used in laboratory investigations to a competitive event lasting several days with multiple stages on some days. Therefore, laboratory measurements may not be entirely applicable to free living conditions. Alternatively, measuring EI and EE in field based studies may prove haphazard due to an inability to provide the accuracy seen in the laboratory. The gold standard for measuring EE in field studies is the doubly labeled water (DLW) technique and the weighed diet-record is considered one of the most precise methods for estimating EI. In fact weighed diet records are frequently used to validate other dietary assessment methods (Gibson, 2005). Using proven and accurate methods to determine EI and EE in the field ensures that subsequent calculation of energy balance will be more precise and a validation technique improves that accuracy. Energy

balance determined in field studies can be validated by accurate measurement of body weight and body composition. If an athlete is found to be in energy balance then he or she will have a stable body weight. Dual x-ray absorptiometry (DXA) scanning provides an accurate estimate of body composition (Prior et al., 1997).

Total daily energy expenditure (TEE) comprises three parts; resting metabolic rate (RMR), the thermic effect of food (TEF) and physical activity (PA). In elite endurance athletes, PA contributes the largest portion of TEE and is most commonly determined by subtracting RMR and TEF from TEE. However, advances in technology have made it possible to directly measure EE in some sports. During cycling a power meter that is built into the hub of a standard bicycle wheel is able to calculate on-the-bike EE. The PowerTap® power meters used in this study have been validated and at submaximal intensities between 100W and 450W validity is very good. However, PowerTap® is less valid during sprinting (Bertucci et al., 2005). The reliability of the PowerTap® during submaximal intensities is also good with a coefficient of variation of 0.9-2.9%. (Bertucci et al., 2005). The power meter measures work during a race and EE can then be estimated. A value for mechanical cycling efficiency needs to be applied as well to ensure EE is accurate.

EI and EE during intense endurance exercise has been studied by a number of groups (Westerterp et al., 1986, Fudge et al., 2006, Hill and Davies, 2002, Sjodin et al., 1994, Trappe et al., 1997). Westerterp et. al. (1986) used DLW to calculate EE in Tour de France cyclists in a study that broke new ground in field based testing (Westerterp et al., 1986). EE was found to be between 29.4 and 36 MJ/day and although the authors acknowledge that the 22 day duration of the study and the requirement to dose subjects with DLW on multiple occasions introduced a margin of error, the findings give valuable insight into the extreme energy demands of the

world's most prestigious cycling tour. In a follow up study, also at the Tour de France, Saris et. al. (1989) estimated EE at 25 MJ/day but EE was not determined by DLW it was estimated from three assumed variables; 1) RMR, 2) estimated non-racing expenditure, and 3) a calculation of EE while racing from speed, time, and altitude change (Saris et al., 1989). In addition EI was estimated based on food diaries which relied on the riders to accurately record their EI. There was no provision of food by the research team and also no weighing of food, meaning that there is probably a large error associated with the reported 24.7 MJ EI.

Regardless, managing TEE with adequate EI is important for replenishment of carbohydrate stores (Burke et al., 2004) and to maintain nitrogen balance (Butterfield and Calloway, 1983, Todd et al., 1984), especially during the course of repeated physical activity. Energy and protein intake have an effect on endurance performance and lack of adequate intake may lead to fatigue and over-reaching (Armstrong and VanHeest, 2002). It is possible that there is a maximal amount of energy that a human can expend in one day and it may be that maximum TEE is set by how much food can be consumed in that day. Kirkwood et. al. (1983) reported that EI was 3 to 6 times the measured RMR in animals. Elite endurance athletes have very large TEE but relatively are in line with the norm for various animals with an EE at 3.6 – 5.3 times RMR (Westerterp et al., 1986) which relates well to the findings presented by Kirkwood. Brouns et. al. (1989) suggest that it is difficult to ingest and digest enough food to maintain an EE of more than 20 MJ/day and therefore it is thought that food intake limits endurance exercise performance (Brouns et al., 1989b, Brouns et al., 1989a). If endurance performance is dependent on an athlete's ability to maintain energy balance and muscle mass, then being unable to consume adequate calories would certainly compromise success.

Although it is known that protein requirements are dependent on energy balance, it is yet unknown if a very high EE changes protein kinetics, independent of energy balance. A large body of evidence supports the premise that resistance exercise increases rates of protein turnover when a person consumes sufficient calories (Balagopal et al., 2001, Phillips et al., 1999, Tipton et al., 1999) but the effect of endurance exercise on whole body protein turnover (WBPT) is not clearly understood. Several studies have reported increases in whole body protein degradation during exercise (Rennie et al., 1981, Wolfe et al., 1984, Wolfe et al., 1982) while others have failed to observe any change (Carraro et al., 1990a, Stein et al., 1989). A tangible understanding of WBPT in endurance athletes has been hampered by methods that, although sound, lack free-living considerations. For example many studies have been performed after an overnight fast, which cannot be extrapolated to a 24-hr period since athletes ingest large amounts of carbohydrate and total calories before, during and after exercise. Additionally, laboratory-based investigations often poorly represent real life because of their tightly controlled nature to ensure validity and therefore findings cannot be well related to actual athletic performance. The relative contribution of protein metabolism to energy provision is approximately 10%. The contribution is small despite the changes in protein synthesis and breakdown, termed *turnover*, that occur during an acute bout of endurance exercise (Tarnopolsky, 2004, Rennie et al., 2006). However, over the course of a multiple day event, requiring a large daily EE, it is possible that the relatively small contribution by protein to energy metabolism could result in a meaningful absolute contribution of protein to energy metabolism and skeletal muscle structural repair. The effect of free-living, prolonged endurance exercise, in this case a 6-day, 10-stage cycle tour, on WBPT has not been investigated previously.

EI has an effect on endurance performance and a lack of adequate intake may lead to fatigue and over-reaching (Armstrong and VanHeest, 2002). During a 6-day event, maintenance of optimal nutrient intake is crucial for continued performance each day and for recovery from the daily stress of exercise. An alteration in mood and elevated plasma cortisol concentrations are widely accepted as markers of over-reaching and consequent overtraining, when found in conjunction with the classical symptoms of profound fatigue, history of frequent illness and deterioration in performance (Ketner and Mellion, 1995). Serum ferritin, although not a known marker of over-reaching or overtraining, is of consequence in fatigue because low levels will alter oxygen carrying capacity and subsequently have an effect on performance. Fry et. al. (1992) demonstrated that a significant reduction in serum ferritin concentration was associated with a significant decline in performance in response to a specific overload training protocol (Fry et al., 1992). In addition, another study found that maintenance of low serum ferritin resulted in no improvement in VO_2max performance in ferritin depleted male and female athletes compared to ferritin depleted athletes who received iron supplementation (Freidmann et al., 2001). There does not appear to be any research that has examined the relationship between fatigue and parameters of over-reaching and energy and protein balance.

3.1.1 Aims

The purpose of this study was to accurately determine EI, EE and WBPT during a multi-day cycling stage race to determine:

1) whether high EE affects WBPT, independent of energy balance

2) energy requirements of such an event and what and how athletes eat during this type of cycling competition, and

3) whether energy and protein turnover relate to measured parameters of fatigue and overreaching.

It was hypothesised that cyclists would be in energy balance by the end of the ToS and that RMR and exercise EE would conform to the measurements of total EE by DLW and that as long as energy balance was maintained, cyclists would maintain protein balance and would not display signs of overreaching or excessive fatigue.

3.2 Methods

3.2.1 Subjects

Five high performance male cyclists (19-23yrs) were recruited by the New Zealand national cycling organisation, Bike NZ, to participate as a team in the ToS, a 6-day, 10-stage cycle tour event. The cycle event covered 883 km and is rated as an International Cycling Union (UCI) category 2.2 race. All procedures were approved by the University of Otago and the University of Auckland Human Participants Ethics Committees (**Appendix 1** & **2**).

3.2.2 Study Overview

Participation in the study was for a 12-day period which included the 6-days of the ToS itself and the entire 12-day study is depicted in **Table 3.1.** Three days prior to the ToS, participants completed baseline testing in the mid-afternoon, that involved a test of maximal oxygen capacity (VO_2max), testing of the PowerTap® power meters (Saris Cycling Group, Madison, Wisconsin) that were used to determine on-the-bike EE and power output during the ToS, and a DXA scan for determination of body composition

Four hours after baseline testing, subjects completed an 18-hr WBPT protocol. Throughout the 6-days of the ToS food intake was *ad libitum* and was measured precisely by supervised weighed diet records and EE was measured using the double labeled water technique. Urine and breath samples were collected on waking every morning and blood was drawn on alternate days. A POMS questionnaire was also administered on alternate days to blood draws. Immediately on completion of the final stage of the ToS subjects returned to the hotel and underwent a second 18-hr WBPT protocol.

Table 3.1: Study Overview – Tour of Southland

Day	Procedure
1	am: Athletes arrive in Dunedin, VO_2 max test, Ht, BM, calibration of power meters pm: Training ride, standardized meal Commencement of 18-hr WBPT study
2	pm: Completion 18-hr WBPT study DXA scan
3	No testing Normal training
4	am: blood draw, Ht, BM, transport to Invercargill for race pm: DLW dose, Commencement of supervised weighed diet records
5	am: BM, urine sample, POMS 8.3km Team TT pm: 81km hill stage
6	am: BM, urine sample, RMR, blood draw 103km flat-rolling hill stage pm: 88km flat-rolling hill stage
7	am: BM, urine sample, POMS 132.8km hill stage
8	am: BM, urine sample, RMR, blood draw 15km individual TT pm: 133km flat-rolling hill stage
9	am: BM, urine sample, POMS 163.6km flat-rolling hill stage
10	am: BM, urine sample, RMR, blood draw 80km flat stage pm: 78.6km flat stage Standardized meal, commencement of 18-hr WBPT study, completion of weighed diet records
11	pm: Completion of 18-hr WBPT study DXA scan
12	Athletes depart

Ht = height, BM = body mass, WBPT = whole body protein turnover, DLW = doubly labeled water, POMS = profile of mood states questionnaire, TT = time trial, RMR = resting metabolic rate

3.2.3 Procedures

3.2.3.1 Exercise Testing: The VO_2max protocol was performed in the University of Otago Exercise Physiology Laboratory on the participants' own bike on a Kingcycle ergometer (High Wycombe, Bucks, UK). Participants performed a 5min warm-up prior to a graded exercise

test consisting of 4-min stages with step increments of 50W each stage, until volitional exhaustion. Gas analysis was performed with a metabolic cart (Sensormedics 2900, Yorbalinda, CA) and VO_2 and VCO_2 were averaged over 30sec intervals. 4-min stages were used to obtain a steady state oxygen consumption and workload in order to calculate mechanical efficiency. The test lasted approximately 20-30 min because of the long stages and therefore probably more correctly represents a VO_2peak not a VO_2max. A shorter protocol would have likely resulted in higher VO_2 values, but because the baseline testing was performed just a few days before the ToS, participants may have been reluctant to exert themselves fully. Ideally, two separate tests, one for mechanical efficiency calculations and one for VO_2max would have been performed but the energy requirements of two tests immediately prior to a major competition may have compromised the riders' performance at the ToS.

VO_2 and VCO_2 were averaged over the last 2-mins of each stage for the calculation of metabolic work (kJ/min) to allow for subsequent calculation of mechanical efficiency:

$$\text{Metabolic work (kJ/min)} = (3.87\ VO_2 + 1.20\ VCO_2) \times 4.186 \qquad \text{(Equation 3.1)}$$

where 3.87 and 1.20 are the calories per litre of oxygen and carbon dioxide respectively and 4.186 is the conversion factor for calories to kilojoules. Physical work performed was calculated from the mean power output, in watts, recorded by the power meter for each stage:

$$\text{Physical work (kJ/min)} = \text{power} \times 0.06 \qquad \text{(Equation 3.2)}$$

Mechanical efficiency was therefore calculated as:

$$\text{Mechanical efficiency} = \text{physical work} / \text{metabolic work} \qquad \text{(Equation 3.3)}$$

The mean mechanical efficiency was then used in calculations of EE throughout the ToS (see section **3.2.3.4** below) to enable an accurate depiction of EE on the bike for each subject

3.2.3.2 Body Composition: Subjects underwent DXA scanning on a Lunar model DPX-L (Madison, WI) at the University of Otago Hospital. The scanning was repeated on the same machine one or two days after the completion of the ToS. DXA measurements of lean mass, bone mineral content (BMC) and fat mass (FM) were obtained and fat free mass (FFM) was calculated as the sum of lean mass and BMC. In addition, nude body mass measurements were taken prior to the ToS, and every morning upon rising, after voiding, using the same scale.

3.2.3.3 Whole Body Protein Turnover: To represent real-life conditions and to measure WBPT post exercise, and during fed and fasted states, the study design approximated those previously described (el-Khoury, 1997). At the completion of baseline testing (explained above) on Day 1, participants performed a 2 hr training ride near the laboratory in a single group. Upon completion of their ride, participants were fed a standardized meal consisting of a roast chicken and salad sandwich, a banana and a 250ml glass of orange juice (Nutrient content: Total Energy 760 kcal, carbohydrate 110.4 g, protein 30.4 g, fat 20.5 g). Two hours after consumption of the meal, participants reported to the University of Otago Exercise Physiology Laboratory for an 18-hr WBPT protocol. On arrival, participants had a catheter inserted into a forearm vein on one arm for isotope infusion and another catheter inserted into a hand vein on the contralateral side for sample collection. A priming dose of [l-^{13}C]-leucine (12µmol/kg) and $NaH^{13}CO_3$ (1.5µmol/kg) was given immediately prior to commencement of a continuous [l-^{13}C]-leucine infusion (8µmol/kg/hr) (**Figure 3.1**). Dosed feeding occurred for the first 2 hrs at half hourly intervals (EnsurePlus 200ml Nutrient content: Energy 299 kcal, carbohydrate 42.3 g, protein 11 g, fat 9.6 g) to simulate the normal evening meal and began again at 12 hrs for another 2-hr

period to simulate a breakfast meal. The dosed feedings were to create a new steady state condition to allow for protein turnover determination during the fed state.

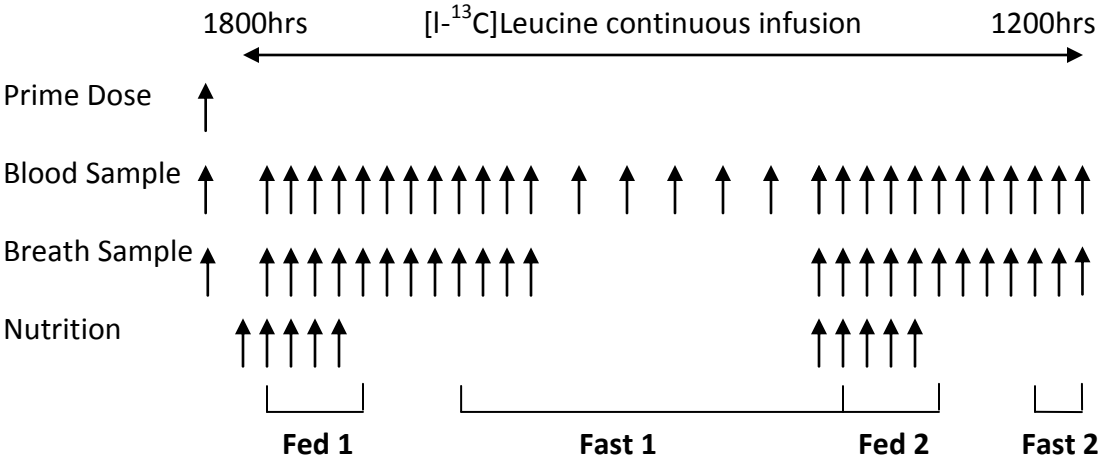


Figure 3.1: Schematic outline of 18-hr WBPT protocol. After a prime dose, [l-^{13}C]-leucine was administered via a continuous infusion for the duration of the study. Blood and breath samples were taken at half hourly intervals, except for during the overnight period when blood sampling was decreased to hourly intervals and breath samples were ceased to minimise participant disruption. Nutrition was given in half hourly bolus feeds in the evening to simulate the normal evening meal and in the morning as the breakfast meal. The protocol provides two fed-state periods and two fasting state periods.

Blood samples were collected into EDTA tubes from a heated hand vein at half-hourly intervals across the 18-hr period except for the 6-hr period from midnight when samples were taken hourly to avoid disruption of sleep to the participants. Breath samples were collected over a 5-min period into Douglas Bags at half-hour intervals until the 'night-time period' when they were ceased for 6 hrs, before re-commencing at half-hourly intervals for the remainder of the protocol. Thirty minutes after the completion of the final stage of the ToS, the participants were fed the same standard meal as had been provided after their training ride previous to the baseline WBPT protocol. The participants were then admitted for a post-ToS 18-hr WBPT study that was identical to the pre-ToS protocol.

Blood samples were immediately centrifuged at 1000g at 4°C for 15 mins. Aliquots of plasma were frozen at -20°C and afterward stored at -80°C for later analysis. Plasma [1- ^{13}C]α-ketoisocaproate (α-KIC) enrichment was determined using a BSTFA-TCMS derivative on a gas chromatograph-mass spectrometer (GC-MS) (DB-17 capillary column, Agilent 19091S-433). Plasma samples were defrosted and deproteinised with ice-cold ethanol. The sample was then transferred to a glass tube and evaporated under nitrogen gas (N_2) at 90°C using a ReactiTherm. Doubly distilled water and *o*-phenylenediamine in 4N hydrochloric acid was then added to the dry tube. Tubes were vortexed and capped and left to incubate for 60 mins at 90°C. Ethyl acetate was added after the incubation period and the upper layer that formed was removed to another glass tube. The second tube was dried under N_2 at 90°C before the addition of equal amounts of the BSTFA-TCMS derivative and pyridine. Tubes were vortexed and capped and incubated for 30 mins at 90°C. At the end of the incubation period, the samples were transferred to GC-MS vials (duplicate) and transported immediately to the GC-MS for analysis. Calculations of protein turnover were modeled off previous publications by el-Khoury et. al. (el-Khoury et al., 1995, el-Khoury, 1997) Briefly, leucine flux (Q) was calculated as:

$$Q\ (\mu mol/kg/hr) = í\ (\mu mol/kg/hr)/E_p \qquad \text{(Equation 3.4)}$$

where í is the infused [1-^{13}C]-leucine (99% enrichment), and E_p is plasma enrichment of [^{13}C]-αKIC (tracer/tracee). Average enrichments were determined as the sum of the two fed and two fasted periods. Fed periods began 30 min after an initial feeding and lasted until 30 min after the final feeding. Fasted periods began 120 min after the final feeding and lasted until the next feeding dose. Leucine appearance from protein breakdown for the fasted period (Leu-PB Fast) was calculated as:

Leu-PB Fast (µmol/kg/hr) = Q (µmol/kg/hr) – í (µmol/kg/hr) (Equation 3.5)

Leucine appearance from protein breakdown during the fed period (Leu-PB Fed) was calculated as:

Leu-PB Fed(µmol/kg/hr)=Q(µmol/kg/hr)–[I(µmol/kg/hr) + í(µmol/kg/hr)] (Equation 3.6)

where I is equal to leucine dietary intake, which was calculated as:

I (µmol/kg/hr) = [(36.53 µmol/ml x 400 ml/hr)/bw (kg)] x (1 – 0.227) (Equation 3.7)

where 36.53 represents the leucine content of the drink per ml (Ensure Plus), 400 ml is the dose per hr, bw is equal to body weight (kg), and (1-0.227) represents a correction for first pass splanchnic extraction (Boirie et al., 1997). Total protein breakdown was calculated from leucine breakdown using the assumption that leucine represents 8% of mixed protein in the body (el-Khoury et al., 1995). Absolute quantities of protein breakdown were calculated for the total 6.5-hr period of feeding and 11.5-hr period of fasting (Equation 3.8). From the sum of the feeding and fasting period, absolute protein breakdown was calculated for a 24-hr period by dividing the 18-hr period by 0.75.

$$\text{Total } 18-\text{hr Protein breakdown (mg)} =$$

$$\left(\left(\frac{LeuPBFed \times 6.5 \times 131.17}{0.08}\right) \therefore 1000\right) + \left(\left(\frac{LeuPBFast \times 11.5 \times 131.17}{0.08}\right) \therefore 1000\right) \quad \text{(Equation 3.8)}$$

where 131.17 represents the molecular weight of leucine

3.2.3.4 Energy expenditure: The DLW technique was used to measure total EE over the 6-days of the ToS. Three baseline urine samples were collected 24 hrs prior, 15 hrs prior and immediately before the dose administration which was at 10 pm the night before the race. Oral

doses of labeled water were given to each participant based on their body weight. Each subject ingested 1.2g 10% $H_2{}^{18}O$/kg and 0.05g 99.9% ^2H_2O/kg, except for one participant (subject 4) who had a reduced dose of 0.967 g 10% $H_2{}^{18}O$ /kg (**Appendix 5**). Subjects were heavier than anticipated and a shortage of $H_2{}^{18}O$ prevented Subject 4 receiving the full dose. The dose was calculated from measured body fat percentage from DXA and was enough to achieve an initial enrichment of approximately 600 times the analytical error (Schoeller, 1988). The dose was ingested using a straw and subjects were instructed to consume all the water in their container by using the straw to draw up any remaining droplets. Following ingestion, two 50-mL washes of the container with tap water were ingested through the straw to ensure all DLW was removed from the container and consumed. Urine samples were subsequently collected from the 2nd void, at approximately 7am every morning of the race. Urine samples collected over the study period were stored in sealed bottles and kept refrigerated prior to transporting to the Department of Chemistry at the University of Otago, Dunedin for analysis. Samples were analyzed in triplicate using an isotope ratio mass spectrometer (ConFlo II; Thermo Finnigan, Bremen, Germany, Delta Plus XP; Thermo Finnigan, Germany). The average background enrichment determined from the three baseline samples was used as the baseline enrichment for each subject. The ^{18}O and ^2H dilution spaces (averages over the race period) were calculated by using the multipoint slope-intercept method (Schoeller et al., 1986a). The ratio of the deuterium to ^{18}O dilution spaces was normalized to 1.034 (Racette et al., 1994). Briefly this method of determining the dilution spaces and the disappearance rate constants for the two isotopes allows total body water, water turnover and carbon dioxide production to be calculated. Total EE was determined from rate of CO_2 production and the food quotient (from weighed diet records)(Weir, 1949):

$$EE\ (kcal/day) = 99\%\ (3.941r_{CO2}/RQ + 1.106r_{CO2}) \qquad \text{(Equation 3.9)}$$

See section **2.6.2** of this thesis for full description and derivation of the DLW equations.

Subjects were provided with PowerTap® power meters for use during the ToS. Different wheels were used for different stages and each wheel had the power meter hub. On-the-bike EE was estimated from the total kJ recorded by the power meter during each stage including the warm-up or cool down periods. The power meters were started when the riders mounted their bike for the first time, sampled at one-second intervals throughout its use, and stopped when the riders got off their bikes. Average, total, and peak power output as well as heart rate were also recorded by the PowerTap® unit. After each stage the power meter files were examined by one of the research team to ensure that the software and meters were working accurately. Work (kJ) on the bike was converted to metabolic EE by dividing by the mechanical efficiency calculated from the baseline exercise test:

Metabolic EE = Work (kJ) / Mechanical Efficiency (Equation 3.10)

In addition, the physical activity level (PAL) was determined by the ratio of TEE:to RMR (methodological description below).

3.2.3.5 Energy Intake: All food and supplements were provided for subjects by the research team and meals were planned and prepared by experienced nutritionists. Food intake during the ToS was *ad libitum* and food choices were based on information obtained through a pre ToS questionnaire about usual food consumption during competition periods. Supervised, weighed diet records were collected for the duration of the ToS at all meals and included snacks and food consumed during and after each stage. Participants selected the amount and type of food and beverages of their choice. Food was weighed cumulatively and after each meal the plate waste was also measured. For combination meals such as spaghetti bolognese, all the raw ingredients,

the complete cooked recipe and the proportion of the recipe selected by subjects were weighed. The quantity consumed for each ingredient was therefore able to be calculated (Gibson, 2005). Foods and supplements used during race stages were selected daily by participants and pre-weighed, any leftovers were weighed on return to the team accommodation. A nutritionist traveled in the team van during each stage of the ToS to ensure subjects consumed only their own food and beverages. The nutritionist carried additional food and supplements in case a rider requested additional nutrition while on the bike. Riders were directed to report any other food or beverages consumed and to hand in any food wrappers and labels to the nutrition team. Available supplements included a carbohydrate-electrolyte fluid replacement beverage, carbohydrate gels and a protein carbohydrate recovery beverage (Pro4 Nutrition, Auckland, NZ).

On the last day of the ToS subjects were transported back to the hotel approximately 1hr after the race finish, to take part in the post-tour WBPT study during which time they no longer had free access to food of choice but were supplied with liquid meal supplements (described above). These supplements were included in the diet records and dietary intake recording ceased at the completion of the 18-hr WBPT study.

Energy and macronutrient intakes were calculated from the weighed diet records using a computerized nutrient analysis program (Diet Cruncher, version 1.2.0, University of Otago). One nutritionist entered and analyzed all diet records while a second nutritionist checked the data for accuracy.

3.2.3.6 Resting Metabolic Rate: Gas exchange was measured every-other day during the tour for the determination of RMR. Upon waking, while the subjects still lay in bed and before any food consumption, expired air was collected into a Douglas bag with one-way Hans Rudolf

valves for 5-mins after a 5-min equilibration period. Expired air was measured for volume (Parkinson-Cowan gas meter) and O_2 and CO_2 content (Cortex, MetaSoft, Germany). RMR was determined by the equation:

$$\text{RMR (kcal/min)} = \left(4.686 + \left[\left[\frac{RER-0.707}{0.293}\right]\right] \times (1-0.707)\right) \times VO2 \qquad \text{(Equation 3.11)}$$

Where 4.686 (kcal/l) is the energy value of 1 litre of oxygen at non-protein respiratory exchange ratio (RER) of 0.707, RER is the measured respiratory exchange ratio, 0.707 is the RER when only fat is being oxidized and VO_2 (l/min) is the rate of oxygen consumption during STPD conditions. RMR was then converted to kJ/day by multiplying by 1440 (mins in a day) and 4.185 (joules in 1 calorie).

3.2.3.7 Blood Measurements: Blood was drawn on the day prior to the ToS and then every second morning during the ToS. Samples were collected on waking after the collection of breath samples, from an antecubital vein, for measurement of hematocrit (Hct), hemoglobin (Hgb), serum ferritin and plasma cortisol. Hct and Hgb were measured to determine plasma volume changes and the latter two as part of the monitoring of over-reaching. Blood was collected into 1 x 8ml EDTA, 1 x 4ml anti-coagulant free and 1 x 4ml SST tube. A small amount of whole blood was used for determination of Hct, in triplicate, by the microhematocrit technique. The remaining whole blood from the second tube was immediately frozen at -20°C and later stored at -80°C for future determination of Hgb. Hgb content was determined in duplicate on a spectrophotometer using the Drabkins method (Drabkin and Austin, 1935). Hct and Hgb were then used for the calculation of percent change in plasma volume (%ΔPV) (Harrison, 1985) according to the equation:

$$\%\Delta PV = \frac{([Hgb1]\times(1-Hct2))}{([Hgb2]\times(1-Hct1))} \quad \text{(Equation 3.12)}$$

Where [Hgb1] is the concentration of Hgb on Day 4 (the day prior to the start of the ToS) and [Hgb2] is the concentration of Hgb on Day 10 (the last day of the ToS) and Hct1 and Hct 2 are the Hcts on Day 4 and Day 10 respectively

Blood collected into EDTA and SST tubes was immediately spun at 1000g at 4°C for 15 mins to separate plasma and serum respectively. Aliquots of plasma and serum were stored in duplicate at -20°C while at the ToS before being transferred to a -80°C storage facility after the ToS for later analysis. Commercially available ELISA assay kits were used to analyse serum ferritin (inter- and intra-run co-efficient of variation (CV) 5.9% and 3.6% respectively) and plasma cortisol (inter- and intra-run CV 3.2% and 5.8% respectively) as per the manufacturer's instructions (Diagnostic Automation Inc, CA). Microwell plates were washed using a mechanical wash system and prepared well plates were tested on a single wavelength (450nm) microwell plate reader.

3.2.3.8 Profile of Mood States Questionnaire: A 37-item modified POMS questionnaire was administered prior to and on 3 mornings during the ToS for the assessment of mood (**Appendix 9**). The POMS questionnaire provides a score for psychological mood state and is well correlated with over-reaching and overtraining (Raglin and Morgan, 1994). Participants were required to complete the questionnaire based on how they felt 'right now'. Each mood item was rated by participants as 'not at all', 'a little bit', 'moderately', 'quite a bit' or 'extremely' and the five descriptors were coded as 0 through 4 respectively for subsequent analysis. Participants spent 5-10 mins before breakfast completing the questionnaire and the data were analysed by grouping mood items into the 6 measured mood states of fatigue, anger, tension, confusion, vigor

and depression and adding the scores to produce a result for each mood state. A mood score was determined by adding the five negative mood states and subtracting vigour (Fatigue + Anger + Tension + Confusion + Depression – Vigour). An athlete who is performing well and is not at risk of overtraining will have a negative mood score. Additionally, scores for each mood state were plotted to identify the presence (or absence) of an Iceberg Profile (Morgan et al., 1987a).

3.2.4 Statistics

When appropriate, pre- and post-ToS measurements were compared by a paired t-test. Changes over time were analyzed with a one-way repeated measures analysis of variance (ANOVA) with a Tukeys post hoc test performed to determine where any differences occurred. Values were considered significant if $P < 0.05$ and data are reported as mean ± standard deviation (SD). A 95% confidence interval (CI) for mean pre/post ToS difference was determined for the standard error of the difference and the t-value for alpha=0.05 at 3 degrees of freedom ($t = 2.353$). Pearson product moment correlations were performed between energy and protein balance and markers of overtraining to determine any relationship between variables

3.3 Results

One subject crashed on the second day of the ToS and was excluded from the study. Therefore the data reported are for the remaining four cyclists. Another cyclist (Subject 1) pulled out of the ToS due to fatigue on day five and thus his data were calculated over 5 days and his post-tour WBPT study was performed on day five immediately on his arrival back from the race. Subject characteristics are presented in **Table 3.2.** Subject 3 did not perform a valid VO_2max test as determined by an exercise RPE of 15, an RER of 0.95 and a maximum heart rate of just 166bpm. Subject 3 did complete a sufficient amount of the test to determine mechanical efficiency but his VO_2 data is not included in the mean.

Table 3.2: Subject Characteristics

	Subject 1	Subject 2	Subject 3*	Subject 4	Mean (SD)
Age	18	19	24	18	20 (3)
Weight(kg)	81.7	73.8	92.3	88.7	84.1 (8.2)
Height (cm)	184	188	197	193	191 (6)
VO_2peak (ml/kg/min)	53.5	61.2	47.4	58.2	57.6 (3.9)
VO_2peak (L/min)	4.4	4.5	4.4	5.2	4.7 (0.4)
LBM^ (kg)	68.9	60.0	72.8	73.4	68.8(6.1)
Body Fat^ %	11.5	14.6	16.9	10.9	13.5(2.8)

*Subject 3 did not achieve a true VO_2peak (RPE = 15, RER = 0.95, and HR_{max} =166) and therefore his VO_2peak is not included in the mean. #Subject 5 crashed on the second day of the ToS and did not complete the ToS. Subject 5's data is not included in any results. ^Lean body mass (LBM) and body fat % as determined by DXA prior to the ToS.

3.3.1 Body Mass, Body Composition, and Plasma Volume Changes

There were no significant changes in body mass from Day 1 of the ToS to Day 6 indicating that subjects remained weight stable (p = 0.332, 95% CI -0.44kg to 1.29kg) (**Figure 3.2**). Body mass at baseline and after the completion of the ToS, measured with the DXA scan (**Table 3.3**) varied from measures made with the scales, but these were calculated from summation of lean and fat mass and bone mineral content and were made three days prior to the

race start and 1-2 days after the race finish. Over the eight-day period between scans only one subject, Subject 3, lost body mass of approximately 3kg although this was not distinguished by the scale measurements from day 1 to day 6. The DXA showed that the loss of mass was a change of about 300 g of lean mass, and the remainder was loss of fat mass. Every other subject gained lean mass and all but Subject 4 lost fat mass over the 6 days. Subject 4 also gained the most weight, approximately 2 kg, of which slightly less than 400 g was fat. On average there was no significant change in body mass with the reduction in BF% ($p = 0.173$, 95% CI -3.07% to 0.42%) and lean mass gain ($p = 0.139$, 95% CI -0.24kg to 3.0kg) equating to relative balance (**Table 3.3**). Three subjects had a plasma volume expansion, and Subject 4 had a slight decrease in plasma volume.

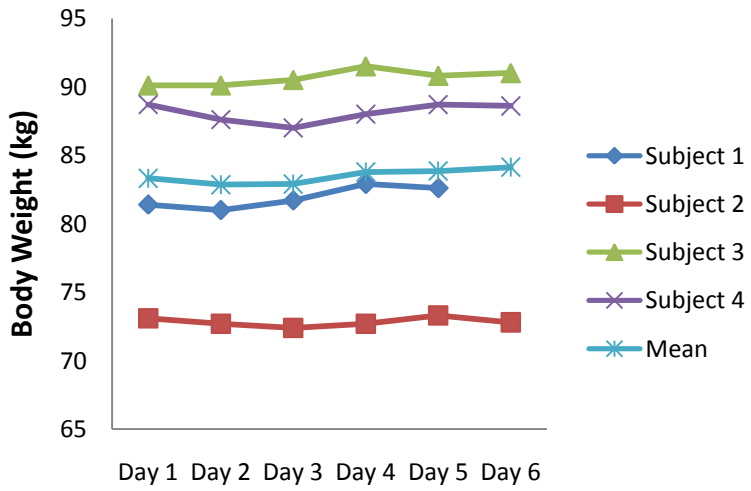


Figure 3.2: Individual and mean changes in body mass over time. Weights were measured by the same scale every morning immediately after voiding. Days indicate days of the ToS and correspond to the 1st through 6th day of racing.

Table 3.3: Body Composition, Plasma Volume Changes and Total Body Water

	Subject 1	Subject 2	Subject 3	Subject 4	Mean (SD)
Body Mass (kg)*					
Prior to race#	82.2	73.8	92.5	86.7	83.8 (7.9)
After race^	83.1	75.3	89.3	88.8	84.1 (6.5)
Change	0.9	1.5	-3.2	2.2	0.3 (2.4)
% Change	1.1	2.0	-3.5	2.5	0.5 (2.7)
Lean Mass (kg)					
Prior to race#	68.9	60.0	72.8	73.4	68.8 (6.2)
After race^	69.8	63.1	72.5	75.2	70.4 (5.5)
Change	0.9	3.1	-0.3	1.8	1.4 (1.4)
% Change	0.3	2.5	2.5	2.0	1.8 (1.0)
Fat Mass (kg)					
Prior to race#	9.5	10.8	15.6	9.5	11.3 (2.9)
After race^	9.4	9.2	12.6	9.8	10.2 (1.6)
Change	-0.1	-1.6	-3.0	0.4	-1.1 (1.5)
% Change	-0.3	-2.4	-2.7	0.1	-1.3 (1.5)
% ΔPV	16.4	16.2	10.9	-4.5	9.7 (9.8)
Absolute ΔPV (ml)˜	323	292	196	-95	179 (190)
Total body water prior to race (kg)>	47.6	49.7	53.5	56.4	51.8 (3.9)

*Calculated from totals of bone mineral content, fat and lean mass by DXA; #measured 3 days prior to race start; ^measured in the afternoon the day after the race finish or the following morning except in subject 1 who dropped out of race on day 5 and thus DXA was two days after his race finish; ˜estimated from a starting volume of 5000 ml and initial Hct; >Calculated from doubly labeled water dilution; No significant changes were noted when comparing pre- to post-competition data.

3.3.2 Energy Balance and Whole Body Protein Turnover

The relationship between energy balance and protein turnover has been described in **Section 2.6** and as such results for both are presented here together for completeness and as a framework for the discussion that follows in **Section 3.5** below. Also, because of the relatively

small subject number and high inter-subject variability, data are expressed on both group and individual bases. Subjects had a mean EI of 27.3 ± 3.8 MJ of which 63.7 ± 1.8% was carbohydrate (CHO), 15.2 ± 0.9% protein, and 17.3 ± 2.3% fat. There was a trend toward a significant increase in mean EI from Day 1 to Day 2 of 8.5 ± 5.5 MJ (p = 0.054, 95% CI 2.0 MJ to 15.0 MJ) but EI became stable thereafter (**Figure 3.3**).

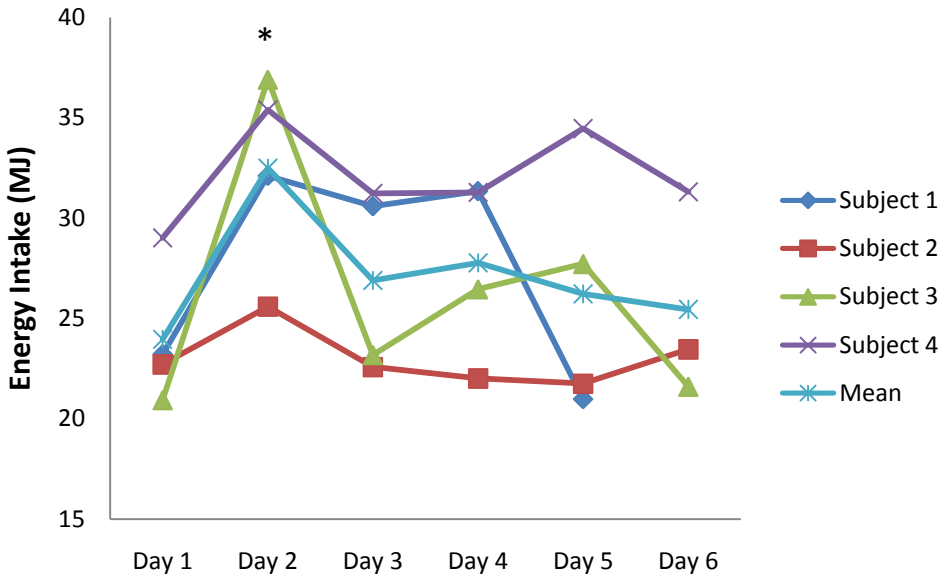


Figure 3.3: Individual and mean EI. EI was measured by supervised weighed diet records. The days indicate the 1st through 6th day of racing at the ToS. There was a trend toward a significantly increased mean EI on Day 2 compared to Day 1 but no other significant differences were detected. *p = 0.054.

Mean macronutrient intakes are presented in **Table 3.4**. Fat consumption was low at just 17.3 ± 2.3% of daily EI. One subject consumed alcohol on one day of the study but no other alcohol was consumed by any other subject. Real, non-supplement food made up the majority of the EI (80 ± 4.1%) with a smaller proportion of EI consumed as supplements (20 ± 4.1%). Mean TEE was 27.4 ± 2.0 MJ/d which is equivalent to the mean EI (27.3 ± 3.8 MJ/d) (Difference

between EI and EE, p = 0.943, 95% CI -6.3 MJ to 5.9 MJ). Measured RMR was 42 ± 2% and exercise EE was 62 ± 10% of TEE and mean PAL over the course of the competition was in the range of 2.3-2.5 (**Table 3.5**). DLW analysis results are presented in **Appendix 6**.

Table 3.4: Mean Energy (EI) and Macronutrient Intakes for the Tour of Southland

	Subject 1	Subject 2	Subject 3	Subject 4	Mean (SD)	Highest Single Day
Mean EI (MJ/day)	27.6	23.0	26.3	32.1	27.3 (3.8)	36.9
Carbohydrate (g/day)	1076	951	1024	1284	1084 (143)	1372
(g/kg/day)	13.2	12.9	11.1	14.5	12.9 (1.4)	15.9
%EI	62.0	66.1	62.7	64.1	63.7 (1.8)	70.8
Protein (g/day)	230	204	249	294	244 (38)	371
(g/kg/day)	2.81	2.76	2.7	3.3	2.9 (0.3)	4.0
%EI	14.1	15.0	16.2	15.6	15.2 (0.9)	21.3
Fat (g/day)	151	95	120	147	128 (61)	218
(g/kg/day)	1.85	1.29	1.30	1.65	1.52 (0.28)	2.36
%EI	20.5	15.3	16.4	16.8	17.3 (2.3)	26.3
Alcohol						
(g/day)	0.0	0.0	10.7	0.0	10.7 (0.0)	10.7
%EI	0.0	0.0	1.2	0.0	1.2 (0.0)	1.2
%EI Food	83.7	75.6	83.5	77.5	80.0 (4.1)	91.6
%EI Supp	16.3	24.4	16.5	22.5	20.0 (4.1)	30.5

EI = energy intake, %EI = percentage of energy intake, %EI Food = percentage of energy intake from food, %EI Supp = percentage of energy intake from supplements

Table 3.5: Energy Expenditure

	Subject 1	Subject 2	Subject 3	Subject 4	Mean^ (SD)
TEE (MJ/day)*	28.6	29.6	25.3	26.2	27.4 (2.0)
RMR (MJ/day)	11.3	12.5	11.0	11.1	11.5 (0.7)
Exercise EE (MJ)#	15.4	16.5	19.3	16.5	16.9 (0.2)
PAL	2.54	2.37	2.30	2.35	2.39 (0.10)
RMR as % of TEE	39%	42%	43%	43%	42% (2%)
Exercise EE as % of TEE	54%	56%	76%	60%	62% (10%)

*TEE was calculated by DLW. #Exercise EE was estimated from total work on the bike (measured by power meters) multiplied by a mechanical efficiency factor. PAL = physical activity level (TEE:RMR) ^Means are based on 6-day measures for Subjects 2-4 and 5-day measures for Subject 1, with the exception of RMR.

There was no significant difference between pre- and post-ToS mean leucine flux during either the fasted (p = 0.605, 95% CI -24.25µmol/kg/hr to 17.71 µmol/kg/hr) or fed (p = 0.202, 95% CI -6.91 µmol/kg/hr to 37.81 µmol/kg/hr) states. In addition there was no significant difference between pre- and post-ToS Leu-PB during either the fasted (p = 0.583, 95% CI -37.14 µmol/kg/hr to 63.35 µmol/kg/hr) or fed (p = 0.783, 95% CI -44.95 µmol/kg/hr to 34.75 µmol/kg/hr) states. 24-hr total protein breakdown was also not different between the pre- and post-ToS studies (p = 0.376, 95% CI -5.75 mg/hr to 14.81 mg/hr) (**Table 3.6**). However, mean leucine flux was 54.03 ± 26.84 µmol/kg/hr higher during the fed, compared to the fasting period of the pre-tour study (p = 0.028, 95% CI 22.45 µmol/kg/hr to 85.60 µmol/kg/hr) and was 74.25 ± 13.39 µmol/kg/hr higher during the fed, compared to the fasting period of the post-tour study (p = 0.001, 95% CI 58.49 µmol/kg/hr to 90.00 µmol/kg/hr). Mean Leu-PB was 81.23 ± 31.57 µmol/kg/hr higher during the fasting period compared to the fed period of pre-tour study (p = 0.014, 95% CI 44.09 µmol/kg/hr to 118.37 µmol/kg/hr) but there was no difference between the states during the post-tour study (mean difference 99.44 ± 77.37 µmol/kg/hr, p = 0.082, 95% CI 8.41 µmol/kg/hr to 190.47 µmol/kg/hr).

Table 3.6: Leucine flux, appearance and total protein breakdown

Leucine Flux (µmol/kg/hr)		Subject 1	Subject 2	Subject 3	Subject 4	Mean(SD)
Pre-Tour	Fed	207.44	223.11	188.63	221.02	210.05(15.88)
	Fasted	162.33	172.31	160.13	129.32	156.02(18.57)*
Post-Tour	Fed	234.18	244.59	215.05	208.19	225.49(16.82)
	Fasted	177.20	172.60	135.74	119.47	151.25(28.16)*
Leucine Appearance (µmol/kg/hr)						
Pre-Tour	Fed	46.86	78.06	60.23	87.41	68.14(18.12)
	Fasted	154.80	165.51	154.11	123.06	149.37(18.30)*
Post-Tour	Fed	70.04	29.13	80.22	72.76	63.04(23.01)
	Fasted	169.51	237.86	129.42	113.12	162.48(55.56)
Protein Breakdown (mg/kg)						
Pre-Tour	18-hr	46.32	48.38	38.44	36.66	42.45(5.76)
	24-hr	61.76	64.51	51.25	48.88	56.60(7.69)
Post-Tour	18-hr	54.61	58.06	37.49	33.24	45.85(12.31)
	24-hr	72.81	77.41	49.98	44.32	61.13(16.41)

*significantly different from fed period ($p<0.05$).

Subject 1 had an overall increase in leucine flux, Leu-PB and total protein breakdown from pre- to post-tour. In addition Subject 1 was in negative energy balance with an EE of 28.6 MJ which was slightly higher than his EI (27.6 MJ). Subject 2 had increased leucine flux and Leu-PB during the fasted period and decreased leucine flux and Leu-PB during the fed period from pre- to post-tour which resulted in an overall increase in total protein breakdown. In addition, Subject 2 had an EE of 29.6 MJ that exceeded his EI (23 MJ). Subject 3 had slightly increased leucine flux and Leu-PB during the fed state but had decreased values for the fasted condition from pre- to post-tour. Subject 3 had no change in total protein breakdown from pre- to post-tour and had an EE of 25.1 MJ which was slightly below his EI (26.3 MJ). Subject 4 had no

change in leucine flux, Leu-PB or total protein breakdown from pre- to post-tour and he also had the highest EI of 32.1 MJ and was in positive energy balance with an EE of 26.2 MJ. Due to an error in the analysis of breath samples by a commercial laboratory, leucine oxidation and consequently non-oxidative leucine disposal (NOLD) were unable to be reported.

3.3.3 Blood Measurements

There was no mean change in Hct (p = 0.631, 95% CI -2.34% to 0.62%), or Hgb (p = 0.824, 95% CI -9.49 g/dL to 16.38 g/dL) in any subject from Day 1 to Day 5.

Table 3.7: Serum Ferritin and Plasma Cortisol Concentrations

	Subject 1	Subject 2	Subject 3	Subject 4	Mean (SD)
Ferritin (ng/ml)					
Baseline	60.5	31.5	94.0	43.0	57.3(27.2)
Day 2	31.5	4.8^	47.0	49.2	33.1(20.5)
Day 4	11.0^	2.8^	39.5	33.0	21.6(17.5)#
Day 6	9.0^	ND	38.5	12.5^	20.0(16.1)#
Cortisol (ng/ml)					
Baseline	246*	121	96	85	110(67.5)
Day 2	123	97	121	106	134(72.5)
Day 4	278*	117	124	103	147(73.5)
Day 6	270*	124	130	41	122(95.0)

Normal range for serum ferritin = 20-300ng/ml, normal range for plasma cortisol = 60-230ng/ml. ^below normal values. * values in excess of normal. # significantly different from baseline $p < 0.05$

Serum ferritin concentrations declined across the ToS in all subjects. The mean concentration at Day 4 was significantly reduced compared to baseline (21.6 ± 17.5ng/ml vs. 57.3 ± 27.2ng/ml; p = 0.040) as was the concentration on Day 6 (25.5 ± 16.1ng/ml; p = 0.027). Mean plasma cortisol concentrations remained stable across the ToS but Subject 1 displayed plasma cortisol concentrations in excess of normal at baseline and on Days 4 and 6 (**Table 3.7)**.

3.3.4 Profile of Mood State

There was no change in mood score over time during the ToS. Subjects 2, 3 and 4 had a negative MS (meaning a positive mood state) and displayed positive iceberg profiles at each time-point measured throughout the ToS with fatigue being the only negative mood state mildly indicated (**Figure 3.4**). Subject 1 had a positive mood score (negative mood state) and displayed negative iceberg profiles at all time points with scores for vigor falling below those for confusion and fatigue. Pearson product correlations did not reveal any significant correlations between leucine flux, Leu-PB or energy balance and any of the over-reaching markers measured.

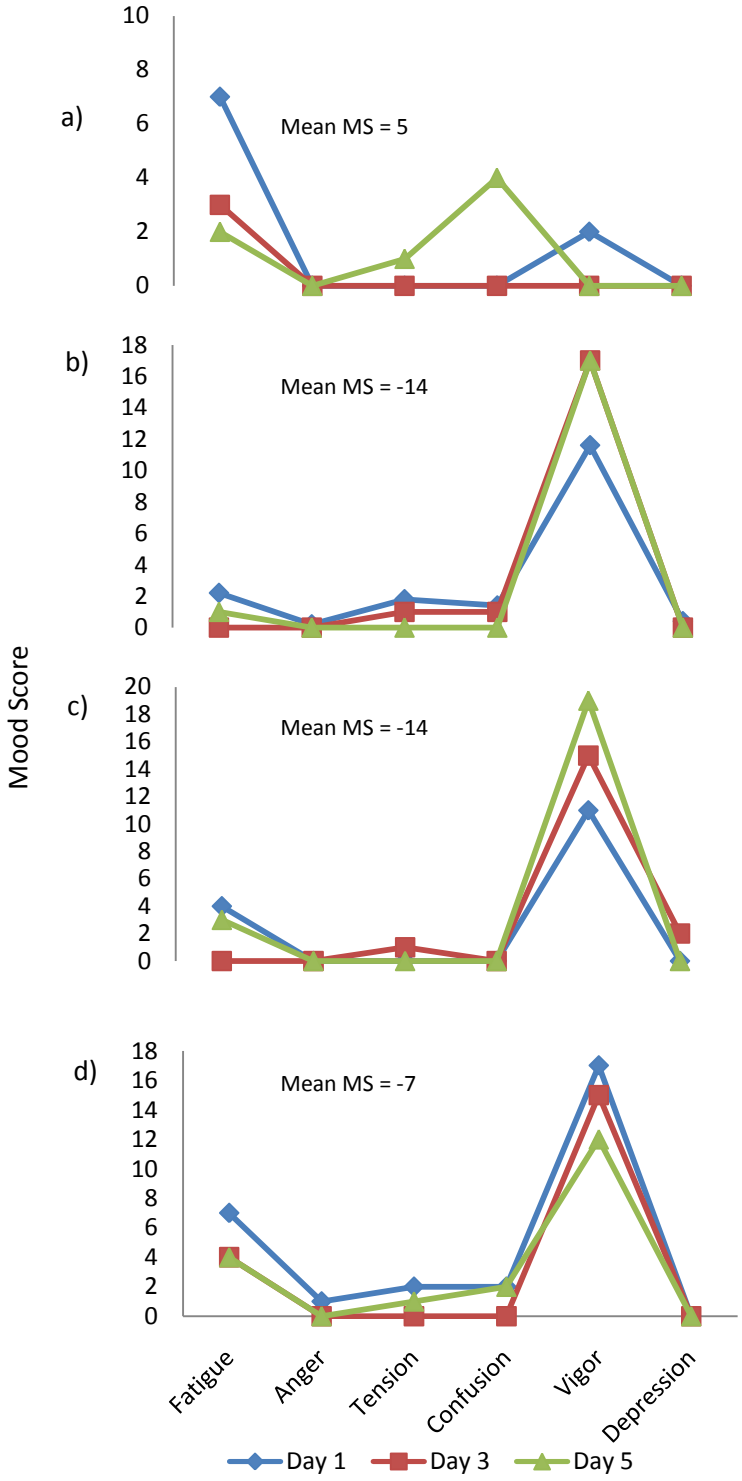


Figure 3.4: Individual iceberg profiles and mood scores (MS) for subjects 1 – 4 (a) – d) respectively). Subjects 2-4 displayed positive iceberg profiles and negative mood scores at all time points while subject 1 had no iceberg profile and a positive mood score which are both indicative of over-reaching/overtraining

3.4 Discussion

The purpose of this study was to examine high performance male cyclists during a 6-day, 10-stage cycle tour to determine EE and EI and if an increase in EE changes protein kinetics, independent of changes in energy balance. Further, the study examined whether measures of protein kinetics relate to values determined for energy balance and markers of over-reaching and overtraining. The main finding was that a high EE over a 6-day period did not significantly change protein breakdown during fed or fasted conditions. In addition it was found that cyclists were able to maintain body weight over the course of the ToS and gain lean mass, despite their large EE. EE reported here match previously reported levels and reported EI were roughly equivalent to those currently recommended for endurance exercise.

3.4.1 Protein Kinetics

Of practical concern in this study is whether the breakdown of protein changed during the course of the study which would potentially increase dietary protein requirements. The catabolic nature of endurance exercise suggests that active individuals may have a higher rate of WBPT and therefore require more dietary intake of protein than sedentary individuals (Lamont et al., 1999, Lemon, 2000). Our data indicate that increased EE, in and of itself, did not increase leucine flux, Leu-PB or total protein breakdown. The athletes in this study were consuming in excess of the daily recommended values for protein (mean protein intake 2.9 ± 0.3g protein/kg) which currently stand at between 1.2 and 1.4g protein/kg (Tipton and Wolfe, 2004) and it could be argued that this covered any increased protein needs and limited protein breakdown. The higher than recommended protein consumption is likely indicative of the eating habits of competitive cyclists and not an effect of food provision because the subjects were allowed to eat

ad libitum. This may mean that athletes who have periods of large EE during training and competition simply consume protein to levels that maintain protein mass when EE is high.

It is well known that energy balance has a dramatic effect on the utilisation and turnover of protein in the body (Calloway and Spector, 1954). When an individual is in positive nitrogen balance, amino acids are being amassed and lean tissue is being synthesised. Nitrogen balance can be maintained with a dietary intake of protein of between 0.8 and 1.2g protein/kg/day (Tarnopolsky, 2004) in normal healthy individuals. However, it has been shown that nitrogen balance cannot be maintained if energy intake is insufficient and that feeding nitrogen to even extreme levels (6.9g N/day which equates to 43g protein/day) is of little benefit to nitrogen balance when calories were deficient (Calloway and Spector, 1954). Energy intake is therefore one of the important factors in maintenance of nitrogen balance and lean mass (Butterfield and Calloway, 1983, Todd et al., 1984). A diet deficient in calories will be unable to maintain nitrogen balance, and thus body protein mass, regardless of the protein content of that diet. Equally, energy from non-protein sources may be able to maintain nitrogen balance, possibly through inhibition of proteolysis that occurs with post feeding hyperinsulinemia, even when protein content is low, as long as the calories are sufficient to maintain energy balance. Despite being unable to measure protein balance in this study, an unchanged mean leucine flux, Leu-PB and total protein breakdown from pre-tour to post-tour suggests unchanged protein kinetics and maintenance of protein mass. Based on previous studies (Phillips et al., 1993, Knapik et al., 1991, Wolfe et al., 1982) it is suggested that an acute bout of endurance exercise is catabolic during the exercise itself although this did not add to a significant difference in protein kinetics when measured pre- and post- competition in our study. It is likely that the anabolic state that occurs after exercise balances the catabolic state of the exercise when calories are available.

As mentioned, exercise induces a catabolic state during an acute bout but the exercise is followed by an anabolic state. Therefore it is important to investigate metabolic affects over a prolonged period of time that includes both metabolic phases. The strength of the current study was that measurements were taken over an 18-hr period that allowed physiological changes in protein metabolism to be characterised over almost an entire day and include various post absorptive and postprandial states. The methods were adapted from El-khoury et. al. who defined a 24-hr pattern of leucine kinetics in subjects fed a healthy diet and who underwent two 90-min periods of exercise (el-Khoury, 1997). They found that when viewed over a whole day, exercise did not affect leucine homeostasis and 24-hr leucine kinetics gave a good representation of total body nitrogen and amino acid status as measured by nitrogen excretion and urea. Protein degradation increases during an acute bout of exercise (el-Khoury, 1997, Phillips et al., 1993, Knapik et al., 1991, Wolfe et al., 1984) but if an athlete is able to maintain energy balance over a day then they can remain in nitrogen balance and the catabolic state that occurs during exercise will have little effect on their physiology or in theory their performance. In support of this notion, Todd et. al. (1984) have demonstrated that exercise helps to preserve body nitrogen stores and that negative energy balance, with adequate protein intake, is better tolerated when the energy deficit is due to exercise as opposed to a reduction in energy intake (Todd et al., 1984).

If the energy-protein relationship is considered in individual subjects it can be seen that when a subject (e.g. Subjects 1 & 2) was in negative energy balance there was a general increase in leucine flux, Leu-PB or total protein breakdown. The increased kinetics, indicative of increased protein breakdown, could be explained by the energy deficit across the 6 days. Protein intake in this study (mean 2.9g/kg) was well above the recommended level for endurance athletes and therefore, as mentioned above, it is likely that the negative energy balance was the

major factor that contributed to the catabolic state. The increased protein breakdown evidenced in Subject 1 may also have been the result of other physiological changes (see discussion in **Section 3.4.4**). Additionally, these findings are in agreement with Todd et. al. (1984) who found that exercise is able to preserve nitrogen so that negative energy balance is better tolerated when protein intake is adequate (Todd et al., 1984). When a subject was in positive energy balance (e.g. Subjects 3 & 4) there was little difference in leucine flux, Leu-PB or total protein breakdown from pre- to post-tour presenting a case that it is possible for a highly trained endurance athlete to be in positive energy balance throughout an intensive period of competition. Additionally, the maintenance of energy balance or in this case the achievement of positive balance as well as adequate protein intake preserve whole body protein stores. The present findings are in agreement with others' who have measured post-absorptive whole body protein breakdown after exercise and found rates of breakdown are either decreased (Tipton et al., 1996) or no different (Rennie et al., 1981) after exercise compared to rest. Our study's strength in this regard is the concurrent measurement of energy balance that was not determined by either Tipton or Rennie. Its inclusion in this study allows clearer conclusions to be drawn about protein breakdown because of the intricate link between energy provision and protein kinetics.

3.4.2 Energy Expenditure and Intake

Westerterp et. al. (1986) made the first measurements of EE by DLW in Tour de France cyclists. Multiple doses of DLW were required throughout the 3-weeks of the tour and samples were measured at three different intervals in four different subjects making the DLW protocol less than optimal. Average daily EE ranged from 28.4 – 38.0 MJ/day, which was somewhat higher than the range of 25.3 – 29.6 MJ/day found in this study although the ToS is admittedly not as arduous as the Tour de France. Saris et. al. (1989) estimated mean EE at 25.4MJ/day in a

later study also at the Tour de France which is slightly less than the mean EE found here of 27.4 ± 2.0 MJ. However EE was in the Saris study was based on calculations and assumed efficiencies and therefore does not have the accuracy of the DLW method used in this study. Findings of EE presented here are similar to those found by Brouns et. al. (1989) of 25.2 – 26.8 MJ/day during a simulated Tour de France race based in a laboratory. However, as discussed previously, laboratory measures may not well reflect free-living conditions because of the extremely controlled environment. Despite the differences in methodological approaches the daily and mean EE‘s for the ToS correspond well with other previously published studies that have determined the energy demands of a cycling tour event.

Although the DLW method is the gold standard for EE determination there is a disadvantage to the technique in that it is unable to measure EE for a single day. Therefore, among other things, EE and EI are unable to be compared on a day to day basis. To account for this limitation RMR and exercise EE were also measured in this study to give an understanding of the separate parts that make up TEE. The rationale for this was that if RMR and exercise EE, determined from the power meters, are added the sum is equivalent to TEE. However, TEF is not accounted for in this equation nor is any other activity that may have occurred throughout the day such as walking, thus some error is apparent when using the simple addition equation.

Decay curves are used in the calculation of TEE by the DLW method and are known to be a valid measure of all unfractionated water loss including fluid lost by sweating (Prentice et al., 1952). Routes of fractionated water loss not accounted for by the DLW decay curve are water loss from breathing and transcutaneous evaporative water loss. A correction factor of 2.1 was used with the data presented here in the determination of the rate of carbon dioxide production to correct for the unmeasured fractionated fluid losses (Prentice, 1990). It is acknowledged that

some error is inherent in the use of the correction factor. This is especially the case in endurance athletes because the correction is based on the premise that transcutaneous losses are related to average rates of water loss per square meter of the body surface area and also average 24-hr ventilatory volumes in the typical adult population. The present subjects were not typical. Ruby et. al. (2002) concluded that the correction factor could account for up to a 4% of the error in the determination of TEE if the assumptions used were not correct under extreme conditions such as firefighting in their study or ultra endurance events as in this study (Ruby et al., 2002).

In addition, the DLW technique requires accurate dosing of subjects to ensure enough enrichment above background levels to make accurate analyses. One subject was given a lower dose than the others due to a shortage of $H_2{}^{18}O$ but this did not affect correct determination of EE. The multipoint method was used to calculate EE and for the subject with the reduced dose the R^2 value for the slope of the ^{2}H and ^{18}O transformed decay curves (7 measured points of enriched urine) was >0.993, a value that ensures that there was sufficient enrichment above background to measure EE. If there had not been enough of the dose the curve would have bottomed out over time thus the smaller dose here does not affect the meaningfulness of the figures reported.

When considering energy balance and providing accurate EI recommendations for endurance athletes, the efficacy and accuracy of the EI recording method is paramount. Westerterp et. al. (1986) used self-recorded, diet records to determine EI in their landmark study and estimated riders intake at between 21.5 MJ – 26.9 MJ/day (Westerterp et al., 1986). It is surprising that when EE was measured using the gold standard, the concurrent EI was estimated using simple diet records which have well known limitations. Saris et. al. (1989), also used self-recorded diaries and estimated mean EI at 24.7 MJ/day with a high value of 32.4 MJ on one day (Saris et al., 1989). A well planned study was conducted at the Vuelta a Espana, another cycling

tour event (Garcia-Roves et al., 1998). Food was weighed and recorded by the research team and mean EI was determined to be 23.5 MJ/day with a range of 17.9 – 28.2 MJ/day. The EI range of the present study was 23.0 – 32.1 MJ/day with a mean of 27.3 ± 3.8 MJ/day and matches well to the Garcia Roves findings where EI methods were almost identical. The findings of all the studies mentioned here are interesting in light of suggestions that it may be difficult to consume sufficient food to maintain an EI of over 20 MJ/day when exercise is prolonged (Brouns et al., 1989a, Brouns et al., 1989b). The ToS is a relatively short period of competition compared to the 3-week Tour de France and perhaps if the ToS duration was longer our subjects may not have been able to maintain such a high EI with continued physiological demands. However, the EI findings in Tour de France riders are well above the supposed 20 MJ/day threshold which suggests that these professional cyclists are able to cope with large energy requirements. Further investigation is warranted on this point with a focus on accuracy of the EI recording.

A factor that needs to be considered when assessing the findings here is that throughout the 6-day ToS, food was *ad libitum.* Previous studies have also been *ad libitum* but from a practical perspective, especially for non-professional cyclists, unlimited availability of food might not be the case at all competitions. In New Zealand there are very few cyclists who are funded to ride and therefore teams are usually subject to budget constraints and eating in less than ideal conditions for example in a restaurant setting. It may be possible that cyclists in this study consumed more food than they normally would in the same situation simply because of the food availability and the regularity of meals. However, the consistency of the total energy and macronutrient intakes across the 6 days suggests that the intakes may have been close to habitual. Also, the possible ceiling of maximal metabolisable energy intake (TEE as a multiple of BMR), mentioned above, is thought to be in the vicinity of 3-6 times RMR in a variety of animals

(Kirkwood, 1983). Westerterp et. al. (1986) found that the energetic ceiling in humans was similar at between 4 and 5 times BMR. The PAL value (TEE:RMR) in our subjects was 2.30 – 2.54 which falls below these reported values (Westerterp et al., 1986). This may be because the measured RMR values were not a true basal determination of metabolic rate as subjects were awake, albeit lying in bed, and may have been aroused in anticipation of the days racing. To evaluate this issue the Harris Benedict (Harris and Benedict, 1919) and Cunningham (Cunningham, 1980) equations were used to calculate RMR and in turn PAL. The mean PAL calculated from the average RMR of the two equations was 3.26 with a range of 2.8 – 3.8 which is more in line with previous findings. Subjects in this study had similar TEE as those in the study by Westerterp, but subjects here had decreased EE/kg (360 kJ/kg vs. 493 kJ/kg). In comparison the differences in EI/kg were similar between studies (322 kJ/kg compared to 329 kJ/kg) indicating that it might be the ability to consume energy that is the important factor in determining performance over long-term strenuous competition. Smaller riders may be better suited to these tour events because of the increased EI requirements needed in heavier riders. Future research could potentially measure EI and EE in riders who are larger than the norm to clarify this issue.

During the days of the ToS, subjects were in energy balance and body weight was maintained. Three of the four subjects gained lean mass and had a 1 kg or less change in body weight over the experimental period. One subject lost 3.2kg of body weight as measured by DXA of which the majority was fat mass. This subject was the heaviest participant at 92.5 kg and had the highest initial fat mass at 15.6 kg. He also had the highest exercise EE (19.2 MJ) but his mean DLW-measured EE was only 25.3 MJ with an EI of 26 MJ. There may be some error in both the EI and EE but it is more likely that the time-lag between the end of the race and the

post-race DXA account for the discrepancy between the EI and EE and body composition measures. It is unlikely that the inherent error associated with DXA was responsible for the discrepancy because the error of measurement for DXA scanning is less than that caused by the time delay (Clasey et al., 1999). It is therefore concluded that on average all participants were in energy balance throughout the ToS.

3.4.3 Macronutrients and Timing of Nutrition

The methodological procedures utilised in this study allowed accurate calculation of EI and specific macronutrient composition of each rider's diet. The data are interesting especially for an understanding of what is required, nutrient-wise, to compete in a multi-day tour event. Daily CHO and protein intakes in this study was above the current recommendations of 10-12 g CHO/kg and 1.2-1.7g protein/kg (Burke et al., 2004). Athletes here consumed 12.9 ± 1.4 g CHO/kg and 2.9 ± 0.3 g protein/kg and protein intake was as high as 4.0 g/kg. These findings are in line with previous reports and with respect to protein, support the view that endurance athletes generally consume more protein than is required (Tarnopolsky, 2004). Fat intake in this study was less than has been reported elsewhere (Saris et al., 1989, Garcia-Roves et al., 1998) with fat content only contributing a mere 17.3% of calories to total energy. The low fat content of the rider's diet may be due to having their food prepared by the research team who developed menus based on nutrition guidelines for endurance athletes.

There has been much discussion in recent years about the timing of energy and protein intake around exercise. Protein synthesis that is stimulated by feeding is a temporary response whereas the anabolic effect of exercise is a more permanent process of physiological adaptation. If food is given post exercise the anabolic stimulation, of muscle protein synthesis, of the exercise is enhanced by the provision of amino acids (Miller, 2007). Riders in this study were

provided with carbohydrate and protein supplements throughout and immediately after each stage of racing. The timing of these supplements in relation to the exercise they performed is likely to have promoted the gains in lean mass seen in 3 of the 4 subjects. The subject who did not gain mass lost 3.2 kg, of mostly fat, of which a negligible 300 g was lean mass. Supplements accounted for 20% of EI which were mostly consumed on the bike or immediately after racing. The remaining 80% of EI was actual food consumption which was mostly at and around meals. Considering the significant amount of EE that was attributed to exercise in this study, it is obviously important that nutrients are provided around and during exercise bouts. Further research into the timing of nutrients in tour-type events would be beneficial.

3.4.4 Relationship between Energy and Protein Balance and Over-reaching

Subject 1 was the participant who pulled out of the ToS due to fatigue on the 5th day. His POMS analysis revealed a positive MS (indicating a negative mood state) at every time point measured and the absence of an iceberg profile. Additionally his plasma cortisol concentration was above the normal range on days 4 and 6. Subject 1 was likely over-trained as determined by his positive MS, lack of an iceberg profile, higher than normal plasma cortisol concentrations and his poorer than expected performance at the ToS (Hooper and MacKinnon, 1995, Fry et al., 1991). It is acknowledged that it is difficult to distinguish a truly overtrained athlete from one who is over-reaching as they exist on a continuum from under-training to optimal training, to over-reaching and then overtraining, but it is clear that Subject 1 was displaying the typical signs of an over-reached and potentially overtrained athlete. In addition, his serum ferritin concentration was within the iron deficient range on days 4 and 6 indicating an inability to adequately carry oxygen, which would have contributed to his fatigue. It is possible however that the 16.4% plasma volume expansion that occurred may have had a dilutional effect on serum

ferritin causing pseudo-anaemia and therefore the findings in regard to ferritin may be inconclusive. Subject 1 provides a case where energy balance was negative, protein breakdown is enhanced and markers of fatigue and overtraining appear with ensuing performance deterioration.

3.4.5 Limitations

The major limitation of this study is the small sample size (n=4) because of the restriction of 5 riders per ToS team. Losing one subject on the second day of the study reduced the original sample from five to four and although another subject withdrew from fatigue later in the week he did complete the study requirements. The study demonstrates the difficulty of performing field-based measurements and the data are novel in that regard. The data are unique in that they come from using a combination of the most technically advanced and valid methods available in a real-life environment with minimal disturbance to competition. To determine WBPT in all five athletes, seven investigators from the research team were required for the 18-hr study period, all requiring skill, experience and buy-in and of course the athletes themselves had to be willing to participate in all testing procedures. Regardless, it is acknowledged that a small sample can over state significant findings and therefore actual p-values have been reported and where appropriate 95% confidence intervals have been stated to indicate the possible spread of the mean differences in a larger group. Further investigations using stable isotope methodologies in the field would prove beneficial despite the likelihood that future studies will also be under powered.

The strength of the study lies with the techniques used. DLW, the gold standard for EE measurement, was utilised in addition to the partitioning of EE with direct measurements of exercise EE via power meters and directly measuring RMR with respiratory gas analyses. Also,

providing food and supplements for the participants and using supervised weighed diet recording to determine EI gives this study an accuracy level that exceeds that of similar studies despite the small sample size.

The second limitation of the study is the loss of oxidation samples due to laboratory error. Although we are not able to report leucine oxidation and leucine disappearance, we believe leucine appearance and protein breakdown is still informative (as discussed above).

3.4.6 Practical Recommendations and Conclusions

In conclusion this study has found that leucine flux and appearance and protein breakdown did not change in fed or fasted periods in response to high EE over a 6-day period. It was also found that despite the high energy requirements of a 6-day, 10-stage cycle tour, on average (3 out of 4) cyclists were able to maintain body weight and gain lean mass in response to the race. Macronutrient intakes approximated or exceeded that recommended for ultra-endurance exercise.

From a practical standpoint there is still no consensus about the optimal amount of protein required to maintain protein balance in response to endurance exercise. Subjects in this study consumed protein in significant excess to the current recommendations for endurance exercise and except for the one subject, who was likely overtrained, all had no change in leucine kinetics despite the considerable energy cost of the exercise they performed. Investigations to determine optimal protein intake for endurance athletes is still warranted and has not yet been addressed even with considerable mention of the discordance in recommendations in the protein metabolism literature.

Maintaining energy balance could be the most important factor in successful stage racing for cyclists. There may be a ceiling to EI, based on limits to digestion and absorption and this may ultimately limit EE over prolonged distances in competitions like a cycling tour event. Macronutrient intake and the timing of that intake may also be crucial in determining recovery rates of energy reserves and in maintaining or enhancing lean mass. The trend of increased lean mass in three of the four subjects is noteworthy. The influence of all food and supplements being provided and readily available, particularly after each stage, would certainly have been considerable. It would be interesting to determine whether the same patterns would be apparent if athletes are independently making choices about food types and quantities. Although there are many studies of nutrient timing, to our knowledge none have studied the effects of timing over a multi-day competition.

There is an interesting link between increased protein breakdown, negative energy balance and the presence of markers of over-reaching, which were not statistically significant but warrant further investigation. Further research would provide both a better understanding of protein kinetics associated with chronic fatigue and also would add to the complex picture that is over-reaching and the overtraining syndrome providing evidence from a protein kinetic viewpoint.

Chapter 4: Taupo Ironman Camp

4.1 Introduction

In 1978 the first Ironman event was held when a group of Navy Seals, stationed in Hawaii, started an argument about who was the fittest athlete out of runners and swimmers. It was suggested that the best way to decide would be to combine the three existing long distance competitions; the Waikiki Rough Water Swim, the Around Oahu Bike Race and the Honolulu Marathon and whoever finished first would be the best athlete – "a real Ironman." The inaugural running of the event saw 15 male competitors start of which 12 completed the race and the winning time was 11hr 46min. Today the Ironman format remains unchanged, and the Hawaiian Ironman is still regarded as the most prestigious triathlon event to win in the world. For the 25th anniversary of the Ironman in October 2003, nearly 1500 athletes participated, with the majority required to have qualified for the event in a previous race. After almost 30 years of the event the race record is significantly faster than that set on the first day in 1978. The current world record is held by Luc Van Lierde of Belgium who completed the Ironman in 8hr 4min 8sec in 1996 (www.ironman.com/mediacentre). In New Zealand, the Ironman has been running since 1985 and was the first international Ironman event. It was raced for 14 years in Auckland before being moved to Taupo in 1999 where it remains today.

Despite the Ironman event being world renowned with professional and elite competitors, there is still very little physiological or metabolic research that specifically focuses on the event itself or even the effect of training for the event. Of particular interest is the energy cost of training for Ironman and consequently the amount of food that needs to be consumed to ensure energy demands are met and an athlete maintains energy balance. Calculation of energy balance

in field studies is precise when proven and precise methods are utilised. Energy balance can be validated by accurate measurement of body weight and body composition and if an athlete is found to be in energy balance then he or she will have a stable body weight. The DLW technique is the gold standard for measuring EE and the weighed diet-record is considered one of the most accurate methods for estimating EI. These methods combined give a precise energy balance determination.

Kimber et. al. (2002) describe EI and EE during an Ironman event. They reported that mean EI for male subjects was 16.5 ± 3.6 MJ and EE was an enormous 42 ± 3.4 MJ leaving the athletes in severe energy deficit (Kimber et al., 2002). However, heart rate recordings were used to predict EE and EI was estimated from a series of interviews with the athletes throughout the race. It is unlikely that an athlete is able to consume 42 MJ of energy during a race to maintain balance but it may be that athletes consumed enough food after the race to correct the negative energy balance reported during the race, or at the very least make it less negative. Kimber et. al.'s findings illustrate the significant energy cost of participation in an Ironman but an emphasis on 24-hour recordings of EE and EI is necessary to draw accurate and practical conclusions from the findings. Knechtle et. al. (2008) studied athletes during multiple Ironman events using heart rate recordings and found that daily EE was 31.6 ± 3.8 MJ/day which matched well with the recorded EI of 32 ± 5.7 MJ/day (Knechtle et al., 2008a, Knechtle et al., 2008b). Significantly lower EIs than those presented by Knechtle have however been reported in triathletes. Elite Australian male triathletes consumed only 17 MJ/day with a weekly training regime that involved 13km of swimming, 323km of cycling and 75km of running (Burke and Read, 1987). In addition, male triathletes participating in the Hawaii Ironman consumed a mere 15 MJ/day during the week prior to the Ironman event (Khoo et al., 1987) and although there are no EE data

from either of these studies the intake values do seem very low in comparison to the study by Knechtle and colleagues and other endurance athletes (Westerterp et al., 1986, Saris et al., 1989).

An understanding of the energy cost of training for Ironman can be gained by examining the findings of studies in endurance runners, cyclists and swimmers. A 1997 study examined the energy cost of endurance swimming in 5 female swimmers (Trappe et al., 1997). Using the DLW technique the study found that swimmers expended 23.4 ± 2.1 MJ/day over a 5-day period when they trained twice per day. The estimated EI of these swimmers was only 13.1 ± 1.0 MJ/day leaving them in energy deficit. The use of the DLW technique gives valuable meaning to the EE findings but the collection of EI information by diet records is not ideal methodology. The under-reporting that often occurs with self report diet records means that the EI reported by Trappe may be too low. However, it is unlikely that the error was in excess of 10 MJ/day which represents the energy deficit of the study and therefore the conclusion that swimmers find it difficult to maintain energy balance is probably accurate. In endurance cycling EE has been estimated at between 25 and 35 MJ/day by heart rate recordings and the DLW method (Westerterp et al., 1986, Saris et al., 1989) with an EI of between 23.5 and 32.4 MJ/day (Westerterp et al., 1986, Saris et al., 1989, Garcia-Roves et al., 1998) using various EI protocols. These values are higher than those seen in swimmers but similar to EE and EI findings in Ironman (Knechtle et al., 2008b). Endurance runners monitored over an 11-day training period had an EE estimated from heart rate recordings of 18 MJ/day (Motonaga et al., 2006). EI was set at only 15 MJ/day based on a survey prior to the study that assessed the normal energy and macronutrient composition of athletes meals. Runners were therefore not able to maintain energy balance. The evidence from studies in swimming, cycling and running suggest that maintenance of energy balance is difficult and that there are differences in methodological approaches when determining energy balance.

Also the difference in EE and EI between the sports is apparent. There is therefore support for specific research with Ironman competitors to ensure that energy balance recommendations are relevant to their event.

In addition, the macronutrient composition of an Ironman's diet is important for sustained performance regardless of energy cost. There is a significant understanding about the role of carbohydrate in training for an Ironman (Coyle et al., 2001, Hawley et al., 1997) and in fact, Kimber et. al. (2002) found that Ironman finishing time is inversely related to carbohydrate intake during the run leg of the race for male athletes. However, the role of protein is unclear and certainly there is no evidence which describes the effect of Ironman training on nitrogen balance and protein kinetics. Again, seeking knowledge by examining studies in runners, cyclists and swimmers can help in the understanding of the issue. Pikosky et. al. (2006) found that endurance trained runners were not able to maintain nitrogen balance when the protein composition of their diet was equivalent to the protein intake recommended for the general population of 0.8g protein/kg/day (Pikosky et al., 2006). This led to recommendations by the authors that endurance athletes should consume more than 0.8g of protein /kg. However, there is significant evidence that states that if EI is adequate to maintain energy balance then protein content of the diet is not the important factor in the maintenance of nitrogen balance (Calloway and Spector, 1954). Runners in the aforementioned study consumed 9988 ± 218kJ/day for a 4-week period. No EE data was collected but the EI is very low for endurance athletes and therefore, the negative nitrogen balance may have been the result of insufficient calorie intake as opposed to insufficient protein.

It may be that the quantity of protein is not the most important factor in maintenance of lean protein mass and that the timing of protein ingestion is important. Nitrogen balance was

found to be positive in endurance cyclists given a protein enriched meal post-exercise and when protein was not given post-exercise, nitrogen balance was negative (Rowlands et al., 2008). These findings are in agreement with others and show that post exercise increases in muscle protein synthesis is in part due to the availability of amino acids (Bennet et al., 1989, Bennet and Rennie, 1991). In addition, the ingestion of a mixed meal induces a hyperinsulinemic response that may have an inhibitory effect of proteolysis (Biolo et al., 1999, Biolo et al., 1995a, Bell et al., 2005). The combined effect of available amino acids, a hyperinsulinemic response and the post exercise anabolic effect in the protein enriched trial probably produced the positive response.

These findings are somewhat helpful for Ironman athletes but neither the study in cyclists or in runners used a protocol that had subjects exercising at a volume equivalent to an Ironman training programme. Also, running and cycling impose different stressors on the body and using one or the other as a descriptor for an event that requires an athlete to perform both, plus one other, is probably not entirely sound. In fact, Kolkhorst et. al. (1994) found that male subjects who participated in both a running and a cycling protocol were in less positive nitrogen balance during running than they were during cycling illustrating that findings for one exercise mode do not readily transfer to another (Kolkhorst et al., 1994).

Another consideration for maintenance of performance both during an Ironman race and during training is the tendency of endurance athletes to over-reach and consequently suffer from the overtraining syndrome. Too much training in conjunction with inadequate recovery from training sessions can yield less than optimal performance improvements and in severe cases a breakdown in the physiological adaptation processes (Budgett, 1998, Ketner and Mellion, 1995). The overtraining syndrome results in stagnation or decrement in athletic performance following a

state of prolonged fatigue and requires significant rest for recovery (Fry et al., 1991). A range of physiological measures have been investigated as possible markers of the overtraining syndrome, however findings are inconsistent and no definitive markers have been set. Two markers that have been regularly identified as reliable indicators of the overtraining syndrome, when found in conjunction with prolonged fatigue and a decline in training and/or competition performance, are an altered mood state and raised plasma cortisol concentrations (Hooper and MacKinnon, 1995). Again, there is no specific research that describes Ironman and overtraining. A 2005 study developed a relationship between carbohydrate consumption and salivary cortisol in male triathletes during a period of high intensity, high volume training (Costa et al., 2005). The conclusion was that a high carbohydrate diet throughout a high intensity training period had a favourable effect on salivary cortisol and therefore reduced an athletes susceptibility to overtraining. However, the triathletes in the study did not develop the overtraining syndrome and the high intensity training period could likely be described as a normal part of a balanced training reigeme. There is currently no research that establishes a relationship between energy and nitrogen balance and markers of overtraining in Ironman populations.

There is a lack of quality research that uses Ironman competitors as subjects. Studies that focus on the energy cost and subsequent EI requirements of an Ironman would be beneficial to produce accurate recommendations for maintaining energy balance. An athlete who trains while in negative energy balance is unlikely to be able to adapt physiologically or metabolically to the training stimulus. In addition, an athlete who is unable to maintain energy balance during an Ironman event is unlikely to perform at an optimal level. Accurate EE and EI information is therefore required. Also, research that determines the amount of protein required to maintain nitrogen balance for Ironman athletes would also be useful so that athletes are aware of how

much protein they need to consume each day while training and also throughout the Ironman race. A description of any relationship that exists between energy and nitrogen balance and markers of over-reaching and overtraining may prove to be helpful in identifying athletes at risk from over-training.

4.1.1 Aims

The Ironman is a grueling triathlon event that requires competitors to swim 3.8km, cycle 180km and run 42km. The training required for Ironman is of critical importance, not only so that a competitor is proficient at all three disciplines but so that he or she can complete the entire distance of the race. This study was conducted at an Ironman training camp held in Taupo, New Zealand which is home to the New Zealand Ironman event. Seven male Ironman competitors attended a 5-day intensive training camp and were tested before, during and after the camp. The purpose of the study was to accurately assess EI, EE and nitrogen balance during an Ironman training camp to determine;

1) the energy cost of training for an Ironman event and whether athletes are able to maintain energy balance when EE is high,
2) the effect of training for an Ironman event on nitrogen balance and whether athletes are able to maintain nitrogen balance while training, and
3) the relationship, if any, between energy and nitrogen balance and measured parameters of overreaching or overtraining.

It was hypothesised that Ironman athletes would be able to maintain energy and nitrogen balance during the training camp, and consequently would not display signs of overreaching or excessive fatigue.

4.2 Methods

Some methods utilised in this study are identical to those described in Chapter 3. Therefore significant similarities in methodological descriptions occur throughout this section.

4.2.1 Subjects

Seven male ironman competitors (29-47yrs) volunteered to participate as research subjects during their involvement in an Ironman Training Camp they were attending in Taupo, New Zealand. The location of the camp was important because the training involved using the actual New Zealand Ironman course. Volunteers had to have an estimated Ironman completion time of between 10 and 14 hours to participate and although it was not a requirement that attendees at the camp had to be involved in the research, all male athletes that attended, participated as subjects. All procedures were approved by the University of Auckland Human Participants Ethics Committee (**Appendix 3** & **4**).

4.2.2 Study Overview

Participation in the study was for a 6-day period and the entire study is depicted in **Table 4.1.** In the week prior to the Ironman camp participants performed a VO_2max test on a treadmill for determination of maximal oxygen consumption but did not perform any other training in the week prior to the camp.

At mid-day on the day of arrival in Taupo (Day 0), prior to the commencement of the training camp, subjects began a 24-hour urine collection protocol for determination of nitrogen excretion. Throughout the 6-days of the study food intake was *ad libitum* and was measured precisely by supervised weighed diet records and EE was measured using the DLW technique. Urine, blood samples and nude weights were collected on waking every morning and a Profile of

Mood State (POMS) questionnaire was also administered on Day 1 and Day 5 in the morning. A second 24-hour urine collection was performed from mid-day on the last day of the training camp for post-camp determination of nitrogen excretion.

Table 4.1: Study Overview - Ironman Camp

Day	Procedure
	VO_2 max test during week prior to camp
0	am: Athletes arrive in Taupo, skinfold assessment, BM
	pm: 24-hour urine collection commences, commencement of supervised weighed diet recording, 10pm DLW dosing
1	am: BM, urine sample, POMS, blood draw 120km cycle, 7km run
	pm: Completion of 24-hr urine collection 4km swim
2	am: BM, urine sample, blood draw 3km swim,
	pm:30km bike, 30km run
3	am: BM, urine sample, blood draw 60km bike, 1km swim
	pm: Running hill drills
4	am: BM, urine sample, blood draw 120km cycle, 7km run
	pm: 24-hour urine collection commences 4km swim
5	am: BM, urine sample, POMS, blood draw 3km swim, 10km run
	pm: Completion of 24-hour urine collection, completion of supervised weighed diet records, skinfold assessment Athletes depart Taupo

BM=nude body mass (kg), DLW = doubly labelled water, POMS = profile of mood state

4.2.3 Procedures

4.2.3.1 Exercise Testing: The VO_2max protocol was performed at the Waikato Institute of Technology Exercise Physiology Laboratory on a Powerjog EG30 Treadmill (Sport Engineering, Birmingham, England). Participants performed a graded exercise test to exhaustion and gas analysis was carried out with a Cortex Metalyser analyser (Cortex Biophysik GmbH, Leipzig, Germany). Treadmill speed was equivalent to each individuals 10km race pace as determined by previous race results but in cases where a recent 10km race result was not available, race pace was determined subjectively in consultation with the athlete and their coach. After a 5 minute warm-up the test commenced at a starting gradient of 1.5% and step increments were 1% every minute until volitional exhaustion.

4.2.3.2 Body Composition: Subjects underwent an 8-site skinfold assessment on arrival in Taupo on the first day of the study and the assessment was repeated on the last day of the study prior to their departure. Body density (BD) was determined from the skinfold measurements by the equations of Withers (Withers et al., 1987) and Durnin & Wormersley (Durnin and Wormersley, 1974). The two BD results were then used to calculate body fat percentage (BF%) from the equation of Siri (Siri, 1961) and the average of the two BF% calculations was reported. In addition, nude body mass measurements were performed prior to the camp on Day 0, and every morning upon rising, after voiding, using the same scale. DXA measurements were unable to be performed due to the absence of a DXA scanner in Taupo. Access to a DXA scanner would have required these measurements to be completed at least 1 week prior to the camp (at the same time as the VO_2max test) and a scan so far in advance of the study would have been of limited value.

4.2.3.3 Twenty four-hour Urine Collection: At midday on the day of arrival in Taupo subjects cleared their bladders and a 24-hour urine collection for determination of nitrogen balance was commenced. All urine produced in the 24-hour period from midday on Day 0 to midday on Day 1 was collected. The volume of each void was measured and recorded, time of void recorded and a 5ml sample from each void was put aside as a continuous sample for later analysis. The 24-hour urine collection was repeated at the end of the training camp, commencing at midday on Day 4 and finishing at midday on Day 5.

Analysis of urine samples for creatinine and urea was performed by LabPLUS at the Auckland City Hospital, Auckland, New Zealand. Creatinine concentration of urine was determined via the picric acid method using a commercially available reagent (Roche Creatinine reagent kit Cat. No. 11936131001V11) and prepared samples were read on a spectrophotometer at 640nm. As provided by the manufacturer, the intra-batch coefficient of variation (CV) ranged between 1.1 and 2.1% and the inter-batch CV ranged between 1.2 and 2.2%. Urea concentration of urine was determined kinetically at 340nm using a commercially available reagent (Roche Urea reagent kit Cat. No. 11729691 216). The intra-batch CV ranged between 0.6 and 1.9% and the inter-batch CV ranged between 1.4 and 2.6%. Nitrogen content of determined creatinine and urea was then established as follows:

Creatinine $C_4H_7N_3O$, atomic mass 113.118g/mol, nitrogen content = 37.20%

Nitrogen from creatinine (g) = measured urine creatinine (g) x 0.3720

Urea $(NH_2)_2CO$, atomic mass 60.07g/mol, nitrogen content = 46.70%

Nitrogen from urea (g) = measured urine urea (g) x 0.4670

Total urine nitrogen was determined as the sum of creatinine and urea nitrogen and was corrected for unmeasured losses. Unmeasured integumentary nitrogen losses were estimated at 5mg/kg/day (Rand et al., 1977), unmeasured fecal nitrogen loss was estimated at 960mg/day (Calloway and Margen, 1971), unmeasured nitrogen loss from blood draws was estimated at 32mg/ml of blood drawn (Calloway et al., 1971) and miscellaneous nitrogen losses were estimated at 400mg/day (Calloway et al., 1971).

4.2.3.4 Energy expenditure: The DLW technique was used to measure total EE. At 5pm on the evening of the Day 0 of the camp, a baseline urine sample was collected. At 7.45pm, after voiding, subjects fasted for two hours before a calculated dose of $^{2}H_{2}{}^{18}O$ was given. The dose was 2g/kg total body water of 10% $H_{2}{}^{18}O$ and 0.12g/kg total body water of 99.9% $^{2}H_{2}O$ for each subject. Total body water was derived from measured nude body weight and body fat percentage estimated from an 8-site skinfold assessment (**Appendix 5**). The dose was enough to obtain an initial enrichment of about 600 times the analytical error described by Schoeller (1988). The dose was given at 9.45pm and was ingested using a straw. Subjects were instructed to consume all the water in their bottle by using the straw to draw up any remaining droplets. Following ingestion, two 50-mL washes of the bottle with tap water were ingested through the straw to ensure all DLW was removed from the bottle and consumed. Urine samples for equillibrium measurements were collected at 7am and 8am the following morning. On each morning of the camp the time of each subject's 1st void was recorded, but the 1st void was not collected. The time of the 2nd void was then recorded and the 2nd void sample was kept for later analysis. During analysis, time of void was taken as the mid-point between 1st and 2nd void times because the kidneys continually produce urine and it is stored in the bladder. Thus the 2nd void sample is made up of urine produced by the kidneys from the time of 1st void through till the time of the

2nd void, therefore taking the midway time point is the average of the urine production and collection time. Urine samples collected for DLW analysis were stored in sealed bottles and kept refrigerated prior to being transported to a -80□C storage facility in the Department of Surgery at the University of Auckland. Samples were analyzed in the Department of Medicine at the University of Sao Paulo, Brazil, in triplicate using a continuous flow isotope ratio mass spectrometer (ANCA20-20, EuropaScientific, Crewe, UK).

The background enrichment determined from the baseline sample was used as the baseline enrichment for each subject. The ^{18}O and ^{2}H dilution spaces (averaged over the camp) were calculated by using the multipoint slope-intercept method(Schoeller et al., 1986b). The ratio of the ^{2}H to ^{18}O dilution spaces was normalized to 1.034 following the method of Racette (Racette et al., 1994). Briefly this method of determining the dilution spaces and the disappearance rate constants for the two isotopes allows total body water, water turnover and carbon dioxide production to be calculated. Total EE was determined from rate of CO_2 production and the food quotient (FQ) (from supervised weighed diet records) using the formula of Weir (Weir, 1949):

$$EE\ (kcal/day) = 99\%\ (3.941r_{CO2}/FQ + 1.106r_{CO2}) \qquad \text{(Equation 3.9)}$$

See section **2.3.1** of this thesis for a full description and derivation of the DLW equations.

Resting metabolic rate (RMR) was calculated by the Harris Benedict equation (**Equation 3.10**) and the Cunningham equation (**Equation 3.11**) and these values were averaged to calculate a final RMR which was converted to kJ/day by multiplying by 4.185 (joules in 1 calorie) (Cunningham, 1980, Harris and Benedict, 1919).

RMR (kcal/day) = 66 + (13.7 x BW) + (5 x Ht) – (6.8 x age) (Equation 3.10)

RMR (kcal/day) = 500 + 22(FFM) (Equation 3.11)

Where BW is body weight in kg, Ht is height in cm and FFM is fat free mass as determined by DLW (see **Equation 2.6** in **Section 2.4.1** for derivation of FFM). In addition, the physical activity level (PAL) was determined by the ratio of TEE:to RMR

4.2.3.5 Energy Intake: Three researchers were responsible for all food purchasing, meal planning, preparation, and for recording the dietary intake data. Food intake during the training camp was *ad libitum* and menus were based on participants' usual food consumption during competition periods, (information obtained through a pre camp questionnaire) and established nutrition guidelines for ultra-endurance exercise. Supervised, weighed diet records were collected from midday on Day 0 until midday on Day 5 of the camp. Diet records for the determination of EI for comparison against EE were taken from breakfast on Day 1 until the last food consumption on Day 4. EI data collected on Day 0 and Day 5 was used to determine N content of the diet (protein intake in grams divided by amino acid nitrogen constant 6.25) for comparison against 24-hour nitrogen excretion for the same time period (e.g. midday Day 0 to midday Day 1 and midday Day 4 to midday Day 5). Supervised, weighed diet-records are considered the most accurate method for estimating dietary intake of individuals and are frequently used to validate other dietary assessment methods (Gibson, 2005).

All meals were consumed at the team accommodation. At all meals, including snack times, participants selected the foods and beverages they wanted to eat and they also selected the amount they wanted. Food items were weighed cumulatively to give a total weight and precise

determination of each food component. After each meal the plate waste was also measured and foods were removed cumulatively from the plate in the same way that they were added. For composite dishes such as stir-fry meals, all raw ingredients, the complete cooked recipe and the proportion of the recipe selected by individual subjects were weighed. This enabled calculation of the quantity consumed for each ingredient (Gibson, 2005). Foods, snacks, supplements and beverages for use before, during and after training were selected daily by participants and pre-prepared and weighed by the research team. Subjects were instructed to report any additional foods and beverages they may have consumed during training and to present any food wrappers. Supplements were the participants own and all supplement labels were collected for analysis.

Energy and macronutrient intakes were calculated from the weighed diet records using a computerized nutrient analysis program (FoodWorks, Xyris Software Ltd, Australia).

4.2.3.6 Blood Measurements: Blood was collected from an antecubital vein on each morning of the training camp. Samples were collected on waking, for measurement of hematocrit (Hct), hemoglobin (Hgb), serum ferritin and plasma cortisol. Hct and Hgb were measured to determine plasma volume changes and the latter two as part of the monitoring of over-reaching markers. Blood was collected into 1 x 8ml EDTA, 1 x 4ml anti-coagulant free and 1 x 4ml SST tube. A small amount of whole blood was used for determination of Hct, in triplicate, by the microhematocrit technique. The remaining whole blood from the second tube was immediately frozen at -20°C and later stored at -80°C for future determination of Hgb. Hgb content was determined on a spectrophotometer using the Drabkins method in duplicate (Drabkin and Austin, 1935). Hct and Hgb were then used for the calculation of percent change in plasma volume (%ΔPV) as described by Harrison (Harrison, 1985).

$$\%\Delta PV = \frac{([Hgb1]\times(1-Hct2))}{([Hgb2]\times(1-Hct1))} \qquad \text{(Equation 3.12)}$$

Where [Hgb1] is the concentration of Hgb on Day 1 and [Hgb2] is the concentration of Hgb on Day 5 and Hct1 and Hct 2 are the Hcts on Day 1 and Day 5 respectively.

Blood collected into EDTA and SST tubes was immediately spun at 1000g at 4°C for 15 mins to separate plasma and serum respectively. Aliquots of plasma and serum were stored in duplicate at -20°C while at the training camp, before being transferred to a -80°C storage facility at the University of Auckland after the camp. Commercially available ELISA assay kits were used to analyse serum ferritin (Calbiotech Inc, USA) and plasma cortisol (R&D Systems, USA) as per the manufacturer's instructions. As provided by the manufacturers the intra-assay CV for ferritin ranged between 4.6% and 7.5% and the inter-assay CV ranged between 6.6% and 9.6%. The intra-assay CV for cortisol ranged between 5.4% and 9.2% and the inter-assay CV ranged between 9.3% and 21.1%. Microwell plates were washed using a mechanical wash system and prepared well plates were tested on a single wavelength (450nm) microwell plate reader.

3.2.3.8 Profile of Mood States Questionnaire: A 37-item modified POMS questionnaire (**Appendix 9**) was administered on the morning of Day 1 and Day 5 of the training camp for the assessment of mood state. The POMS questionnaire provides a score for mood state and is well correlated with over-reaching and overtraining (Raglin and Morgan, 1994). Participants were required to complete the questionnaire based on how they felt 'right now'. Each mood item was rated by participants as 'not at all', 'a little bit', 'moderately', 'quite a bit' or 'extremely' and the five descriptors were coded as 0 through 4 respectively for subsequent analysis. Participants spent 5-10 mins before breakfast completing the POMS and the data were analysed by grouping mood items into the 6 measured mood states of fatigue, anger, tension, confusion, vigour and depression and adding the scores to produce a result for each mood state. A mood score was

determined by adding the five negative mood states and subtracting vigour (Fatigue + Anger + Tension + Confusion + Depression – Vigour). An athlete who is performing well and is not at risk of overtraining will have a negative mood score or positive mood state. Additionally, scores for each mood state were plotted to identify the presence (or absence) of an Iceberg Profile (Morgan et al., 1987a).

4.2.4 Statistics

When appropriate, pre- and post-camp measurements were compared by a paired t-test. Changes over time were analyzed with a one-way repeated measures analysis of variance (ANOVA) with a Tukeys post hoc test performed to determine where any significant differences occurred. Values were considered significant if $P < 0.05$ and data are reported as mean ± standard deviation (SD). A 95% confidence interval (CI) for mean pre/post training camp difference was determined for the standard error of the difference and the t-value for alpha = 0.05 at 6 degrees of freedom ($t = 2.40$). Pearson product moment correlations were performed between energy and nitrogen balance and markers of overtraining to determine any relationship between variables.

4.3 Results

All subjects completed the prescribed training for the camp which consisted of 15km of swimming, 330km of cycling and 54km of running over the study period. Subject characteristics are presented in **Table 4.2**. There was a non-significant mean reduction in body weight over the camp of -0.7 ± 1.0 kg (p = 0.090, 95% CI -1.69kg to 0.15kg). The decrease in body weight was associated with a non-significant mean reduction in the sum-of-8 skinfolds of -4.8 ± 5.8mm (p = 0.074, 95% CI -0.50mm to 10.14mm) and a mean non-significant decrease in BF% of -0.83 ± 0.95% (p = 0.059, 95% CI -1.69% to 0.03%) (**Figure 4.1**).

Table 4.2: Subject Characteristics

	Mean (SD)
Age (yrs)	39 (6.6)
Body Weight (kg)	84.1 (8.2)
Height (cm)	191 (6)
VO_2max (ml/kg/min)	51.1 (2.2)
VO_2max (L/min)	4.3 (0.5)
Lean Body Mass (kg)*	65.9 (2.6)
Body Fat (%) ^	14.8 (6.0)
Sum of Skinfolds (mm)†	85.4 (42.1)

*Lean body mass as determined by DLW. ^ Body fat % as determined by the equation of Siri (1961). †Skinfolds measured at 8 sites.

4.3.1 Energy Intake and Expenditure

Mean energy and macronutrient intakes are reported in **Table 4.3**. Supervised weighed diet records were collected from midday on Day 0 until midday on Day 5 as per **Table 4.1** above but mean energy and macronutrient intakes are reported from breakfast on Day 1 through until the end of food consumption on Day 4 to match with the period of energy expenditure measurement with DLW. The mean energy intake for the period of the camp was 20.27 ± 2.91 MJ/day. There was a significant mean increase in energy intake of 5301.74 ± 4530.51 kJ on Day 4 compared to Day 1 (p = 0.021, 95% CI 1183.09 kJ to 9420.39 kJ) which was accounted for by

a significant increase in carbohydrate intake (p = 0.012, 95% CI 56.95 g to 289.99 g) and a trend toward an increase in fat consumption intake (p = 0.050, 95% CI 0.67 g to 77.87 g) but not protein (p = 0.124, 95% CI -22.89 g to 157.42 g). The majority of energy consumed was as real (non-supplement) food (87.06 ± 3.13%) and the percentage of energy attributable to each of the macronutrients was at levels expected of endurance athletes (carbohydrate = 63.69 ± 4.59%, protein = 17.08 ± 2.08%, fat = 22.98 ± 3.09%). There was a negligible level of alcohol consumption which was due to red wine being used as an ingredient in the evening meals. Individual data for energy intake are tabulated in **Appendix 7**.

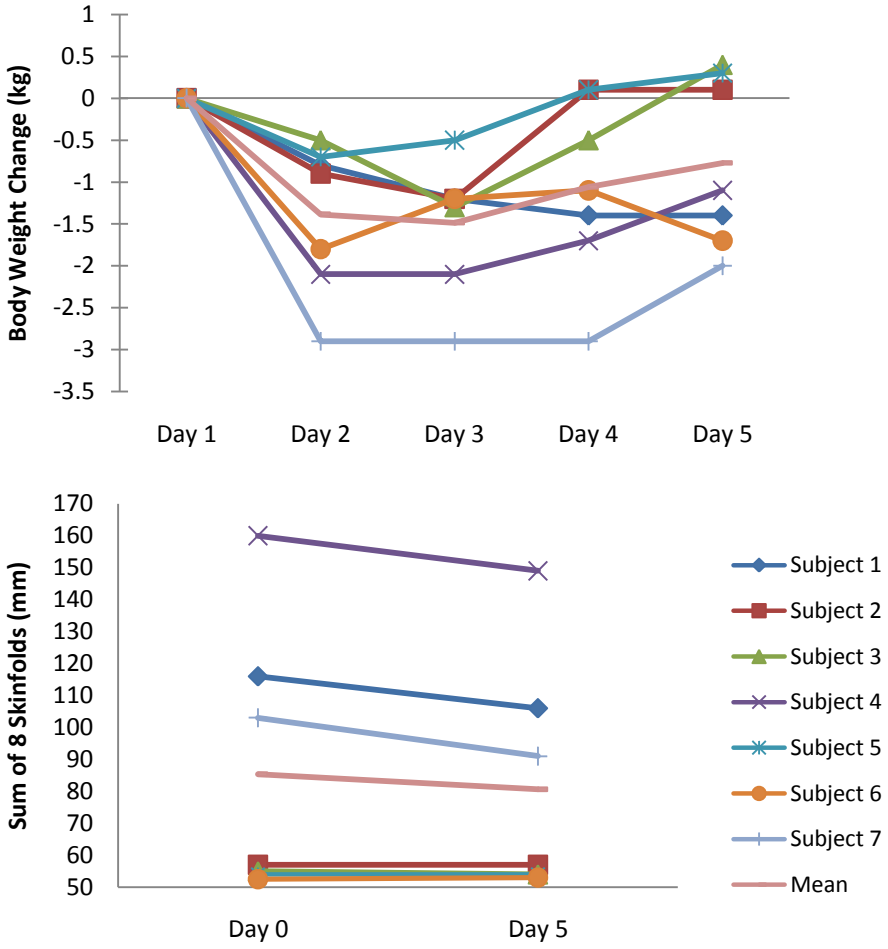


Figure 4.1: Mean and individual body weight and sum-of-8 skinfold changes. Nude weights were measured by the same scale every morning immediately after voiding. Skinfold measurements were taken on Day 0 and Day 5. No significant differences.

Table 4.3: Mean Energy and Macronutrient Intakes

Mean (SD)	Day 1	Day 2	Day 3	Day 4	Week
EI (MJ/day)	19.87 (3.28)	17.78 (4.37)	19.91 (3.29)	25.17* (5.25)	20.27 (2.91)
Carbohydrate					
(g/day)	747.75 (105.04)	643.28 (189.76)	681.68 (140.81)	921.22* (192.55)	763.16 (136.86)
(g/kg/day)	8.76 (0.80)	7.68 (1.61)	7.99 (1.61)	10.70 (1.27)	8.90 (0.91)
%EI	63.34 (5.27)	59.96 (3.73)	69.05 (18.80)	61.35 (3.35)	63.69 (4.59)
Protein					
(g/day)	168.70 (39.00)	189.48 (35.65)	203.85 (26.56)	235.96 (73.81)	201.96 (20.10)
(g/kg/day)	2.01 (0.53)	2.30 (0.38)	2.43 (0.51)	2.75 (0.72)	2.38 (0.30)
%EI	14.22 (2.78)	18.15 (1.90)	20.66 (3.91)	15.80(3.62)	17.08 (2.08)
Fat					
(g/day)	122.94 (38.38)	109.87 (24.05)	97.32 (36.07)	162.60*(45.63)	124.85 (30.45)
(g/kg/day)	1.43 (0.38)	1.33 (0.33)	1.13 (0.35)	1.88 (0.39)	1.46 (0.26)
%EI	22.95 (3.77)	23.49 (1.64)	21.73 (7.50)	24.08 (2.95)	22.98 (3.09)
Alcohol					
(g/day)	3.49 (1.46)	5.10 (1.31)	1.66 (0.74)	0.21 (0.56)	2.67 (0.64)
%EI	0.51 (0.18)	0.87 (0.23)	0.30 (0.14)	0.03 (0.07)	0.42 (0.11)
%EI Food	82.18 (6.06)	85.66 (3.67)	95.68 (2.22)	83.49 (6.86)	87.06 (3.13)
%EI Supp	17.42 (6.22)	13.63 (3.56)	4.07 (2.16)	16.48 (6.90)	12.61(3.24)

EI = energy intake, %EI = percentage of energy intake, %EI Food = percentage of energy intake from food, %EI Supp = percentage of energy intake from supplements * Significantly different from Day 1 ($p<0.05$)

Mean EE was 20.50 ± 3.40 MJ/day which was not significantly different from the mean EI for the period indicating that athletes were in energy balance ($p = 0.808$, 95% CI -2343.27 kJ to 1894.06 kJ). **Figure 4.2** shows the mean and individual energy intakes against energy expenditures. The graph shows that three subjects were in slight positive energy balance (Subjects 1, 3 and 7) with one subject (Subject 2) in considerably positive balance with a mean EI that exceeded their EE by 3.29 MJ/day. Two subjects were in slight negative energy balance (Subjects 4 and 5) and one subject (Subject 6) had a large energy deficit of 4.06 MJ/day. DLW analysis results are presented in chart form in **Appendix 6**.

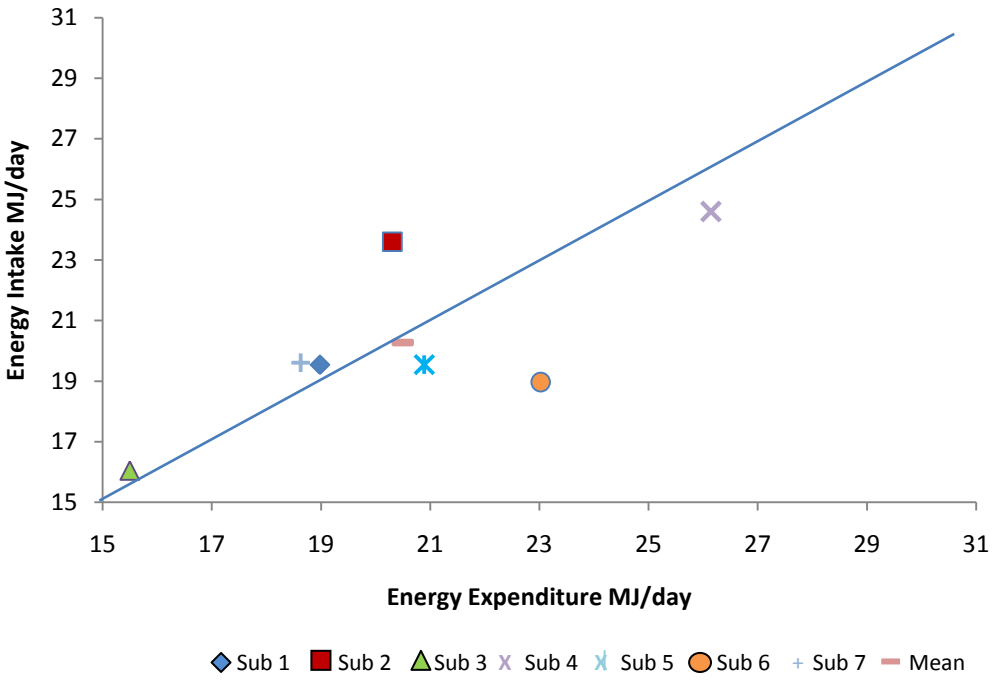


Figure 4.2: Mean and individual energy intake and expenditure. The line represents energy balance. The mean sits almost on the energy balance line. Four subjects are in positive energy balance and three subjects are in negative energy balance.

RMR and PAL are presented in **Table 4.4**. Calculated mean RMR was approximately 40 ± 6% of EE and PAL was in the range of 2.04 - 3.16.

Table 4.4: Energy Expenditure

	Mean (SD)	Range
EE (MJ/day)	20.50 (3.40)	15.50 – 26.14
RMR (MJ/day) Harris Benedict	7.70 (0.48)	7.07 – 8.28
RMR (MJ/day) Cunningham	8.16 (0.24)	7.86 – 8.59
Average RMR (MJ/day)	7.93 (0.33)	7.60– 8.40
RMR (% EE)	40.56 (6.21)	31.69 – 49.06
PAL	2.58 (0.41)	2.04 – 3.16

EE was calculated by DLW. RMR was determied from the average of the RMR calculated from the Harris Benedict and Cunningham equations. PAL = physical activity level (EE:RMR).

4.3.2 Nitrogen Intake and Excretion

Nitrogen intake data were collected from midday on Day 0 until midday on Day 1 and again from midday on Day 4 through until midday on Day 5. These times coincided with the 24-hour urine collections used to determine nitrogen excretion. Mean nitrogen intake prior to the camp was 22.98 ± 2.45 g/day which was greater than the mean nitrogen excretion of 17.27 ± 1.54 g/day, resulting in a significant positive mean nitrogen balance of 5.72 ± 2.36 g/day (p = 0.0007, 95% CI 3.58 g to 7.86 g). Mean nitrogen intake over the last 24-hour period of the camp was 28.92 ± 5.62 g/day compared to a mean nitrogen excretion of 23.93 ± 4.24 g/day but the resulting positive nitrogen balance of 4.99 ± 5.80 g/day was not significant (p = 0.063, 95% CI -0.27 g to 10.25g) although the 95% confidence interval suggests that in a larger sample that the positive balance may be significant. Mean nitrogen intake during the post-camp study was 5.93 ± 4.25g higher compared to pre-camp (p = 0.010, 95% CI 2.08g to 9.79g). Mean nitrogen excretion was 6.66 ± 4.08g higher post-camp compared to pre-camp (p = 0.005, 95% CI 2.96g to 10.36g) (**Figure 4.3**).

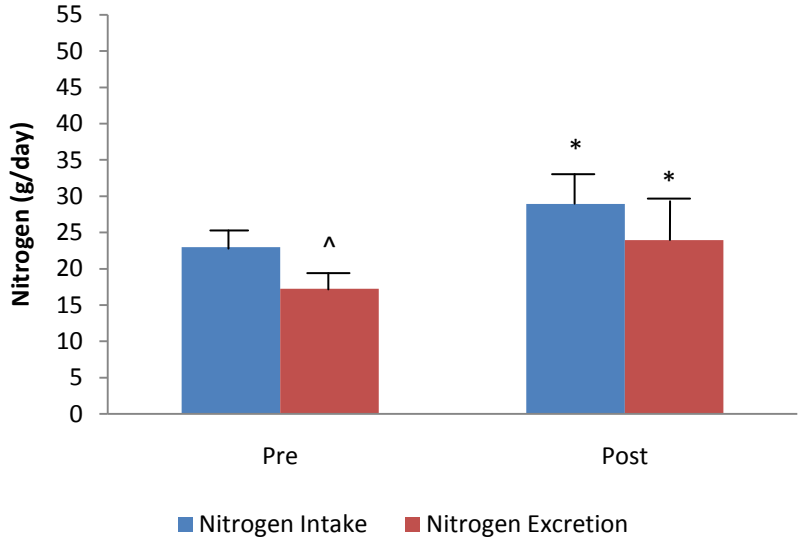


Figure 4.3: Mean nitrogen intake and excretion prior to and at the end of the training camp. ^Significantly different from pre camp nitrogen intake (p = 0.0007). *Significantly different from pre camp values (intake p = 0.010, excretion p = 0.005)

There was considerable individual variation in nitrogen balances both before and after the camp. All subjects were in positive nitrogen balance prior to the camp. Post camp testing showed that two subjects (4 and 6) had moved into negative nitrogen balance while all others remained positive with Subjects 1 and 3 having a positive nitrogen balance that was in excess of their pre-camp measurement (**Figure 4.4**).

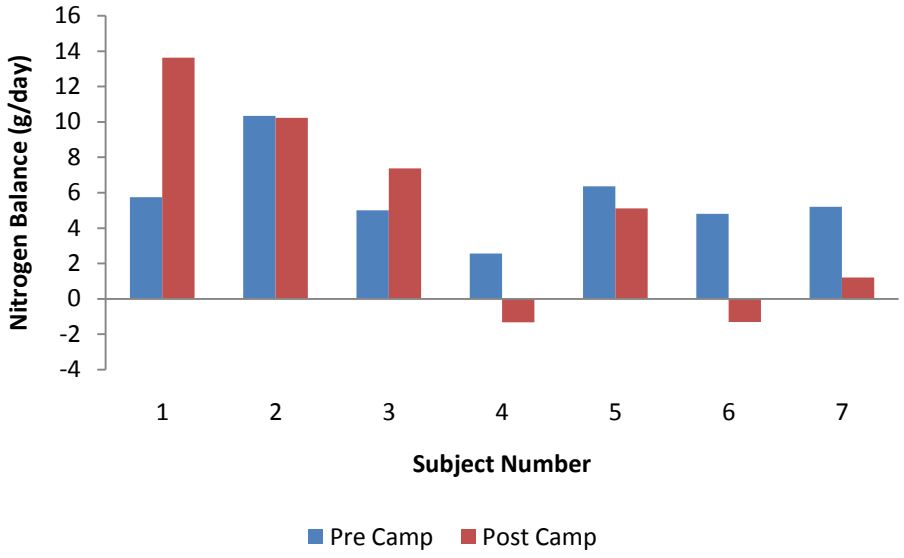


Figure 4.4: Individual nitrogen balances. All subjects were in positive nitrogen balance prior to the camp. Two subjects moved into negative nitrogen balance by the end of the camp.

4.3.3 Blood Measurements

There was no mean change in Hct ($p = 0.631$) or Hgb ($p = 0.824$) at any timepoint as determined by ANOVA. As a result there was no change in plasma volume (mean change 0.96 ± 0.23%). Mean serum ferritin and plasma cortisol concentrations are presented in **Table 4.5**. Mean serum ferritin was within the normal range on all days and mean cortisol values were increased above normal on all days. There were no significant differences in either ferritin ($p = 0.315$) or cortisol ($p = 0.134$) concentrations at any time-point, as determined by ANOVA. There was no correlation between either serum ferritin or plasma cortisol concentration and post-camp

nitrogen balance and in addition there was no correlation between energy balance and plasma cortisol concentration. However, there was a positive correlation between serum ferritin concentration and energy balance (r = 0.740).

Table 4.5: Serum Ferritin and Plasma Cortisol Concentrations

	Day 1	Day 2	Day 3	Day 4	Day 5	Week
Serum Ferritin (ng/ml)						
Mean	209.60	219.41	221.74	158.78	283.49	261.38
(SD)	(137.53)	(134.73)	(145.57)	(129.17)	(108.06)	(99.14)
Plasma Cortisol (ng/ml)						
Mean	249.14*	243.90*	252.42*	293.13*	266.20*	255.78
(SD)	(23.69)	(56.09)	(77.18)	(46.94)	(20.61)	(31.45)

Normal range for serum ferritin = 20-300ng/ml, normal range for plasma cortisol = 60-230ng/ml. * values in excess of normal. No significant differences.

4.3.4 Profile of Mood State

A positive mean Iceberg Profile resulted from the POMS questionnaire analysis on both Day 1 and Day 5 of the camp (**Figure 4.5**). The mean profile was slightly improved on Day 5 with a higher score for vigour and lower scores for fatigue and tension although these changes were not significant (**Table 4.6**). Mood scores were not altered from Day 1 to Day 5 with a mean mood score of 0.14 ± 13.26 on Day 1 and a mean mood score on Day 5 of -4 ± 13.14 (p = 0.516, 95% CI -18.55 to 10.26). Individual Iceberg Profiles are presented in **Appendix 10**.

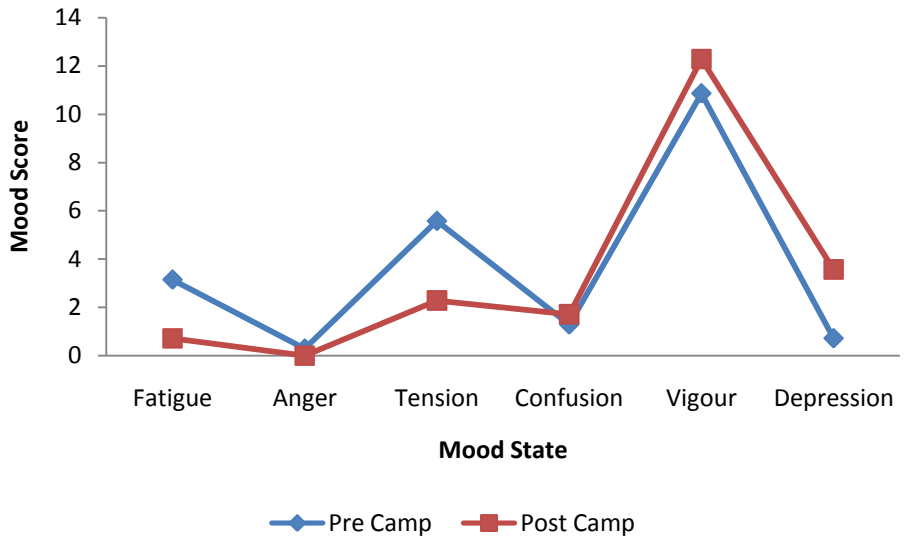


Figure 4.5: Iceberg Profile. Scores for vigour are above scores for the five negative mood states producing a positive iceberg profile and indicating optimal mood.

Table 4.6: Mean Difference in Mood State

Mood State	Mean Difference Day 1 – Day 5	SD	P value	95% Confidence Interval	
Fatigue	-2.43	5.77	0.308	-7.66	2.80
Anger	-0.29	0.76	0.356	-0.97	0.40
Tension	-3.29	4.82	0.121	-7.66	1.09
Confusion	0.43	2.82	0.702	-2.13	2.99
Vigour	1.42	8.62	0.676	-6.39	9.25
Depression	2.86	3.29	0.061	-0.13	5.84

Mean difference between Day 1 and Day 5 for each of the six mood states measured by the POMS questionnaire. A negative mean difference indicates that the score for that mood state decreased and a positive mean difference indicates that the score for that mood state increased. There were no significant differences in any of the mood states. SD = standard deviation

There was a correlation between the difference in mood score from Day 1 to Day 5 and both nitrogen ($r = -0.679$) and energy balance ($r = -0.765$) meaning that as mood scores improved (became more negative) energy and nitrogen balance were more positive. There was however no correlation between the presence of an iceberg profile and values for energy and nitrogen balance.

4.4 Discussion

The purpose of this study was to assess EI, EE and nitrogen balance during an Ironman training camp to determine the energy cost of training for an Ironman event and whether athletes are able to maintain energy balance when EE is high. In addition, the study aimed to determine the effect of training for an Ironman event on nitrogen balance and whether athletes are able to maintain nitrogen balance while training. Any relationship between markers of overtraining and energy and/or nitrogen balance was also explored. The main finding of this study was that on average Ironman athletes were able to maintain energy balance during a period of high EE which was associated with a positive nitrogen balance.

4.4.1 Energy Expenditure and Intake

There is very little evidence which describes the energy cost of training for an Ironman and subsequently the amount of dietary energy required to sustain that training. The average energy cost of Ironman training in this study was 20.5 ± 3.4 MJ/day which is lower than values reported previously in Ironman competitors. Kimber et. al. (2002) reported a substantial EE of 42 ± 3.4 MJ/day during an ironman event and Knectle et. al. (2008) reported EE was 31.6 ± 3.8 MJ/day during multiple ironman events (Kimber et al., 2002, Knechtle et al., 2008b). Subject characteristics were similar between the three studies and all athletes had Ironman finishing times between 10.5 and 14 hours (See **Appendix 11** for Ironman results for participants of this study). The major difference in methodological approach was that this study used the gold standard for measurement of EE, DLW, while the other studies used heart rate recordings. The DLW method is considered to be valid and reliable in the determination of EE (For discussion about assumptions used in the calculation see **Section 3.4.2**). The other difference between this

study and the aforementioned is that the energy component of this study was training while the others were during Ironman events. EE reported here is also lower than values reported in endurance cyclists of between 25 and 35 MJ/day at the Tour de France (Saris et al., 1989, Westerterp et al., 1986) but similar to daily EE reported in endurance swimmers (23.4 MJ/day) and runners (18 MJ/day) (Trappe et al., 1997, Motonaga et al., 2006).

A disadvantage of the DLW method is the inability to measure daily EE to enable a direct comparison with daily EI. In addition it is not possible to partition EE to explain the proportion of EE that occurred with exercise, rest or that was attributed to the thermic effect of food (TEF). In studies with cyclists, power meters imbedded into the hub of the rear wheel or in the crank can be used to determine exercise EE. Unfortunately there is no practical means of measuring specific EE during swimming or running outside of the laboratory environment where a swimming flume or treadmill can be used to determine work performed. Power meters would have been helpful for determination of EE during the bike component of the camp but the expense meant that there was not the budget to supply one meter to each of the 7 subjects. Exercise EE is therefore not reported. The Harris Benedict and Cunningham equations were applied to determine RMR. Mean RMR was 7.9 ± 0.3 MJ/day and mean PAL (TEE:RMR) was subsequently calculated at 2.6 ± 0.4. The PAL value in our subjects falls below those reported previously in cyclists and is lower than the reported energetic ceiling of maximal metabolisable energy, which is 4-5 times BMR in humans (Westerterp et al., 1986). The concept of maximum metabolisable energy comes from the premise that there must be a ceiling to how much energy can be expended in one day and that ceiling is probably determined by how much food a person can consume in that day. It has been reported in animals that EI is 3 to 6 times the measured RMR (Kirkwood, 1983) which relates well to the findings by Westerterp in cyclists (Westerterp

et al., 1986). The subjects in this study were heavy for endurance athletes and three of the seven had relatively high body fat, both of which would have contributed to the low RMR and PAL. Lighter, leaner athletes who were in race condition would have RMR and PAL values higher than those reported here and more in line with the findings of Westerterp and colleagues whose subject group were professional cyclists at the Tour de France (Westerterp et al., 1986).

Supervised weighed diet records are considered to be more accurate than self report diet recording for determining EI (Magkos 2003). Self report diet records are prone to under-reporting and rely on the participants to accurately remember what they have eaten and the portion (Hill and Davies, 2001, Schoeller, 1995). Supervised weighing of food by a research team removes a large amount of the guess work from the EI reporting and allows for an accuracy that is often lacking in nutritional investigations. The measured mean EI in this study was 20.27 ± 2.91 MJ/day which was equivalent to the mean EE producing energy balance across the 4 days of the camp. Ironman participants in a recent study were also in energy balance during a series of Ironman events although their reported mean EI and EE was in excess of 10 MJ/day more than those reported here (Knechtle et al., 2008b). Interestingly, a study that assessed EI in elite Australian triathletes during a week of training that was almost identical to the training performed in this study, found that EI was only 17 MJ/day for male participants (Burke and Read, 1987). Although, if it is assumed that under-reporting of dietary intake occurred because diet records were used to determine EI, then EI values of the two studies may well be similar and indicative of the energy requirements of training for an Ironman. Applegate (1989) suggests that ultra distance triathletes require between 184 and 391 kJ/kg/day (Applegate, 1989). If the mean body weight from subjects in this study (84.1 kg) is used, then Applegate is suggesting that a total EI of between 15.5 and 33.4 MJ is appropriate. The findings here indicate that the lower end

of the range would not be enough to maintain energy balance and the higher end is well in excess of requirements. The subjects in this study consumed on average 241 kJ/kg/day which is similar to the reported mean EI of 247 kJ/kg/day in other ultra distance triathletes (Burke et al., 1991).

Some suggest that it may be difficult to consume energy in excess of 20 MJ/day to meet high energy requirements of endurance exercise and therefore the ability to consume food may be the limiting factor in the maintenance of energy balance (Brouns et al., 1989b, Brouns et al., 1989a). The mean EI of this study falls within those limits and the two subjects (4 & 6) who expended more than 20 MJ of energy were unable to match that expenditure with a similar EI leaving them in energy deficit. If the 20.5 MJ/day EE reported here accurately represents the energy cost of training for a 10.5 – 14hr Ironman for male athletes then it may be that these athletes are more easily able to maintain energy balance compared to other endurance athletes who have an energy cost that is significantly higher than 20 MJ/day.

It is not only the total EI that is important for endurance athletes. The macronutrient composition of the diet is essential to consider when giving nutrition recommendations. 600 - 650 g/day or 8 - 10g/kg/day of carbohydrate is generally accepted as the required intake to maintain glycogen stores in the liver and skeletal muscle for endurance athletes (O'Toole and Douglas, 1995). In a normal, non-athletic diet this equates to approximately 60-70% of calories. In athletes with significantly higher calorie intakes (e.g. 19 – 25 MJ) the carbohydrate requirement can be met from a diet that is 55-60% carbohydrate. These requirements hold true for this study where on average carbohydrate made up 63.7% of total EI with an average daily intake of 763 g/day or 8.9 g/kg/day.

Carbohydrate status has an effect on protein status in endurance athletes. Protein metabolism typically contributes about 10% to EE but when carbohydrate stores are low, amino

acid oxidation increases thereby increasing the protein contribution to about 15% of total EE. Protein content of the diet is therefore important for maintenance of protein mass (Dohm et al., 1986, Dohm et al., 1982b). The current recommendations for protein intake by endurance athletes are between 1.2 and 1.4 g/kg (Tipton and Wolfe, 2004) and subjects in this study were well in excess of those recommendations. Average protein intake here was 2.4 g/kg which equates to 17% of total EI. Other findings in ultra distance triahtletes have reported protein intake to be between 1.65 g/kg (Khoo et al., 1987) and 2.0 g/kg (Burke et al., 1991) which are lower than in this study but still in excess of recommended requirements. These findings do not suggest that protein intake in endurance athletes needs to be higher than the recommendations. In fact, some researchers suggest that it is the ability to maintain energy balance by consuming sufficient total calories that determines maintenance of protein stores and not the protein content of the diet itself (Calloway and Spector, 1954).

Food was *ad libitum* and subjects were instructed to eat normally during the study. However, it is possible that confounding of habitual intake may have occurred because the research team supplied and prepared the food, using good quality protein and ingredients. Despite the ability to choose what, and how much they ate, athletes may have been eating better than their habitual diets due to the foods that were available for consumption. A similar study in free-living athletes who provide their own meals would be interesting to determine whether athletes can maintain energy balance if they had to provide and prepare their own food. A study performed as described would also provide insight into how well athletes understand nutrition recommendations and whether their knowledge of how much and what to eat is easily transferred to optimal nutrition on their plate.

4.4.2 Energy and Nitrogen Balance Relationship

Mean nitrogen balance was positive both at the start of the training camp and at the end, beginning at 5.72 ± 2.36 g/day on Day 0 and finishing at 4.99 ± 5.80 g/day on Day 5. There was a non significant mean reduction in body weight of -0.7 ± 1 kg which was likely due to a reduction in fat stores as opposed to lean mass loss, although without specific body composition information from DXA scans it is difficult to draw definite conclusions. There are a number of limitations associated with the nitrogen balance method (see **Section 2.3.3** for a full description). These limitations suggest that estimated nitrogen balances are probably higher than in reality due to the combined effect of over-estimation of nitrogen intake and under-estimation of nitrogen excretion. There is likely some element of over-estimating of EI in this study despite the strict supervised weighing of all foods. However, the degree of overestimation will be minimal and related to the loss of recorded nitrogen on cutlery and crockery, although food wastes and plate scraps were weighed and factored into the analyses. Under-estimation of nitrogen excretion is probably the main factor in the potential confounding of the positive nitrogen balance results here. Urinary nitrogen was the only excreted nitrogen measured and despite corrections for blood, fecal, integumentary and miscellaneous losses, no assurance can be given that these values are correct for these subjects. The correction values used (integumentary nitrogen loss; 5mg/kg/day (Rand et al., 1977), fecal nitrogen loss; 960mg/day (Calloway and Margen, 1971), nitrogen loss from blood draws; 32mg/ml of blood drawn (Calloway et al., 1971) and miscellaneous nitrogen losses; 400mg/day (Calloway et al., 1971)) are widely accepted and as such it is likely that the overall positive balances found in this study are real.

There was considerable inter-subject variability in the nitrogen balances and for this reason consideration of individuals is warranted. Subjects 1, 2, 3 and 7 were able to maintain energy balance over the duration of the camp with EE's that were 20 MJ/day or less (19.0, 20.3,

15.5, 18.6 MJ/day respectively). All were also in positive nitrogen balance and Subjects 1 and 3 had improved nitrogen balances after, compared to prior to, the camp. The positive nitrogen and energy balances were associated with a minimal increase in body weight in Subjects 2 and 3 (0.1kg and 0.4kg respectively) and these findings support the accepted ascertion that when a person is in energy balance they are weight stable. Subjects 1 and 7 had a reduction in body weight (-1.4 and -2 kg respectively) but both had a large reduction in BF% over the camp (-1.68% and -2.14% respectively) and it is likely that the decreased body weight was solely the result of the body fat loss as opposed to lean mass, although lack of accurate body composition findings from DXA means that conclusions cannot be made. Subjects, 1, 2, 3 and 7 support the notion that it is possible to balance energy output with adequate intake when EE is 20MJ/day or less (Brouns et al., 1989a, Brouns et al., 1989b).

Subject 5 was in slightly negative energy balance (-1350kJ) that was associated with a minimal 0.3kg increase in body weight and a nitrogen balance which decreased from pre- to post-camp but still remained positive. Subject 5 could be described as 'borederline' with his negative energy balance related to a reduction in body weight and a decrease in nitrogen balance status.

Subjects 4 and 6 had EE's well in excess of 20 MJ/day (26.1 and 23.0 MJ/day respectively) and were not able to maintain energy balance during the camp. Under these conditions nitrogen balance was negative and both subjects lost body weight (-1.1 and -1.7 kg respectively). A minimal amount of weight loss can be attributed to fat mass loss in Subject 4 who had a decrease in BF% of -1.63% but it is likely that the majority of weight loss for Subject 6 was lean mass loss because there was no change in BF%. These findings suggest that it is difficult to consume enough food to maintain energy balance when EE is in excess of 20 MJ/day.

The subsequent negative nitrogen balance leads to a loss of lean mass despite adequate protein intake (2.0 and 2.8 g protein/kg respectively).

Many have reported that endurance athletes require a higher amount of dietary protein than sedentary controls or even strength based athletes. Protein intakes ranging from 1.14 g/kg/day through to 1.80 g/kg/day have been reported as necessary to maintain nitrogen balance in trained endurance athletes (Brouns et al., 1989b, Brouns et al., 1989a, Meredith et al., 1989, Tarnopolsky et al., 1988, Friedman and Lemon, 1989). The average protein intake in this study was 2.38 g/kg, well in excess of the level suggested by previous authors. The findings from this study cannot test the appropriateness of the recommended protein intake but the high positive nitrogen balances do suggest that intake at a level greater than 2.0 g/kg is beyond the requirement. It is interesting to note however that despite the excessive protein intake the percentage of EI that is attributable to protein is similar to other studies in endurance athletes. Westerterp et. al. (1986) and Saris et. al. (1989) have both reported that at extreme EE's protein intake remains approximately 17% of total daily calories which is equivalent to the findings presented here. In addition, Cheng et. al. (1987) have reported that elite and amateur endurance athletes from a variety of sports habitually consume over 2.0g/kg of protein per day which supports the notion that the subjects here were in fact consuming protein at habitual levels despite the food provision and preparation by the research team.

The provision of amino acids stimulates muscle protein synthesis and the provision of a mixed meal that induces hyperinsulinemia results in an inhibition of proteolysis (Bennet et al., 1989, Manchester and Young, 1958). These responses occur with normal feeding, but the timing of nutrient ingestion after exercise is thought to be crucial in the maintenance of protein stores. The anabolic effect of exercise is thought to be enhanced by the ingestion of a mixed supplement

immediately after that exercise. It has been shown that the post endurance exercise ingestion of a mixed meal increases amino acid uptake and protein synthesis compared to later ingestion (Levenhagen et al., 2001). In the same study, protein breakdown was unchanged with immediate post exercise ingestion but when ingestion was delayed there was a net amino acid release from muscle and net loss of protein. In the Tour of Southland study described in Chapter 3 of this thesis, riders were provided with carbohydrate and protein supplements throughout and immediately after each stage of racing. The timing of these supplements in relation to the exercise they performed was postulated to have promoted the gains in lean mass seen in 3 of the 4 subjects. Subjects in the current study were not instructed to eat immediately post exercise and there was no provision of specific carbohydrate or protein supplements by the research team. Most subjects did however consume either their own supplements which they brought with them to camp or real food as soon as they arrived back at the team accommodations after training. It may be that the immediate post exercise nutrition contributed to the positive nitrogen balances seen in 5 out of the 7 subjects.

4.4.3 Relationship between Energy and Protein Balance and Overtraining

There was a statistical relationship between the change in mood score over the week of the camp and both nitrogen and energy balance. The correlation confirms that the more negative the mood score became over the week (an indication that mood state was becoming more positive) the more positive or toward positive the energy and nitrogen balances. In that regard, from the perspective of monitoring an athlete, it is possible that the presence of a measured negative mood state (positive mood score) may be an indicator that an athlete is at risk of being unable to maintain energy and nitrogen balance and the initiation of a nutritional assessment or intervention may be prudent. There was also a strong positive correlation between serum ferritin

concentration and energy balance in that the higher the serum ferritin the greater the likelihood of maintaining or being in positive energy balance. Low serum ferritin levels have definite implications for fatigue and altered performance for an athlete because of the potential for a reduction in oxygen carrying capacity. If low ferritin is associated with less than optimal energy balance as the correlation suggests then regular monitoring of ferritin levels may be warranted for at risk athletes. It is acknowledged however that all subjects in this study had serum ferritin values within the normal range. The correlation may be different for values outside the normal range although the strength of the relationship here ($r = 0.740$) suggests that it would be similar. It would be beneficial to determine any relationship between energy balance and altered iron status in subjects whose ferritin levels were below the normal range.

From a non-statistical viewpoint there are certainly interesting findings when subjects are assessed on an individual basis. Two subjects (Subject 4 and 6) were in negative energy balance and were also in negative nitrogen balance at the end of the camp in comparison to the other five subjects. These two subjects had the lowest serum ferritin concentrations in addition to the lowest scores for vigour and the absence of iceberg profiles on Day 5 of the camp. These factors are potential markers of overtraining and despite the lack of statistically significant correlation, it is important to note these findings. It would be incorrect to suggest that Subjects 4 and 6 were overtrained but the statistical findings in addition to the individual analyses suggest that these athletes need to be closely monitored and given nutritional interventions to ensure energy and nitrogen balances are maintained. Their performance at the camp was very good and fatigue was not a concern but because the overtraining syndrome develops over an extended period of training, identifying at risk athletes well in advance of symptoms is essential. There is definite merit in the monitoring of overtraining variables and the use of nutritional interventions to ensure

energy and nitrogen intakes are optimal. There is evidence that suggests that poor nutritional status is a factor in overtraining (Fry et al., 1991) and therefore if the relationship between energy and nitrogen balance and overtraining is strong then ensuring optimal nutrition practices may be preventative in the development of the syndrome. This relationship needs to be explored in more depth with a study that specifically focuses on the effect of suboptimal energy and protein balance on the appearance of overtraining markers.

4.4.4 Limitations

An obvious limitation of the study is the low subject number and consequent low power. The research personal required to perform the study was a significant factor in the small subject number. Four researchers were present for the duration of the camp to ensure all data was collected with accuracy. In addition the expense of the study was substantial as food and accommodation for the participants, their coach and the research team was paid for by the research team and there was considerable expense related to the methodologies used. In fact, the combined expense of this study and the previous research described in Chapter 3 meant that the WBPT technique used at the ToS was unable to be used here. Instead the nitrogen balance technique was used where, although not inexpensive, analysis of the nitrogen content of urine was cheaper than the purchase, preparation and analysis of ^{13}C-leucine. The subject number is however, not unusual for this type of energy balance field investigation (Westerterp et al., 1986, Saris et al., 1989, Kimber et al., 2002). In addition the early nitrogen balance studies also had small samples sizes (Todd et al., 1984, Butterfield and Calloway, 1983, Calloway, 1975). Regardless, it is acknowledged that a small sample has the tendency to over state significant findings and therefore specific p-values have been reported and where appropriate 95%

confidence intervals have been determined to indicate the possible spread of the data in a larger group. Further investigations using stable isotope methodologies would be beneficial although it is unlikely that such projects will be run with large samples in the near future.

The accurate assessment of body composition is a vital component of any energy and protein balance study, particularly as verification of DLW and protein or nitrogen balance results. The inability to access a DXA scanner in Taupo was a significant limitation of this study and indicates the difficulty in accessing appropriate technologies when performing studies in the field. Subjects underwent VO_2max testing in the week prior to the camp in Hamilton, 2 hours north of Taupo. DXA scans were not performed at the same time because subjects performed their tests 4-5 days prior to the first day of the camp and the time period was considered inappropriate for the DXA findings to be meaningful. In addition, subjects would have had to make a return trip to Hamilton immediately after the camp for a post-camp scan and this was not possible for most. Due to the lack of complete body composition findings any discussion that has been made regarding changes in LBM over the period of the camp are assumptions only, despite the accuracy of the measured body fat changes.

4.4.5 Practical Recommendations and Conclusions

In conclusion, this study found that Ironman athletes are able to maintain energy and nitrogen balance throughout a period of high EE. In addition, there are selected correlations between negative energy and nitrogen balance and the appearance of overtraining markers which with further investigation may provide a major role for nutritional intervention in the prevention of overtraining.

It is particularly apparent that the EE required to train for a 10.5 – 14 hour Ironman is on average 20 MJ/day. The EI necessary to sustain the energy cost must of course be equivalent.

This study supports the findings previously described in Ironman athletes (Burke et al., 1991) that shows that EI values of approximately 20 MJ/day are indicative of the energy requirements for Ironman training and maintenance of energy balance. These absolute energy values are equivalent to approximately 241 kJ/kg/day.

This study also concludes that it is likely that Ironman athletes are more easily able to maintain energy balance compared to other endurance athletes who have an energy cost in excess of 20 MJ/day. 20 MJ/day is the suggested level of maximal metabolisable energy intake and therefore because the EE required to train for an Ironman is equivalent to 20 MJ/day, Ironman athletes should be able to maintain energy balance better than their counterparts in other sports that have a higher EE.

The provision and preparation of all food by the research team is likely to have had some effect on the findings here. It would be interesting and beneficial to perform an energy and protein balance study with endurance athletes who had to provide and prepare their own foodstuffs. This would determine if they were able to maintain energy and protein balance without expert support. Findings from a study as described would identify how much knowledge athletes have about optimizing performance through nutritional practices and if they are able to transfer the knowledge to appropriate calories and nutrients on their plate.

Endurance athletes consume protein in excess of dietary recommendations but from a practical point of view there is still no agreement about what the optimal daily intake of protein is that will maintain protein balance in response to high energy cost exercise. Subjects in this study were in positive nitrogen balance when energy intake was adequate to maintain the energy cost of exercise. However, investigation to determine the optimal protein intake for endurance athletes is still warranted but requires a rigorous approach. The use of stable isotope

methodologies, accurate body composition measurement, precise methodologies for determining EE and EI and ideally a free-living environment where exercise is performed under normal training or competition conditions would provide a setting that optimises the research rigour and allows for findings to have practical application.

There is an interesting link between negative energy and nitrogen balance and the presence of markers of overtraining. None of the subjects in this study were overtrained and therefore establishing a clear relationship was difficult. Further research that investigates specifically the association between energy and protein/nitrogen balance and overtraining markers would need to be performed in athletes identified as at risk of developing the overtraining syndrome. The syndrome is complex and despite a body of overtraining research there is still no agreement about a set of markers that are predictive. Further research would also provide an understanding of energy and protein kinetics associated with chronic fatigue and would be of considerable benefit to the endurance sport population.

Chapter 5 Summary

5.1Overview

Road cycling and Ironman are endurance sports which differ in their physiological and metabolic requirements but are similar in their ultra-distance nature and the need for optimal nutrition to ensure performance can be sustained for the duration of a training session or competition period. The overall goal of this thesis was to collect and interpret accurate energy, protein and nitrogen balance evidence in free-living endurance athletes with a view to building a better understanding of the energy and protein requirements of ultra-endurance sport. In addition the purpose was to determine whether energy balance and protein balance parameters had any relationship to factors related to over-reaching and the risk of overtraining. This relationship was developed in an attempt to provide recommendations that not only ensure optimal performance but which help in the prevention of under-performance that occurs in an overtrained athlete. The specific aims of the thesis were to determine:

whether high energy expenditure, independent of energy balance, affects whole body protein turnover during a 6-day cycling tour

whether high energy expenditure, independent of energy balance, affects nitrogen balance during a 6-day Ironman camp

whether athletes are able to maintain energy balance over a period of increased energy expenditure

energy requirements of a 6-day cycling tour and a 6-day Ironman camp and exactly what athletes eat during this time, and

whether energy balance, protein turnover or nitrogen balance relate to measured parameters of fatigue and overreaching.

The following experimental hypotheses were tested:

High energy expenditure during a 6-day cycling tour event will not have any effect on whole body protein turnover

High energy expenditure during a 6-day Ironman training camp will not have any effect on nitrogen balance

Athletes will be able to maintain energy balance during their 6-day event

Energy requirements will be in excess of 20MJ/day and both cyclists and Ironman athletes will consume protein in excess of the recommendations for endurance athletes

Negative energy, nitrogen and protein balance will be associated with increased presence of overtraining markers

5.1.1 Answering Aims

Aim 1: To determine whether high energy expenditure, independent of energy balance, affects whole body protein turnover during a 6-day cycling tour.

During the Tour of Southland cyclists were required to expend a considerable amount of energy every day for 6 days. There was no significant effect of the extreme energy cost on whole body protein turnover and importantly there was no significant increase in protein breakdown during either the fed or fasted states.

Aim 2: To determine whether high energy expenditure, independent of energy balance, affects nitrogen balance during a 6-day Ironman camp.

An intensive period of specific Ironman training did not have any significant effect on nitrogen balance in Ironman competitors. Mean nitrogen balance was positive after the training camp.

Aim 3: To determine whether athletes are able to maintain energy balance over a period of increased energy expenditure.

Endurance cyclists participating in the Tour of Southland were on average able to maintain energy balance over the duration of the tour. However, in specific cases where a subject was in negative energy balance, the energy cost was associated with a general increase in leucine flux, Leu-PB and total protein breakdown.

Ironman athletes on average were also able to maintain energy balance throughout the days of an intensive training camp. Again, in the cases where energy balance was negative, there was a non-significant effect of increased protein breakdown as evidence by negative nitrogen balances.

Aim 4: To determine the energy requirements of a 6-day cycling tour and a 6-day Ironman camp and exactly what athletes eat during this time.

The average energy cost of a 6-day, 10 stage cycle tour is 27.4 ± 2.0 MJ/day. To maintain energy balance cyclists consumed a diet that consisted of 63.7 ± 1.8% carbohydrate, 15.2 ± 0.9% protein and 17.3 ± 2.3% fat. On average carbohydrate intake equated to 12.9 g/kg/day and protein intake averaged 2.9 g/kg/day both of which are in excess to the

nutrient recommendations for endurance athletes. 80% of the cyclists dietary intake was in the form of real food and the remaining 20% was consumed as supplements mostly during and immediately after the race.

The average energy cost of an Ironman training camp was 20.5 ± 3.4 MJ/day. Ironman athletes consumed 63.7 ± 4.6% carbohydrate, 17.1 ± 2.1% protein and 23.0 ± 3.1% fat to maintain energy balance. On average carbohydrate intake was 8.9 g/kg/day and average protein intake was 2.4 g/kg/day which are lower than intakes recorded at the Tour of Southland but approximate the carbohydrate recommendations and exceed the protein recommendations for endurance athletes. Approximately 87% of dietary intake was in the form of real food with the remaining nutrients coming from the athletes own supplement supply.

Aim 5: To determine whether energy balance, protein turnover or nitrogen balance relate to measured parameters of fatigue and overreaching.

There was a tendency toward a relationship between negative energy and protein balance and the presence of overtraining markers. However, the low subject numbers and the fact that athletes were not overtrained (except 1 subject participating at the Tour of Southland) meant that this relationship can only be implied here not confirmed. There is evidence to support further research into the correlation between a negative mood state and high plasma cortisol concentrations and negative energy and protein balance which arose from findings in one subject at the Tour of Southland. There is also support for the correlation between negative mood and negative energy and nitrogen balance from the Ironman camp. In addition, there may also be a correlation between low serum ferritin

concetration and negative enrgy and nitrogen balance although assessment of this realtionship at below normal ferritin levels is required.

5.1.2 Explanation of Study Differences

There were many similarities in the methodological approaches used in both the Tour of Southland and the Ironman camp studies. It is accepted that it would have been much easier to make direct comparisons between the studies had the methods used been identical. It was the intention at the outset to perform the same studies in two different endurance populations and it may have been interesting to have used cyclists for both and have a female subject sample in the second study. Nevertheless, a number of constraints including cost, availability of subjects, availability of skilled research assistants and time restrictions associated with the PhD process meant that an identical study was relinquished. An explanation of the differences in the studies approaches in answering essentially the same questions follows.

The use of ^{13}C-leucine and the whole body protein turnover technique in the Tour of Southland study was ambitious especially when considering the doubly labelled water methodology for measurement of energy expenditure and the rigour of the collection of dietary intake information. It was enormously disappointing to all involved when it was revealed that by no mistake of our own we were unable to obtain findings that would complete the picture of the effect of endurance exercise on protein kinetics. Without leucine oxidation we were unable to determine non-oxidative leucine disposal or the protein synthesis response to endurance exercise. The findings presented within this thesis are novel and informative but the lack of an entire picture of protein kinetics is frustrating and the lack of protein synthesis findings was part of the reason why the same procedure was not used for the Ironman camp.

The limitations of the nitrogen balance method have been described in Chapter 2 of this thesis and provide reason for the preferential use of stable isotopes in the assessment of protein kinetics. However, there is large body of traditional nitrogen balance evidence which was instrumental in the early understanding of protein kinetics. There is certainly still a place for nitrogen balance methodologies particularly from a practical view point when it is necessary to be aware of the protein status of an athlete. In such a situation stable isotope methodologies would be inappropriate because of their expense and the invasiveness required (IV line for whole body methods and biopsy for regional skeletal muscle) to obtain meaningful results. The nitrogen balance method used in the Taupo Ironman study was sound and the supervised weighed measurement of dietary intake coupled with the use of correction values for unmeasured nitrogen losses provides some assurance that the balance values found were accurate. Hindsight provides a valuable means of reflection and it would have been beneficial to both the knowledge pool and practically for Ironman athletes to have protein balance evidence based from a stable isotope method.

RMR was not measured directly during the Taupo Ironman camp mainly because of the confounding that occurred during the Tour of Southland study where measured RMR was higher than expected due to probable arousal of subjects in anticipation of the days competition. It was also convenient to simply calculate RMR using the Harris Benedict and Cunningham equations because, as was the issue with the DXA scanner, Taupo was not equipped with a facility that held a volume meter and gas analyser. It is obvious of course that the research team could have used portable devices sourced from an out of town venue but at the time of planning it was decided that RMR was not an essential component of the study and considering the arousal issue, the direct analysis was forgone.

During the Tour of Southland study there was a deliberate effort to provide on-the-bike and post exercise supplementation. The main reason for this was to follow optimal nutritional practices for endurance athletes. All athletes had their on-the-bike supplements prepared and weighed prior to leaving for the race each day and a nutritionist travelled in the team van each day to provide additional food and supplementation as required. In addition, post exercise supplements were also prepared prior to the race each day meaning that athletes had access to immediate post exercise nutrition. In some cases athletes had to be transported back to the team accommodations by vehicle (as opposed to biking back from some of the local stages) and during this time they consumed their post race nutrition, making the immediacy of their supplement consumption post exercise an interesting discussion point in the study. The supplements used as part of the Tour of Southland study were sponsored by a New Zealand supplement company and there was a significant amount available for use. There was no supplement provision during the Taupo Ironman camp and athletes were advised prior to the camp that all food would be provided for them but that they needed to bring with them their own supplements for consumption. In the same way, supplements for training were prepared and weighed by the research team each morning prior to a training session but only on the request of the athlete. Any post exercise supplementation was prepared for the athletes upon return to team accommodations at their request, but there was no preprepared supplementation as in the Southland study. The difference in methodological approach in this respect is evident in the difference in the percentage of energy intake that came from supplements during the Tour of Southland compared to the Ironman camp, with 20% of total energy intake coming from supplements in the Southland study compared to only 15% in Taupo. The Ironman camp

probably better reflects endurance athletes free living consumption of supplements because they had to provide their own and request their preparation.

5.2 Limitations

The major limitation of the thesis was the small sample size for both studies. Both studies illustrated the difficulty of performing accurate field-based measurements and the data are novel in that regard. There was considerable cost involved in the provision of food and accommodation for athletes and researchers in both studies and in addition the cost of the methodological procedures was significant. The data are unique however, in that they come from using a combination of the most technically advanced and valid methods available in a real-life environment with minimal disturbance to training and competition. To determine WBPT in all five athletes at the Tour of Southland, seven investigators from the research team were required for the 18-hr study period, all requiring skill, experience and buy-in and of course the athletes themselves had to be willing to participate in all testing procedures. The supervised weighed diet records also required skilled staff and enough people weighing and preparing to ensure that meals were eaten at appropriate times. The time required to accurately collect weighed diet records must be considered by researchers who want to use the technique because it is substantial. The research personal available to assist in the Taupo Ironman camp was less than for the Tour of Southland due to the remote location. Four researchers were present for the duration of the camp to ensure all data was collected with accuracy. Regardless, it is acknowledged that the small samples can over state significant findings and therefore actual p-values and where appropriate 95% confidence intervals have been reported to indicate the spread of the mean differences in a larger group.

The loss of oxidation samples in the Tour of Southland study due to laboratory error was a major unforeseen limitation. Although leucine oxidation and leucine disappearance have not been reported, leucine appearance and protein breakdown findings are still informative and novel.

The accurate assessment of body composition is a vital component of any energy and protein balance study, particularly as verification of DLW and protein or nitrogen balance results. The inability to access a DXA scanner in Taupo was a significant limitation of this study and indicates the difficulty in accessing appropriate technologies when performing studies in the field. Subjects underwent VO_2max testing in the week prior to the camp in Hamilton, 2 hours north of Taupo. DXA scans were not performed at the same time because subjects performed their tests 4-5 days prior to the first day of the camp and the time period was considered inappropriate for the DXA findings to be meaningful. In addition, subjects would have had to make a return trip to Hamilton immediately after the camp for a post-camp scan and this was not possible for most. Due to the lack of complete body composition findings any discussion that has been made regarding changes in LBM over the period of the camp are assumptions only, despite the accuracy of the measured body fat changes.

5.3 Conclusions

In conclusion this study has found that leucine flux and appearance and protein breakdown did not change in fed or fasted periods in response to high EE over a 6-day period. It was also found that despite the high energy requirements of a 6-day, 10-stage cycle tour, on average (3 out of 4) the cyclists were able to maintain body weight and gain lean mass in response to the race. Macronutrient intakes approximated or exceeded that recommended for ultra-endurance exercise.

From a practical standpoint there is still no consensus about the optimal amount of protein required to maintain protein balance in response to endurance exercise. Subjects in this study consumed protein in significant excess to the current recommendations for endurance exercise and except for the one subject, who was likely overtrained, all had no change in leucine kinetics despite the considerable energy cost of the exercise they performed. Investigation to determine optimal protein intake for endurance athletes is still warranted and has not yet been addressed even with considerable mention of the discordance in recommendations in the protein metabolism literature.

Maintaining energy balance could be the most important factor in successful cycling stage racing. There may be a ceiling to EI, based on limits to digestion and this may ultimately limit EE over prolonged distances in competitions like a cycling tour event. Macronutrient intake and the timing of that intake may also be crucial in determining recovery rates of energy reserves and in maintaining or enhancing lean mass. The increase in lean mass in three of our four subjects is noteworthy. The influence of all food and supplements being provided and readily available, particularly after each stage, would certainly have been considerable. It would be interesting to determine whether the same patterns would be apparent if athletes are independently making choices about food types and quantities. Although there are many studies of nutrient timing, to our knowledge none have studied the effects of timing over a multi-day competition.

In conclusion, this study found that Ironman athletes are able to maintain energy and nitrogen balance throughout a period of high EE. In addition, there are selected significant correlations between negative energy and nitrogen balance and the appearance of overtraining marker which

with further investigation may provide a major role for nutritional intervention in the prevention of overtraining.

It is particularly apparent that the EE required to train for a 10.5 – 14 hour Ironman is on average 20 MJ/day. The EI necessary to sustain the energy cost must of course be equivalent. This study supports the findings previously described in Ironman athletes (Burke et al., 1991) that shows that EI values of approximately 20 MJ/day are indicative of the energy requirements for Ironman training and maintenance of energy balance. These absolute energy values are equivalent to approximately 241 kJ/kg/day.

This study also concludes that it is likely that Ironman athletes are more easily able to maintain energy balance compared to other endurance athletes who have an energy cost in excess of 20 MJ/day. 20 MJ/day is the suggested level of maximal metabolisable energy intake and therefore because the EE required to train for an Ironman is equivalent to 20 MJ/day, Ironman athletes should be able to maintain energy balance better than their counterparts in other sports that have a higher EE.

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is that will maintain protein balance in response to high energy cost exercise. Subjects in this study were in positive nitrogen balance when energy intake was adequate to maintain the energy cost of exercise. However, investigation to determine the optimal protein intake for endurance athletes is still warranted but requires a rigorous approach. The use of stable isotope methodologies, accurate body composition measurement, precise methodologies for determining EE and EI and ideally a free-living environment where exercise is performed under normal training or competition conditions would provide a setting that optimises the research rigour and allows for findings to have practical application.

There is an interesting link between negative energy and nitrogen balance and the presence of markers of overtraining. None of the subjects in this study were overtrained and therefore establishing a clear relationship was difficult. Further research that investigates specifically the association between energy and protein/nitrogen balance and overtraining markers would need to be performed in athletes identified as at risk of developing the overtraining syndrome. The syndrome is complex and despite a body of overtraining research there is still no agreement about a set of markers that are predictive. Further research would also provide an understanding of energy and protein kinetics associated with chronic fatigue and would be of considerable benefit to the endurance sport population.

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Appendix 1: Participant Information Sheet – The Tour of Southland

DEPARTMENT OF SPORT AND EXERCISE SCIENCE

THE UNIVERSITY OF AUCKLAND
NEW ZEALAND

Building 734, Tamaki Campus
Morrin Road, Glen Innes
Auckland, New Zealand
Telephone 64 9 373 7599 ext 86887
Facsimile 64 9 373 7043
The University of Auckland
Private Bag 92019
Auckland, New Zealand

PARTICIPANT INFORMATION SHEET

Project title: **Energy and protein requirements of elite endurance athletes during competition**

Researcher name: Benjamin F Miller, PhD

To: Potential research participant:

I am a researcher and lecturer at the University of Auckland's Department of Sport and Exercise Science. You are invited to participate in a research study attempting to determine the interaction of energy intake, expenditure, and daily protein requirements during endurance sport competition. The study is funded by Sport and Recreation New Zealand (SPARC) and the University of Auckland. Your participation is voluntary; you do not have to take part if you do not wish to.

Why are we doing this study?

Whether endurance athletes have increased protein requirements remains debatable. The confusion in the literature has lead to daily protein recommendations for endurance athletes of 1.0 – 1.9 g/kg body weight. Actual daily practice of endurance athletes is complicated even further because of the psyche that more is better. However, twenty years ago it was demonstrated in endurance athletes protein requirements are not increased, and may even be less, as long as the athlete has enough energy. However, this hypothesis has not been rigorously tested over a variety of training or competition states. Particularly interesting is whether protein requirements change in an intense short term training period or competition. Since training is cyclic by nature with occasional periods of intense training and competition the question of whether protein requirements increase in this period is important.

Who may participate in this study:

To be part of this study, you must be 18-35 years old, physically healthy and invited by Michael Flynn, High Performance Director of Bike NZ. You must not have or have had any problems with bleeding, or be on medication that prolongs bleeding time, or be allergic to local anaesthetics. Because smoking is known to affect the factors being investigated, you cannot be a smoker.

What is involved?

Your participation in this project will take place over the period of Thursday, 3 November to Sunday, 13 November. Participation is roughly divided into pre-race testing, race testing, and post-race testing. It is important to note that outside a controlled experiment pre-race and immediately post-race, you will be allowed to eat as much or as little food as you prefer as this will be recorded but not restricted.

Your commitment to the experiment is detailed in the following table:

Table of commitment

Thursday, 3 November:	Morning: Arrive Dunedin
	VO_2 max testing, Ht, Weight, Constant workload test
	15:00: "Bolus" Feeding
	18:00: Begin overnight protein turnover study.
Friday, 4 November:	12:30 Finish overnight study
	14:00 DEXA
	Afternoon: normal training.
Saturday, 5 November:	Normal training.
Sunday, 6 November:	Morning: weight, blood draw, transport from Dunedin to Invercargill.
	Afternoon: Normal training.
Mon, 7 November:	Weight, questionnaire of mood (POMS), urine sample
	10:00 8.3 Km prologue TTT
	13:00 78.3 Km
Tuesday, 8 November:	Morning Basal metabolic rate (BMR), Weight, blood draw
	09:00 113.8 Km
	13:00 88.3 Km
Wednesday, 9 November:	Weight, POMS, urine sample
	10:00 132.8 Km
Thursday, 10 November	Morning BMR, Weight, blood draw
	10:00 15.0 Km ITT
	13:00 127.3 Km
Friday, 11 November:	Weight, POMS, urine sample
	10:00 163.6 Km
Saturday, 12 November:	Morning BMR, weight, blood draw
	09:30 79.0 Km
	13:30 64.4 Km
	16:00 Transfer to Dunedin (or in hotel room)
	18:30 Begin overnight protein turnover study
Sunday, 13 November:	12:30 Urine sample, finish overnight protein turnover study
	14:00 DEXA scan
Monday, 14 November:	Morning departure

As you can see, there will be minimal disturbance to you during the race with investigations concentrated pre- and post-race. As mentioned above, we will not restrict your diet, but will record everything you eat during this period.

The overnight protein turnover study will require placement of a catheter for infusion of a stable isotope and periodic sampling of blood. You will also receive periodic breath sampling, although this will be discontinued during the overnight period to allow you to sleep.

You are likely to experience a minor and brief (<30s) physical discomfort during the insertion of the catheters. There is also a small risk, as with any incision, of local infection. In the unlikely event that you have any unanticipated physical injury during or following the procedures, you will be directed to a physician for medical advice and/or treatment. If any abnormal or unusual results are found during the analyses of your samples, we will notify you of this and refer you to appropriate medical personnel.

What is a stable isotope? It is important that you realize that a stable isotope is **NOT** radioactive. Stable isotopes are naturally occurring and at any given time are present in your body at variable amounts. The amount of stable isotope in your body at any given time is largely determined by what you eat. For instance corn (maize) has a large amount of the stable isotope of carbon. We will be infusing stable isotopes of leucine (an amino acid) and water. Again, both the stable isotopes are naturally occurring in your body and we are simply increasing the amount. You will feel nothing nor is it possible for you to detect that it is there. The amount we infuse will be out of your system with 1 week.

During the race, you will be awoken on three mornings just prior to your normal scheduled time. At this point you will breathe into a mouthpiece connected to a balloon for approximately 10 minutes. This will allow us to measure your basal metabolic rate. After collecting breath, we will do a small (≈10 ml) blood draw and measure body weight.

On the alternate mornings we will have you complete a questionnaire that asks questions related to your mood. These questionnaires have been demonstrated to be reliable and valid predictors of overtraining. On these mornings you will also be weighed and your first void of the day (urine) will be collected.

What are the benefits for you? During the study you will ride your own bicycle equipped with a prototype wireless PowerTap® power meter built into Zipp® wheels. The data from these meters will be downloaded after every stage. You will also receive full sponsorship and race support from the research staff. In exchange for study participation you will receive all transportation, food, supplements, lodging, race clothing, race registration, and race support free of charge.

Your participation in this research project requires a commitment of 11 days before, during, and after the Tour of Southland. Actual investigation time will be only a fraction of that period and all efforts have been made to limit disturbance to your racing. However, you are free to withdraw under your own free will at any time.

Samples from your experiment will be stored in a low temperature freezer in the Exercise Physiology Laboratory, Dept of Sport and Exercise Science, University of Auckland Tamaki, Building 734.134 until use or for up to six years. Most samples will be disposed of during their analyses. Remaining samples will be destroyed according to standard biological sample disposal methods

The research will be conducted by researchers from the Dept of Sport and Exercise Science, at the University of Auckland and School of Physical Education, University of

Otago, Dunedin. Pre- and post-race investigations will take place in the School of Physical Education at the University of Otago, while race investigations will take place during the normal scheduled race location.

Your rights:

- Your participation is entirely voluntary.
- You may have your own data with some comparative values from normal and athletic populations sent to you upon completion of the study.
- Your identity will be kept strictly anonymous, and no identification of you or your data will be made in any subsequent publication of the research findings.
- You are free to withdraw from the project at any time, without giving any reason, and without any disadvantage to yourself of any kind.
- You may withdraw information that you have provided at any time prior to completion of data collection (12/11/05).

Your information and data will be used for this project only.
Data identifiable to you will be stored in a locked office for up to six years, after which it will be destroyed. You will not be identified on any electronically stored data, data that will be retained indefinitely and used for publication.

Your consent to participate will be indicated by your signing and dating the consent form. Signing the consent form indicates that you have freely given your consent to participate, and that there has been no coercion or inducement to participate.

Thank you very much for your time and help in making this study possible. If you have any queries, wish to know more, or have medical concerns, please phone Dr. Miller at: 373 7599 extn 86607, or write to:

Dr. Benjamin Miller
Department of Sport and Exercise Science
The University of Auckland
Private Bag 92019
Auckland Tel. 373-7599 extn. 86607
b.miller@auckland.ac.nz

The Head of Department is:

Dr. Alan Lee
Department of Sport and Exercise Science
The University of Auckland
Private Bag 92019
Auckland Tel. 373-7599 extn 86846

If you have any queries or concerns about your rights as a participant in this study you may wish to contact:

The Chair
Human Participants' Ethics Committee
University of Auckland
Private Bag 92019
Auckland Tel. 373-7599 extn 87830

APPROVED BY THE AUCKLAND HUMAN PARTICIPANTS ETHICS COMMITTEE ON 15 June 2005 FOR 3 YEARS, (2005/219)

Appendix 2: Participant Consent – The Tour of Southland

DEPARTMENT OF SPORT AND EXERCISE SCIENCE

THE UNIVERSITY OF AUCKLAND
NEW ZEALAND

Building 734, Tamaki Campus
Morrin Road, Glen Innes
Auckland, New Zealand
Telephone 64 9 373 7599 ext 86887
Facsimile 64 9 373 7043
The University of Auckland
Private Bag 92019
Auckland, New Zealand

THIS CONSENT FORM WILL BE HELD FOR A PERIOD OF SIX YEARS

Research Project: **Energy and protein requirements of elite endurance athletes during competition**

Researcher: Dr Benjamin Miller

I have read and understand the information in the Participant Information Sheet. I have had the opportunity to ask any questions about the project and have had them answered. My participation in this research is voluntary.

I have been given and have understood the explanation of this research project and my role as a participant. In particular, I have been informed and understand that:

1. If I request, I can obtain results regarding the outcome of the project upon completion of the study.
2. My personal information and my identity will remain anonymous.
3. Data from my participation will be kept secure and used for research purposes only.
4. I will not be identified on any electronically stored data, data that will be retained indefinitely and used for publication
5. I am free to withdraw from participation at any time, without giving a reason, and to withdraw any data traceable to me until 12/11/05.

I agree to take part in this research.

Signed: _____

Name (please print): _____

Date : _____

APPROVED BY THE AUCKLAND HUMAN PARTICIPANTS ETHICS COMMITTEE ON 15 June 2005 FOR 3 YEARS, (2005/219).

Appendix 3: Participant Information Sheet – Taupo Ironman Camp

DEPARTMENT OF SURGERY

THE UNIVERSITY OF AUCKLAND
NEW ZEALAND

Department of Surgery
Level 12
Auckland City Hospital Support Building
Park Rd
Grafton
Telephone 09 373-7599 extn.86820
Fax 09 373-9656

The University of Auckland
Private Bag 92019
Auckland, New Zealand

PARTICIPANT INFORMATION SHEET

Project title: **Energy and protein requirements of endurance athletes**

To: Potential research participant:

My name is Anna Rolleston and I am a PhD candidate within the Department of Surgery at the University of Auckland. You are invited to participate in a research project attempting to determine the effect of endurance exercise on energy balance and daily protein requirements. You have been selected as a participant due to your involvement in the Ironman training camp in January 2008, being run by your coach Carolynn Margan. Your participation in this research is voluntary and there is no obligation for you to take part in the study.

The rationale for this research comes from the question about whether endurance athletes have increased dietary protein requirements compared to other athletes and also compared to the general population. There is much debate about protein requirements for endurance athletes and the confusion in the literature has led to daily protein recommendations for endurance athletes of between 1.0 – 1.9 gram of protein per kilogram of body weight (g/kg). This is in comparison to recommendations for the general population of approximately 0.8g/kg. However, twenty years ago it was demonstrated in endurance athletes that protein requirements are not increased, and may even be less, as long as the athlete has enough energy. This hypothesis has not been rigorously tested over a variety of training or competition states. Particularly interesting is whether protein requirements change in an intense short term training period or competition. Since training is cyclic by nature with occasional periods of intense training and competition the question of whether protein requirements increase in this period is important.

The training camp in January will involve all of Carolynn's Ironman athletes and there will be some who participate as research subjects and some who do not. Your involvement in this research will not disrupt the training and information giving sessions that Carolynn has planned for the camp. In turn, athletes who are not research participants will not be disrupted by your participation in this study. Confidentiality of all training camp participants (research participants and non-research participants) will be preserved in any reports or publications that result from this study.

Your participation in this project will take place over the period of Wednesday 23 January to Monday 28 January, 2008. Additionally, in the week prior to Wednesday 23 January you will undergo a VO_2max test on a cycle ergometer for determination of your maximal oxygen capacity. This will take place at the New Zealand Academy of Sport

facility at Wintec in Hamilton. Participation is thus roughly divided into pre-camp testing, in-camp testing, and post-camp testing.

Your commitment to the research is detailed in the following table:

Table of commitment

1 week prior	VO_2 max testing
Wednesday, 23 January:	12:00 Arrive in Taupo, Commencement of supervised eating 13:00 Begin 24-hour urine collection 22:00 Doubly labelled water dosing
Thursday, 24 January:	06:30 Body weight, blood draw, resting metabolic rate, mood state questionnaire 08:00 Anthropometry Assessment Training Camp Activities 13:00 Finish 24-hour urine collection
Friday, 25 January:	06:30 Urine sample, body weight, blood draw, resting metabolic rate, mood state questionnaire Training Camp Activities
Saturday, 26 January:	06:30 Urine sample, body weight, blood draw, resting metabolic rate, mood state questionnaire Training Camp Activities
Sunday, 27 January:	06:30 Urine sample, body weight, blood draw, resting metabolic rate, mood state questionnaire Training Camp Activities 13:00 Begin 24-hour urine collection
Monday, 28 January:	08:00 Anthropometry Assessment 13:00 Finish 24-hour urine collection, end of supervised eating Athletes free to depart

As you can see, there will be minimal disturbance to you during the camp with investigations concentrated pre- and post-camp and in the mornings only, on each day. We will not be restricting your diet. You will be able to eat as much, or as little, as you like but we will record everything you eat during the camp.

You will be ingesting a stable isotope of water, more commonly called doubly labelled water. What is a stable isotope? It is important that you realize that a stable isotope is **NOT** radioactive. Stable isotopes are naturally occurring and at any given time are present in your body in varying amounts. The amount of stable isotope in your body at any given time is largely determined by what you eat. For instance corn (maize) has a large amount of the stable isotope of carbon. Both the stable isotopes of hydrogen and oxygen are naturally occurring in your body and we are simply increasing the amount. You will feel nothing nor is it possible for you to detect

that either are there. The amount we give you will be removed from your system within about 1 week.

During the camp, you will be awoken each morning and at this point you will breathe into a mouthpiece connected to a balloon for approximately 10 minutes. This will allow us to measure your resting metabolic rate. After collecting the breath sample from you, we will take a small (≈10 ml) blood draw, measure your body weight, take a urine sample and ask you to fill in a mood questionnaire while you have breakfast.

What are the benefits for you? Some of the research information we will collect can be used to better inform your training. You will know, when all the data has been analysed, if your energy intake matches your energy expenditure, whether training leads to any decline in your body protein stores and if you eat appropriate amounts of each of the major macronutrients – carbohydrate, protein and fat. The VO_2max test will also give you an indication of your oxygen carrying capacity and in exchange for study participation you will receive all food, supplement and lodging free of charge during the camp.

Your participation in this research project requires a commitment of 7 days in total – 1 day for the VO_2max test in the week prior to the training camp and 6 days during the camp. Actual investigation time will only be a fraction of that period and all efforts have been made to limit disturbance to the training camp.

Your samples from this research will be stored in a low temperature freezer in the Department of Surgery until analysis or for up to six years. Most samples will be disposed of during the analysis process and any remaining samples will be destroyed according to standard biological sample disposal methods.
Your information and data will be used for this project only.

Data identifiable to you will be stored in a locked cabinet for up to six years, after which it will be destroyed. You will not be identifiable on any electronically stored data or any data that will be retained indefinitely and used for publication.

Your rights:

- Your participation in this research is entirely voluntary.
- Your identity will be kept strictly confidential, and no identification of you or your data will be made in any subsequent publication of the research findings.
- You are free to withdraw from the project at any time, without giving any reason, and without any disadvantage to yourself of any kind.
- You may withdraw information that you have provided at any time prior to completion of data collection (27/01/08).

This research will be conducted by researchers from the Department of Surgery and the Department of Sport and Exercise Science, at the University of Auckland. Funding for this project has been obtained from the University of Auckland.

If you have any queries or wish to know more please contact Anna Rolleston by phoning (09) 373-7599 ext 88559 or email to a.rolleston@auckland.ac.nz

Anna Rolleston MSc(Hons)
PhD Candidate
Department of Surgery
The University of Auckland
Private Bag 92019
Auckland
Tel. 09 373-7599 extn. 88559
a.rolleston@auckland.ac.nz

My PhD Supervisor is:
Dr. Lindsay Plank
Associate Professor
Department of Surgery
The University of Auckland
Private Bag 92019
Auckland
Tel. 09 373-7599 extn 86949

My Head of Department is:
Dr. John Windsor
Department of Surgery
The University of Auckland
Private Bag 92019
Auckland
Tel. 09 373-7599 extn 89820

For any queries regarding ethical concerns you may contact:
The Chair
The University of Auckland Human Participants Ethics Committee
The University of Auckland
Office of the Vice Chancellor
Private Bag 92019
Auckland 1142
Tel. 09 373-7599 extn. 87830

APPROVED BY THE AUCKLAND HUMAN PARTICIPANTS ETHICS COMMITTEE for 3 years on 14 November 2007 REFERENCE NUMBER 2005/219

Appendix 4: Participant Consent – Taupo Ironman Camp

DEPARTMENT OF SURGERY

THE UNIVERSITY OF AUCKLAND
NEW ZEALAND

Department of Surgery
Level 12
Auckland City Hospital Support Building
Park Rd
Grafton
Telephone 09 373-7599 extn.86820
Fax 09 373-9656

The University of Auckland
Private Bag 92019
Auckland, New Zealand

THIS CONSENT FORM WILL BE HELD FOR A PERIOD OF SIX YEARS

Researcher: Anna Rolleston MSc (Hons)

Research Project: **Energy and protein requirements of endurance athletes**

I agree to take part in this research

I have read and understand the information in the Participant Information Sheet. I understand the nature of the research and why I have been selected to participate. I have had the opportunity to ask any questions about the project and have had them answered. My participation in this research is voluntary.

I have been informed and understand that:

1. My personal information and my identity will remain anonymous.
2. Data from my participation will be kept secure and used for research purposes only.
3. I will not be identified on any electronically stored data, data that will be retained indefinitely and/or used for publication
4. Some minor (<30sec) discomfort may be experienced during blood sampling procedures and as with any incision there is a minimal risk of infection
5. Stable isotopes are not radioactive and occur naturally in my body and the amount that will be used in this study (doubly labelled water) will be gone from my body in about one week
6. My commitment to this research requires 7 days – 6 days during my participation in Carolynn Margan's Ironman training camp and 1 day in the week prior when I have to perform a VO_2max test
7. I will benefit from participation in this project by receiving information that will better inform my training and also that all food, supplement and lodging costs will be covered for me during the camp
8. I am free to withdraw from participation at any time, without giving a reason, and to withdraw any data traceable to me until 27/01/08.

Signature: _____ Date:_____

APPROVED BY THE AUCKLAND HUMAN PARTICIPANTS ETHICS COMMITTEE for 3 years on 14 November 2007 REFERENCE NUMBER 2005/219

Appendix 5: Doubly Labelled Water Dose Determination

Mixture Preparation:

Mix 10% $H_2{}^{18}O$ with 99.9% deuterium oxide

If mix	**Final Concentration**
90g 99.9% deuterium oxide	0.056604g 99.9% $^{2}H_2O$/g
1500g 10% $H_2{}^{18}O$	0.943396g 10% $H_2{}^{18}O$/g

Dose Calculation:

Want to dose at	2g/kg TBW with 10% $H_2{}^{18}O$	
	0.12g/kg TBW with 99.9% $^{2}H_2O$	
	18%	% BW
	70kg	BW
	12.60kg	FM
	57.40kg	FFM
	42.0168	Derived body water (assuming hydration constant 0.732)
	89.075g	Dose to be used

The information contained in Appendix 7 is modified from a calculations spreadsheet (Rush, personal communication). Grey shaded areas indicate data to be entered into the spreadsheet and yellow shaded areas indicate calculated results. TBW = total body water, BW = body weight, FM = fat mass, FFM = fat free mass

Appendix 6: Doubly Labelled Water Data

The Tour of Southland

	Subject 1	Subject 2	Subject 3	Subject 4
FQ	0.906	0.924	0.915	0.919
Energy Equivalent	22.634	22.301	22.466	22.392
TDEE (kJ) Shoeller*	28586	29632	25306	26154
TDEE (kJ) Coward	23036	21768	18779	17614
TDEE (kJ) Livingstone	22520	20752	17076	16482
TDEE (kcal) Shoeller	6832	7082	6048	6251
TDEE (kcal) Coward	5506	5203	4488	4210
TDEE(kcal)Livingstone	5382	4960	4081	3939
BW (kg)	81.9	73	90.6	88.4
Water (kg)	47.6	49.7	53.5	56.4
LBM (kg)	65.3	68.1	73.2	77.3
FAT (kg)	16.6	4.9	17.4	11.1
FAT%BW	20.3	6.7	19.2	12.6
Space ratio	1.094	1.100	1.077	1.106

Taupo Ironman Camp

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7
FQ	0.912	0.911	0.904	0.920	0.920	0.910	0.930
Energy Equivalent	22.52	22.54	22.67	22.41	22.32	22.50	22.28
TDEE (kJ) Shoeller*	18982	20306	15500	26140	20893	23025	18626
TDEE (kJ) Coward	12977	15510	15333	20047	18495	21266	20371
TDEE (kJ) Livingstone	11522	14348	14731	18924	17694	20287	19156
TDEE (kcal) Shoeller	4537	4853	3704	6248	4993	5503	4452
TDEE (kcal) Coward	3102	3707	3665	4791	4420	5083	4869
TDEE(kcal)Livingstone	2754	3429	3521	4523	4229	4849	4578
BW (kg)	87.6	87.6	73.1	94.7	79	77	93.1
Water (kg)	46.1	48.3	45.7	49.1	48.1	48.2	51.4
LBM (kg)	63.1	66.1	62.6	67.3	65.9	66.0	70.5
FAT (kg)	24.5	21.5	10.5	27.4	13.1	11.0	22.6
FAT%BW	28	24.5	14.4	28.9	16.6	14.3	24.3
Space ratio	1.080	1.075	1.037	1.084	1.058	1.050	1.023

FQ = food quotient, TDEE = total energy expenditure, BW = body weight, LBM = lean body mass, FAT%BW = fat as a percentage of body weight. *TDEE used in findings

Appendix 7: Individual Mean Energy and Macronutrient Intake Data for Ironman Camp

Mean (SD)	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7
EI (MJ/day)	19.5 (2.9)	23.6 (7.0)	16.0 (2.2)	24.6 (4.3)	19.5 (3.4)	19.0 (1.5)	19.6 (7.3)
EE (MJ/day)	19.0	20.3	15.5	26.1	20.9	23.0	18.6
Carbohydrate							
(g/day)	742.5(132.1)	950.2(86.4)	572.0(98.2)	936.4(178.8)	725.4(128.6)	669.9(43.5)	745.6(209.8)
(g/kg/day)	8.5 (1.5)	10.2 (0.9)	7.8 (1.3)	9.9 (1.9)	9.2 (1.6)	8.7 (0.6)	8.0 (2.3)
%EI	63.4 (2.1)	72.9 (25.7)	59.4 (2.5)	63.7 (3.0)	62.1 (2.2)	59.2 (1.5)	65.0 (7.1)
Protein							
(g/day)	198.8(25.6)	231.9(27.1)	170.9(37.2)	185.0(21.8)	212.5(19.3)	214.3(10.1)	200.1(126.)
(g/kg/day)	2.3 (0.3)	2.5 (0.3)	2.3 (0.5)	2.0 (0.2)	2.7 (0.2)	2.8 (0.1)	2.2 (1.4)
%EI	17.1 (1.2)	17.7 (5.9)	17.9 (3.4)	12.8 (2.4)	18.7 (4.0)	18.0 (1.5)	16.3 (5.1)
Fat							
(g/day)	106.4(15.8)	173.8(34.5)	95.8 (13.3)	162.5(46.0)	109.6(35.1)	118.0(23.0)	107.8(54.8)
(g/kg/day)	1.2 (0.2)	1.9 (0.4)	1.3 (0.2)	1.7 (0.5)	1.4 (0.4)	1.5 (0.3)	1.2 (0.6)
%EI	20.6 (2.5)	28.9 (5.8)	22.7 (3.1)	24.7 (3.5)	20.7 (3.8)	23.3 (2.7)	20.0 (3.3)
Alcohol							
(g/day)	1.9 (2.1)	2.6 (2.5)	2.3 (1.8)	2.5 (2.5)	4.0 (2.6)	2.6 (2.0)	2.7 (3.1)
%EI	0.31(0.38)	0.3 (0.3)	0.5 (0.4)	0.3 (0.3)	0.6 (0.4)	0.4 (0.3)	0.5 (0.5)
%EI Food	83.2 (12.4)	87.5 (5.9)	83.5 (6.8)	88.0 (5.2)	91.9 (3.3)	89.4 (6.1)	85.9 (8.1)
%EI Supp	22.4 (12.4)	12.5 (5.9)	16.0 (6.9)	11.6 (5.3)	7.5 (3.1)	10.2 (6.0)	13.6 (8.1)

Individual mean energy and macronutrient intakes averaged over 4 days. EI = energy intake, EE = energy expenditure, %EI = percentage of energy intake, %EI Food = percentage of energy that was from food, %EI Supp = percentage of energy that was from supplements

Appendix 8: Nitrogen Balance Data for Ironman Camp

Pre-Camp 24-hour Nitrogen Balance

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7
Urea N (g/day)	14.85	14.85	13.98	15.64	11.66	16.76	14.18
Creatinine N (g/day)	0.59	0.58	0.56	0.74	0.83	0.59	0.76
Urate N (g/day)	11.67	5.82	10.15	10.49	17.13	8.16	24.95
Integumentary N Loss	0.44	0.46	0.37	0.47	0.40	0.39	0.47
Blood N loss	0.256	0.256	0.256	0.256	0.256	0.256	0.256
Misc loss	0.115	0.115	0.115	0.115	0.115	0.115	0.115
Total N loss (g/day)	27.92	22.09	25.42	27.71	30.33	26.26	40.72
Total N intake (g/day)	23.24	27.85	21.54	21.03	20.86	24.16	22.22
N Balance (g/day)	-4.68	+5.76	-3.88	-6.68	-9.47	-2.10	-18.50

Post-Camp 24-hour Nitrogen Balance

	Subject 1	Subject 2	Subject 3	Subject 4	Subject 5	Subject 6	Subject 7
Urea N (g/day)	18.86	23.62	13.95	19.73	22.75	26.98	17.61
Creatinine N (g/day)	0.52	0.68	0.52	0.76	0.75	0.67	0.85
Urate N (g/day)	8.17	21.96	17.63	29.63	5.82	19.81	25.12
Integumentary N Loss	0.43	0.46	0.37	0.47	0.40	0.38	0.46
Blood N loss	0.256	0.256	0.256	0.256	0.256	0.256	0.256
Misc loss	0.115	0.115	0.115	0.115	0.115	0.115	0.115
Total N loss (g/day)	28.34	47.09	32.84	50.96	30.09	48.21	44.41
Total N intake (g/day)	35.06	36.61	23.83	21.24	30.62	28.34	26.73
N Balance (g/day)	+6.71	-10.48	-9.01	-29.72	+0.53	-19.86	-17.69

Integumentary N loss estimated as 5mg/kg/day (Rand 1977), Blood N loss estimated as 22mg/ml of blood drawn (Calloway 1971), Miscellaneous N loss estimated at 115mg/day (Calloway 1971).

Appendix 9: Profile of Mood State Questionnaire

Name: _____ Date: _____

Below is a list of words that describe the feelings people have. Please read each word carefully and then CIRCLE ONE NUMBER THAT BEST DESCRIBES HOW YOU FEEL RIGHT NOW

	Not At All	A Little	Moderately	Quite a Bit	Extremely
01. Hopeless	**0**	**1**	**2**	**3**	**4**
02. Resentful	**0**	**1**	**2**	**3**	**4**
03. Blue	**0**	**1**	**2**	**3**	**4**
04. Annoyed	**0**	**1**	**2**	**3**	**4**
05. Discouraged	**0**	**1**	**2**	**3**	**4**
06. Unable to concentrate	**0**	**1**	**2**	**3**	**4**
07. Nervous	**0**	**1**	**2**	**3**	**4**
08. Worthless	**0**	**1**	**2**	**3**	**4**
09. Fatigued	**0**	**1**	**2**	**3**	**4**
10. Miserable	**0**	**1**	**2**	**3**	**4**
11. Uneasy	**0**	**1**	**2**	**3**	**4**
12. Bitter	**0**	**1**	**2**	**3**	**4**
13. Peeved	**0**	**1**	**2**	**3**	**4**
14. Exhausted	**0**	**1**	**2**	**3**	**4**
15. Cheerful	**0**	**1**	**2**	**3**	**4**
16. Energetic	**0**	**1**	**2**	**3**	**4**
17. Anxious	**0**	**1**	**2**	**3**	**4**

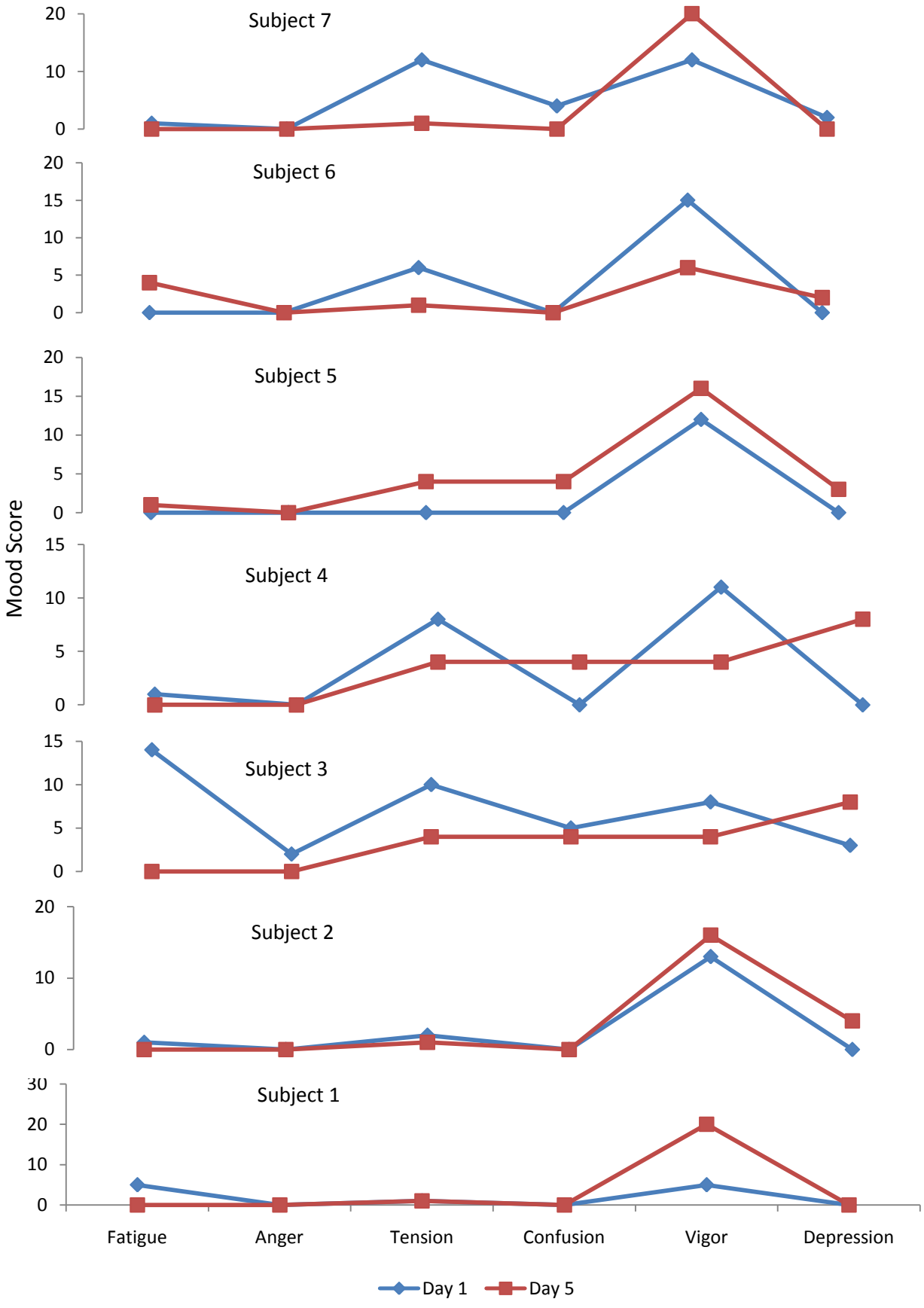
Please turn over

	Not At All	A Little	Moderately	Quite a Bit	Extremely
18. Helpless	**0**	**1**	**2**	**3**	**4**
19. Grouchy	**0**	**1**	**2**	**3**	**4**
20. Weary	**0**	**1**	**2**	**3**	**4**
21. On-Edge	**0**	**1**	**2**	**3**	**4**
22. Active	**0**	**1**	**2**	**3**	**4**
23. Bewildered	**0**	**1**	**2**	**3**	**4**
24. Sad	**0**	**1**	**2**	**3**	**4**
25. Full of Life	**0**	**1**	**2**	**3**	**4**
26. Furious	**0**	**1**	**2**	**3**	**4**
27. Confused	**0**	**1**	**2**	**3**	**4**
28. Lively	**0**	**1**	**2**	**3**	**4**
29. Restless	**0**	**1**	**2**	**3**	**4**
30. Forgetful	**0**	**1**	**2**	**3**	**4**
31. Unhappy	**0**	**1**	**2**	**3**	**4**
32. Vigourous	**0**	**1**	**2**	**3**	**4**
33. Worn Out	**0**	**1**	**2**	**3**	**4**
34. Uncertain about things	**0**	**1**	**2**	**3**	**4**
35. Angry	**0**	**1**	**2**	**3**	**4**
36. Tired	**0**	**1**	**2**	**3**	**4**
37. Tense	**0**	**1**	**2**	**3**	**4**

Entered into spreadsheet by: _____

Date of entry: _____ **Time of entry: _____**

Appendix 10: Individual Iceberg Profiles for Ironman Camp



Appendix 11: Ironman Results

Subject	Time	Age Group Rank	Out of
1	13:42:33	107	125
2	11:52:01	66	125
3	DNS		
4	13:18:52	130	151
5	11:33:31	54	125
6	12:10:09	46	94
7*	13:36:37	139	160

Results of the 2008 Ironman for the 7 participants of the Taupo Ironman Camp held in January 2008. DNS = did not start. *Results from 2007 Ironman because Subject 7 did not compete in 2008 due to injury