

**The effects of environment, diet and exercise on the reliability and  
validity of measurements of resting metabolic rate and body  
composition in athletes.**

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## Statement of Sources

This thesis contains no material that has been extracted in whole or in part from a thesis that I have submitted towards the award of any other degree or diploma in any other tertiary institution.

No other person's work has been used without due acknowledgment in the main text of the thesis.

All research procedures reported in the thesis received the approval of the relevant Ethics/Safety Committees (where required).

Unless otherwise stated, all work comprising of this thesis has been undertaken by the candidate.

This candidature was completed as a joint venture between the Australian Catholic University (ACU) and the Australian Institute of Sport (AIS).

There have been several collaborations and ancillary staff involved in the studies of this thesis. These are listed in order of importance / contribution:

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- Standardised diets were designed by Accredited Sports Dietitian Nikki Jeacocke and other dietitians in the Sports Nutrition Department at the AIS. This team also completed diet entry and dietary analysis.
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## **Supervisory Panel**

It is acknowledged that the work within this thesis was supervised by the following ACU staff:

**Principal Supervisor:** Professor Louise M Burke (ACU, Fitzroy, VIC / AIS, Canberra, ACT)

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## List of Publications

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**Bone, J L**, Ross, M L, Tomcik, K A, Jeacocke, N A, Hopkins, W G, & Burke, L M. (2017). Manipulation of muscle creatine and glycogen changes dual x-ray absorptiometry estimates of body composition. *Med Sci Sports Exerc.*, 49, 1029-1035.

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## List of Abbreviations

AIS	Australian Institute of Sport
ANOVA	analysis of variance
BEE	basal energy expenditure
BM	body mass
BMC	bone mineral content
BMR	basal metabolic rate
CHO	carbohydrate
CI	confidence interval
DXA	dual energy x-ray absorptiometry
EA	energy availability
EE	energy expenditure
EEE	exercise energy expenditure
EI	energy intake
EPOC	excessive post exercise consumption
FT3	free triiodothyronine
FAT	Female athlete triad
FFM	fat free mass
FM	fat mass
ICC	intraclass correlation coefficient
IGF-1	insulin growth factor-1
LCHF	low carbohydrate high fat
LEA	low energy availability
LM	lean mass
LOA	limits of agreement

MRI	magnetic resonance imaging
PICP	procollagen type I C-terminal propeptide
RED-s	relative energy deficiency syndrome
REE	resting energy expenditure
RER	respiratory exchange ratio
RMR	resting metabolic rate
RQ	respiratory quotient
SD	standard deviation
SEE	sleeping energy expenditure
SMR	sleeping metabolic rate
SWA	SenseWear activity monitor
T <sub>3</sub>	triiodothyronine
TE	typical error
TEA	thermic effect of activity
TEF	thermic effect of food
TEM	thermic effect of a meal
USG	urine specific gravity
$\dot{V}CO_2$	carbon dioxide production
$\dot{V}O_2$	oxygen consumption
VO <sub>2</sub> max	maximal oxygen consumption
y	years of age

## Abstract

Resting energy expenditure (REE) can be reduced in situations of Low Energy Availability (LEA) in athletes, providing both a diagnostic sign of LEA and a potent risk factor for illness, injury and sub-optimal health. Current protocols regarding pre-measurement standardisation for REE are based on non-athlete populations, often following stringent rest and fasting protocols that would not be practical in a high performance environment. Furthermore, the reliability of measurements derived from these protocols has often been assessed in general and clinical populations and is unknown in an athlete population. Characteristics of the test protocol which alter an athlete's presentation (e.g. the location of the test, the duration of recovery from the last exercise bout) and changes in the athlete's own characteristics (changes in intramuscular solute and water content) were identified as variables that could affect the reliability of measurement of REE. This thesis presents a series of distinct but related studies which examine the how these variables affect the measurement and interpretation of REE in athletes.

*Study 1* examined the effect of testing location on REE in 32 elite and sub-elite athletes. REE was measured either at their bedside upon waking (inpatient) and as an outpatient (laboratory) protocol in a cross-over design following 8 h overnight fasts prior to each measurement. The day to day variation and reliability of each protocol was also assessed. There was no difference in REE when measured under the inpatient or outpatient protocols ( $7302 \pm 1272$  and  $7216 \pm 1119$  kJ/d respectively). Both protocols showed good day to day reliability (inpatient 96%, outpatient 97%), however, the outpatient protocol was found to have a greater typical error (TE) (478 kJ/d) and to be less sensitive to changes in REE than the inpatient protocol (336kJ/d).

*Study 2* was a pilot study that investigated the effect of acute exercise on REE. A cross-over intervention was used in ten male athletes. Measurements were undertaken

following training sessions in the morning and afternoon to determine REE approximately 12, 24, 36 and 48 h post exercise. There was a trend for REE to decrease with increasing rest time from exercise, with REE measured 48 h post exercise being significantly lower than REE measured at 12 h. However, the difference of 375 kJ/d was within the typical error determined in *Study 1*.

*Study 3* focused on the reliability of DXA estimates of lean mass (LM), which is important in the interpretation of REE relative to fat free mass (FFM). Intramuscular solutes and fluid were manipulated through a series of glycogen depletion, glycogen loading and creatine loading protocols in 18 male cyclists. Main outcome measures were total body and leg LM measured by dual x-ray absorptiometry (DXA), and total body water (TBW) measured by bioelectrical impedance spectroscopy (BIS). Changes in the mean were considered substantial if they reached the threshold for the smallest worthwhile effect of the treatment. There were substantial increases in TBW (2.3 and 2.5%), total body (2.1 and 3.0%) and leg LM (2.6 and 3.1%) following glycogen loading and the combined glycogen-creatine loading protocols respectively. Glycogen depletion caused a substantial decrease in leg LM (-1.4%) and trivial decrease in total body LM (-1.3%). Creatine loading resulted in substantial increases in TBW and in trivial increases in LM measures.

*Study 4* addressed the potential development of a practical method to determine an athlete's glycogen stores in combination with DXA-derived estimates of LM by investigating the validity of measuring muscle glycogen via a non-invasive ultrasound technique. The same cohort and design involved in *Study 2* was used in this investigation, with the ultrasound derived estimates of muscle glycogen concentration and changes in glycogen concentration being compared with results derived from direct (biopsy-derived) measurements. Poor correlations and substantially large or unclear errors were determined for the ultrasound estimates of muscle glycogen compared to muscle biopsy. Therefore, under the conditions

employed in the current study design, the ultrasound technique was unable to accurately predict either single measures of muscle glycogen or changes in muscle glycogen stores.

*Study 5* applied the findings from *Study 3* to investigate how changes in muscle glycogen influence the measurement and interpretation of REE in athletes, with particular interest in its effect on the sensitivity to detect changes in REE over time or as a result of an intervention. The investigation was undertaken within a larger study of the effect of adaptation to a low carbohydrate, high fat (LCHF) diet during a 21 day training camp for endurance athletes. Nineteen elite male race-walkers participated in this study; ten were assigned to the control group (CHO) where they received a diet providing 60% of energy from carbohydrate while nine were in the intervention group in which carbohydrate was restricted to <50g per day and fat comprised >70% of their energy intake for this period. Before and after the dietary interventions, measurements were made of REE, body composition (DXA) and TBW (BIS). Information derived from *Study 3* to distinguish acute changes in TBW associated with changes in intramuscular glycogen and its bound water, from true (chronic) changes in muscle mass, was applied to the baseline and post-intervention measures of LM in all athletes. There was a significant decrease in FFM between Baseline and uncorrected Post-Intervention values FFM (-1.4; 95% CI -2.0, -0.80 kg). Using the uncorrected measures of FFM, we interpreted that no change in relative REE between baseline and post intervention occurred in either group. However, when the correction factor was applied to FFM of the LCHF group, correcting for the artefact of reduced muscle glycogen levels associated with restricted carbohydrate intake, we detected a decrease in relative REE post intervention measurements compared to baseline.

The conclusions from this series of studies are; 1) Inpatient and outpatient protocols should not be used interchangeably when tracking changes in REE over time. 2) An 8 h overnight fast has good day to day reliability for both inpatient and outpatient protocols. 3) Rest time from exercise should be kept consistent between measures of REE for longitudinal

monitoring. 4) Manipulations of muscle glycogen and creatine supplementation cause an artefact in the DXA which changes the estimate of LM accordingly. 5) Measurement of TBW via BIS is better suited to track changes in muscle glycogen than proprietary ultrasound technology. 6) A reduction in muscle glycogen stores, such as that achieved by the consumption of a LCHF diet, creates an artefact in the DXA-derived measurement of FFM, which could potentially alter the interpretation of relative REE. This knowledge should be integrated into best practice guidelines for the measurement of REE in athletes to enhance the reliability and validity of measurement as well as the interpretation of the results.



## 1.0 Introduction and Overview

Low energy availability (LEA) describes situations in which a mismatch between an individual's energy intake and the energy cost of their exercise program leaves insufficient energy to support optimal function of many other body systems (Loucks et al., 2011). Operationally, energy availability can be calculated from information about energy intake and exercise energy expenditure, and is expressed relative to the body's fat free mass (FFM). There is a growing literature on the association between LEA and an increased risk of illness and injury in athletes (Drew et al., 2017; Mountjoy et al., 2014). These factors have been shown to increase the number of lost training days, creating a negative impact on athletic performance (Raysmith & Drew, 2016). There is also evidence in female athletes that LEA directly impairs the response to training, leading to a reduction in performance gains (Vanheest et al., 2014). Therefore, early detection and management of LEA in males as well as females is important for not only maintaining the athlete's health and well-being but also for achieving sporting success under Australia's Winning Edge program.

Resting metabolic rate can be suppressed in situations of LEA and can be used as part of a spectrum of tools in the diagnosis of LEA (Melin et al., 2015). This is convenient, since calculating energy availability for a free-living individual is laced with difficulties ranging from getting accurate information on energy intake from self-reported methods to defining what constitutes exercise and quantifying its energy expenditure. However, if measurements of resting metabolic rate (RMR) or daily resting energy expenditure (REE) are to be used as stand-alone tools in the assessment of energy availability, it is important that the protocols are practical to implement in the daily training environment, and that the measurements are reliable (sensitive enough to detect meaningful changes or differences) and valid (not being influenced by artefacts or variables that artificially alter the results).

The current best practice protocols for measuring REE in athletes are based on research on healthy sedentary individuals or clinically ill populations (Compher et al., 2006; Fullmer et al., 2015). Factors that could influence the variability or error of REE in these populations have been well investigated (Compher et al., 2006; Fullmer et al., 2015). However the same considerations have not been applied to athletes or some of the characteristics that define them. For example, REE is often expressed relative to FFM as it explains between 70-80% of the variability between individuals (Cunningham, 1991; Sparti et al., 1997). However, the validity and reliability of the tools used to assess body composition are not considered in the best practice protocols for REE assessment. Although it has been shown that DXA estimates of lean mass (LM) are influenced by recent food and fluid intake (Nana et al., 2012b), the effects of larger or longer term changes in body water content achieved through diet, exercise or supplements on DXA estimates of LM and consequent calculations of REE are largely unknown. Since athletes can undertake extreme changes in dietary intake and exercise in the day/s preceding the measurement of REE, it is important to understand how these would influence the assessment or interpretation of REE. Furthermore, in the case where it is not possible or practical to standardise these behaviours, it is of interest to develop tools that could correct for the potential measurement artefacts that they introduce. Meanwhile, the effect of other factors that need to be included in practical protocols for measurement of REE in free-living high performance athletes (e.g. testing location, period since the last exercise session, shorter fasting periods) have yet to be systematically studied.

The implications of the current lack of clarity about the reliability and validity of REE measurements in high performance athletes include a failure to manage athlete preparation needs as well as to undertake research. Problems may occur when a single measurement of REE is used to diagnose LEA, when serial measurements are used to track real life changes over time, or when there is investigation of whether an intervention poses a meaningful help or harm in the management of energy availability goals. Thus there is a need to develop or

validate protocols that are evidence based and athlete specific for use by sport science/medicine practitioners and researchers.

The aim of this thesis was to investigate the reliability of protocols used to assess and interpret resting metabolism in an athletic population. The work presented in the following chapters consists of five independent but related studies that identify and examine key variables that influence the measurement and interpretation of metabolic rate in athletes.

*Study 1:* Comparison of inpatient and outpatient protocols for measurement of resting energy expenditure in athletes (Chapter 4). *Study 2:* Investigation of the effect of prior exercise on measures of resting metabolic rate in trained men (Chapter 5).

*Study 3:* Investigation of the effect of altering muscle solute and water content on DXA measurements of lean mass (Chapter 6).

*Study 4:* Investigation of a practical and indirect method of assessment of muscle glycogen concentrations in athletes to allow changes to be considered in assessments of lean mass and REE. (Chapter 7).

*Study 5:* Investigation of the significance of artefacts in DXA measurements of lean mass in assessment of REE in athletes (Chapter 8).

## **2.0 Literature review**

### **2.1 Introduction**

The concept and measurement of resting metabolic rate (RMR) is important in sports nutrition. The management of energy intake and physique, which is important in athlete health and performance, requires an appreciation of basal metabolism within total energy expenditure. Indeed, in the case of elite athletes, values for each of these features may be found at each end of the normal continuum and require special expertise in measurement and interpretation of what is optimal.

More importantly, over the past decade, there has been interest in sports nutrition research and practice in the concept of energy availability (EA), which represents a potential interrelationship between physique management and basal metabolism. Energy availability is defined as the amount of energy that remains for body metabolic functions after accounting for the energy cost of exercise (Loucks et al., 2011). Low energy availability (LEA), in which energy intake relative to the energy cost of exercise is inadequate to support optimal body function, can occur because energy intake is reduced and/or the exercise load is increased without adequate adjustment of dietary energy. Since basal metabolism can be suppressed as a compensatory response to situations of low EA, it can therefore be used as a marker to support a diagnosis (Loucks et al., 1998).

Functional outcomes of LEA and its metabolic and hormonal adjustments include menstrual dysfunction in female athletes, impairment of protein synthesis and bone metabolism and disruption to many other body systems (Mountjoy et al., 2014). These factors can impair athletic performance by increasing the risk of illness and injury (therefore causing the loss of training days or interruption to full training loads), as well as by causing difficulties in maintaining ideal physique and decreasing the effectiveness of the response to a training stimulus (Vanheest et al., 2014). While exercise is known to produce an acute

increase on RMR (Bullough et al., 1995; Dolezal et al., 2000; Sjodin et al., 1996; Tremblay et al., 1988), this is not well characterised in terms of the range of training or competition activities undertaken by well-trained athletes. Furthermore, the time course of any chronic changes in RMR as a result of low EA or the restoration of a healthy EA in athletes has not been well studied, in isolation or as an interaction with the acute effects of the daily exercise load.

When used as a marker of LEA, RMR can be interpreted by comparing absolute values in kilojoules per day (kJ/d), against predicted equations (De Souza et al., 2007) or against pre-determined criteria relative to fat free mass (kJ/kg FFM/d) (Loucks, 2003). However, current values used to classify someone with optimal, reduced and low energy availability are still debated, particularly when used with male athletes (Koehler et al., 2016) as the majority of research has been conducted in female athletes. The reliability and validity of the protocols used to measure/estimate FFM are equally vital as the introduction of error on an artefact may cause an over or underestimation of the true EA and relative RMR (Melin & Lundy, 2015). Dual energy x-ray absorptiometry (DXA) has become the preferred tool for assessment and monitoring of body composition in athletes when conducted under standardised conditions such as the presentation of the subject in a fasted, rested and euhydrated state, and controlled placement on the scanning bed (Nana et al., 2015). However, it is suspected that changes in muscle glycogen concentrations, due to manipulations of diet and/or exercise, may be interpreted by DXA scans as changes in lean mass (Rouillier et al., 2015; Sherman et al., 1982) and may introduce an artefact in measurements of body composition. Since this scenario is likely to occur in athletes, an ideal approach would be either to standardise muscle glycogen and total body water (TBW) content, or at least be able to assess any perturbations for consideration in the interpretation of body composition measurements. Currently, it is possible to assess TBW using bioelectrical impedance spectroscopy (BIS), (Kerr et al., 2015), however, the assessment of muscle glycogen is either

very invasive (biopsy techniques) or expensive (magnetic resonance imaging) (Harris et al., 1974; Nygren et al., 2001). Ultrasound technology using proprietary software has been used to assess muscle glycogen, however this technology is still in its infancy and further independent validations have yet to occur (Hill & Millan, 2014; Nieman et al., 2015).

This literature review will provide background information about the measurement, interpretation and manipulation of body composition and muscle glycogen in relation to basal metabolism in athletes. It will include a summary of techniques and protocols, with insights in to the suitability of these techniques in athletic populations. Furthermore, it will review the reciprocal relationship between RMR and athletic performance; how alterations in RMR may affect the outcome of the athlete's goals for optimal health and performance while activities undertaken by an athlete in their sports pursuits may affect RMR. To undertake this review, literature was sourced from Internet databases including but not limited to Medline via Ovid, Web of Knowledge, PubMed and Sports Discuss. Keywords used were BMR; RMR; REE; SMR; resting metabolic rate; resting energy expenditure; athlete/s; trained; sport; diet; TEM; exercise; male/s; injury; illness and performance. The Australian Catholic University (ACU) Library catalogue was also used to source reference texts on metabolism and injury. Articles were limited to those written in the English language, abstracts and conference proceedings were excluded as were articles involving, clinical populations and weight loss interventions in obese individuals. The last literature search was performed on 17 May 2017.

## 2.2 Overview of basal metabolism

Basal metabolism is one of three components of total daily energy expenditure alongside the thermic effect of food (TEF; the energy cost of eating, digesting and absorbing food) and the thermic effect of activity (TEA; energy expended during planned physical activity and non-exercise activity (Burke & Deakin, 2009; Mahan & Escott-Stump, 2008; Mann & Truswell, 1998). Basal metabolism is defined as the minimum energy required to perform metabolic and chemical processes when awake, in order for the body to function at its optimum (Institute of Medicine, 2005; Ravussin et al., 1986). In a sedentary population, basal metabolism is the largest component of total daily energy expenditure (Mahan & Escott-Stump, 2008; Mann & Truswell, 1998), while physical activity is the most variable component, ranging from 20-30% of total energy expenditure (2005; Ravussin et al., 1986). However, this is often not the case in elite athletes where the energy expenditure from exercise (EEE) is often larger than basal metabolism (Beidleman et al., 1995; Rontoyannis et al., 1989; Thompson et al., 1993). There are small intra-individual changes in basal metabolism however there are large inter-individual variations, mainly due to differences in body composition (Nelson et al., 1992; Ravussin et al., 1986).

### 2.2.1 Terminology used to describe basal metabolism

Although a number of terms are used, often interchangeably, to describe basal metabolism, there are differences in the definition of various metrics, according to the protocols used to collect measurements as well as units of measurement (Institute of Medicine, 2005).

**BMR (Basal metabolic rate):** measured in kJ/min, quantifies a measurement of metabolic rate, taken from the bedside of a lying-supine individual who has just woken up and

has followed a 9-12 h fast (Institute of Medicine, 2005), abstained from alcohol and caffeine intake and in a thermoneutral environment (Institute of Medicine, 2005; Levine, 2005).

**RMR (Resting metabolic rate):** measured in kJ/min, quantifies a measurement of metabolic rate when any one or more of the conditions listed under BMR are not met (Institute of Medicine, 2005; Levine, 2005).

**SMR (Sleeping metabolic rate):** measured in kJ/min or kJ/h, quantifies the measurement of basal metabolism in a sleeping individual. It is reported to be 5-10% lower than BMR since it excludes the metabolic cost of arousal or being awake (Institute of Medicine, 2005). There are differing protocols in the literature for monitoring SMR with the most common involving assessment over a continuous 60 min or 3 h period (Goldberg et al., 1988; van Marken Lichtenbelt et al., 2001).

**BEE (Basal energy expenditure):** measured in kJ/d, quantifies the extrapolation of BMR to a 24 h period

**REE (Resting Energy Expenditure):** measured in kJ/d, quantifies the extrapolation of RMR to a 24 h period.

**SEE (Sleeping energy expenditure):** measured kJ/d, quantifies the extrapolation of SMR to a 24 h period.

## 2.3 Methods to assess basal metabolism

In its simplest terms, our metabolic rate is the amount of heat produced over a (set) time.

Energy is not destroyed, but transformed from one form to another (Schutz, 1995). Chemical and mechanical energy in humans is released as heat, which allows us to quantify energy expenditure (Schutz, 1995); the science known as calorimetry. Therefore, the heat released and measured when we are at complete rest represents our basal metabolism. Heat loss can be difficult to measure and is often impractical, particularly in large groups (Compher et al., 2006). Although there are several different ways to measure or predict basal metabolism, the



proxy measurement of heat production, through indirect calorimetry, is the most common method used in both research and clinical settings (Compher et al., 2006; Mahan & Escott-Stump, 2008; Mann & Truswell, 1998). This section will review the different methods for quantifying basal metabolism.

### ***2.3.1 Direct calorimetry***

Direct calorimetry is the gold-standard technique of measuring metabolic rate and quantifies the basic principle of measurement of heat dissipated over a 24 h period (Mann & Truswell, 1998). Energy expenditure is calculated from the difference in heat stored compared to heat lost through mechanical work and evaporation, convection and radiation (Mann & Truswell, 1998). Direct calorimetry requires a specialist room-sized chamber that is able to detect the heat loss. Such chambers are expensive to construct and operate, require a high degree of technical expertise to ensure precision of measurement and are not universally available. Furthermore, energy expenditure is not able to be measured in real time due to the time lag between heat production and measurement by the chamber. They also impose a high participant and investigator burden as participants have to be in the chamber for at least 24 h while being constantly monitored (Mahan & Escott-Stump, 2008; Mann & Truswell, 1998). If exercise ergometers are placed in the chamber, they also produce heat when used, which needs to be accounted for in subsequent analysis. In addition, as only heat is measured, it is not possible to obtain substrate oxidation rates. Due to these disadvantages direct calorimetry is rarely used to measure basal metabolism in both research and clinical settings. Indirect calorimetry, when performed under stringent conditions, is considered the criterion method for measuring energy expenditure in real-life settings (Henry et al., 2003; Leonard, 2012; Levine, 2005).

### ***2.3.2 Indirect calorimetry***

The principle of indirect calorimetry is that knowledge of respiratory gas exchange can be used to accurately estimate heat production, and hence, energy expenditure. The measurement of the gas exchange requires the collection of respiratory gas (pulmonary ventilation), and analysis of the volumes, flow rates and composition of the gas, when standardised for ambient temperature and pressure, saturated with water vapour (ATPS) conditions. Specifically, the consumption of oxygen ( $O_2$ ) and production of carbon dioxide ( $CO_2$ ) is measured and the respiratory quotient (RQ) and energy expenditure are calculated using formulae, such as the Haldane Transformation (Haldane, 1918). Although most techniques for indirect calorimetry capture the respiratory gases from subjects through mouthpieces or ventilation hoods, the construction of respiration chambers has allowed measurements of metabolic rate to be undertaken on sleeping subjects without being interrupted by the gas collection process (Rising et al., 2015)

The traditional method of indirect calorimetry undertook collection of expired pulmonary gases into large polyvinyl chloride ‘Douglas’ bags (Douglass 1911). While this method is very laborious, it is inexpensive, highly robust (<3% ‘small’ error; (Levine, 2005)) and is still considered the most accurate method for respiratory gas collection under steady-state conditions (Roecker et al., 2005). To address the earlier absence of a commercially available alternative, the Australian Institute of Sport custom-built a fully automated version of this system (Russell et al., 2002). It continues to use this system since it provides complete control of the algorithms used to calculate oxygen consumption (Gore et al., 2013). A large range of metabolic carts are now available for purchase, with some showing more reliable and valid results than others. These typically involve breath-by-breath and mixing-chamber systems.

### ***2.3.3 Activity monitors***

Activity monitors estimate energy expenditure via algorithms which use data from a variety and combination of sources, including heart rate, movement patterns and other biological factors (King et al., 2004; Koehler et al., 2013b; Welk et al., 2007). While early activity monitors used single inputs, more recent technology has produced more sophisticated units that integrate data from multiple sources. For example, SenseWear (SWA) is a multisensory monitor that is claimed to have improved accuracy in estimating energy expenditure from multiple factors such as heat flux, skin temperature, galvanic skin response and tri-axel accelerometer (Liden et al., 2002). The SWA monitor is worn on the upper left arm and is transportable, practical and reported to be of little discomfort when being worn (Fruin & Rankin, 2004). It is therefore particularly suited for use in free-living populations for collecting field measures, and presents another option for measuring SMR since it can be worn by the individual while they are sleeping.

Energy expenditure derived from SWA has been compared with RMR measured by indirect calorimetry with mixed results (El Ghoch et al., 2012; Elbelt et al., 2012; Malavolti et al., 2007). In healthy untrained individuals, the resting energy expenditure measured by the SWA correlated well ( $r= 0.86$ ,  $p < 0.0001$ ) with RMR measured by indirect calorimetry (Malavolti et al., 2007). In contrast, SWA overestimated RMR compared to indirect calorimetry in anorexia nervosa populations (El Ghoch et al., 2012; Elbelt et al., 2015). Meanwhile, in obese individuals, Papazaoglu et al. (2006) reported that SWA had a good correlation ( $r=0.88$ ,  $p<0.0001$ ) with indirect calorimetry but tended to underestimate RMR, with errors increasing in magnitude with the increase in RMR. Elbelt et al. (2012) compared overnight measurements of sleeping energy expenditure (SEE) via SWA to the RMR measured by indirect calorimetry in 20 healthy individuals and 61 patients who were either obese or diagnosed with fatty liver syndrome. Although SEE is generally regarded as being 5-10% lower than RMR, the SWA-derived SEE was found to be 7% higher than the measured

RMR (Elbelt et al., 2012; 2005). However, all these studies used an older version of the SWA monitor, based on a 2-axel accelerometer and older firmware (El Ghoch et al., 2012; Papazoglou et al., 2006). It would be of interest to see if these errors persist with the updated 3-axel accelerometer and when applied to athletic populations.

### ***2.3.2 Prediction equations***

A number of prediction equations have been developed to calculate basal energy expenditure of individuals in the absence of the equipment, expertise or opportunity to undertake measurements. These equations have typically been derived via regression analysis from studies of RMR in sedentary populations, with input factors including age, body mass, height and fat-free mass (FFM) (Cunningham, 1980; Harris & Benedict, 1918). The most widely used equation for predicting RMR in normal populations comes from the work of Harris and Benedict (1918), while other popular algorithms are provided by Cunningham et al. (1980), Westerterp et al. (1995) and Mifflin et al. (1990). Prediction equations derived from athletic populations include the work of De Lorenzo and colleagues (De Lorenzo et al., 1999). Whereas, the equations of Harris-Benedict, Mifflin-St Jeor and De Lorenzo use body mass and height to predict RMR with different equations for men and women, the Westerterp equation takes into account both fat-free mass (FFM) and fat mass (FM) (Westerterp et al., 1995) while Cunningham only accounts for FFM (Cunningham, 1980). Thomas and Manore (1996) investigated the accuracy of applying commonly used prediction equations to athletic populations, finding that they generally underestimate RMR in an athletic population, particularly if athletes have a high FFM. According to their work, the Cunningham equation was the most accurate in predicting RMR in well-trained individuals (Thompson & Manore, 1996).

As well as providing an estimate of RMR for clinical use, it has been proposed that prediction equations could be used to assess the “normality” of measurements of basal metabolism within athletic populations. Here, the comparison of measured RMR to expected RMR could be used to classify an individual into categories of “normal” or “suppressed” basal metabolism for further investigation (De Souza et al., 2007). Of course, this assumption requires an understanding of the accuracy or bias of various prediction equations, as well as the use of valid and reliable protocols for measuring basal metabolism. Therefore, techniques for measuring RMR, particularly the most commonly available method of indirect calorimetry, should be systematically investigated in high performance athletes to assess measurement variability and to refine protocols to minimise avoidable errors.

## **2.4 Sources of variability measurements of basal metabolism in athletes**

As summarised in section 2.3.2, indirect calorimetry involves the measurement of RMR from respiratory gases collected from a resting individual. Sources of error or variability in the measurement of basal metabolism can be divided into three main categories: those that derive from the instruments used to collect data, those associated with the conditions under which the subject is assessed, and inherent biological variability within the individual.

### ***2.4.1 Technical***

Technical error may be associated with any of the processes involved in collecting respiratory gases and then analysing the composition, volume and flow rate of these gases.

*Ventilation hoods vs mouthpieces*

There are three main apparatuses used for the collection of respiratory gases: nose clip and mouthpiece (mouthpiece), facemask and ventilation hood. In the first two cases, the individual is required to breathe into a device that is either attached directly to the Douglas Bag or metabolic cart or connected through a short hose of known volume (Haugen et al., 2007). The ventilation hood consists of a transparent bubble, placed over the face, attached to a fabric skirt that gets tucked in around the participant to create a tight seal. Room air enters the hood through a one-way valve while air is extracted via a pump into the mixing chamber for analysis as an air-tight seal is often difficult to achieve (Isbell et al., 1991). An error could be added to RMR measurements if there is any physical cost (e.g. body movement or breathing effort) involved with the use of any of these apparatuses; particularly the mouthpiece or facemask.

Several studies have measured the variability of RMR measurements between and within the different protocols of collecting respiratory gases. Roffey and colleagues (Roffey et al., 2006) measured the daily variation in gases collected via nosepiece-mouthpiece and ventilation hood protocols in ten sedentary individuals. The volume of oxygen consumption ( $\dot{V}O_2$ ), and hence RMR was significantly higher when the mouthpiece system was used compared with the ventilation hood (Roffey et al., 2006). However, different types of gas analysers were used in conjunction with these protocols; therefore, it is unknown how much the gas collection method contributed to the error (Roffey et al., 2006). Conversely, Isbell et al. (1991) reported no difference in mean RMR over a three-day measurement period in 30 females. In this study, resting expirate was collected using a Hans Rudolph mouthpiece (Hans Rudolph, Inc, Kansas City, MO), a facemask (Concord Laboratories, Inc., Keane, NH) and a ventilated hood (Medical Graphics Corporation, Minneapolis, MN), but were analysed using a standardised calibrated Medical Graphics Critical Care Monitor (CCM), (Medical Graphics Corporation, Minneapolis, MN). However, confounding methodological parameters

were identified; the relatively short periods of time required for fasting (5 h) and post-exercise rest (5 h) and exposure to external noise (participants were permitted to listen to classical music). Therefore, future studies are needed to better understand the effect of different types of gas collection equipment on RMR measurements, when confounding factors are standardised.

### *Gas analysis systems*

Under ideal practices, the validation of a gas analysis system should be compared against the criterion technique; the Douglas bag method. Under resting conditions, as performed with RMR testing, it is common for one gas analysis system to be compared with another in a randomised crossover research design where the participants have their RMR measured using each of the apparatuses (Ashcraft & Frankenfield, 2014; Blond et al., 2011; Cooper et al., 2009; Haugen et al., 2007; Macfarlane & Wu, 2013).

Although the Deltatrac (Sensormedics, Yorba Linda, CA) metabolic cart (Ashcraft & Frankenfield, 2014; Blond et al., 2011) is no longer commercially available, two studies were conducted comparing energy expenditure measured using this device with that of the Quark metabolic cart (Cosmed, Rome, Italy) and found that there was no difference between measures of  $\dot{V}O_2$ ,  $\dot{V}CO_2$  and RMR. Kistorp et al (1999) compared the Medigraphics CCM System (Medical Graphics, Minneapolis, USA) to a respiration chamber and concluded that RMR measured under the ventilated hood was consistently lower by 13%. (Kistorp et al., 1999). However, there was no clear reason stated that could help explain these differences.

A number of other confounding variables have been identified among the available studies of commercial metabolic carts, making it difficult to make clear interpretations of the validity of specific systems. Such limitations include low sample sizes ( $n=1$ , (Ashcraft &

Frankenfield, 2014)); the engagement of participants in passive activities (reading, watching movies (Blond et al., 2011)); measurements performed in a non-randomised order (Kistorp et al., 1999); and the lack of uniform standardisation of the pre-test period (Ashcraft & Frankenfield, 2014; Bassett et al., 2001; Blond et al., 2011; Cooper et al., 2009; Macfarlane & Wu, 2013). As such, future studies, comparing the different gas analysis systems against the criterion, are warranted.

#### ***2.4.2 Subject preparation***

Across all related studies, there are large variations in the practices included in the pre-test preparation of subjects that could affect the RMR assessment. Although some practices are generally controlled or standardised (e.g., subjects are rested and fasted according to a standard protocol believed to achieve a resting steady-state (Compher et al., 2006; 2005; Matarese, 1997), the protocols for achieving these conditions vary between studies (Compher et al., 2006). A summary of the variations in protocols can be found in **Table 2.1** and will be discussed further below. These have been considered in a review written by Compher and colleagues (Compher et al., 2006) and recently updated by the Academy of Nutrition and Dietetics (Fullmer et al., 2015) to provide Guidelines for Best Practice in undertaking RMR measurements in healthy and non-critically ill individuals; special considerations for elite athletes or well-trained individuals have not received such attention.



**Table 2.1.** Standardisation of RMR protocols used in studies for measurement of RMR in an athletic, trained or exercising population.

Study	Population	Standardisation of Participant				Standardisation of measurement technique & technician						
		Time Rested – exercise	Time fasted	Body Composition - Assessment	Positioning	Lab Temp	Gas – analysis system	Expirate collection device	Rest upon arrival	Data collection time	Time of day	Equation for conversion to EE
Burleson et al 1998	15 M resistance trained	NS	NS	NS	Supine	NS	Metabolic Cart	NS	30 min	3 x 5 min	NS	NS
Binzen et al 2001	10 F – resistance trained.	48h	4-5	Skinfolds – 7 sites	Reclining	25-27°C	Metabolic Cart	FMK	20 min	20 min	Throughout day	Weir
Bullough et al 1995	8 M trained –type NS	*First measure – NS	12 h	Hydrostatic weighing	Supine	23°C	Metabolic Cart	V	15 min	30 min	6.30am	Weir
Carlsohn et al 2011	8 M and 9 F – rowing and canoe international	24 h* vigorous only, low intensity training allowed	NS - O/N	Skinfolds – 10 sites	Supine	22-25°C	Metabolic Cart	FM	10 min – sitting	30 min	Morning	Weir
De Lorenzo et al 1999	51 M – water polo, judokas, karatekas	36 h no training	10-12 h	DXA	Supine	23-26°C	Metabolic Cart	V	30	30 min	NS	Weir
De Souza		24 h	12 h	DXA	Supine	20-24°C	Metabolic Cart	V	45	30 min	8.30-11.00am	Weir
Doyle et al 2010	15 F professional dancers	24h Vigorous exercise	10-12 h	DXA	NS	NS	Metabolic Cart	V	10 min	45 min	6.30am	NS

Study	Population	Standardisation of Participant				Standardisation of measurement technique & technician						
		Time Rested – exercise	Time fasted	Body Composition - Assessment	Positioning	Lab Temp	Gas – analysis system	Expirate collection device	Rest upon arrival	Data collection time	Time of day	Equation for conversion to EE
Freedman-Akabas et al. 1985	N=12 highly active	24 h	4 h	Weight only	NS	25°C	Metabolic Cart	MP	30 min	12 min	Mid-morning/ mid-day	NS
Guebels et al 2014	17 F - endurance trained	19 h (range 11-24h)	8 h	DXA	Supine	NS	Metabolic Cart	V	20-30 min	8-10 min	Morning	Weir
Herring et al. 1992	9 F – highly trained runners	15 h	10-12 h	Hydrostatic weighing	Own home – prior to getting up	Variable	Kofranyi-Michaelis gasometer – rubber bladder	MP	Done in subject's own home	15-20 min	Morning	NS
LaForgia et al. 1999	16 M: various – trained	36 h	12 h	2,3,4 Compartment models	Supine	24.0±0.5°C	DB	MP	50 m	2 x 10 min	7.20am	Elia & Livesey
Lebenstedt et al. 1999	33 F – regional track and field	24 h	O/N	BIA	Supine	NS	Metabolic Cart	V	20 min	20 min	7.30 am	Weir
Melin et al. 2014	40 F – endurance – teams/ club level	60 h* Hard prolonged	O/N	DXA	NS	NS	Metabolic Cart	V	15 min	35 min	7-8.30am	Weir
Myerson et al. 1991	27 F Competitive runners	O/N	12 h	Hydrostatic weighing	Seated	21-24°C	DB	MP	30 min	2 x 6 min	8.00am	Weir
O'Donnell et al.	14 F – recreational	NS	NS	DXA	Supine	NS	Metabolic Cart	V	45 min	30 min	8.30-11.00am	Weir

Study	Population	Standardisation of Participant				Standardisation of measurement technique & technician						
		Time Rested – exercise	Time fasted	Body Composition - Assessment	Positioning	Lab Temp	Gas – analysis system	Expirate collection device	Rest upon arrival	Data collection time	Time of day	Equation for conversion to EE
Paoli et al. 2012	18 M resistance trained	O/N	N/S	SF – 9 sites, bone breadths 4 sites, girths 2 sites	Seated	24°C	computerised spirometer	N/S	15 min	30 min	7-8.00am	Weir
Poehlman 1988 – DIT	9 M endurance runners	24 h	12 h	Hydrostatic weighing	Supine	24°C	Metabolic cart	V	30 min	30 min	0630	Weir
Ribeyre et al. 2000	15 M and 11 F adolescents	NS	10 h	Skinfolds 4 sites	Supine	N/S	Respiratory Chamber^	NA	N/A	1 h	7-8am	Weir
Schmidt et al. 1993	25 M – collegiate wrestlers	12 h	10 h	Hydrostatic weighing Skinfolds – 6 sites	Supine	24-25°C	Metabolic Cart	V	15 min	30 min	6-9.00am	Weir
Schuenke et al. 2002	7 M – resistance trained	Baseline: 4 d	Baseline N/s others 4 h	N/S	Supine	N/S	Metabolic Cart	FMK	30 Min	30 Min	Baseline – 7.00am	N/S
Short & Sedlock 1997	7 M and 5 F, competitive running, cycling, swimming	Replicated	12 h	H & W only	Seated	22.4±0.9°C	Metabolic cart	MP	15 min	15 min	6:30-7am	N/S
Ten Haaf & Weijs 2014	103 M & F recreational	12 h (non vigorous)	4 h	BodPod	Supine	N/S	Metabolic Cart	V	30 min	30 min	N/S	Manufacturer Software

Study	Population	Standardisation of Participant				Standardisation of measurement technique & technician						
		Time Rested – exercise	Time fasted	Body Composition - Assessment	Positioning	Lab Temp	Gas – analysis system	Expirate collection device	Rest upon arrival	Data collection time	Time of day	Equation for conversion to EE
Thomson & Manore 1996	24 M and 13 W	12 h	12 h	Hydrostatic weighing	NS	NS	Metabolic cart	V	30 min	30 min	6-8am	Weir
Thomson et al. 1993	24 M runners, tri or bi athletes	12 h	12 h	Hydrostatic weighing	NS	NS	Metabolic Cart	V	30 min	30 min	5-8am	Weir
Thomson et al. 1995	10 M endurance	12 h	12 h	Hydrostatic weighing	N/S	N/S	Metabolic cart^	V	30 k	30 min	Morning	Weir
Tremblay et al. 1987S	8 M – endurance runners and x-country skiers	15-18h >72 h	12 h	Hydrostatic Weighing	Reclined	N/S	Metabolic Cart	V	30 Min	15 min	Morning	Weir
Vanheest et al. 2014	10 F swimmers (15-17 y)	NS	NS – assume ON	Skinfolds – 4 sites *DXA considered impractical and not safe	NS	22.2-23.3°C	Metabolic Cart	V	45 min	30 min (min 20 min used for assessment)	NS	Weir
Westrate et al. 1990*	9 – M recreational	10-12h +*	12-14h	Hydrostatic weighing	Supine	24°C	Metabolic Cart	V	30 min	1 h	8-9.00am	Jéqueir

V=ventilation hood, FMK=Face mask, ON=overnight, M=male, F=female; NS=not stated; DB=Douglas Bag; MP=mouthpiece and nose clip; BIA=Bioelectrical Impedance Analysis; MC=metabolic cart; DXA=dual energy x-ray absorptiometry;

\*Participants exercised on cycle ergo at either 25-35% or 60-7-% VO<sub>2</sub>max after dinner, were not allowed to eat anything until after RMR measure next day – allowed to watch a video during measure.

^=BMR measurement.

### *Inpatient vs outpatient (RMR vs BMR)*

As previously mentioned, the defining difference between BMR and RMR is the circumstance under which the measurement of metabolic rate is made: in particular, whether the test is conducted at the bed in which the participant has slept overnight, or whether the participant has reported to the laboratory with minimal arousal possible after waking at home (2005). It is of interest to understand the differences in magnitude and variability between measurements of BMR (hereafter referred to as “inpatient”) and RMR (hereafter referred to as “outpatient”), particularly in athletic populations when measured under the criterion Douglas Bag method. In one study, Berke et al. (Berke et al.) reported that outpatient RMR measurements were 8% higher than BMR in elderly subjects, although there was no attempt to identify the factors that explained this difference (Berke et al., 1992).

More recent studies have failed to determine any statistical difference between RMR measured simulating the outpatient/inpatient method (Bullough & Melby, 1993; Turley et al., 1993). Bullough & Melby (1993) studied nine recreationally active males, which may offer greater application to a high performance athlete population compared with Berke et al. (1992), who conducted their study on elderly patients. Bullough & Melby (1993) repeated the evening meal prior to the overnight fast that preceded testing both inpatient and outpatient settings, and compared this to an outpatient setting where participants were allowed to self-select another meal. Again there were no differences detected in RMR brought about by the different meal selection (Bullough & Melby, 1993). Interestingly, in this study participants were chauffeured from their home to the laboratory with a travel time of 5 min. Furthermore, participants were permitted to rise, void and had their height and body mass measured before returning to the bed they slept in for the inpatient RMR (Bullough & Melby, 1993). It is unknown if the lack of difference would have remained if the participants had transported

themselves (Bullough & Melby, 1993) or if they had remained in their beds until after the measurement. At sports institutes or clinics with dormitories nearby, the effect of a short walk on RMR is of interest. Furthermore, exposure to varying outdoor ambient temperature due to the required transfer to the laboratory may also affect athletes who are measured using an outpatient protocol. Indeed, Kashiwazaki et al. (1990) reported that RMR was 6-7% higher after a 10-min outdoor walk in winter compared to summer. This suggests that the time of year when measures are undertaken should be considered for longitudinal monitoring of RMR.

### *Length of fast*

The typical increase in metabolic rate following food ingestion is termed the thermic effect of food (TEF) or the thermic effect of a meal (TEM) (hereafter referred to as TEM) (Institute of Medicine, 2005; Manore & Thompson, 2009). In sedentary individuals this accounts for up to 10% of daily energy expenditure. The time to peak of the TEM is reported to occur 1-3 hours following the consumption of a meal, with obese and older-adults peaking later than non-obese adults and younger people (Compher et al., 2006).

Poehlman and colleagues (Poehlman et al., 1988) demonstrated the TEM in trained individuals was lower than untrained individuals, but the TEM was still elevated above resting levels at the end of the measurement phase (3 h post-prandial)(Poehlman et al., 1988). Thompson et al. (1993) reported similar findings with TEM peaking 1 h post-prandial in male endurance athletes. In contrast, Reed et al. (1996) reported that energy expenditure was still elevated 9% above resting levels 5 h after food was ingested (Reed & Hill, 1996). Kinabo and Durnin (1990) reiterated these findings, reporting that metabolic rate had not returned to resting levels after 5 h of measurement. The TEM typically lasts 4-8 h and is dependent on

the size of the meal. Therefore, it appears that RMR testing within 5 h of a meal, as suggested by Compher et al. (2006), will not accurately reflect resting metabolism. D'Alessio and colleagues (D'Alessio et al., 1988) reported that by 8 h the TEM has dissipated in lean and obese men, except when they ate at energy intakes as high as 133 kJ/kg FFM. Twelve hours of fasting is typically used in studies involving RMR measurements. However, a 12 h fast may be impractical for high performance athletes, due to their high-energy intake requirements and frequent training demands.

#### *Prior exercise*

It is generally accepted that exercise temporarily increases RMR (Compher et al., 2006) and is primarily due to the excessive post-exercise oxygen consumption (EPOC) and increased metabolic cost of repairing and resynthesising muscle (Manore & Thompson, 2009). The effect of exercise on metabolic rate post-exercise has been extensively studied in both trained and untrained individuals (Herring et al., 1992; Hunter et al., 2006; Hunter et al., 2003; Kelly et al., 2013; Knab et al., 2011; Lee et al., 2009; Melby et al., 1993; Osterberg & Melby, 2000; Paoli et al., 2012; Pratley et al., 1994; Schuenke et al., 2002; Tremblay et al., 1988; Williamson & Kirwan, 1997). However, there is currently no consensus on the amount of rest time following an exercise session that is sufficient before a valid RMR can be measured, as the majority of studies only measure EPOC for up to 3 h (Binzen et al., 2001; Freedman-Akabas et al., 1985; Short & Sedlock, 1997). A subset of studies has looked at the effect of exercise up to 72 h later (Bullough et al., 1995; Dolezal et al., 2000; Hunter et al., 2006; Tremblay et al., 1988; Williamson & Kirwan, 1997), however, we question the practicality of this in elite athletes, as it is highly likely that a subsequent training session will fall within that period. It is estimated that the longest rest period for an elite athlete might be 18-36 h (Burke, L; 2014-personal communication).

Studies investigating EPOC indicate that energy expenditure returns to baseline 40-60 min following aerobic-based exercise involving 20-30 min at  $\sim 70\%$   $\dot{V}O_{2\text{peak}}$ , regardless of training status (Freedman-Akabas et al., 1985; Short & Sedlock, 1997). Meanwhile, another study which compared the effect of steady state, resistance and high intensity intermittent training sessions on measurements of RMR taken 12 and 21 h post exercise (Greer et al., 2015) found that both resistance and high intensity sessions increased RMR above baseline at each time point (Greer et al., 2015). Indeed, a number of studies have shown a sustained (14-38 h) and elevated (16-22% over 24 h) EPOC following an acute bout of high-intensity resistance exercise in trained men (Gillette et al., 1994; Paoli et al., 2012; Schuenke et al., 2002). Investigations by Gillette et al (Gillette et al., 1994), Paoli et al (2012) and Schuenke et al (2002), who used resistance training protocols of varying reps, sets, percent of maximal effort and number of exercises, determined that RMR was higher for 14-, 22- and 38 h, respectively, post exercise compared to a non-exercise baseline RMR. Conversely, Abboud et al. (2013), reported no increase in RMR above baseline for up to 48 h following resistance exercise in eight trained men, however, only a 4 h fasting period was used prior to RMR. While these studies have looked at RMR in response to an exercise protocol, only two studies have investigated the effect of the cessation of exercise in chronically trained individuals (Herring et al., 1992; Tremblay et al., 1988). RMR was lower by 6-8% in both male and female athletes following three days without exercise (Herring et al., 1992; Tremblay et al., 1988), compared to the morning after a 90 min aerobic exercise bout. No study has systematically investigated the combination of the type of exercise and the period of recovery from exercise on RMR in highly trained individuals.



It is difficult to find common ground in the methodologies of studies of the effect of prior exercise on RMR. There are differences in study populations (athletes vs non-athletes), the techniques of undertaking RMR measurements (e.g. equipment and protocols), and the conditions of pre-measurement standardisation. Furthermore, even among the studies of athletes, there are different criteria for defining the participants as ‘well-trained’: whereas one study required a training history of six months (Schuenke et al., 2002), in another, the minimum commitment was at least two years (Dolezal et al., 2000). These factors make it difficult to apply the results of the current literature to a high performance environment where athletes train 6-8 h per day for 5-6 days per week, year-round. Further work is needed to identify standards for the rest period between the last exercise session and an RMR test which will allow a meaningful measurement to be collected.

#### ***2.4.3 Measurement of Body composition***

Although measurements of RMR and REE are typically assessed in absolute terms (kJ/min or kJ/d respectively), REE is often expressed relative to FFM (kJ/kg FFM/d). Fat Free Mass (FFM) can be determined by a range of protocols including anthropometry (skin folds), hydrostatic (underwater) weighing, air displacement plethysmography (BodPod), body mass index (BMI), bioelectrical-impedance and dual energy x-ray absorptiometry (DXA) (Kelly et al., 2013; Knab et al., 2011; Lee et al., 2009; Melby et al., 1993; Osterberg & Melby, 2000; Paoli et al., 2012). In the athletic population, the validity of some of these measures has come under scrutiny, with doubly indirect methods such as skinfolds and bioelectrical impedance being deemed unsuitable for the assessment of FFM (Ackland et al., 2012). Whole body DXA measurements have been validated as a measure of body composition in athletes when undertaken according to a standardised protocol (Nana et al., 2012b; Nana et al., 2016; Nana et al., 2015). Lean mass and bone mineral content are the components that comprise FFM

when assessed by DXA. Obviously, there is a risk of inaccurate measurement and misinterpretation of relative RMR if there are errors in the assessment of the components of FFM due to either a lower precision technique or measurement error (Korth et al., 2007).

The timing of the assessment of body composition used in the calculation of relative RMR is important; indeed, the measurement of FFM should be undertaken as close as is practical to the assessment of RMR, especially in well-trained and/or adolescent athletes who can experience changes in lean mass over short periods of (Garthe et al., 2011; Kerr & Ackland, 2010). Changes in measurements of lean mass can occur due to real changes in muscle mass (which would be expected to change RMR), but can also reflect acute fluctuations in intra-muscular substrate and associated fluid storage which can add variability to assessments of FFM (without affecting RMR) (Nygren et al., 2001). For example, an investigation using Magnetic Resonance Imaging (MRI) showed increases in muscle cross-sectional muscle area following dietary-based carbohydrate loading (Nygren et al., 2001). Furthermore, carbohydrate loading was reported to increase DXA estimates of lean mass in healthy untrained men (Rouillier et al., 2015; Toomey et al., 2017). There is also another report of lean mass change induced by a 10-day creatine monohydrate loading protocol, when measured by DXA in 12 untrained men (Safdar et al., 2008). Since changes in muscle glycogen would be a common occurrence in this population as part of their daily training and competition regime, it is of interest to see how much error they add to assessments of FFM and how this may alter an assessment and interpretation of RMR relative to FFM.

#### *Assessment of muscle glycogen*

Current techniques to assess intramuscular substrates involve either the invasive muscle biopsy technique (Harris et al., 1974) or use of expensive and non-portable equipment (MRI) (Nygren et al., 2001) both of which require trained personal to conduct. Recently, a

non-invasive protocol to measure muscle glycogen via ultrasound technology has been developed (MuscleSound<sup>®</sup>) (Hill & Millan, 2014; Nieman et al., 2015), based on the hypothesis that measurement of the echo-intensity of the ultrasound image reflects the fluid bound with the storage of glycogen (Hill & Millan, 2014; Utter et al., 2010). Specifically, muscle glycogen is indirectly assessed and assigned an arbitrary score, via the use of a proprietary algorithm which assumes that the presence of muscle water makes the fluid darker or hypoechoic, representing “high glycogen” (Hill & Millan, 2014; Nieman et al., 2015), while the breakdown of glycogen and subsequent release of fluid results in a brighter or hyperechoic image (Nieman et al., 2015). Two validation studies, investigating muscle glycogen concentrations pre- and post-exercise, reported high correlations between the ultrasound-derived score and estimates derived from the gold standard muscle biopsy technique from rectus (Hill & Millan, 2014) and vastus lateralis (Nieman et al., 2015) respectively. However, no analysis on the limits of agreement or Bland-Altman plots were reported and the statistical analysis used of student t-tests and Pearson’s correlation coefficients are deemed to be inappropriate for assessing validation (Watson & Petrie, 2010). Furthermore, the authors did not address potential confounders which may also cause changes in intramuscular fluid such as muscle damage, or changes in cellular metabolites such as creatine or carnitine. Whether the ultrasound technology can detect changes in glycogen associated with carbohydrate loading has also yet to be explored.

Using the same concept, the measurement of total body water may also be able to assess whether or not a change in muscle glycogen has occurred. Bioelectrical impedance spectroscopy (BIS) has been validated for measuring total body water and distinguishing between the intracellular and extracellular fluid compartments against the criterion dilution method (Matias et al., 2016; Moon et al., 2010). Bioelectrical impedance, has been used

assess changes in total body water following creatine supplementation (Brilla et al., 2003). However, its use for confirming a change in muscle glycogen, due to exercise depletion or dietary loading, has been achieved has yet to be explored.

#### ***2.4.4 Biological variation***

Biological variation refers to errors or variability introduced due to innate characteristics of the participant and includes factors such as chronic exercise and training history, menstrual cycle and body composition (Compher et al., 2006).

##### *Body Composition*

The majority (70-80%) of the inter-individual variation in RMR measurements can be explained by FFM (Cunningham, 1991; Gibney et al., 2002; Sparti et al., 1997). An individual's FFM is sum of the mass of organs, skeletal muscle, bone and fluid in the body; since these tissues have greater metabolic activity than FM, individuals with a higher skeletal muscle mass and FFM have a higher absolute RMR (Gibney et al., 2002; Ravussin et al., 1982). This has been reported in studies comparing males and females (Arciero et al., 1993; Buchholz et al., 2007) and young adults compared to elderly individuals (Bosy-Westphal et al., 2003; Poehlman et al., 1993). For this reason RMR is often expressed relative to FFM (kJ/kg FFM/d), when interpreting results, especially when evaluating the impact of an intervention on RMR.

##### *Chronic training status*

It is commonly believed that athletes have a higher RMR than their sedentary counterparts. The difference in RMR is attributed to the higher skeletal muscle mass relative

to body mass (Poehlman et al., 1988; Sjodin et al., 1996; Tremblay et al., 1986). However Sjodin et al., (1996) reports that RMR remained significantly higher by 16% in trained individuals compared to untrained after adjusting for FFM. Conversely, Broeder et al., (1992) report no difference in RMR when participants are matched for FFM. The concept of energy flux, describing the combination of a high (or low) energy intake and exercise energy expenditure (EEE), has been used to explain the differences between the studies (Bullough et al., 1995). In one study RMR was only higher in trained individuals after two to three days of a high energy intake and EEE. There was no difference between trained and sedentary groups following a period of low EEE and low energy intake.

### *Menstrual status*

There is conflicting evidence regarding the change in RMR during the different phases of the menstrual cycle (Bisdee et al., 1989; Henry et al., 2003; Piers et al., 1995; Solomon et al., 1982). Bisdee et al., (1989) reports that mean BMR was higher by 619 kJ/d during the late luteal phase compared with the late follicular phase, with six of the eight women in their study cohort showing differences between phases (Bisdee et al., 1989). SMR was also significantly higher during the late luteal phase compared with the late follicular phase however, these differences appear to be minimal as they were not reflected in the total daily energy expenditure while in a respiratory chamber (Bisdee et al., 1989). These findings support those of Solomon and colleagues (Solomon et al., 1982) who also reported an increased RMR in the late luteal phase. On the other hand, Piers et al. (1995) reported no change in RMR between the follicular and luteal phases in 13 women. The individual data from this study were not reported and may be important to consider. For example, Henry et al. (2003) noted the individuality of change in RMR over the menstrual cycle; of the 19 participants in this study, 10 showed no variation while others exhibited a large variation over the cycle.

### *Altitude training*

Altitude training may influence metabolic rate, through a variety of reasons. RMR may decrease due to decreased appetite and decreased nutrient absorption resulting in a decreased energy intake and loss of FFM (Westerterp, 2001). Alternatively, greater muscle catabolism at altitude could lead to an increase in RMR through increased muscle building and repair (Westerterp, 2001). It is unknown if undertaking the collection of respiratory gases at different altitudes influences the results of RMR measurements; in such a case, a technical error would also be introduced.

## **2.5 Energy availability and RMR in athletes**

Within the field of sports nutrition, there is a renewed interest in RMR as a marker of the “healthiness” versus “impairment” of an athlete’s metabolic function. This interest has grown out of the need to explain, prevent and treat the apparently high incidence of amenorrhea in female athletes; a problem that was first recognised in the 1990s (Loucks, 2003). The concept of energy availability (EA) was first hypothesised by Anne Loucks (Loucks et al., 1998) in an attempt to unify the theories that this phenomenon was caused by low levels of body fat or high training volumes. Rather than focusing on energy balance (the difference between energy intake and energy expenditure), EA considers the role of energy intake in meeting the needs of the various physiological processes it must support within a biological system.

The construct of EA was developed from the field of bioenergetics, where it is proposed that dietary energy used in one of the processes of thermoregulation, cell

maintenance, locomotion (exercise), growth, immunity and reproduction cannot be used for the others (Loucks et al., 2011). From an exercise physiology perspective, EA is defined as the energy available to support other physiological processes once the energy expenditure associated with exercise (EEE) has been deducted from energy intake (EI) (Loucks et al., 2011). Since fat mass has a much lower metabolic activity, Energy availability (EA) is expressed relative to Fat Free Mass (FFM). Therefore:

$$EA = (EI - EEE)/FFM$$

An EA of 45 kcal/kg FFM/d (188 kJ/kg FFM/d) is considered to provide sufficient energy to maintain energy balance and physiological processes in healthy athletes (Loucks, 2003). However when energy balance is disturbed in scenarios in which dietary energy is less than total energy expenditure (TEE), the body can respond either by contributing its energy reserves (e.g. adipose tissue, body proteins) to meet the energy deficit and/or by reducing the energy expenditure involved with other body functions. EA is not synonymous with energy balance, since a situation in which energy availability is reduced in the face of a chronic energy deficit ( $EI < TEE$ ) leading to adaptations to reduce energy expenditure may return the body to a new situation of energy balance ( $EI = TEE$ ). Although such energy conservation is an evolutionary adaptation favourable for survival, it comes at the cost of physiological processes such as growth, cellular maintenance, reproduction and thermoregulation (Loucks et al., 2011).

Loucks proposed that in situations in which a female athlete either reduced energy intake and/or increased EEE to the extent that other physiological systems (e.g. the reproductive system) sensed low energy availability (LEA), there would be a reduction in expenditure and function by that system. She and her colleagues then undertook a systematic series of studies, manipulating energy availability in stepwise changes via changes in dietary

intake and/or exercise energy expenditure, to determine the level of EA at which reproductive function was impaired (Loucks, 2006; Loucks & Thuma, 2003; Loucks & Verdun, 1998; Loucks et al., 1998). These studies identified an EA threshold of 30 kcal/kg FFM/d (126 kJ/kg FFM/d) below which reproductive function in females is impaired, with the effect being independent of whether the reduction in EA was achieved by dietary restriction or a large volume of exercise (Loucks & Thuma, 2003). It was later realised that this threshold of 30 kcal/kg FFM/d corresponded to RMR in an athlete of mean body size (Loucks et al., 2011).

This work coincided and contributed with the evolution of the understanding of the Female Athlete Triad (FAT), with changes to this concept including an expansion of the “dietary corner” of the Triad beyond its previous assumption of eating disorders to encompass any situation of reduced EA (Otis et al., 1997). Indeed, it was recognised that a number of scenarios in athletic populations can lead to inadequate energy intake (EI) and/or high exercise energy expenditure and thus contribute to LEA (Loucks et al., 2011). Disordered eating is often the underlying cause of low EI, especially given its prevalence in sports where a low body mass or body fat level is considered important (e.g. endurance sports, gymnastics). However, LEA can also occur due to mismanagement or excessive manipulation of diet and/or training when an athlete is trying to reduce body fat, or to the failure to sufficiently increase energy intake during a period of intensified training or competition (Burke, 2015; Mountjoy et al., 2014). Indeed, these behaviours may be well-intentioned or unintentional rather than innately pathological, with the athlete being unaware of the negative health and performance consequences of allowing a mismatch between energy intake and exercise energy expenditure to occur. At times, energy deficit and its accompanied reduction in EA is required/necessary for an athlete to optimise body composition. However, it is important that the athlete is monitored and provided with appropriate nutritional strategies to spare lean



muscle tissue and to create an energy deficit that maintains energy availability at a sufficient level to avoid overly negative effects on performance and physiological processes; typically an EA within the range of 30-45 kcal/kg FFM can be tolerated for modest periods (Loucks et al., 2011).

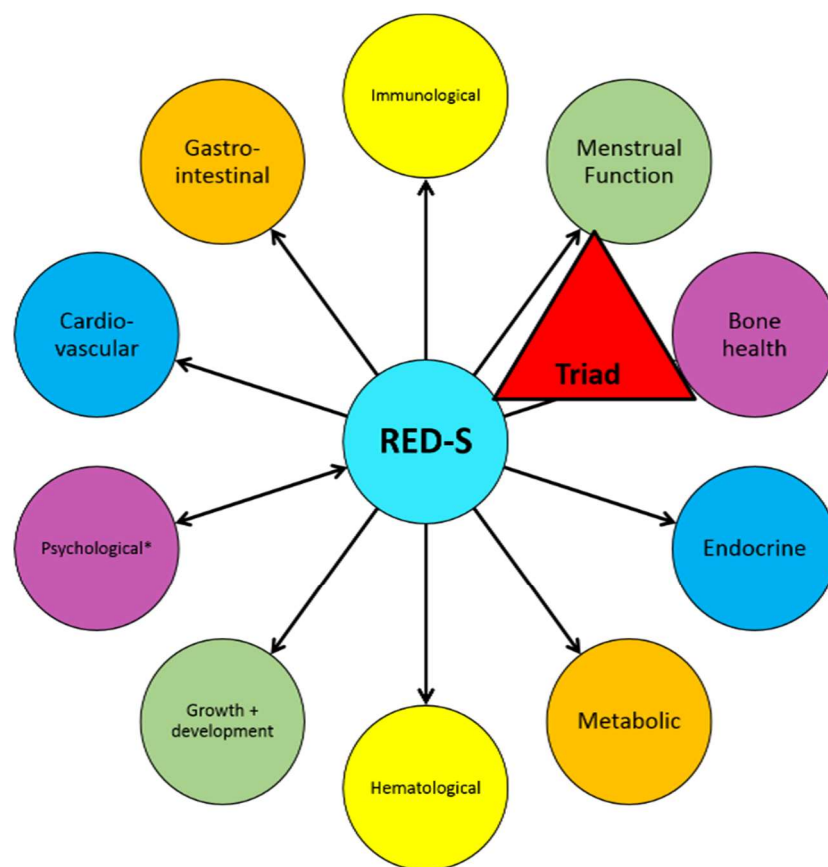
Over the past decade, there has been a concentrated effort to measure the prevalence, causes and effects of LEA in athletic populations. The practical direction of this work may have commenced with the assumption that tools could be found to measure EA accurately and that a universal threshold of EA exists above which athletes can maintain good health and performance (i.e. >30 kcal/kg FFM/d). However, further studies have revealed some problems with these assumptions as well as other extensions of our knowledge regarding impaired basal metabolism in athletes. These will now be briefly reviewed.

#### ***2.5.1 Impairment of many body systems with low EA***

The primary work in the area of what has become known as the FAT focussed on the inter-related impairments of menstrual function and bone health in female athletes (Anderson et al., 2000; Manore et al., 2007; Nattiv et al., 2007). There is now strong evidence that LEA causes menstrual and reproductive dysfunction underpinned by disruption of the pituitary secretion of luteinizing hormone secondary to disruption of the pulsatile secretion of gonadotropin-releasing hormone (Loucks et al., 1994; Loucks & Thuma, 2003; Loucks et al., 1998). Although impairments of bone mineral density and architecture with FAT were first attributed to reduced oestrogen concentrations associated with menstrual dysfunction, later studies identified that LEA is an independent and additive factor in the deterioration of bone health. Indeed, LEA has been shown to reduce bone formation via reduction in procollagen

type I C-terminal propeptide (PICP) and osteocalcin, as well as an increase in bone resorption (Ihle & Loucks, 2004).

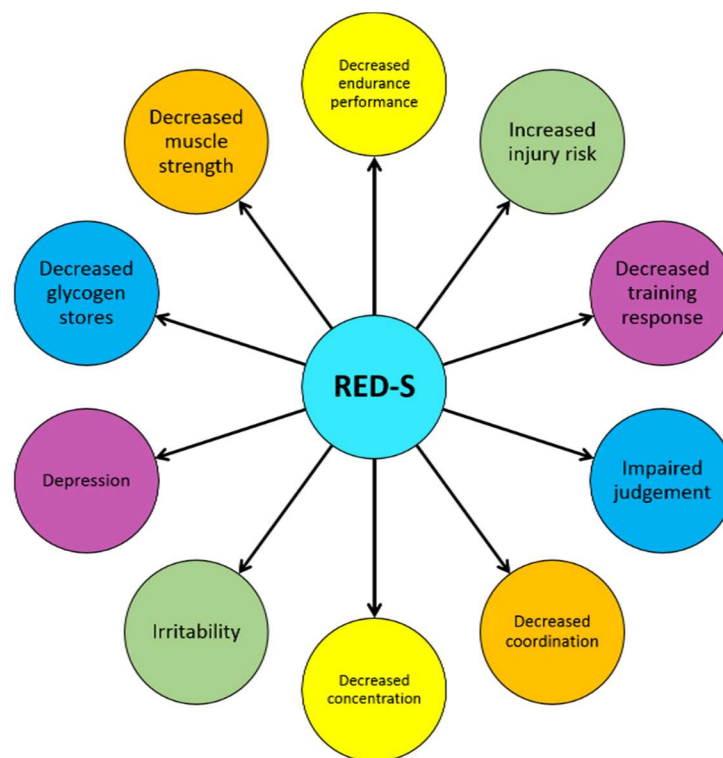
The recognition of the expanding range of metabolic systems that are impaired by low EA has led to a proposal to expand FAT into a wider syndrome termed RED-S (Relative Energy Deficiency in Sport) (Mountjoy et al., 2014). **Figure 2.1** and **2.2** taken from a review paper underpinning this proposal summarises that low EA can impair health and performance of athletes in a variety of ways.



**Figure 2.1.** Health consequences of Relative Energy Deficiency in Sport (RED-S) (Mountjoy et al., 2014).

Evidence for the range of additional indices of impaired health and optimal function now associated with low energy availability (**Figure 2.1**) includes the following observations:

- Hyperlipidaemia and endothelial dysfunction have both been reported in athletic females with amenorrhea (Rickenlund et al., 2005) increasing their risk of a cardiovascular event.
- Metabolic deficiency indicated by low 3,5,3'-triiodothyronine (T<sub>3</sub>) suppressed RMR in females with an EA of <30kcal/kg FFM/d (Loucks & Heath, 1994; Melin et al., 2015).
- Attenuation of muscle protein synthesis at rest following 5 days at an EA of 30kcal/kg FFM/d compared with 45kcal/kg/FFM (Areta et al., 2014).
- Type 1 immunity decreases in an energy deficiency state, making athletes more susceptible to viral infections (Hagmar et al., 2008).



**Figure 2.2.** Potential performance effects of RED-S (Mountjoy et al., 2014).

The potential for a direct impact of LEA on sports performance is of particular importance to athletes, and includes a range of issues identified in **Figure 2.2**. Evidence that LEA has a measureable effect on training adaptations and competition performance is emerging. Indeed, Vanheest and colleagues (Vanheest et al., 2014) recently reported that a sub-group of adolescent female swimmers with LEA, estimated from food and training diaries

and confirmed by direct measurement of ovarian hormones and RMR, showed no improvement in swimming performance following a 12 week training block while a cohort with adequate EA achieved a 8% improvement in 400 m swimming time over the same training program. Of further interest to many athletes, this study observed that the swimmers with LEA had higher body fat levels, which remained static over the training, in comparison to the swimmers with healthier RMR and EA. A range of mechanisms identified in **Figure 2.1** and **2.2** may contribute to the reduced ability to adapt to a training response.

### ***2.5.2 Extension to male athletes***

The negative physiological effects of relative energy deficiency are not confined solely to female athletes (Areta et al., 2014; Dolan et al., 2012; Hackney, 2008; Vogt et al., 2005), since factors such as the pressure to be lean and light or the engagement in training and competition activities involving high energy expenditure also occur in male athletes and may lead to situations of LEA (Tenforde et al., 2015). Indeed, observational data in male cyclists have reported EA of as low as 8kcal/kg FFM per day (Vogt et al., 2005). Although there have been fewer studies of the effect of LEA in male subjects, it is known that important growth hormones associated with bone formation such as testosterone and insulin growth factor-1 (IGF-1) are down regulated in males with low energy intake (Dolan et al., 2012). A reduction in resting levels of muscle protein synthesis and its response to protein intake was reported in male athletes when following a diet providing EA of 30kcal/kg FFM/d compared with 45kcal/kg FFM/d. Meanwhile, Koehler et al. (Koehler et al., 2016), reported reductions in serum leptin and insulin when LEA of 15 kcal/kg FFM/d was induced with or without exercise in six healthy men. However, no changes in testosterone, IGF-1 and T<sub>3</sub> were observed. Although there has been some debate that the effects of LEA in male athletes may have a different outcome in the nature and severity of symptoms, there is clearly a case for

further research in male athletes (Koehler et al., 2016; Mountjoy et al., 2015; Tenforde et al., 2015).

### ***2.5.3 Challenges to the use of EA as a diagnostic or assessment tool***

Although EA and the role of LEA in impaired health and performance of athletes are robust concepts, there are several issues that prevent the assessment of EA from being used as a diagnostic tool.

#### *Difficulties in accurate and reliable calculation of EA*

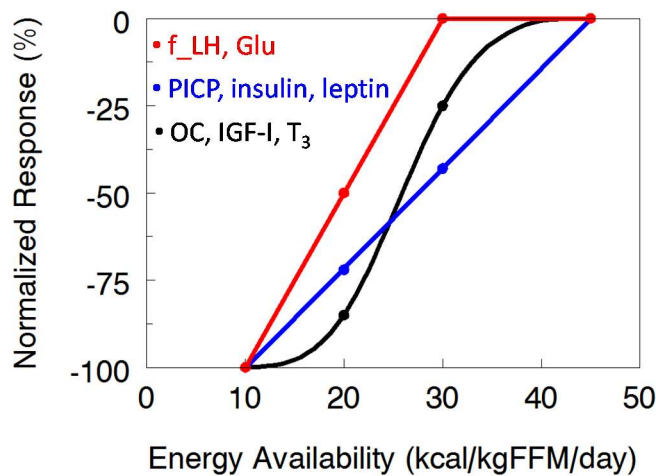
The calculation of EA requires accurate measurements of EI, EEE and FFM. This is straightforward in short-term studies where a desired EA is devised as an intervention: in these situations there is the equipment and capability to measure each component of the EA equation and an opportunity to control the events that contribute to EI and EEE for a brief period (Burke, 2015). However, the assessment of EA in free-living individuals is more problematic from several viewpoints. First, there is no standard protocol for the time period over which it should be measured, as a reflection of the individual's current EA status (Burke, 2015). Second, it involves the assessment of two metrics that are problematic (EI and EEE) and one that may require access to specialised equipment such as DXA technology to measure accurately (FFM). Finally, the assessment of each of the components is time consuming.

The collection of information on dietary intake via self-reported methods is challenged by issues of reliability and accuracy, with a particular bias to under-reporting in individuals who possess the characteristics that are likely to be associated with LEA (Loucks et al., 2011). There are also several challenges to the accuracy and reliability of measurements of EEE,

particularly during activities that involve high-intensity exercise, intermittent exercise or activities involving skills (King et al., 2004; Loucks et al., 2011); these are likely to contribute to the sporting activities of many athletes. Furthermore, the definition of exercise in free-living athletes is unclear: for example: should recreational or incidental activity (e.g. walking or cycling for transport) be included as well as the athlete's primary training or competition activities (Burke, 2015). It is important for a standardised protocol to be developed for the assessment of each parameter otherwise it will lead to different calculations and interpretations of EA (Burke, 2015).

#### *Lack of universal threshold of LEA at 30 kcal/kg FFM*

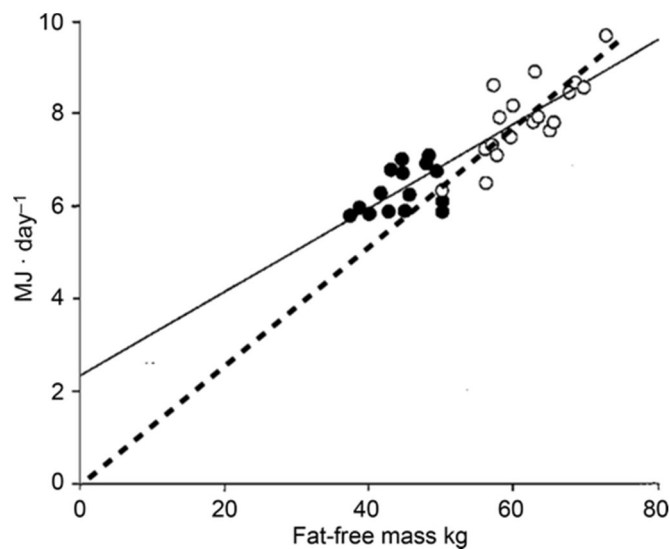
The pioneering work undertaken by Loucks and colleagues studies suggested a dose-response between EA and impairment of menstrual reproductive function, in which function was maintained until a threshold at 30 kcal/kg FFM/d beyond which there was a rapid decline (Loucks et al., 1994; Loucks et al., 1998). However, further investigations involving step-wise changes in EA have identified differences in the response of other systems to LEA in terms of the absence of a threshold and different rates of decline in relation to the reduction in EA (Hilton & Loucks, 2000; Ihle & Loucks, 2004). This issue has been elegantly summarised by Loucks in **Figure 2.3** which plots the impairment of a number of important metabolic hormones in response to difference degrees of reduced energy availability. While it is of interest to gain such understandings with other parameters of body function, the current message from this work is that it is impossible to set universally “safe” levels of reduced EA or to expect uniformity around the reduction in health and performance that might accompany a specific level of LEA. Rather than assuming or predicting a reduction in function associated with a certain reduction in EA, it remains more prudent to collect direct measurements of the altered function.



**Figure 2.3.** Dose-response effects of energy availability on LH pulse frequency ( $f_{LH}$ ), plasma glucose (Glu), markers of bone protein synthesis (PICP) and mineralisation (OC), and anabolic hormones that regulate bone formation (insulin, leptin, tri-iodothyronine [T<sub>3</sub>], and insulin-like growth factor-1 [IGF-1]). Created by Loucks – Clinical Sports Nutrition- (2015) from data in (Ihle & Loucks, 2004; Loucks & Thuma, 2003).

#### *Lack of scaling of low EA to RMR*

A further impediment to the use of EA as an indicator of the risk of impaired function is the lack of linear scaling of EA and RMR. Although the cut-off for impaired reproductive function at an EA of  $\sim 30$  kcal/kg FFM/d was found to be equivalent to RMR in an athlete of average body mass, this relationship does not exist across the spectrum of BM which athletes may span. As body size changes, there is also a change in the relative proportion of vital organs with high metabolic rate (e.g. liver and brain) to total FFM. As shown by Loucks (**Figure 2.4**), the relationship between measured sleeping energy expenditure and FFM observed by Westerterp (2003) fails to track EA, with the line of identify for an EA of  $\sim 30$  kcal/kg FFM/d resulting in an underestimation of SMR in smaller individuals and an overestimation in athletes with greater FFM (Loucks et al., 2011; Westerterp, 2003).



**Figure 2.4.** by Loucks et al (Loucks et al., 2011). Sleeping metabolic rate plotted as a function of fat-free mass (FFM). • = females; (○ = males. The solid regression line has a significant non-zero intercept. The dashed line is 30 kcal.kg FFM<sup>-1</sup>.day<sup>-1</sup>. Figure modified from Westerterp (2003).

#### 2.5.4 Direct measurement of suppressed RMR to confirm or diagnose LEA

Several studies of the FAT have reported the lack of association between measurements of LEA, impaired menstrual function and poor bone health (Koehler et al., 2013a; Reed et al., 2013) confirming that the correlation or inter-relatedness is more complicated than originally thought and/or that there is a lack of precision in the measurements of these indices. Therefore, it is of interest to find additional ways to measure or confirm the presence of problematic levels of EA in athletes which are practical to achieve and reliable in outcome and interpretation. The direct measurement of RMR as evidence of energy conservation that is likely to signal a reduction in metabolic function is one such strategy.

In support of the value of an impaired RMR as a signal of reduced activity in important body systems, there is evidence that measured reductions in RMR are associated with impaired health and function in athletes (Melin et al., 2015; Vanheest et al., 2014). A



cross-sectional study in well-trained female runners found that those with menstrual deficiency and LEA also had a 7% lower RMR compared with runners who were eumenorrheic and had optimal EA (Melin et al., 2015). Total cholesterol was also higher and concentrations of fasting glucose and luteal hormones lower in the group with the lower RMR. Vanheest (Vanheest et al., 2014) and colleagues also found that the adolescent swimmers with low EA and ovarian suppression also had a suppressed RMR.

In order for RMR to be used as a diagnostic tool for LEA in athletes, it must be able to be measured with sufficient accuracy and reliability such that changes in RMR that are linked with functional outcomes can be detected. This literature review has identified a variety of sources of variability in the measurement of RMR, including factors that are either likely to be controllable and/or to contribute to substantial error. Furthermore, there is a paucity of work on LEA on male athletes. The goal of this body of work is to better identify and quantify the influence of these factors, towards the development of a reliable standardised protocol for assessment of RMR in both female and male athletes that optimises the practicality of measurement and the utility of the results. This will provide a useful diagnostic tool for those working to optimise the health and performance of athletes as well as aid future research into the role of energy availability in achieving such goals.

## **2.6 Determining reliability and validity**

In order to have confidence that measurements of RMR are reflective of a true metabolic rate it is important to have an understanding of measurement error and reliability; particularly as this can affect the ability to make a valid judgement about a single measurement, or detect the difference or change between two measurements, in the same or different individual, respectively. Measurement error causes the observed or tested value to

deviate from the true value (Hopkins, 2000). If the deviation from the true value is large then the test is said to have poor validity or low precision (Hopkins, 2000). While, large variations in the score when the same test is repeated indicate poor reliability. There are multiple sources of error categorised into technical or instrumental, biological variability, tester error and participant error.

Reliability has important implications when monitoring a change in a variable over time. It enables researchers, practitioners and coaches to determine if a change from the previous test is a “real” change rather than the error inherent in the test or measurement. If a test or protocol has poor reliability, a larger magnitude of change is required to be confident of detecting a “true” change. In turn, tighter reliability is able to identify changes of smaller magnitude (Hopkins, 2000). The reliability compares the variability within the same participants to the variability between participants from repeated measurements of the same test (Hopkins, 2000; Peat & Barton, 2014). It is possible to decrease the error of measurement and increase the reliability by standardising certain aspects of the testing procedure to increase repeatability between tests.

Reliability is grouped into different categories: intra-tester (within one’s self); inter-tester (consistency between different testers); or test-retest reliability (of the measurement itself regardless of tester) (Peat & Barton, 2014). In sport science the focus is often on test-retest reliability, which can be determined using several different statistical methods (Atkinson & Nevill, 1998; Hopkins, 2000; Weir, 2005). It is outside the scope of this thesis to provide an in depth review of each method, rather an overview of four common methods will be provided; the intra-class correlation coefficient (ICC), the typical error (also known as the standard error of measurement), the Bland-Altman assessment and the change in the mean (Atkinson & Nevill, 1998; Hopkins, 2000; Weir, 2005).

### ***2.6.1 Intra-class correlation coefficient***

The ICC is a ratio calculated from the between participant variance to the between and within participant variance. Its values range from 0 to 1, and is presented with a 95% confidence interval. A value of one (e.g.  $ICC = 1.0$ ) indicates no within-participant variance, meaning any difference in scores is due to a true change and not measurement error. An ICC of  $>0.90$  indicates good reliability (Hopkins, 2000). The ICC is a relative measure of reliability dependent on the amount of difference between repeated measures and also the reliability of the sample. Therefore, a 1-way or 2-way analysis of variance (ANOVA) model is used with either fixed or random effects (Weir, 2005). The chosen ANOVA model will indicate if the results from that specific study are generalisable to other populations. Therefore, it should always be stipulated in the written description of the statistical methodology of an article.

### ***2.6.2 Typical error***

The typical error (TE) is a measure of within-subject variation, and is also known as the standard error of measurement. The TE is expressed in the units of the measurement and is thus an indicator of absolute reliability (Atkinson & Nevill, 1998). The use of the within-subject standard deviation as the TE removes susceptibility to sample size but permits susceptibility to sample heteroscedasticity (Atkinson & Nevill, 1998); a phenomenon commonly seen in sports science where the TE of a measurement increases as the mean value increases (Hopkins, 2000). However, expressing the TE as a percentage or the coefficient of variation (CV) nullifies this phenomenon (Hopkins, 2000). The TE and CV can then be used to determine effect sizes for the Magnitude Based Inference statistical approach and also

calculation of the smallest worthwhile effect which can be used to monitor an individual or estimate individual differences (Hopkins, 2000).

### ***2.6.3 Bland-Altman and Limits of Agreement***

The Bland-Altman method calculates that there is 95% probability that the difference between two tests in an individual lie within specific values or limits of agreement (LOA) (Atkinson & Nevill, 1998). This is visualised by plotting the individual participant test mean against the corresponding difference between two tests (Atkinson & Nevill, 1998). The limits of agreement are the standard deviation of the differences between the test multiplied by 1.96, which provides the 95% probability range (Atkinson & Nevill, 1998; Hopkins, 2000). Bland-Altman deals with heteroscedasticity by using a natural log transformation prior to calculating the limits of agreement. The significance of the LOA can be dependent on the sample size and there is disagreement whether 95% limits are applicable to all measures (Hopkins, 2000).

### ***2.6.4 Change in the mean***

The change in the mean value between two or more tests is the simplest measure of reliability, however, it should not be used as the sole measure of reliability (Atkinson & Nevill, 1998; Hopkins, 2000). A change in the mean is more commonly used to determine if any systematic bias has occurred (Atkinson & Nevill, 1998). For example, a learning effect may be indicated by the second test value being significantly lower or higher than the first (Atkinson & Nevill, 1998; Hopkins, 2000). In the case of RMR, a participant may hyperventilate on a first test because they are not familiar with the breathing apparatus. This would cause them to “blow off” CO<sub>2</sub> which decreases  $\dot{V}CO_2$  and increases  $\dot{V}O_2$  thus inflating RER above the RQ at the cell. This results in an inflated energy value per litre of oxygen

(McClave & Snider, 1992). Bias can be assessed by conducting a paired t-test or ANOVA to test the null hypothesis that there is no change in the mean between tests. (Atkinson & Nevill, 1998).

## **2.7 Conclusions**

The measurement of RMR has the potential to become a widespread diagnostic tool in research and servicing activities of sports nutrition and sports science. Currently there is no consensus on a variety of features that should be included in a standardised protocol for measuring RMR in athletes via indirect calorimetry: these include the duration of the period since the last exercise session and the value of inpatient vs outpatient measurement scenarios. Since, the measurement of FFM is included in the assessment of RMR, it is also important to continue previous activities of our research group to improve the precision of measurement via DXA techniques by understanding the effects of changes in glycogen concentrations in the muscle and the liver. Further evidence is also required to assess the reliability and validity of novel software to non-invasively measure muscle glycogen at the time of body composition assessment. The studies presented in this thesis will directly address these gaps in the literature.

### 3.0 Methodology and Design

The following chapter expands on the methodology and study design presented in the forthcoming study chapters. It provides further detail beyond what has been provided in the manuscripts for publication.

#### 3.1 Resting energy expenditure

All RMR measures were conducted in the morning between 05:00 and 08:00 h, following an 8 h overnight fast and overnight rest from exercise. Participant standardisation prior to RMR has been detailed in the methods section of chapters four, five and eight respectively.

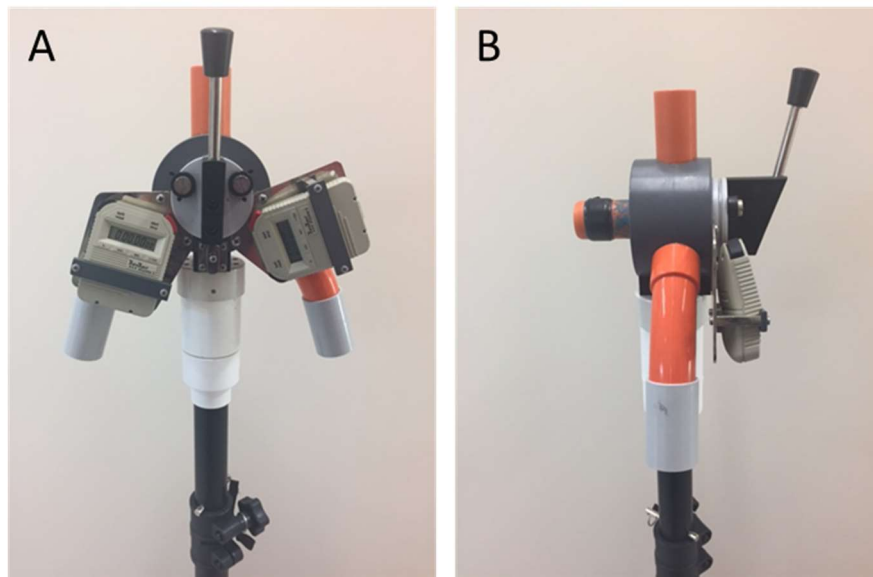
Resting metabolic rate was measured using open circuit indirect calorimetry. Participants wore a two-way valve mouthpiece (series 2700, Hans Rudolph Inc, Shawnee, KS, USA) and nose clip during expirate collection. Expired air was collected into 120 L foil Douglas Bag (Scholle, Edinburgh North, SA, AUS) for 10 min in duplicate.

For *Studies 1* and *5* the mouthpiece was connected directly to one Douglas Bag modified with a tap and sampling line (**Figure 3.1**).



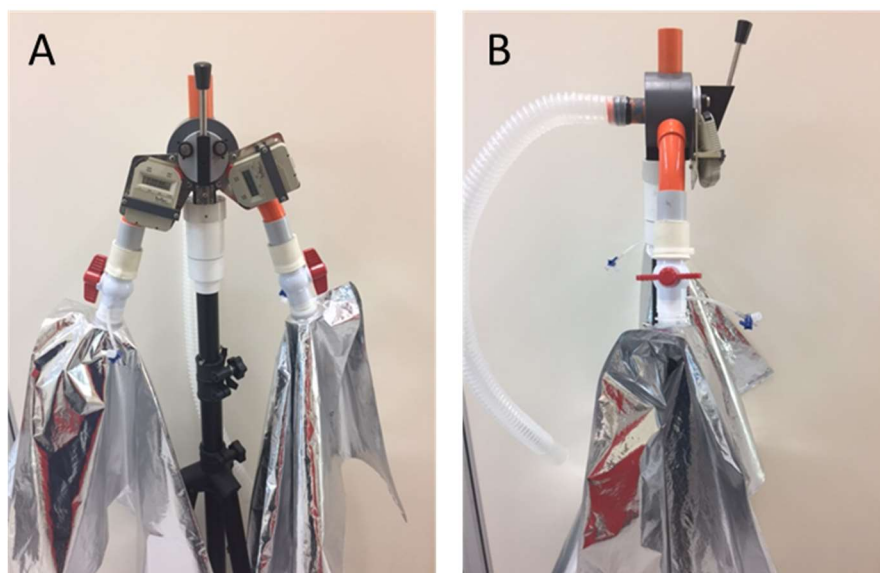
**Figure 3.1.** Douglas Bag.

For study 2, the mouthpiece was connected by a plastic hose to an in-house designed four-way tap apparatus known as “The Bob” (**Figure 3.2**).



**Figure 3.2.** Four way tap “The Bob” front (A) and side (B) on views.

The hose was connected to the back valve of The Bob while two Douglas Bags were connected to valve 1 and 2 respectively (**Figure 3.3**). Three towels were used to support the hose or mouthpiece plus the Douglas Bag.



**Figure 3.3.** Douglas Bag with Bob. Front (A) and side (B) views with the hose attached.

Following collection, the gas fraction of the expirate was analysed first followed by the bag volume. The Douglas Bag was connected to Ametek O<sub>2</sub> and CO<sub>2</sub> analysers (Ametek Inc, PA, USA) via a silicone hose which was connected to calcium chloride crystals (Unilab, Ajax Fredman, NSW, Australia) to dry the expirate. Oxygen and CO<sub>2</sub> was recorded after 1 min which was deemed appropriate time for the gases to stabilise throughout the analysers. The flow rate was recorded alongside laboratory pressure (mmHg), temperature (°C) and relative humidity (%).

Volume was measured using a 350 L Tissot Spirometer (Warren Collins, Braintree, MA, USA). The starting value of on the Tissot Spirometer was recorded to the nearest 0.1cm. A hose connected the tank to a pump and the Douglas Bag. Once connected the tank was opened, the pump turned on and the bag opened in this sequence. The expirate was pumped into the tank and any excess air in the Douglas Bag was rolled out using a solid plastic pipe. The final value of the Tissot was then recorded. The volume of the expired air ( $V_E$ ) was calculated by multiplying the Tissot scaling factor of 3.244 which is the cross-sectional area of the tank. This gives the Tissot tank a precision of volume to the nearest 300 ml. The same process was repeated for the second bag.

The gas fractions, Tissot displacement and environmental conditions were placed into a spreadsheet where the Haldane equation was used to determine  $\dot{V}O_2$  and  $\dot{V}CO_2$ ,  $\dot{V}_E$ . The Weir (Weir, 1949) equation was used to determine energy expenditure in kJ/min for RMR and extrapolated to 24 h to provide REE.

### **3.1.1 Calibration**

The Ametek analysers were calibrated prior to gas analysis each morning. The flow meter was turned on for a minimum of 30 min prior to calibration until the O<sub>2</sub> analyser temperature was  $6.790 \pm 0.05$  °C. Calcium chloride crystals were replaced with fresh ones each morning. For *Study 1* two known gases with the following concentrations were used;



14.56% O<sub>2</sub> and 2.50% CO<sub>2</sub>; and 16.01% O<sub>2</sub> and 4.00% CO<sub>2</sub>. For *Studies 2* and *5* three known gases with the concentrations of O<sub>2</sub> 14.63% and CO<sub>2</sub> 2.56%; O<sub>2</sub> 16.17% and CO<sub>2</sub> 3.99%; and O<sub>2</sub> 15.30 and CO<sub>2</sub> 3.38 were used. The analysers were calibrated within 0.03% of the gas concentrations. The system was checked for leaks prior to calibration.

### **3.2 Body composition**

All body composition assessments were completed using dual energy x-ray absorptiometry (DXA). For *Studies 1* and *4* a GE Lunar Prodigy was used to capture the scans, with the images analysed using GE enCore v12.30 software. For *Studies 2* and *5* a GE Lunar iDXA was used with images analysed using enCore v16.0 software (GE, Madison, WI, USA). Both versions of the software use the reference population database Lunar-Geelong for bone mineral density and body composition (Pasco et al., 2012).

The DXA was calibrated the morning of each body composition assessment according to manufacturer instructions. The Quality Assurance process calibrates and checks the functionality, accuracy and precision of the DXA (GE Healthcare Lunar, 2015). A black block consisting of three bone simulating chambers of known bone mineral content and tissue equivalent material is used for the Quality Assurance process (GE Healthcare Lunar, 2015). A Spine phantom with BMD of  $1.252 \pm 0.03 \text{ g/m}^2$  was then scanned for quality control to monitor drift.

A pre-DXA questionnaire (Appendix: 11.6-A) was completed by all participants prior to measurement. This collected descriptive information as well as enquired about recent radiation exposure within the previous 12 months, their ability to lie still for 10 min and if they had any metal or prosthesis, as per radiation safety requirements of Australian Radiation Protection and Nuclear Safety Agency.

Participants presented for DXA following an overnight fast and without exercising prior to arrival (Nana et al., 2015). Participants voided their bladder and were then weighed in their underwear (underwear or boxer shorts for men and underwear and non-wired crop top for women). Participants then lay supine on the scanner bed with their head towards the head of the scanner. Custom made positioning aids to standardise feet width separation at 15cm and hand separation of 3 cm from the body were used (Appendix 11.7). All scans were run on standard mode which provides a surface radiation dose of 3 $\mu$ Gy per scan. The enCore software automatically generates regions of interest (ROIs) to separate the arms, legs and trunk (left and right). The ROIs were checked by a qualified and experienced DXA technician. Two DXA technicians were used. One completed all the scans in *Studies 1* and *5*, while another completed all the scans for *Studies 2* and *3*.

The technical error of measurement for the Lunar Prodigy was  $\sim 0.1\%$  for total mass, 0.4% for total lean, 1.9% for total fat and 0.7% for total bone mineral content (Nana et al., 2012a). The test-retest technical error of measurement for the iDXA was determined during the pilot study in Chapter 4. Participants were scanned once, and then they got up from the bed and were re-positioned and scanned once again. The technical error of measurement for the iDXA was, 0.1% for total mass, 0.4% for lean mass, 1.6% for fat mass and 0.4% for bone mass.

Hydration status was assessed by urine specific gravity (USG). Participants collected a mid-stream urine sample from their first morning void (Nana et al., 2012a). One drop of urine was placed on the refractometer (UG-a, Atago Refractometer, Japan) and USG recorded to the nearest 0.001 g/L.

### **3.3 Total Body Water**

Total body water and the fluid compartments were estimated using Bioelectrical Impedance Spectroscopy (BIS). All measures were conducted using an IMP SFB7 (ImpediMed Limited, Queensland, Australia) and analysed using BioImp Analysis 5.4.0 Software (ImpediMed Limited, Queensland, Australia). BIS measures were taken while participants lay in a supine position with arms at 30 degree angle from the torso and legs separated at 30 degree angle. Participants were required to rest in this position for 10-15 min prior to measurement in order to allow body water to stabilise prior to measurement (Moon et al., 2008). Part of this rest time was made up of the DXA scan. Dual-tab electrodes (L-Dex, (ImpediMed Limited, Queensland, Australia) were placed on the dorsal surface of the left wrist at the site of the styloid and on the left ankle at the medial and lateral malleoli (IMP SFB7, Instructions for use, ImpediMed, QLD, Australia). The sites were prepared by cleaning with alcohol swabs and shaving if necessary prior to placing the electrode tabs. Site preparation and placement of electrodes was done during the 15 min rest period. Prior to measurement, the four leads of the BIS were calibrated/tested as per manufacturer instructions. The BIS measures were then taken in triplicate, with the mean of the values used for analysis. The values in litres and percent for total body water (TBW), extra cellular fluid (ECF) and intracellular fluid (ICF) were recorded.

### **3.4 SenseWear**

The SenseWear Mini-arm band is a multi-sensory activity monitor (Liden et al., 2002). The monitor was worn on the mid left triceps. Monitors were pre-programmed with the participant's date of birth, height (in cm), weight (in kg), sex, dominant hand and smoking status prior to wearing the monitor. Participants were advised to wear the monitor at all times

except when showering or swimming (Liden et al., 2002). Following the study completion the SWA data were downloaded using SenseWear Professional (version 8, Body media). SenseWear estimates energy expenditure via a proprietary algorithm that combines what it directly measures (heat flux, skin temperature skin galvanic response and accelerometry data) with the individual age, height, weight, sex, dominant hand and smoking status. The raw data which included the measured variables and those derived from it (laying down time, sleep time, energy expenditure and METs) were then exported into a Microsoft Excel file. Data is provided in one minute intervals. Energy expenditure was determined by selecting the required date and time using the filter feature in Microsoft Excel (2010, Redmond, WA, USA) and summing the energy expenditure within the specified time frame.

### **3.5 Muscle Biopsy**

All Muscle biopsies were taken from the vastus lateralis of either the left or right leg by registered medical professionals. The site was anaesthetised with 1% xylocaine and once it was numb (participant reported no response to sharp test), the area was sterilised using Iodine (povidone-iodine, Betadine antiseptic solutions, Sanofi-aventis Consumer Healthcare, QLD, AUS) prior to an incision being made with a scalpel through the dermal layer and fascia of the leg. A 5 mm Bergstrom needle modified with suction was then inserted into the vastus lateralis. The muscle tissue was frozen immediately in liquid nitrogen and stored at -80°C until analysis.

#### **3.5.1 Muscle Glycogen Analysis**

Muscle glycogen was hydrolysed to glycosyl units prior to using enzymatic analysis to determine glycogen concentrations (Churchley et al., 2007). Approximately 20g of freeze-dried muscle was powdered and cleaned of connective tissue and blood prior to the addition

of 500  $\mu\text{L}$  of hydrochloric acid. The sample was then heated to  $100^{\circ}\text{C}$  for 2 h to hydrolyse the glycogen to glycosyl units. 1.5 mL of 0.67 M sodium hydroxide was then used to neutralise the sample prior to determining glycogen concentrations with fluorometric detection (Jasco FP-750 spectrofluorometer, Easton, MD) at excitation 365 nm/emission 455 nm (Tomcik, 2016).

## Pre-face to Chapter 4

Chapter 2 identified and reviewed aspects of RMR measurement protocols that have the potential to increase the error and reduce the reliability of the measurement of RMR in an athlete population. The following chapter (*Study 1*) investigates the effect of testing location and day to day variability while taking into consideration aspects of an athlete's life that would make controlled laboratory protocols for measurement of metabolic rate impractical for the routine assessment of RMR in exercising individuals. Reliability is assessed using the intra-correlation coefficient and the typical error. The TE is expressed in units and as a coefficient of variation in order to be comparable with the current literature.

#### **4.0 Study 1: No difference in young adult athletes resting energy expenditure when measured under inpatient or outpatient conditions.**

This chapter is comprised of the following paper which has been accepted and is in Press in International Journal of Sports Nutrition and Exercise Metabolism:

Bone, J L, & Burke, L M. (2017). No difference in young adult athletes resting energy expenditure when measured under inpatient or outpatient conditions. *Int. J. Sport Nutr. Exerc. Metab. In Press.*

## 4.1 Abstract

Low energy availability can place athletes at increased risk of injury and illness and can be detected by a low metabolic rate. The lowest metabolic rate is captured at the bedside, after an overnight fast and termed inpatient REE. Measurements done in a laboratory with a shorter overnight fast are termed outpatient REE. Although important to know what the lowest energy expenditure is, a bedside measure and/or 12 h fast is not always practical or logistically possible particularly when you take into account an athlete's training schedule. The aim of this investigation was to compare a bedside measure of resting metabolism to a laboratory measure in athletes following an 8 h fast. Thirty-two athletes (24 females, 8 males) underwent measures of resting metabolism using indirect calorimetry once at their bedside (inpatient) and once in a simulated laboratory setting (outpatient). Paired T-Test was used to compare the mean ( $\pm$  standard deviation) differences between the two protocols. Inpatient REE was 7302 ( $\pm$ 1272) kJ/d and outpatient REE was 7216 ( $\pm$  1119) kJ/d ( $p=0.448$ ). Thirteen participants repeated the outpatient protocol and 17 repeated the inpatient protocol to assess the day to day variation. Reliability was assessed using the intra-correlation coefficient and typical error. The inpatient-protocol variability was 96% with a typical error of 336 kJ/. For the outpatient-protocol the ICC and typical error were 87% and 478 kJ/d respectively. Results indicate no difference in REE when measured under inpatient and outpatient conditions; however the inpatient protocol has greater reliability.



## 4.2 Introduction

The measurement of basal metabolism, the lowest amount of energy required for the body to function, is of interest to athletes due to its potential as a diagnostic tool to detect low energy availability (Mountjoy et al., 2015). The terms basal energy expenditure (BEE) and resting energy expenditure (REE) are used interchangeably, however, this is not appropriate as they reflect difference in the protocols under which measurements were obtained. BEE is a measurement of metabolic rate, extrapolated to 24 h, taken from the bedside of a supine individual who has just woken up following a 12 h fast and abstinence from alcohol and caffeine intake (Institute of Medicine, 2005). Protocols that deviate from one or more of these conditions are deemed to measure REE. The most common deviations are the collection of data from time-points other than “on waking” (i.e. bedside measurement) and shorter fasting periods. Indeed, in real-life, it is usually most practical to measure REE in a patient who reports to the laboratory in fasted, rested condition and further undertake a standardised period of rest (outpatient protocol) (Fullmer et al., 2015). Although ideal, a 12 h overnight fast is not always practical and considerations on the reliability of REE following a shorter overnight fast are yet to be done.

An outpatient REE is considered to be higher than inpatient REE (which would be closer to BEE) (D'Alessio et al., 1988). However, investigations in healthy and elderly populations which directly compared inpatient and outpatient REE have found conflicting results (Berke et al., 1992; Bullough & Melby, 1993; Turley et al., 1993). Furthermore, no comparison has been done in an athlete population nor employed a more practical 8 h overnight fast. This is of interest in athletes given they have a higher metabolic rate and different training and dietary habits that could impact on the variability of REE (Poehlman et al., 1988; Sjodin et al., 1996). Therefore, the aims of this study are to, 1) investigate the variability in measurements of REE which results from a bedside versus outpatient protocol

following an 8 h overnight fast in athletes and 2) determine the daily variability and reliability of each protocol.

## 4.3 Methods

### *Participants*

Twenty-four female and eight male athletes who compete at state or national level (**Table 4.1**) participated in this study after providing informed consent. Participants were recruited from a convenient sample of athletes who visited the Australian Institute of Sport for training purposes. Approval was granted by the Australian Institute of Sport and Australian Catholic University Ethics Committees.

**Table 4.1.** Participant characteristics by sport and sex, presented as mean  $\pm$  SD.

Characteristic	All	Males	Females	
Sport		Gymnastics	Cycling	Athletics
n	32	8	19	5
Age (y)	23.6 $\pm$ 4.3	20.4 $\pm$ 2.14	24.5 $\pm$ 4.1	25.4 $\pm$ 5.05
Weight (kg)	58.2 $\pm$ 6.9	63.3 $\pm$ 6.95	57.9 $\pm$ 5.6	51.0 $\pm$ 4.6
Height (cm)	166.8 $\pm$ 6.6	165.4 $\pm$ 7.84	167.0 $\pm$ 6.7	168.6 $\pm$ 4.1
Fat free mass (kg)	49.4 $\pm$ 7.6	59.2 $\pm$ 6.4	47.1 $\pm$ 4.6	42.6 $\pm$ 2.4
Fat mass (kg)	9.2 $\pm$ 3.6	5.0 $\pm$ 0.8	11.1 $\pm$ 2.9	9.0 $\pm$ 2.6

### *Study Design*

REE was measured for each participant under an inpatient and outpatient protocol in a cross-over study design. The conditions were measured on separate days with 24-48 h between measurements and random allocation of protocol order. Participants stayed overnight at the AIS residences for the study duration.

#### *Pre-testing standardisation:*

Participants were required to undertake a similar training load and macronutrient composition of their diet for the 24 h prior to each REE measurement. REE was collected following an 8 h overnight fast (including caffeine) and a minimum 10 h after training for both protocols (Fullmer et al., 2015). Dietary intake was recorded using a written food diary with analysis completed by an accredited sports dietitian. Training activities were recorded and SenseWear™ activity monitors (BodyMedia, Mini-MF) were worn to monitor compliance to training and to estimate total energy expenditure (BodyMedia, SenseWear Version 8).

#### *REE collection:*

Resting energy expenditure was measured using indirect calorimetry. Measurements were collected at the same time each morning between 5-7am. Participants wore a nose clip and breathed into a two-way valve mouth-piece (Hans Rudolph R2700). Their expired air was collected in 10 min duplicates into two 120 L gas-impermeable Douglas Bags (Haugen et al., 2007; Woods et al., 2016).

#### *Intervention Protocols:*

*Inpatient:* Expired air was collected at the bedside after participants had been woken and without them rising from their bed. Following 10 min of familiarization with the mouthpiece, the expirate was collected into the Douglas Bags.

*Outpatient:* To minimise the effect of potential confounding factors such as a different bed and laboratory sounds, the outpatient protocol was conducted in the same room as the inpatient protocol after the participant had slept overnight. However, to simulate the situation of reporting to a laboratory, participants were required to wake themselves, dress and

walk a return distance of 500m back to their bed. They then lay supine for 25 min prior to connecting the Douglas Bags. The mouth-piece was provided 10 min into the rest period for familiarization.

#### *Determination of REE:*

A sample of air from the Douglas Bag was measured for the gas fractions of oxygen consumption ( $\dot{V}O_2$ ) and carbon dioxide production ( $\dot{V}CO_2$ ) using Ametek O<sub>2</sub> and CO<sub>2</sub> analysers (Ametek Inc, Pennsylvania, USA) and the sampling duration and flow rate recorded. The analysers were calibrated against two known gases (14.56% O<sub>2</sub>, 2.501% CO<sub>2</sub> and 16.01% O<sub>2</sub> and 4.001% CO<sub>2</sub>) prior to measurement. Following the sample measurement the Douglas Bag was attached by hose to a water sealed 350 L Tissot Spirometer (Warren Collins, Braintree, MA, USA) to measure gas volume.  $\dot{V}O_2$  and  $\dot{V}CO_2$  were determined using the Haldane transformation (Haldane, 1918) and converted to kJ/min based on the Weir equation (Weir, 1949). The process was repeated for the second Douglas Bag and the mean value of the duplicate bags was extrapolated to kJ/d and kJ/kg FFM/d.

#### *Body Composition:*

Body composition was assessed via a standardised protocol using a narrowed fan-beam DXA (Lunar Prodigy, GE Healthcare, Madison, WI) with technical error of measurements of ~ 0.1% for total mass and 0.4% for lean mass and 1.9% for fat mass (Nana et al., 2016).

#### *Reliability of protocols*

Thirty participants were randomised to complete a third REE measure under either the inpatient (n=17) or the outpatient (n=13) condition in order to determine the day to day variation and reliability for each protocol. This assessment was completed no more than 48 h

after the last intervention protocol and was completed at the same time of the morning and following the same pre-testing standardisation protocols.

#### *Statistical Analysis:*

Energy intake, energy expenditure and REE data were all normally distributed for both protocols as assessed by Shapiro-Wilk's test ( $p > 0.05$ ). Paired t-tests were used to compare REE, energy intake and energy expenditure between inpatient and outpatient protocols, and between days for the same protocol. Reliability and day to day variation for the inpatient and outpatient protocols was determined using a two-way random intra-correlation coefficient (ICC) using absolute agreements and the typical error (TE) (Hopkins, 2000; Weir, 2005). Paired t-test and ICC were determined using SPSS (Version 23 for Windows, SPSS Inc, Chicago, IL). All values are mean  $\pm$  standard deviation (SD) unless otherwise stated.

## 4.4 Results

Results were combined as there was no statistical difference in mean absolute REE between the male and female athletes. There was no statistical difference in absolute REE (kJ/d) or relative REE (kJ/kg FFM/d) between the inpatient and outpatient protocols (**Table 4.2**).

**Table 4.2.** REE for inpatient and outpatient conditions for all participants (mean  $\pm$  SD).

	Inpatient (n=32)	Outpatient (n=32)	p-value
REE (kJ/d)	7302 $\pm$ 1272	7216 $\pm$ 1116	0.448
REE (kJ/kg/FFM/d)	148.5 $\pm$ 20.5	146.8 $\pm$ 17.2	0.459
RER	0.86 $\pm$ 0.05	0.84 $\pm$ 0.04	0.009*

\*Significantly different between Inpatient and Outpatient conditions

REE: resting energy expenditure; FFM: fat free mass; RER: respiratory exchange ratio.

For kCal/d divide by 4.18

Reliability results are presented in **Table 4.3**. Mean day to day variation in REE for the inpatient and outpatient protocol were  $92 \pm 456$  kJ/d ( $p=0.417$ ) and  $382 \pm 716$  kJ/d ( $p=0.107$ ) respectively. The outpatient protocol had a lower ICC and a higher TE by 155 kJ/d.

**Table 4.3.** Inter-day Reliability and Typical Error of Inpatient and Outpatient protocols.

	Inpatient (n=17)	Outpatient (n=13)
ICC (95% CI)	0.96 ( 0.896, 0.986)	0.87 (0.58, 0.96)
SEM (95% CI) (kJ/d)	323 (240, 491)	478 (342, 788)
Typical error as CV (%)	4.9 (3.6, 7.6)	5.9 (4.2, 9.9)

SD: Standard deviation; ICC: Intra-class correlation coefficient, SEM: Standard error of the mean; CI: 95% Confidence limits; CV: coefficient of variation

ICC determined using two-way random model with absolute agreements. SEM calculated mean squares from ANOVA For kCal/d divide by 4.18

Three athletes failed to comply with diet and energy expenditure monitoring for the 24 h preceding each REE measure and were excluded from this analysis. There was no significant difference in energy intake, macronutrient intake or total energy expenditure in the 24 hours prior to measurement between protocols (**Table 4.4**), nor when the same protocol was repeated.

**Table 4.4.** Energy intake, macronutrient intake and estimated total EE in the 2 4h preceding the REE measurement for inpatient and outpatient conditions, n=29 (mean  $\pm$  SD).

	Inpatient	Outpatient	p-value
Total EE (kJ/d)	12109 $\pm$ 4419	12929 $\pm$ 3733	0.557
Energy Intake (kJ/d)	11022 $\pm$ 3100	10694 $\pm$ 2812	0.223
Protein intake (g/d)	146 $\pm$ 47	140 $\pm$ 48	0.318
Fat intake (g/d)	77 $\pm$ 33	76 $\pm$ 26	0.771
Carbohydrate intake (g/d)	324 $\pm$ 115	313 $\pm$ 104	0.412

EE: Energy expenditure. For kCal/d divide by 4.18

## 4.5 Discussion

Our results indicate that there is no difference in REE when measured following either an inpatient or outpatient protocol, with this being the first investigation of this issue in athletes. This is a useful finding since it means that practitioners and researchers can obtain a measure of metabolic rate in athletes under conditions that are more practical and economical to administer. The further novelty of our work in determining the reliability of these measurement protocols indicates that there is greater noise associated with the outpatient protocol; thus it has reduced capacity to detect small but potentially important changes in REE when longitudinal measurements are taken on the same athlete. Given the wide variation in the TE, the results indicate that multiple REE measures over consecutive days should be collected in order to determine an individual athlete's TE, however this may not always be logistically possible.

Previous work (Berke et al., 1992) reported an 8% higher REE following outpatient conditions compared with the inpatient conditions in elderly men and women. In contrast, other studies have found no differences in REE and BEE measurements in elderly populations (Fredrix et al., 1990) or young active men (Bullough & Melby, 1993); a cohort more similar to the current investigation. More recently, REE was found to have returned to resting levels 20 min after a 300m walk. However, the initial resting REE was not measured following an inpatient protocol (Frankenfield & Coleman, 2009). One difference between these studies and the current investigation is our use of an 8 h overnight fasting period for both inpatient and outpatient protocols, which is shorter than the 12-14 h period which is often used to define a true fast (Institute of Medicine, 2005). Others (D'Alessio et al., 1988) have reported that metabolic rate increases associated with the thermic effect of food return to baseline by 8 h in lean men. Furthermore, updated guidelines for conducting indirect calorimetry by the Academy of Nutrition and Dietetics state that a minimum of 7 h of fasting is required prior to

measuring REE in general and clinical populations (Fullmer et al., 2015). Given the impracticality of achieving a 12 h fast in athletic populations whose training schedules are often linked with food intake during the evening and early morning rising, an 8 h fast is considered by the authors as adequate to achieve non-absorptive conditions and a strength of the current investigation.

The current investigation required athletes to undertake a 10 min walk protocol within their outpatient preparation, and contrasts with the conditions of other studies in which participants have been driven to the laboratory or used transport that has not been noted. We note that REE is purported to be higher than BEE due to the energy costs for the activities of waking, dressing and the neural activation /mental activity required to get to the testing location. Further research investigating the effect of different modes of transport (e.g. driving different distances, walking or taking public transport) would have application not only to athletes but to general populations. However, the procedures used in the current protocol represent the major activities involved in conducting research in an outpatient setting, and is deemed to be practical and representative of real-life.

Having a reliable protocol for measuring REE in athletes is important for some of its practical applications, such as its use as a diagnostic tool for low energy availability (Melin et al., 2015). Although the reliability of both protocols was high, the ICC of the outpatient protocol was lower and the TE higher than that of the inpatient protocol, indicating lower reliability. This difference may be of importance when monitoring REE over time or in response to an intervention to interpret if a real change in REE has occurred. Further research should investigate changes in REE in response to other likely artefacts in the measurement protocols (e.g. environmental noise and temperature, effects of prolonged/intense exercise on the previous day) to determine real-life variability in measurement versus changes that indicate a physiological alteration in baseline metabolic health and function.



### *Limitations*

Participants were recruited from a convenience sample, therefore we were unable to schedule REE collection for the same phase of the menstrual cycle in female athletes. Furthermore, it was unknown if participants were in energy balance upon study commencement. However, as the study was completed within 3-5 days it was presumed that there would be minimal changes in weight.

### **4.6 Novelty and Practicality Statement:**

This study is the first to compare measurements of REE via inpatient and outpatient protocols in athletes, using protocols that represent conditions that are realistic or practical for the implementation of this measurement in athletic populations. Although both protocols were shown to have high reliability, the inpatient protocol would provide greater sensitivity to monitor real changes that might occur in an athlete over time. Although not always possible logistically, obtaining multiple (two to three) REE measures should be conducted to establish an athlete's individual TE.

## Preface to Chapter Five

The results from Chapter 4 (*Study 1*) indicate that the 8 h overnight fasted outpatient protocol is reliable for measuring REE in a young adult athletic population when measurements are taken on the morning after a training day. However the validity of such measurements needs to be addressed since exercise has been previously shown to elevate REE for a period of 39- to 72 h after the session (Herring et al., 1992; Tremblay et al., 1988).

Previous studies that have investigated the effect of exercise on REE have either used endurance or resistance trained participants who completed a standardised bout of exercise in their habitual exercise mode. Whether the acute effect of exercise remains in conditions that better reflect the variety of exercises and athletes in the daily training environment has yet to be investigated. In addition there have been few attempts to track this acute post-exercise effect in a stepwise fashion at specific time intervals following exercise. Although *Study 1* was conducted on both male and female athletes, the majority of participants were female. There is a lack of literature in male athletes where REE has been used as a proxy for determining or diagnosing low EA. Therefore, this population should be targeted when conducting research in this area and that of REE reliability. For this reason, the remaining studies of this thesis have solely used male athletes as participants. The following chapter summarises *Study 2*, an investigation of the acute effect of exercise on REE in a heterogeneous male athlete population.

## **5.0 Study 2: The effect of prior exercise on measures of resting metabolic rate in trained men.**

The following chapter consists of a pilot study and will not be submitted for publication. However, the outcomes and limitations of this study are important to the central theme/aim of this thesis and thus worthy of inclusion.

This pilot study was funded in part by a grant from Sports Dietitians Australia awarded to myself, and the Australian Institute of Sport PhD Program Student Budget.

## 5.1 Abstract

### *Background:*

The increase in resting energy expenditure (REE) following exercise has been reported to last up to 39 or 72 h in homogeneously trained populations. The aim of this pilot study was to profile REE when measured at 12 h intervals up to 48 h post exercise in a heterogeneous group of athletes undertaking self-selected training sessions.

### *Methods*

Ten trained males (age,  $30.32 \pm 6.15$  y; weight  $81.45 \pm 11.64$  kg; height,  $187.82 \pm 6.94$  cm) from a variety of sports participated in this study. Participants undertook a standard training session of their choosing on Day 0, either in the morning or afternoon. The next morning they presented for their 1st REE (post-exercise-day) and DXA with no exercise being permitted following the REE on Day 1. Participants returned on Day 2 for their second REE (post-rest-day). They then repeated this process the following week, however with the training session completed at the other time of day. Participants were assigned to train either in morning or the afternoon in the first week and in the alternative time in the second week. This provided REE approximately 24 and 48 h post exercise following morning training session and 12 and 36 h post exercise following the afternoon training session. The training session and diet was replicated from week to week. Linear mixed modelling was used to determine the difference between the different time points. The typical error for REE is 478 kJ / d.

### *Results:*

There was a statistically significant decrease in REE of 345 kJ/d when measured 48 h post exercise compared to 12 h. There was a trend for REE to be lower with increasing rest

from exercise. All differences between time-points were within the typical error for the outpatient protocol.

*Conclusion:*

There was a trend for REE to decrease with increasing rest period from exercise. However, no clear effects of acute exercise on subsequent morning measures of REE could be detected in participants from a mixed sport population undertaking self-selected training sessions.

## 5.2 Introduction

Resting energy expenditure (REE) is one tool that can be used to support the diagnosis of low energy availability (LEA). However to accurately measure REE and to be able to detect changes between measurements, it is important to investigate how certain variables may artificially increase the REE, potentially masking evidence of a suppressed metabolic rate (Manore & Thompson, 2009). One such variable is exercise.

It is well documented that oxygen consumption is increased acutely after exercise in what is termed excess post exercise oxygen consumption (EPOC) (Gore & Withers, 1990; Manore & Thompson, 2009). The increase in oxygen consumption translates to a temporally elevated metabolic rate (Gore & Withers, 1990). Whether this elevated metabolic rate remains high for measurement of REE in athletes the following morning is inconclusive with reports ranging from a return in REE to resting levels by 3 h post exercise (Binzen et al., 2001; Freedman-Akabas et al., 1985; Short & Sedlock, 1997) to elevations in REE taking up to 72 hours to return to normal (Dolezal et al., 2000; Tremblay et al., 1988).

Furthermore, the majority of studies have been undertaken in untrained persons or populations with a short training history (6 month to 2 years) (Schuenke et al., 2002). One study investigated elite cross-country skiers and reported that REE was lower when measured three days after training compared to the morning after training (Tremblay et al., 1988). However, REE was not measured in the mornings in between, so the time course of the return to baseline metabolic rate is unknown.

It could also be argued that refraining from exercise for three days is not reflective of an athlete's habitual lifestyle, and that undertaking REE the day after exercise would better reflect the athlete's typical metabolic state. However, it is common for athletes to have 12-36 h of rest between exercise sessions during a training cycle. Conducting an REE measurement within 12-24 h of a training session may cause the measurement to be made during a period of

elevated metabolic rate, either masking or diluting the apparent degree of suppression of metabolic rate in an athlete. In turn, this would interfere with an appropriate diagnosis and management plan for cases in which true low energy availability was in effect outside these times of post-exercise metabolic elevation. (Melin & Lundy, 2015; Mountjoy et al., 2014). Although laboratory studies provide good control over the measurement of REE, it is important that any findings can be applied practically.

Therefore, the aim of this pilot study was to determine if there is a difference in REE when measured the morning after an exercise day compared to the morning after a rest day and if the time of the exercise (morning or afternoon) plays a role in these differences. We hypothesised that REE will be lower when measured at the period of longest recovery from exercise.

### **5.3 Methods**

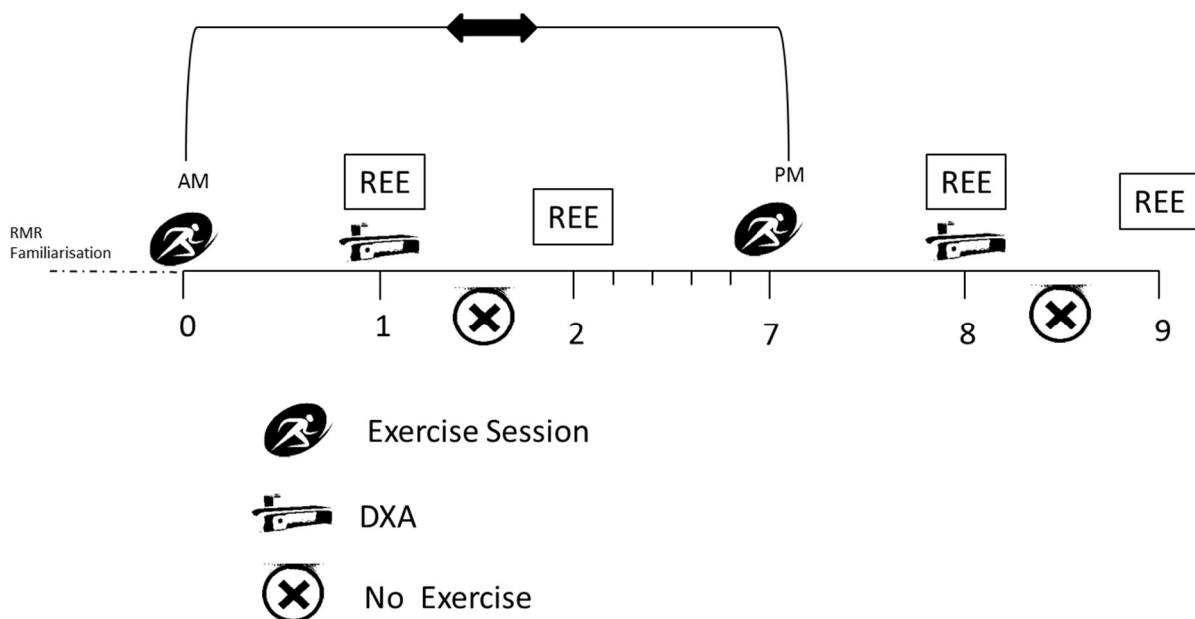
#### *Participant characteristics:*

Male athletes (age,  $30.3 \pm 6.2$  y; weight  $81.5 \pm 11.6$  kg; height,  $187.8 \pm 6.9$  cm) who trained a minimum of 10 hours per week in their respective sport were recruited for this pilot study. Participants ranged from triathletes, cyclists, endurance runners, middle distance runners, Australian football players and swimmers. Written informed consent was provided prior to commencing the study which was approved by the ethics board of the Australian Catholic University.

#### *Study design:*

This study was conducted as a cross-over intervention. Participants had their REE measured on four occasions, over a nine day period (**Figure 5.1**). The time of the

training/exercise session was manipulated so that in the first week they trained in the morning (AM REE measurements) and in the second week they trained in the afternoon (PM REE measurements) or vice versa. The AM session had to be completed by 1000 h, while the afternoon (PM) session started after 1600. Within each week, REE was measured on two consecutive mornings. The first measurement (REE-1) was taken the morning after the training day. The second measurement (REE-2) was taken the morning after REE-1 which was a rest day. Following REE-1, participants were not permitted to exercise for the remainder of the day and were instructed to limit strenuous activity (e.g. running upstairs, riding to work). This was repeated in week 2 for REE-3 and REE-4. Therefore, REE measurements approximately 12-, 24-, 36- and 48 h post exercise were obtained.



**Figure 5.1** Cross over study design. Time of exercise sessions were assigned as either Week 1: AM - Week 2 PM or Week 1 PM - Week 2 AM. REE: resting energy expenditure

#### *Diet and Training standardisations:*

Participants were required to complete the same training session each week with the time of the session being the only manipulated variable. Participants recorded their sessions



in a food and activity diary and were also required to wear SenseWear Armband (BodyMedia, Mini-MF) activity monitors over the measurement period (three days) each week. Participants wore the SWA during exercise and recorded exercise duration and intensity using a 20 point RPE scale (Borg, 1970). Dietary intake for the 24 h prior to each REE measurement was recorded and participants were instructed to replicate this meal plan in week 2. All diets were analysed using Foodworks (version 8, Xyris, Australia) by the same Accredited Sports Dietitian. SenseWear data were analysed using Professional software version 8.1 and analysed for total energy expenditure, total exercise energy expenditure ( $> 3$  METs) and energy expenditure of moderate (3-6 METs), vigorous (6-9 METs) and very vigorous ( $> 9$  METs) activity.

#### *REE Protocol:*

Resting energy expenditure was determined using indirect calorimetry as previously described in Chapter 3.1 (p. 50). Briefly, participants arrived at the lab for an outpatient measure of REE following an 8 h overnight fast (Bone & Burke, 2017). Participants were required to lie supine for 25 min upon arrival to “rest” before collection of expirate into Douglas bags in 10 min duplicates. After 10 minutes of this rest period, participants were given a Hans Rudolph (2700 series, country) mouthpiece for familiarisation. Tests of REE commenced between 0530 – 0700 and were at the same time of the morning for each participant’s set of measures. Laboratory conditions and patient behaviour (e.g. refraining from listening to music) were also kept consistent.

#### *Determination of energy expenditure:*

Oxygen and carbon dioxide gas fractions were determined using Ametek analysers (Ametek Inc, Pennsylvania, USA) for a period of one min with the flow rate recorded. Gas volume was determined using a Tissot Spirometer (Warren Collins, Braintree, MA, USA). Gas fractions and volumes were converted to energy expenditure based on the Weir equation (Weir, 1949). Bags were excluded if the respiratory exchange ratio was greater than 0.95 (Compher et al., 2006; Fullmer et al., 2015). The mean of the duplicate bags was used to calculate energy expenditure, expressed as kJ/day and kJ /kg FFM/day.

### *Body Composition*

As REE is commonly interpreted relative to fat free mass, participants underwent a total body composition assessment via dual x-ray absorptiometry (DXA, GE, Lunar iDXA, Wisconsin, IL) each week. Scans were conducted following REE collection on the first morning of each week when participants were still rested and fasted (Nana et al., 2012a). Although the diets and training were replicated, differences in the period of recovery from the last exercise bout meant muscle glycogen may have replenished to different concentrations which could impact on the measurement of fat free mass (Bone et al., 2017). One experienced DXA technician conducted and analysed all DXA scans. The test-retest technical error of measurement for the iDXA is, 0.1% for total mass, 0.4% for lean mass, 1.6% for fat mass and 0.4% for bone mass.

### *Statistical Analysis:*

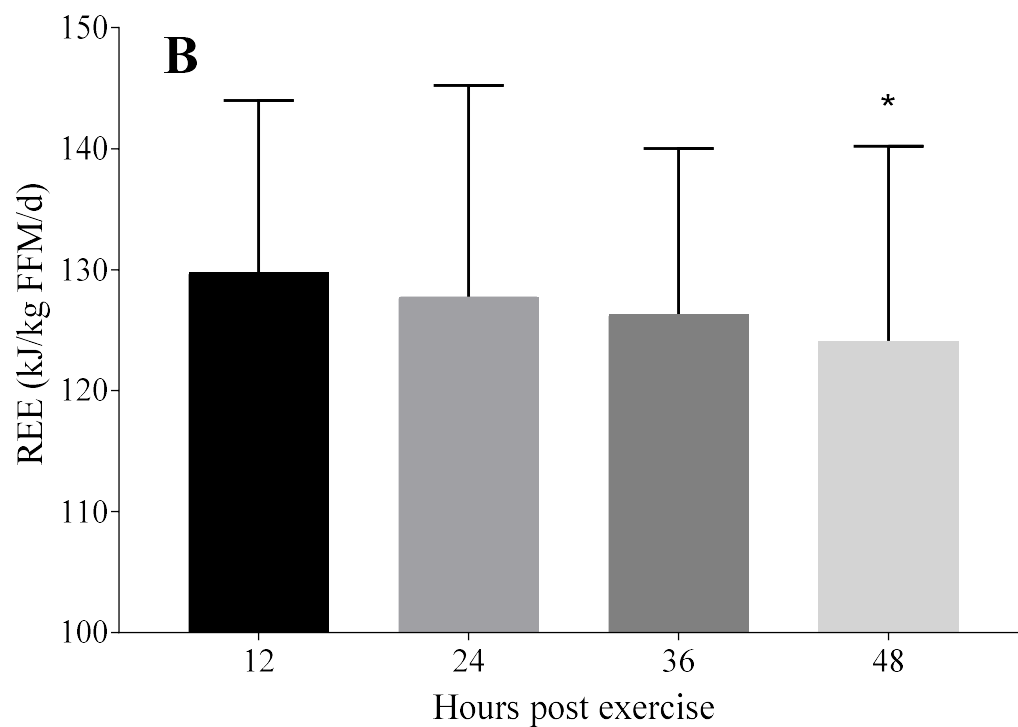
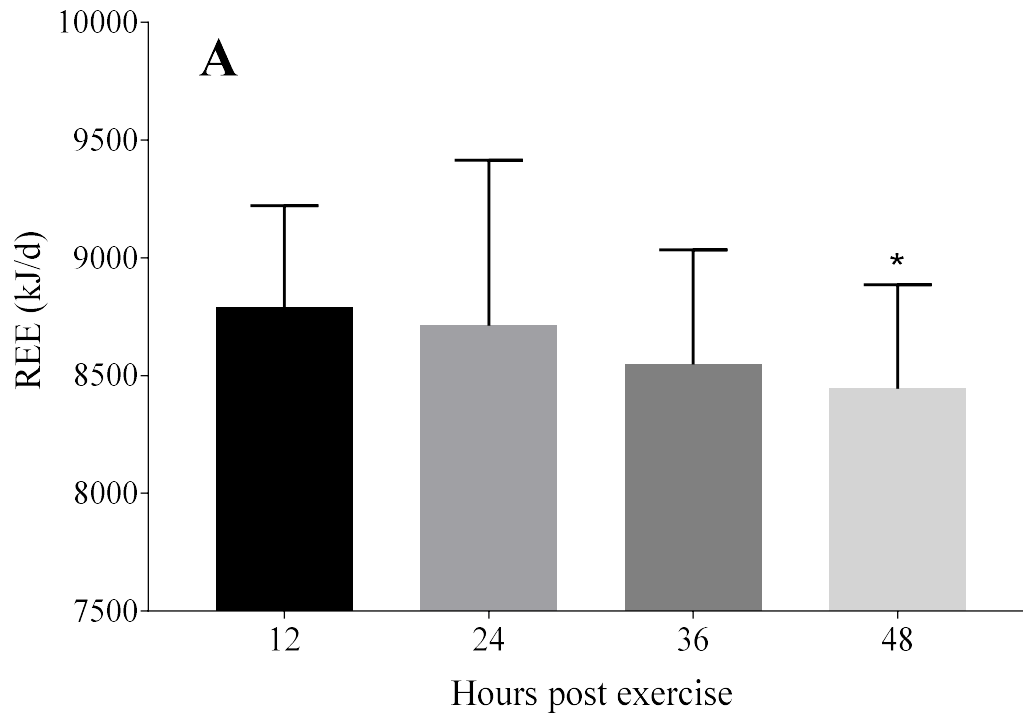
Data were checked for normality and outliers prior to interpretation. Data at all time points were normally distributed as per the Shapiro-Wilks test ( $p > 0.05$ ). Repeated linear mixed models with pairwise comparisons were conducted for REE kJ/d, REE kJ kg FFM/d,

respiratory exchange ratio (RER), energy intake and total energy expenditure. Paired t-tests were used to compare body composition between week 1 and week 2. Data are presented as mean  $\pm$  standard deviation (SD). All data was analysed using SPSS (Version 23 for Windows, SPSS Inc, Chicago, IL, USA). Graphs were constructed using GraphPad Prism (version 7.03, GraphPad Software, Inc, La Jolla, CA).

## 5.4 Results

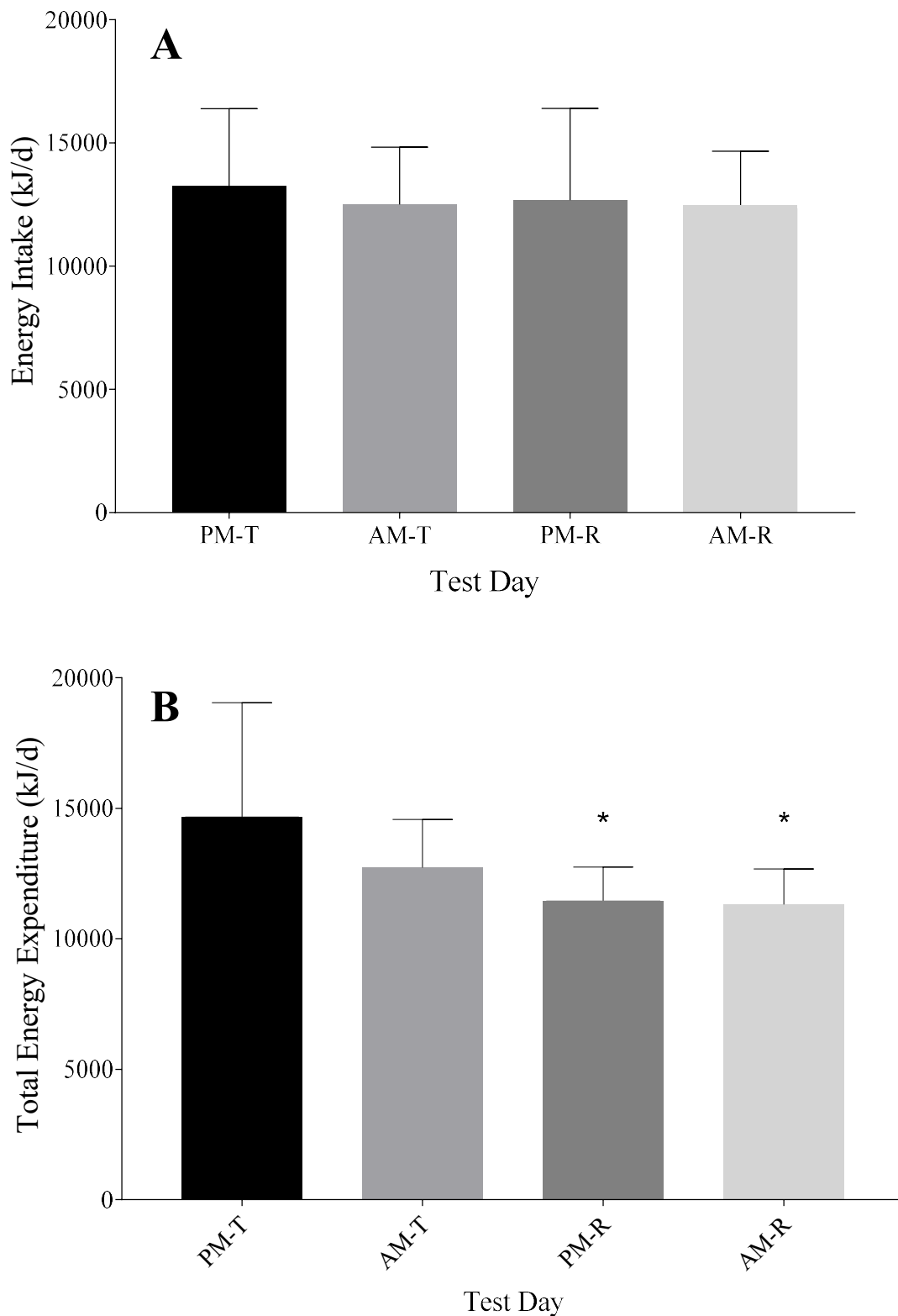
The results of investigations on nine participants are presented below. One participant failed to comply with the resting/no vigorous activity protocol on the rest day and was therefore excluded from statistical analysis. The exercises varied from long distance running, road cycling, swimming and high intensity interval sessions.

**Figure 5.2** shows the mean time course for absolute and relative REE. Comparison of results at 48 h post exercise to 12 h post exercise showed a reduction in absolute REE by 345 kJ/d, ( $p=0.015$ ) while relative REE was lower by 5.7 kJ/kg FFM/d ( $p=0.012$ ).



**Figure 5.2.** Mean  $\pm$  SD for absolute (A) and relative (B) REE measured after 12, 24, 36 and 48 h post exercise. \* indicates statistically significant difference between REE measured 12 h post exercise.

**Figure 5.3** shows energy intake and total energy expenditure for the 24 h periods preceding each REE measurement. Total energy expenditure was significantly lower on the rest day compared to measurements taken in the afternoon ( $p=0.023$  and  $0.013$  for rest day after afternoon and morning training, respectively). There was no difference in mean values for self-reported energy intake in the 24 h periods prior to each RMR measurement.



**Figure 5.3.** Mean  $\pm$  SD energy intake (A) and total energy expenditure (B) in the 24 h preceding REE measurement. PM-T, afternoon training day; AM-T, morning training day; PM-R, rest day following afternoon training; AM-R, rest day following morning training. \* indicate statistically significant difference in total energy expenditure compared to PM-T.

There was no significant difference in RER between the different time points (**Table 5.1**), nor was there a difference in body mass, lean mass, fat free mass and fat mass between weeks 1 and 2 of the study (**Table 5.2**).

**Table 5.1.** Mean  $\pm$  SD RER measured 12, 24, 36 and 48 h post exercise.

Study Week	12 h	24 h	36 h	48 h
RER	0.84 $\pm$ 0.04	0.83 $\pm$ 0.04	0.84 $\pm$ 0.04	0.86 $\pm$ 0.03

RER: respiratory exchange ratio

**Table 5.2.** Mean  $\pm$  SD of body composition between weeks 1 and 2.

Study Week	Body mass (kg)	Lean mass (kg)	Fat Free mass (kg)	Fat mass (kg)
1	79.4	65.4	68.8	11.5
2	79.5	65.4	68.8	11.4

## 5.5 Discussion

The results from the current investigation suggest that REE is lowest when the time interval from the last exercise session is at its greatest (~48 h post exercise). It appeared that REE decreased in a stepwise fashion with each 12 h period from exercise, however, the differences in REE measured between 12-, 24- and 36 h were not significant. Although the difference in REE measured 48 h after exercise was statistically different to that measured 12 h post exercise, the mean difference of 345 kJ/d was within the daily variation for the outpatient 8h fasting protocol (Bone & Burke, 2017). The range in the difference in REE between 12 and 48 h was -90 to 785 kJ /d or -1.8 to 13.9 kJ/kg FFM/d, indicating that there

would be clinical implications for some individuals (i.e. a change in interpretation of the normalcy of a REE measurement according to the time that it was taken). Surprisingly, there was no difference in reported energy intake between training and non-training days. As expected estimated total energy expenditure was significantly lower on rest days compared to exercise days confirming that participants complied with the study protocols. There was no correlation between the magnitude of change in total daily energy expenditure and the RPE of the exercise session between 12 and 36 h and 24 and 48 h post exercise.

It appears that total energy intake and expenditure can both have an impact on REE. Although no significant difference in REE was reported between 36 and 12 h post-exercise, it is possible that if energy intake had been lower on rest days it would have resulted in a decreased REE, as per the “energy flux” theory (Bullough et al., 1995). This concept theorises that metabolic rate is highest when there is a high energy intake and high energy expenditure compared to when both energy intake and energy expenditure are low (Bullough et al., 1995). However, in the current study, energy intake was not different between exercise and rest days and may be one reason why the magnitude of the difference in REE was not as large as previous studies.

Previous studies have reported significant decreases in metabolic rate of 6.6% and 8.8% following 72 h and 39 h rest from exercise in trained men and women respectively. In the case of work from Tremblay et al. (Tremblay et al., 1988), the first measurement of REE did not occur until 72 h post exercise, therefore, the time course of the decrease in REE is unknown. Meanwhile, Herring et al. (Herring et al., 1992) found that in women, REE had decreased ~39 h post exercise and remained low for up to 87 h post training. In the current study a significant decrease was observed ~48 h post exercise however, this was below the



typical error for the outpatient protocol as determined in *Study 1* (Chapter 4) (Bone & Burke, 2017). The discrepancy in the magnitude of the differences reported in the previous studies and those from current investigation could reflect the greater consistency of the effects seen in the former studies which employed 12 h overnight fasting protocols and a homogenous (endurance trained) population who all completed the same mode of exercise (Herring et al., 1992; Tremblay et al., 1988). In the current investigation, greater variability in the effect of exercise on REE was expected, since study participants ranged from triathletes to Australian Rules Football players who undertook a range of exercise sessions. Increased variability in the effects of REE on exercise may have reduced the ability to detect consistent differences at different time points. This feature of the methodological design is not viewed as a limitation, since it provides the study with ecological validity in its goal of trying to identify a common point at which it is best to measure REE in a real world high performance environment. In such a scenario, REE will be measured on a range of different athletes and it is unlikely that the clinician who is investigating REE will be able to limit or prescribe the exercise or training session that the athlete will undertake on the day prior to measurement. Therefore, this study reflects the challenge of trying to incorporate an ideal testing point (i.e. period of rest from the previous exercise bout) or develop a correction factor that might be applied to the results of an REE measurements when an assessment of a potential situation of LEA is required.

Although no attempt was made in this study to track indicators of potential mechanisms underpinning the spike and reduction in REE following an exercise session, several theories have been presented: these include differences in circulating catecholamines and cortisol, as well as the energy cost associated with inflammatory responses and protein synthesis. In the case of catecholamines, elevations have been observed the morning after

strenuous exercise (Maron et al., 1977; Sagnol et al., 1989), while in other studies, a decrease in catecholamines coincided with a decrease in REE following cessation of exercise in female endurance athletes (Herring et al., 1992) and noradrenaline concentrations in male endurance athletes who were in a state of low energy flux (Bullough et al., 1995).

#### *Limitations:*

The small sample size and the lack of inclusion of elite athletes among the study participants prevented this pilot study from achieving its intended outcome of clearly quantifying the difference in REE when measured at different durations from exercise. The recruitment of high performance athletes who were willing to refrain from training (which often includes multiple sessions in a day) was not possible, and it was even difficult to find athletes at a lower level who were willing to interfere with their exercise routines. Therefore, even if a larger sample size had allowed the effects of exercise on REE in the current study to become clearly detected, we would have experienced some difficulty in applying the results to our population of interest. The logistics of the study design (containing REE measurements to a two week period) prevented us from involving female athletes and adding the confounding variable of effects of the menstrual cycle on REE.

In conclusion, this pilot study is the first to measure REE in a stepwise fashion over different periods of recovery from an exercise bout using the 8 h overnight outpatient protocol. Future studies involving greater participant numbers and in elite athlete populations are required before the real life effects of exercise on REE can be clearly detected and factored into best practice protocols for the measurement of REE in clinical practice.

## Preface to Chapter Six

In the following chapter the focus moves from the reliability of athlete presentation protocols for assessment of REE via indirect calorimetry, to the reliability of the assessment of body composition by DXA. One study has highlighted the difference in relative REE when different methods of body composition assessment such as DXA, BIA and surface anthropometry are used (Korth et al., 2007). DXA has become a mainstream tool for the assessment of body composition in athletes, with standardised protocols now in place (Nana et al., 2016). When using DXA to assess body composition FFM is composed of LM and bone mineral content. LM estimates by DXA are susceptible to changes caused by fluid and food intake. It stands to reason that changes in intramuscular solutes that change the fluid content of the cell, such as glycogen and creatine, would also cause changes in the LM component of FFM by DXA.

One study has investigated the effect of acute high carbohydrate intake on LM estimates of DXA (Rouillier et al., 2015). However, they did not have a depletion protocol, used a non-athlete population and used different statistical methods from recent DXA reliability studies (Nana et al., 2012a, 2012b, 2013). Changes in muscle glycogen and creatine concentration influence the LM estimate by DXA would have implications not only for the longitudinal monitoring of body composition but also for resting energy expenditure. In order for *Study 3* to be comparable with the literature on the reliability of DXA assessments of body composition in active populations, magnitude based inference statistics will be used to interpret the effects of creatine and glycogen manipulation on DXA estimates of LM.

## **6.0 Study Three: Manipulation of muscle creatine & glycogen changes DXA estimates of body composition**

This chapter is comprised of the following paper which has been published in Medicine & Science in Sports and Exercise:

**Bone, J L,** Ross, M L, Tomcik, K A, Jeacocke, N A, Hopkins, W G, & Burke, L M. (2017). Manipulation of muscle creatine and glycogen changes dual x-ray absorptiometry estimates of body composition. Medicine and Science in Sports and Exercise, 49, 1029-1035.

This study was funded by a grant from the Australian Catholic University Research Fund awarded to Prof Louise M Burke.

## 6.1 Abstract

Standardising a dual x-ray absorptiometry (DXA) protocol is thought to provide a reliable measurement of body composition.

Purpose: We investigated the effects of manipulating muscle glycogen and creatine content independently and additively on DXA estimates of lean mass.

Method: Eighteen well-trained male cyclists undertook a parallel group application of creatine loading (n=9) (20 g/d for 5 d loading; 3 g/d maintenance) or placebo (n=9) with crossover application of glycogen loading (12 v 6 g/kg BM/d for 48 h) as part of a larger study involving a glycogen-depleting exercise protocol. Body composition, total body water, muscle glycogen and creatine content were assessed via DXA, bioelectrical impedance spectroscopy and standard biopsy techniques. Changes in the mean were assessed using the following effect-size scale: >0.2 small, >0.6, moderate, >1.2 large and compared with the threshold for the smallest worthwhile effect of the treatment.

Results: Glycogen loading, both with and without creatine loading, resulted in substantial increases in estimates of lean body mass (mean  $\pm$  SD;  $3.0 \pm 0.7$  % and  $2.0 \pm 0.9$  %) and leg lean mass ( $3.1 \pm 1.8$  % and  $2.6 \pm 1.0$  %) respectively. A substantial decrease in leg lean mass was observed following the glycogen depleting condition ( $-1.4 \pm 1.6$  %). Total body water showed substantial increases following glycogen loading ( $2.3 \pm 2.3$  %), creatine loading ( $1.4 \pm 1.9$  %) and the combined treatment ( $2.3 \pm 1.1$  %).

Conclusions: Changes in muscle metabolites and water content alter DXA estimates of lean mass during periods in which minimal change in muscle protein mass is likely. This information needs to be considered in interpreting the results of DXA-derived estimates of body composition in athletes.

## 6.2 Introduction

Dual x-ray absorptiometry (DXA) is recognised as a criterion technique for the measurement of body composition and has become a routine part of the preparation and monitoring of athletes (Nana et al., 2015). Strategies which improve the precision of measurement can have real-life importance in sports nutrition; we have previously shown that the use of a standardised protocol which allowed the detection of small but worthwhile changes in total lean body mass and body fat that would have otherwise been missed if measured under non-standardised conditions (Nana et al., 2016). Although the current recommendations for standardizing DXA scanning protocols aim to reduce the error/variability associated with gastrointestinal content from recent meals, general hydration status and fluid shifts associated with exercise (Nana et al., 2012a, 2013), we have proposed that alteration of intramuscular solutes (e.g., glycogen, creatine, carnosine) and their associated water binding may cause another source of biological variation. Indeed, even with the implementation of a Best Practice Protocol, we sometimes observe within-athlete differences in lean body mass estimates of up to 2 kg over an acute time frame, which are unlikely to be explained by real changes in muscle mass.

It has previously been shown that changes in cellular substrates achieved by common practices in sports nutrition can cause detectable changes in muscle size and mass. For example, an investigation using Magnetic Resonance Imaging (MRI) showed increases in muscle cross-sectional area following a carbohydrate loading diet (Nygren et al., 2001). Similarly, a 10 day creatine loading protocol in untrained individuals was shown to increase body mass and DXA estimates of lean mass (Safdar et al., 2008). A recent study reported an increase in the DXA estimate of lean mass in healthy males following the intake of a high carbohydrate in the three days prior to a DXA scan (Rouillier et al., 2015). However, how the variety of changes in muscle solutes and water content commonly experienced by athletes

interact to alter estimates of muscle mass has not been considered. Therefore, it is of interest to undertake a systematic investigation of the variability in DXA measurements of body composition that can be attributed to acute changes in muscle creatine, glycogen and their effect on total body water. We undertook such an investigation, within a larger study of creatine and glycogen loading, with the aim of further refining Best Practice Protocols for body composition assessment by DXA and/or allowing better interpretation of the results. We hypothesized that activities that increased muscle solutes and water would create an artefact in measurement of body composition by increasing the estimate of lean mass, while depletion would be associated with a decrease in the estimate of lean mass.

### **6.3 Methods**

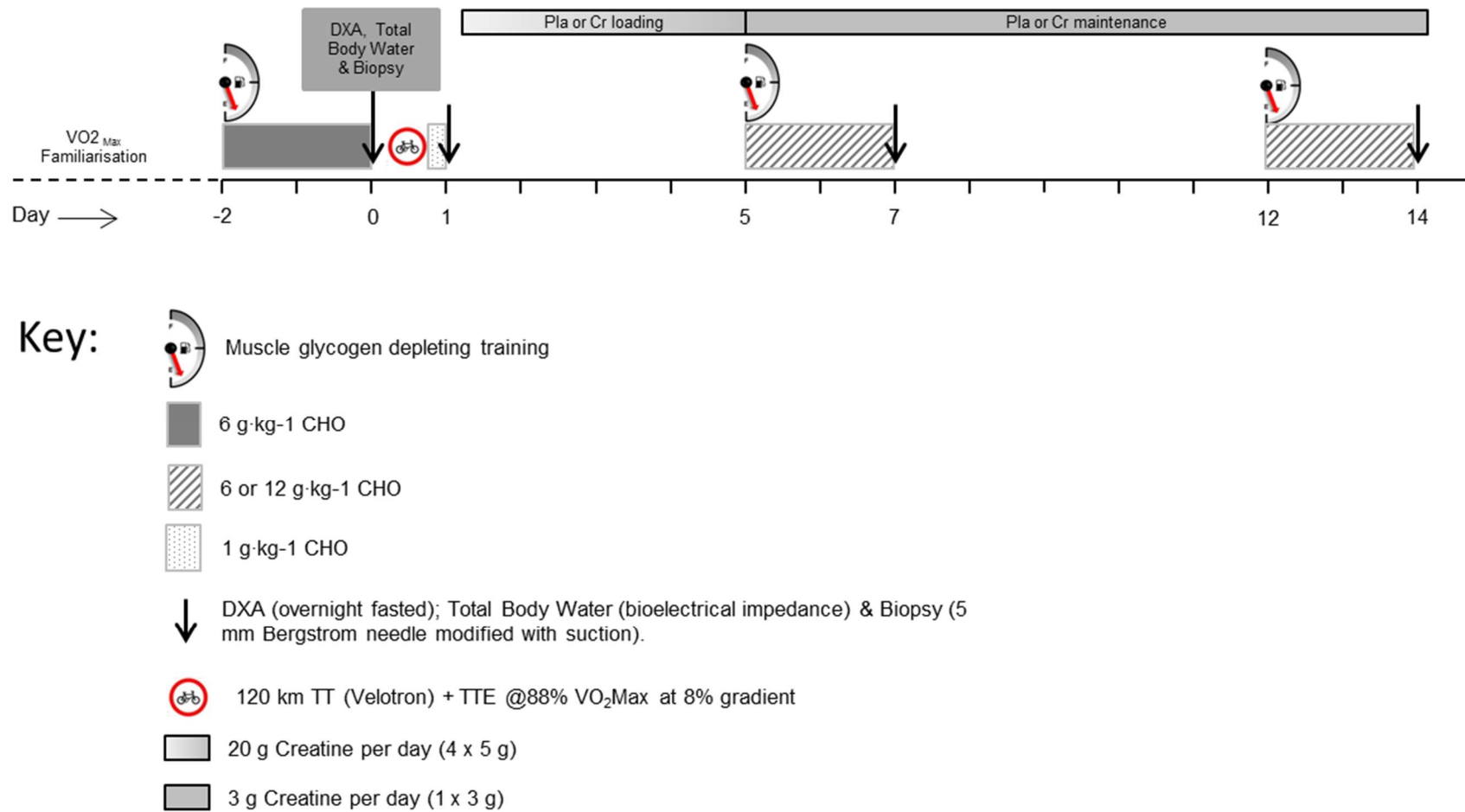
#### *Participants:*

Eighteen competitive male cyclists (age  $31.4 \pm 5.6$  y; body mass (BM)  $78.2 \pm 8.8$  kg; height  $182.7 \pm 7.2$  cm;  $\text{VO}_{2\text{max}}$   $65.2 \pm 7.1$  ml/kg/min) participated in this study which was approved by the human research ethics committees of the Australian Institute of Sport (20140612) and the Australian Catholic University (2014 254N). Participants were informed of protocols and risks of the study before providing written informed consent.

#### *Study Design:*

This study, which was part of a larger investigation of creatine and glycogen loading on cycling performance, employed a parallel group design to investigate the effect of creatine loading, followed by a within-group cross-over application of carbohydrate loading.

All participants underwent baseline measurements on day 0, followed by tests in the Glycogen Depleted state on day 1. Following Day 1 measurements, participants were randomized into either the creatine loading or placebo group and returned for two subsequent testing days one week apart (day 7 and day 14) (see **Figure 6.1**).



**Figure 6.1.** Overview of study design. Pla, placebo; Cr, creatine; CHO, carbohydrate; TT, time trial.



### *Creatine and Glycogen Loading:*

Creatine loading was achieved by intake of 20 g/d of creatine monohydrate (Musashi Creatine Monohydrate, Vitaco, NSW, Australia) for five days using a split dose regimen (4 x 5 g/d, consumed at the same time as a carbohydrate-containing meal or snack) followed by creatine maintenance (3 g/d) (Harris et al., 1992). Normalised glycogen stores were achieved by consuming a pre-packaged diet providing a carbohydrate intake (6 g/kg/d) for 48 h as well as imposing a standardised training protocol including a rest day prior to the DXA scan. Meanwhile, glycogen loading was achieved by providing a pre-packaged diet providing 12 g/kg/d of carbohydrate for the same standardised time period (Burke et al., 2011). Hydration status was standardised by implementing a standardised fluid intake for the 24 h period prior to the DXA scans. Glycogen depletion was achieved by undertaking a cycling protocol in the laboratory lasting ~ 3 h 30 min, with consumption of a pre-packaged low carbohydrate diet following completion of the protocol until the next morning's DXA scan.

The achievement of these protocols provided scenarios to reflect normal-creatine normal-glycogen (n = 18; Baseline), normal-creatine glycogen-depleted (n = 18; Glycogen-Depleted), creatine-loaded glycogen-loaded (n=9; Creatine-Glycogen-Loaded), normal-creatine glycogen-loaded (n = 9; Glycogen-Loaded), and creatine-loaded normal-glycogen (n = 9; Creatine-Loaded).

### *Dietary Standardization:*

An individualised two day menu was constructed for each participant using FoodWorks Professional Edition, Version 7.0 (Xyris Software, Brisbane, Australia) based on their body mass and food preferences. Prior to the baseline trial, subjects received a moderate-carbohydrate diet providing 6 g/kg BM/d carbohydrate; 1.5 g/kg BM/d protein; 1.5 g/kg BM/d fat, with a total energy goal of ~215 kJ/kg/BM per day. The participants were

then randomised to receive either a repeat of the moderate-carbohydrate diet (6g.kg<sup>-1</sup>BM/d) or a carbohydrate-loading diet (12 g/kg BM/d) in the two days prior to the Glycogen Loaded and Glycogen Normal trials (Day 7 and Day14) in a cross-over allocation. These dietary treatments were implemented using a placebo-controlled design, whereby the overall menu for the day was kept constant, but key items were provided either as a low-kilojoule/low carbohydrate option or an indistinguishable carbohydrate-enriched/high kilojoule. Protein and fat intake each remained constant at 1.5 g/kg BM/d in these diets, but energy intake was increased in the carbohydrate-loading diet (~320 kJ/kg/d). Participants refrained from any intake of alcohol during the dietary standardisation period. Caffeine and fluid intake was allowed ad lib two days prior to the baseline trial and up to two standard serves (e.g. 1 cup of coffee or 1 can caffeinated soft drink) the day before the experimental trial. Participants recorded their caffeine and fluid intake and this was repeated during the dietary standardisation period of subsequent trials. Following the glycogen depleting exercise (Day 0), participants were fed a pre-packaged standardised low carbohydrate diet (<1 g/kg BM) for the remainder of the day to minimise resynthesis of muscle glycogen stores. Subjects were provided with all foods and most of their fluids in a standardised menu in portion controlled packages, and were given verbal and written instructions on how to follow the diet. Checklists were used to record each menu item as it was consumed, and to note any deviations from the menu. An analysis of all the actual diets consumed by participants was undertaken on completion of the study using the same software.

#### *Muscle Biopsy:*

Each participant underwent four biopsies over the course of the study, with each being collected from the same leg from an incision that was at least 2 cm from the previously biopsied site. All biopsies were conducted by medical practitioners using a 5 mm Bergstrom needle modified with suction (Evans et al., 1982). The site was anaesthetised using 1%

xylocaine prior to an incision being made through the dermal layer and fascia on the quadriceps. Muscle tissue was immediately frozen in liquid nitrogen and stored at -80°C for later analysis.

#### *Biochemical Analysis:*

Muscle creatine and glycogen concentrations were measured as described previously (Churchley et al., 2007; Harris et al., 1974). Glycogen concentrations were determined via enzymatic analysis with fluorometric detection (Jasco FP-750 spectrofluorometer, Easton, MD) at excitation 365 nm/emission 455 nm. Concentrations were expressed as millimoles of glycogen per kilograms of dry weight (mmol/kg d.w). Muscle tissue was analyzed in duplicate for free creatine, creatine phosphate, and adenosine triphosphate (ATP) using fluorometric techniques. Total creatine was measured as a sum of free creatine and creatine phosphate (Harris et al., 1992).

#### *DXA and Total Body Water Protocol:*

For each of the four different conditions, participants reported to the laboratory in the morning after an overnight fast and undertook a total body DXA scan as per a standardised protocol (Nana et al., 2015). Body composition was assessed using a whole body scan on a narrowed fan-beam DXA (Lunar Prodigy, GE Healthcare, Madison, WI) with analysis performed using GE Encore 12.30 software (GE, Madison, WI). The DXA technical error of measurement (TEM) was ~ 0.1% for total mass, 0.4% for total lean, 1.9% for total fat and 0.7% for total bone mineral content (Nana et al., 2012a). Following 15 min of rest, total body water and fluid compartments were assessed using Bioelectrical Impedance Spectroscopy (BIS) (IMP SFB7, ImpediMed Limited, Queensland, Australia) and analysed using BioImp Analysis 5.4.0 Software (ImpediMed Limited, Queensland, Australia) according to the

protocol described by Moon et al. (Moon et al., 2008). The BIS has a TEM of 0.81 L. Hydration status was monitored by measurement of urine specific gravity (UG-a, Atago Refractometer, Japan) from a sample collected upon waking.

#### *Statistical Analysis:*

We used a mixed linear model (Proc Mixed in version 9.4 of the statistical Analysis System; SAS Institute, Cary, NC) to estimate the effect of the treatments on muscle glycogen concentration, muscle creatine concentration, the mass of each component of body and leg composition, and the mass of intracellular, extracellular and total body fluids. Treatment was a fixed effect in the model (nominal, with six levels), while random effects were the athlete identity and its interaction with dummy variables to estimate error additional to the residual (individual responses) to glycogen depletion, glycogen loading, creatine loading, and combined glycogen and creatine loading. All dependent variables were log transformed for analysis. The smallest important change was determined as per Nana et al. (Nana et al., 2012b). By using the default approach of standardization with an appropriate between-subject standard deviation, here the baseline standard deviation. The magnitudes of changes the resulting effects were assessed using the following scale: <0.2 trivial, >0.2 small, >0.6 moderate, >1.2 large (Hopkins et al., 2009). Small or larger changes were considered substantial when the threshold for the small effect was reached ( $\geq 0.2$ ). Uncertainty in the changes is shown as expressed by 90% confidence limits when the upper and lower confidence limits represented substantial increases and decreases, respectively. Owing to the considerable number of effects investigated, the effects were assessed as clear or unclear using 99% confidence limits. All other effects were deemed clear, and shown with the probabilities that the true effect was a substantial decrease, a trivial change, or a substantial increase.

## 6.4 Results

Baseline values and percentage changes with the different treatments are presented in a series of tables: total body composition (**Table 6.1**), leg regional body composition (**Table 6.2**), body water (**Table 6.3**) and muscle glycogen concentrations (**Table 6.4**).

Body mass (**Table 6.1**): There was a substantial increase in body mass in the combined Creatine–Glycogen Loaded treatment compared to baseline, the observed effect being small. Changes in the separate Glycogen Loaded and Creatine Loaded treatments on body mass were clearly not substantial. Additionally, there was no substantial change in body mass following the Glycogen Depleted condition.

Lean Mass (**Table 6.1 and 6.2**): There were substantial increases in lean body mass following the Creatine-Glycogen-Loaded and the sole Glycogen-Loaded treatments compared with baseline measurements, with the observed effects being small. Similar results were observed for leg lean mass with a small but substantial increase with both treatments. There was no substantial decrease in lean body mass following the Glycogen-Depleted condition but there was a substantial decrease in leg lean mass. The effects of the Creatine-Loading condition on lean body mass and leg lean mass were likely trivial.

Fat mass and Bone mass (**Table 6.1 and 6.2**): Compared to baseline measurements, changes in total fat mass and leg fat mass in Glycogen-Depleted and Glycogen-Loaded conditions were not substantial and produced trivial effect sizes relative to the smallest important effect. The effects of Creatine-Loading and the combined Creatine-Glycogen-Loading conditions on total body fat mass and leg fat mass were also not-substantial. Changes in total bone mass and leg bone mass for all treatment conditions were not substantial.

**Table 6.1.** Baseline values, smallest important change, and percent changes from baseline following various treatments for total body composition. Also shown are magnitude-based inferences for the mean changes with each treatment.

		Change (%) from baseline (mean; $\pm$ CL)				
Measure	Baseline	SIE	Glycogen	Glycogen	Creatine	Creatine-
	(mean $\pm$ SD)		Depleted	Loaded	Loaded	Glycogen
Body mass	77 $\pm$ 9 kg	2.3	-1.3; $\pm$ 0.3	2.1; $\pm$ 0.7	1.2; $\pm$ 0.5	2.8; $\pm$ 0.5 $\uparrow^{**}$
DXA whole body mass						
Total	78 $\pm$ 8 kg	2.2	-1.3; $\pm$ 0.3	2.3; $\pm$ 0.6 $\uparrow^*$	1.3; $\pm$ 0.6	3.0; $\pm$ 0.5 $\uparrow^{***}$
Lean	84 $\pm$ 6 %BM	1.5	-1.3; $\pm$ 0.3	2.1; $\pm$ 0.5 $\uparrow^{**}$	1.3; $\pm$ 0.5	3.0; $\pm$ 0.4 $\uparrow^{***}$
Fat	12 $\pm$ 6 %BM	8.6	-2.0; $\pm$ 1.5	4.5; $\pm$ 3.4	3.3; $\pm$ 5.7	5.2; $\pm$ 3.9
Bone	4.2 $\pm$ 0.3 %BM	1.8	-0.2; $\pm$ 0.5	0.4; $\pm$ 1.1	0.0; $\pm$ 0.5	-0.2; $\pm$ 0.5

%BM, percent of baseline body mass; CL, 90% confidence limits;

SIE, smallest important effect. This is 0.2 of the between subject SD and the percent change a variable has to meet to be considered a substantial change;  $\uparrow$  indicates substantial increase;  $\downarrow$ , indicates substantial decrease;

Asterisk/s indicates how clear the change is at the 99% confidence level, \*possible clear change,

\*\*likely clear change, \*\*\*very or likely clear change.

**Table 6.2.** Baseline values, smallest important change, and percent changes from baseline following various treatments for regional leg composition. Also shown are magnitude-based inferences for the mean changes with each treatment.

Measure	Baseline (mean $\pm$ SD)	SIE	Change (%) from baseline (mean; $\pm$ CL)			
			Glycogen	Glycogen	Creatine	Creatine-
			Depleted	Loaded	Loaded	Glycogen Loaded
Total	35.3 $\pm$ 2.0 %BM	1.1	-1.4; $\pm$ 0.6 $\downarrow$ **	2.9; $\pm$ 0.8 $\uparrow$ ***	1.1; $\pm$ 1.2 $\uparrow$ *	2.9; $\pm$ 1.3 $\uparrow$ ***
Lean	29.4 $\pm$ 2.3 %BM	1.5	-1.4; $\pm$ 0.7 $\downarrow$ *	2.6; $\pm$ 0.8 $\uparrow$ ***	1.2; $\pm$ 1.0	3.1; $\pm$ 1.2 $\uparrow$ ***
Fat	4.3 $\pm$ 2.5 %BM	8.5	-2.5; $\pm$ 1.7	6.2; $\pm$ 1.8	2.4; $\pm$ 6.1	3.2; $\pm$ 4.1
Bone	1.65 $\pm$ 0.15 %BM	2.0	0.0; $\pm$ 0.3	0.7; $\pm$ 0.5	-0.2; $\pm$ 0.7	-0.2; $\pm$ 0.5

%BM, percent of baseline body mass; CL, 90% confidence limits;

SIE, smallest important effect. This is 0.2 of the between subject SD and the percent change a variable has to meet to be considered a substantial change;  $\uparrow$  indicates substantial increase;  $\downarrow$ , indicates substantial decrease;

Asterisk/s indicates how clear the change is at the 99% confidence level, \*possible clear change,

\*\*likely clear change, \*\*\*very or likely clear change.

**Body water (Table 6.3):** There were likely substantial effects of Glycogen-Depletion and Glycogen-Loading treatments on total body water. There was a likely decrease in extracellular fluid in the Glycogen-Depletion treatment. An increase in total body water and intracellular fluid with the combined Creatine-Glycogen-Loaded condition was very likely, with a possible increase in extracellular fluid. The Creatine-Loading condition was associated with a possible likely increase in total body water and intracellular fluid, but no clear effect on extracellular fluid.

**Muscle glycogen (Table 6.4):** The effects of Glycogen-Depletion, Glycogen-Loading and the combined Creatine-Glycogen loading treatments on muscle glycogen concentration were clear. There was no clear effect of the Creatine-Loading treatment on muscle glycogen concentrations.

**Table 6.3.** Baseline values, smallest important change, and percent changes from baseline following various treatments for total body water and water compartments. Also shown are magnitude-based inferences for the mean changes with each treatment.

Measure	Change (%) from baseline (mean; $\pm$ CL)					
	Baseline (mean $\pm$ SD)	SIE	Glycogen	Glycogen	Creatine	Creatine-
			Depleted	Loaded	Loaded	Glycogen Loaded
Total						
body	61.2 $\pm$ 3.8 %BM	1.3	-2.0; $\pm$ 1.1 $\downarrow$ **	2.3; $\pm$ 1.3 $\uparrow$ **	1.3; $\pm$ 1.7 $\uparrow$ *	2.5; $\pm$ 1.0 $\uparrow$ ***
Intra-						
cellular	36.1 $\pm$ 3.0 %BM	1.4	-1.3; $\pm$ 1.6 $\downarrow$ *	2.2; $\pm$ 1.9 $\uparrow$ *	1.4; $\pm$ 2.0 $\uparrow$ *	6.8; $\pm$ 4.5 $\uparrow$ ***
Extra-			-3.5; $\pm$ 1.2			
cellular	25.3 $\pm$ 1.4 %BM	1.0	$\downarrow$ ***	2.2; $\pm$ 1.5 $\uparrow$ **	0.3; $\pm$ 1.8	1.4; $\pm$ 1.9 $\uparrow$ *

%BM, percent of baseline body mass; CL, 90% confidence limits;

SIE, smallest important effect. This is 0.2 of the between subject SD and the percent change a variable has to meet to be considered a substantial change;  $\uparrow$  indicates substantial increase;  $\downarrow$ , indicates substantial decrease;

Asterisk/s indicates how clear the change is at the 99% confidence level, \*possible clear change,

\*\*likely clear change, \*\*\*very or likely clear change.



**Table 6.4.** Baseline values, smallest important change, and percent changes from baseline following various treatments for muscle glycogen and total creatine. Also shown are magnitude-based inferences for the mean changes with each treatment.

Measure	Change from baseline (mean; $\pm$ CL)					
	Baseline	Glycogen		Creatine		Creatine-
	(mean $\pm$ SD)	SIE	Depleted	Loaded	Loaded	Glycogen Loaded
Muscle	580 $\pm$ 140					
Glycogen	mmol/kg d.w.	1.9	-57; $\pm$ 7.3 $\downarrow$ ***	22; $\pm$ 12.6 $\uparrow$ ***	-2; $\pm$ 15.4	20; $\pm$ 15.9 $\uparrow$ ***
Muscle						
Creatine	136 $\pm$ 17 $\mu$ mol/g	2.2	0; $\pm$ 6.2	-2; $\pm$ 8.5	6; $\pm$ 10.1	6; $\pm$ 4.6 $\uparrow$ **

%BM, percent of baseline body mass; CL, 90% confidence limits;

SIE, smallest important effect. This is 0.2 of the between subject SD and the percent change a variable has to meet to be considered a substantial change;  $\uparrow$  indicates substantial increase;  $\downarrow$ , indicates substantial decrease;

Asterisk/s indicates how clear the change is at the 99% confidence level, \*possible clear change,

\*\*likely clear change, \*\*\*very or likely clear change.

## 6.5 Discussion

This study is the first to systematically investigate the effect of glycogen loading, creatine loading and their interaction on DXA estimates of body composition. Estimates of lean body mass were substantially higher with glycogen loading translating to a mean 1.3 kg increase in lean body mass and 1.7 kg increase in leg lean mass following our glycogen loading treatment and a 1.9 kg increase in lean body mass and 2.0 kg increase in leg lean mass following a combined creatine glycogen loading treatment. On the other hand, glycogen depleting exercise resulted in a mean decrease of 1.0 kg and 0.8 kg of lean body mass and leg lean mass which was deemed very likely trivial. The changes in the DXA estimates of lean body mass and leg lean mass were reflected by the changes in total body water and intracellular fluid. Our findings of increased total body water, and more specifically intracellular fluid, with glycogen loading were expected. However, we have demonstrated, for the first time that this creates an artefact in DXA-derived measurements of body composition in well trained athletes.

It is well accepted that water is bound to the glycogen molecule in the cellular environment. Indeed, a ratio of three grams of water to one gram of muscle glycogen is commonly stated, based primarily on a single rat study from the 1940s which determined that 1 g of liver glycogen was associated with 2.7 g of water over a range of concentrations (McBride et al., 1941). Olsson and colleagues assessed body water by tritium trace in dilution in males before and after glycogen loading, reporting that each gram of glycogen was stored with 3-4 g of water (Olsson & Saltin, 1970). They observed a mean increase in body mass of 2.4 kg, of which 2.2 L was attributed to the increase in total body water. (Olsson & Saltin, 1970). However, Sherman et al. completed studies of rat skeletal muscle and failed to find a consistent relationship between glycogen and water content over a range of glycogen concentrations (Sherman et al., 1982). More recently, Fernández-Elías and colleagues reported different ratios of muscle glycogen to water following post-exercise glycogen

repletion under different fluid intakes. A ratio of 1:3 was found when only 400 ml of water was consumed, while a ratio of 1:17 was determined when participants replaced the fluid lost during exercise (Fernandez-Elias et al., 2015). Although anecdotes and studies have noted that glycogen loading is associated with a gain in total body mass (Brotherhood & Swanson, 1979), and that changes in glycogen can confound the results of weight loss programs in the general community (Kreitzman et al., 1992), few studies have investigated how changes in glycogen loading (and consequently body water) would affect interpretations of body composition in athlete populations.

An increase in body mass is considered a direct side effect of the creatine supplementation during the initial loading phase (Hultman et al., 1996; Juhn & Tarnopolsky, 1998; Volek & Kraemer, 1996). The mass increase is often attributed to water retention, as five days is considered too short a period to detect real changes in myofibrillar protein content (Hultman et al., 1996; Juhn & Tarnopolsky, 1998; Powers et al., 2003; Volek & Kraemer, 1996). Since creatine is an osmotic particle, increases in its concentration in muscle could induce cellular swelling leading to fluid retention (Bemben et al., 2001; Francaux & Poortmans, 1999). Indeed, acute decreases in urinary output (Hultman et al., 1996) and increases in total body and intracellular water have both been reported following creatine loading protocols. Furthermore, creatine supplementation of 20-25 g per day for 5-7 days has been associated with increases of 1.0 to 2.0 kg (Kinugasa et al., 2004; van Loon et al., 2003; Volek & Kraemer, 1996) in body mass and 1.3 to 2.3 L in total body water (Deminice et al., 2016; Powers et al., 2003; Safdar et al., 2008). However, not all studies have found a concurrent increase in the intracellular water compartment (Powers et al., 2003).

To our knowledge, only a handful of studies has have investigated the effect of carbohydrate loading or creatine loading on body composition or muscle size (Balon et al., 1992; Nygren et al., 2001; Rouillier et al., 2015), and we are the first to investigate the interaction of these two strategies. Another novel aspect of our study was the assessment of

total body water as an adjunct to the measurement of body composition; to our knowledge, no other study has reported on the effect of glycogen loading on lean body mass and total body water. Our findings support those of Nygren et al. (Nygren et al., 2001) and Rouillier et al. (Rouillier et al., 2015), with substantial increases in muscle glycogen, lean body mass and total body water observed following 48 h of glycogen loading. Nygren et al. (Nygren et al., 2001) reported an increase in the vastus lateralis cross sectional area by MRI following four days of carbohydrate loading in healthy males. The increase in muscle cross sectional area was attributed to the increase in glycogen (281 to 634 mmol/kg d.w.) along with the binding of the water (Nygren et al., 2001). However, neither body water nor measures of body composition were assessed in this investigation. Meanwhile Balon and colleagues found no increase in muscle girth following a three day high carbohydrate diet (80% carbohydrate) compared with a low carbohydrate diet (10% carbohydrate) with concurrent resistance training (Balon et al., 1992).

A recent study investigating three days of increased carbohydrate intake on DXA estimates of body composition reported a mean 0.9 kg increase in lean body mass and 1.4 kg increase in appendicular lean mass (arms and legs) (Rouillier et al., 2015). Although the authors attributed the increase in appendicular lean mass to increased glycogen storage, no biopsies were conducted to verify changes in muscle glycogen content (Rouillier et al., 2015). Furthermore, dietary intake was not prescribed and although carbohydrate intake achieved the stated goal of exceeding 75% of total energy intake, this amounted to a total daily intake of 8 g/kg, compared to 12 g/kg in our study. Some concerns regarding the standardization of the methodology of the DXA scans are also noted: although not clearly stated, the DXA scans were conducted following an overnight rest and fast (Bone & Burke, 2016) but it is unknown whether carbohydrate intake was standardized prior to the baseline scan.

Several studies have investigated the effect of creatine supplementation on body composition, however they are often for longer supplementation periods and taken concurrently with resistance training (Bemben et al., 2001; Earnest et al., 1995; Francaux & Poortmans, 1999; Kutz & Gunter, 2003; Ziegenfuss et al., 2002). Currently only one other study has assessed the sole effect of short term creatine supplementation on body water and body composition (Safdar et al., 2008). Safdar et al. (2008) reported increases in lean body mass by DXA following a 10 day creatine supplementation period in untrained individuals. Furthermore, measurement of total body water by BIS revealed an increase in intracellular fluid compartment, although the magnitude of this increase was not provided (Safdar et al., 2008). Our creatine loading treatment resulted in only trivial changes in muscle creatine content and lean mass, and showed only possible increases in total body water and intracellular fluid. Due to our study design, the assessment of all these parameters occurred on either Day 7 or Day 14 of the supplementation protocol, where participants had changed to a reduced creatine dosage (3 g/d), believed to maintain elevated creatine stores (Preen et al., 2003), for two or nine days respectively. However, since van Loon et al. (van Loon et al., 2003) recently reported that this “maintenance” dose is not always sufficient for maintaining creatine levels, it is possible that a reduction in creatine content occurred over the longer maintenance period, obscuring any earlier effects.

We note some other real and apparent limitations of this study. Due to the requirements of the larger study, we were unable to add further measurements such as an assessment of body composition and body water under a Creatine-Loaded-Glycogen-Depleted condition. Furthermore, we anticipate the criticism that the Glycogen-Depleted condition was monitored 15-18 h after the completion of the glycogen-depleting task. However, as we conducted our scans using a standardised protocol based on Nana et al. (Nana et al., 2016) which require fasted and rested conditions to standardize gut contents and hydration status, we needed to undertake these measurements on the morning following the exercise session.

However, we attempted to minimize glycogen resynthesis during the recovery period by providing participants with a diet providing < 1 g/kg carbohydrate. This was successful in maintaining glycogen content below pre-exercise levels, and indeed may mirror the real-life practices of athletes who “sleep low” (restrict carbohydrate intake) after quality training sessions to prolong the adaptive response to exercise by delaying muscle glycogen storage (Marquet et al., 2016). We acknowledge that BIS is an indirect measurement of total body water, however, the use of the criterion dilution methods did not fit within the constraints of the larger study. Additionally, BIS has been recently validated against criterion methods in athletes and was considered appropriate in this setting (Kerr et al., 2015; Matias et al., 2016).

In summary, the results from this study provide further evidence of daily variability in the DXA assessments of body composition of athletes due to factors frequently experienced in sport. Recent work by our centre has developed techniques to standardize DXA assessment protocols (Nana et al., 2012a, 2013; Nana et al., 2016), showing that the implementation of overnight fasted and rested conditions can reduce variability to allow greater sensitivity in the detection of real and interesting changes in body composition (Nana et al., 2016). The present study expands on this work and indicates that when DXA is used for longitudinal monitoring of physique, scans should be undertaken with consideration of recent practices of training and diet that might be expected to manipulate muscle glycogen stores. Where standardization of these practices is impractical, the interpretation of the results of DXA assessments of body composition should take into account the likely artefacts with respect to lean mass. Future studies should also investigate the effect of other sources of changes in intramuscular fluid and substrate such as muscle damage or carnitine supplementation, alongside those caused by exercise or dietary manipulation.

## Preface to Chapter Seven

The previous study in Chapter 6 highlighted how manipulations of muscle glycogen concentration (whether depleted or loaded) had a likely substantial influence on lean mass estimates by DXA. The following chapter is *Study 4* of this thesis and investigates the validity and reliability of ultrasound technology for the use of estimating and tracking changes in muscle glycogen. If valid, this technique would assist practitioners in the interpretations of REE relative to FFM and possibly allow for a “correction” for this artefact in the DXA estimate of lean mass if the ultrasound detects a change muscle glycogen concentrations.

## **7.0 Study Four: Ultrasound Technology Fails To Provide Indirect Estimate of Muscle Glycogen Concentration**

The following chapter is comprised of the following manuscript under preparation to be submitted to *Nutrients*:

**Bone, JL.** Ross, ML, Hopkins, WG, Tomcik, KA, Jeacocke, NA, Burke, LM.  
Ultrasound Technology Fails To Provide Indirect Estimate of Muscle Glycogen Concentration

This study was funded by a grant from the Australian Catholic University Research Fund awarded to Prof Louise M Burke.



## 7.1 Abstract

Quantification of muscle glycogen traditionally requires muscle biopsies or nuclear magnetic resonance. These procedures limit opportunities to conduct research in the field or with elite athletes. We undertook a validation study of measurement of muscle glycogen with new ultrasound technology in 12 highly-trained male cyclists. Muscle water and solutes were manipulated via glycogen depletion (~3.5 h of cycling) and combinations of carbohydrate loading (12 g/d per kg body mass for 48 h) and creatine loading (20 g/d for 5 d; 3 g/d for 9 d). Glycogen was measured in biopsies from the vastus lateralis on one leg and with ultrasound on the non-biopsied leg. Ultrasound images were captured at the midpoint of the vastus lateralis and contralateral to the biopsy site, then uploaded to proprietary software (MuscleSound® 2015), which provided an ultrasound glycogen score. Changes in mean glycogen concentration in the glycogen-loaded and -depleted conditions were tracked clearly by biopsy, but not by ultrasound. Scatterplots showed poor relationships between biopsy and ultrasound score, and the error in glycogen concentration predicted by ultrasound score was either unclear or ranged in uncertainty from very large to extremely large when evaluated by standardization after removal of the contribution of error of measurement in glycogen concentration. Mixed modelling showed similar errors in prediction of changes in glycogen concentration by changes in ultrasound score. We conclude that the ultrasound-based technique failed to predict muscle glycogen under conditions where muscle solutes and water vary across more extreme ranges experienced by athletes than original validation conditions.

## 7.2 Introduction

Determination of muscle glycogen content is of interest in sports nutrition research and practice due to the important role of this compound as a fuel source in athletic performance and a regulator of muscle metabolism and adaptation (Philp et al., 2012). Typically, in research settings, muscle glycogen concentrations are determined via direct but invasive techniques (muscle biopsy) or by specialized techniques (nuclear magnetic resonance) that are expensive and not commonly available. More recently, a commercial application of ultrasound technology has been promoted as a practical and portable technique to measure muscle glycogen, suitable for both laboratory and field use (Hill & Millan, 2014; Nieman et al., 2015). The MuscleSound® methodology uses high-frequency ultrasound technology and image analysis from patented cloud-based software to estimate muscle glycogen content based on the associated muscle water content, through the image grey scale (Hill & Millan, 2014; McBride et al., 1941; Nieman et al., 2015). Changes in the brightness (echo intensity) of the image indicate greater amounts of fluid and therefore glycogen in the muscle while a darker image represents less fluid and glycogen (Nieman et al., 2015). If this technique can be shown to provide accurate measurements of muscle glycogen, it would have valuable applications in sports nutrition practice and research, particularly in field conditions and among elite athletes where the traditional techniques of glycogen measurement are not suitable.

Previous validation studies comparing direct and MuscleSound® estimates of muscle glycogen from the quadriceps of well-trained cyclists prior to and after a prolonged cycling bout reported significant correlations between values for pre-exercise, post-exercise and change scores in muscle glycogen between the two measurement techniques (Hill & Millan, 2014; Nieman et al., 2015). We undertook a further independent validation study of the MuscleSound® system within a larger investigation of the manipulation of muscle glycogen and creatine content in well-trained athlete. This provided a novel opportunity to assess if

changes in intramuscular fluid and solutes from a range of strategies affect the precision and reliability of MuscleSound® assessments of muscle glycogen. We hypothesised that MuscleSound® estimates of muscle glycogen would not be valid when manipulations of intramuscular solutes and fluids were more extreme and were not related directly to changes in muscle glycogen.

### 7.3 Methods

#### *Participants:*

Twelve competitive male cyclists participated in this study which was approved by the human research ethics committees of the Australian Institute of Sport (20140612) and the Australian Catholic University (2014 254N). Participants were informed of protocols and risks of the study before providing written informed consent. Participants' characteristics are presented in **Table 7.1**. These subjects represent a sub-group of a larger cohort who undertook the main project under which this study was performed (Bone et al., 2017).

**Table 7.1.** Descriptive characteristics of the 12 study participants. Data are mean  $\pm$  SD.

Age (y)	32.6 $\pm$ 5.1
Body mass (kg)	79.2 $\pm$ 9.5
Height (cm)	183.4 $\pm$ 5.0
VO <sub>2</sub> max (L/min)	5.1 $\pm$ 0.6
Maximum power output (W)	639 $\pm$ 115
VO <sub>2</sub> max: maximum oxygen consumption	

#### *Study Design:*

This study, which was part of a larger investigation of creatine and glycogen loading, employed a parallel group design to investigate the effect of creatine loading, followed by a within-group cross-over application of carbohydrate loading on muscle substrate and water content and performance (see **Figure 6.1**). Participants came in for four separate biopsy and ultrasound measurements; baseline (Day 0, 6g carbohydrate /kg BM/d for 48 h), Glycogen Depleted (Day1) and either Glycogen Loaded or Glycogen Normal (6 g carbohydrate /kg BM/d for 48 h) with or without creatine supplementation (Days 7 and 14). Manipulations of creatine and glycogen stores were achieved by implementing “Best Practice Protocols” of creatine loading (20 g/d for 5 d loading; 3g/d maintenance) (Harris et al., 1992) and glycogen loading (12 g carbohydrate /kg BM/d for 48 h) (Burke et al., 2011) through a standardised pre-packaged diet. Furthermore, a supervised cycling protocol of ~ 3.5 h, including a series of higher intensity efforts, was undertaken to deplete muscle glycogen stores with consumption of a low carbohydrate diet (<1g CHO / kg BM) to prevent repletion of glycogen stores before a further assessment of muscle glycogen content the following morning. This provided situations where muscle glycogen was measured under baseline normalised conditions, depleted condition, and also glycogen loaded with or without creatine loading (Bone et al., 2017).

#### *Muscle Biopsy:*

Four biopsies were conducted over the course of the study, with each being collected from the same leg from an incision that was at least 2 cm from the previously biopsied site. All biopsies were conducted by medical practitioners. The skin and fascia at the site was anaesthetised using 1% xylocaine. An incision was then made through the dermal layer and fascia on the quadriceps before a modified 5 mm Bergstrom needle, with suction, was used to collect the sample (Evans et al., 1982). Muscle tissue was immediately frozen in liquid nitrogen and stored at -80°C for later analysis.

### *Biochemical Analysis:*

Creatine and glycogen concentrations in the muscle samples were measured as previously described (Churchley et al., 2007; Harris et al., 1974). Glycogen concentrations were determined via enzymatic analysis with fluorometric detection (Jasco FP-750 spectrofluorometer, Easton, MD) at excitation 365 nm/emission 455 nm. Concentrations were expressed as millimoles of glycogen per kilograms of dry weight (mmol/kg d.w) (Churchley et al., 2007). Samples for creatine concentration were analyzed in duplicate for free creatine, creatine phosphate, and adenosine triphosphate using fluorometric techniques. Total creatine was measured as a sum of free creatine and creatine phosphate (Harris et al., 1974).

Error of measurement of glycogen concentration was estimated from individual values provided by the authors of a previous study (unpublished data), in which 10 athletes similar to those in the present study were biopsied twice for measurement of glycogen concentration in a high glycogen and low glycogen condition using the same biochemical methods. Each biopsy had been divided into two samples and analyzed separately. To obtain an estimate of error of measurement appropriate for the present study, we treated the data for the high and low glycogen conditions as if they came from separate subjects. We then performed a one-way reliability analyses with the first split sample, a separate one-way analysis for the second sample, then averaged the error of measurement (via an average of the variances) from the two analyses. The analyses were performed with a published spreadsheet using the cells for log-transformation (Hopkins, 2015); the resulting error of measurement was 22%. For purposes of parametric bootstrapping (see below), the error was assumed to have 20 degrees of freedom.

### *Ultrasound Imaging & Processing:*

Two of the researchers involved with this study were trained to capture ultrasound images on the vastus lateralis using a portable ultrasound machine. They practised this

technique to achieve acceptable reliability with repeat images and were assigned to the study roster such that each participant was scanned by a single technician over the duration of their study involvement. Thereafter, on each occasion that a muscle biopsy was performed, ultrasound images were captured on the contra-lateral leg using the same machine and by the same technician (Terason t3000, TeraTech Corporation, MA, USA). Five ultrasound images were captured at each time-point, at two different sites of the vastus lateralis. One site, which stayed constant over the four treatment days, was located at the midpoint of the thigh (Mid) between the anatomical landmarks of the distal ischemic bone and the anterior, superior patella. The second site tracked the location of the specific incision on the biopsied leg (Contra). The ultrasound procedures were performed according to MuscleSound® guidelines with images captured on the transverse plane at a depth of 4 cm and a gain of 45 with the muscle relaxed. The transducer head was manipulated to achieve a bright fascia which defined the muscle boundary for the region of interest. Images were then uploaded to the MuscleSound® software (v.2015, MuscleSound, LLC, Denver, CO) which provided a glycogen score (0-100) in arbitrary units (a.u.) (Nieman et al., 2015). Scores were provided in bands of 5 a.u. with a typical error of  $\pm 5$  a.u. Ultrasound scores for US-Contra and US-mid were obtained by averaging the score of the five images captured at each site.

### *Muscle Fuel Scores*

The ultrasound images were re-analysed using MuscleSounds updated algorithm (v 2017). The output from this analysis is termed the “Muscle Fuel Score” to differentiate it from glycogen score from the original analysis. Briefly, the “Muscle Fuel Score” is an estimated fuel level determined placing an image in context of the maximum (100) and minimum (0) percent obtained for a specific participant. The maximum and minimum values are determined from bank of images captured for that participant.

### *Statistical Analysis:*

Complex statistical models were used to determine the correlation and error of prediction for the ultrasound's ability to predict single muscle glycogen concentrations and changes in muscle glycogen concentration against the criterion biopsy method.

Specifically, scatterplots of glycogen concentration vs ultrasound score showed weak relationships that were best quantified with a simple linear model. Log transformation before analysis was deemed appropriate for glycogen concentration, since its values are positive and expected to show more uniformity of error when the error is expressed in percent rather than absolute units. The ultrasound score and Muscle Fuel Estimate were therefore also log-transformed. The analyses were performed with the Statistical Analysis System (version 9.4, SAS Institute, Cary, NC).

The utility of the ultrasound score and Muscle Fuel Estimate to measure glycogen concentration in single measurements of individuals was investigated with a novel analysis, in which the magnitude of the error in glycogen concentration predicted by the linear relationship was assessed by standardization. The prediction error includes error of measurement in glycogen concentration (see above), which was removed by subtracting the square of the error of measurement from the square of the prediction error and taking the square root. The appropriate between-subject standard deviation (SD) for standardization of this net prediction error was the SD of the predicted values of glycogen concentration, which represents the typical differences between subjects free of prediction and measurement error. This SD was derived by multiplying the between-athlete SD of the ultrasound score by the slope of the glycogen-ultrasound relationship. The standardized net prediction error was then derived by dividing the net error by the SD of the predicted values. Thresholds for trivial, small, moderate, large very large and extremely large values of the standardized error were <0.1, 0.1, 0.3, 0.6, 1.0 and 2.0 respectively, which are half the usual thresholds for interpreting magnitudes of differences in means (Smith & Hopkins, 2011). Confidence limits (at the 90% level) for the net prediction error and the standardized error were derived by a

combination of usual and parametric bootstrapping: the analysis was repeated for 3000 bootstrap samples, but for each sample a value of error of measurement of glycogen concentration was generated with a value chosen randomly from the chi-squared sampling distribution (by dividing the randomly-chosen value by the degrees of freedom for the error of measurement, taking the square root, then multiplying by the error of measurement). The slope of the linear relationship between glycogen concentration and ultrasound score (in percent per percent units) and the correlation between the variables were also derived via the usual linear modelling procedures applied to the original sample, but their magnitudes were not interpreted.

This analysis was performed first with the data for two conditions (baseline and glycogen-depleted) by treating the repeated measurements as if they came from different athletes. In this way the resulting range in glycogen concentration would be similar to that expected in athletes sampled before or after a hard training bout or endurance competition. The analysis was repeated for four conditions (Baseline, Glycogen Depleted, Glycogen Loaded) and for all six conditions (Baseline, Glycogen Depleted, Placebo, Creatine Loaded, Glycogen Loaded, Glycogen-Creatine Loaded). All analyses were also performed separately for biopsies sampled from the two sites (Contra and Mid).

A similar novel analysis was performed to investigate the utility of the ultrasound score and Muscle Fuel Estimate to measure changes in glycogen concentration within individuals for the same three combinations of conditions and biopsies from the two sites. A mixed linear model was used to predict glycogen concentration, with the ultrasound score/Muscle fuel estimate as a simple linear fixed effect and athlete identity as a random effect. This model effectively fits a straight line to the data, with the same slope but a different intercept for each athlete. To display graphically what this analysis achieves, each subject's values for glycogen concentration and ultrasound score were rescaled to a mean of zero and then plotted on one set of axes. The line of best fit for this scatterplot is effectively the line



provided by the fixed effect in the mixed model. The SD of the residuals in the mixed model is the error in changes in glycogen concentration predicted by changes in ultrasound score or changes in the Muscle Fuel Estimate. As above, the net prediction error was obtained by removing error of measurement in glycogen concentration, and the magnitude of the error was standardized with the SD of glycogen concentration predicted by multiplying the slope of the relationship by the between-athlete SD in ultrasound scores and Muscle Fuel Estimate. The SD of ultrasound scores and the Muscle Fuel Estimates was the overall SD of all athletes from all treatments, to represent the typical differences between athletes in a randomly chosen sample from randomly chosen treatments. Bootstrapping was performed by generating random samples of athlete identities, to retain the repeated-measures structure of the data.

## 7.4 Results

Baseline muscle biopsy concentrations, ultrasound scores and the mean percent change from baseline for each measurement under the different treatment conditions are presented in **Table 7.2**. The glycogen depleting exercise had the largest effect on changing muscle glycogen with a mean decrease of 330.6 mmol/kg d.w. from baseline. There were moderate substantial increases of 104.4 and 156.6 mmol/kg d.w. in muscle glycogen following glycogen loading with and without creatine loading respectively. There was no effect of creatine loading on muscle glycogen concentrations.

**Table 7.2.** Muscle glycogen concentrations and ultrasound scores at baseline and percent changes from baseline for all treatments. Data are mean  $\pm$  SD. Also shown are magnitude-based inferences for the changes from baseline.

		Changes from baseline (%)					
Measure	Baseline (n=12)	Smallest important (n=12)	Glycogen depleted (n=11)	Normal glycogen (n=7)	Glycogen loaded (n=6)	Creatine loaded (n=5)	Glycogen- Creatine loaded (n=5)
Muscle glycogen	580 $\pm$ 140						
	mmol/kg d.w.	1.9	-57 $\pm$ 42 $\downarrow$ ***	-7 $\pm$ 26	18 $\pm$ 25 $\uparrow$ ***	7 $\pm$ 43	27 $\pm$ 35 $\uparrow$ ***
Ultrasound contra	55 $\pm$ 10						
	units	3.9	-4 $\pm$ 15 $\downarrow$ *	-3 $\pm$ 11	3 $\pm$ 18	4 $\pm$ 6 <sup>00</sup>	10 $\pm$ 9 $\uparrow$ **
Ultrasound mid	56 $\pm$ 9						
	units	4.2	-9 $\pm$ 17 $\downarrow$ **	-1 $\pm$ 11	3 $\pm$ 14	4 $\pm$ 6 <sup>00</sup>	7 $\pm$ 10 $\uparrow$ *

Contra, images captured at the site contralateral to the biopsy; mid, images captured at the midpoint of the thigh.  
, \*possible, \*\*likely, \*\*\*very or most likely clear substantial increase ( $\uparrow$ ) or decrease ( $\downarrow$ ).  
<sup>00</sup>likely, <sup>000</sup>very or most likely clear trivial change.

Changes in muscle glycogen concentration measured by the ultrasound technique were less clear. The Contra and Mid sites showed small but substantial increases of 5.5 a.u. and 3.9 a.u. in ultrasound scores respectively following the combined Glycogen-Creatine Loading condition. No change in ultrasound scores were observed following the Creatine Loading condition. The Glycogen Depleted condition resulted in small but substantial decreases of 2.2 a.u. and 5.0 a.u. for images captured at the Contra and Mid sites respectively. No substantial increases in ultrasound score were observed following the Glycogen Loaded condition. The changes in the Muscle Fuel Estimate across the various conditions are not provided as they were not able to be determined by the statistical model, meaning that errors or effect sizes could not be calculated.. The Muscle Fuel Estimate values provided could not estimate errors or effects.

The scatterplot for predicting single muscle glycogen measurements from ultrasound showed a moderate relationship for the Baseline and Glycogen Depleted conditions at the mid ultrasound site (**Figure 7.1**). When all conditions were assessed the relationship ranged from

moderate to poor across both ultrasound sites (**Table 7.3**). The error in predicting muscle glycogen from ultrasound varied from unclear to extremely large at (**Table 7.3**).

**Table 7.3.** Linear relationships for prediction of single glycogen concentrations and changes in glycogen concentration by ultrasound scores derived with data from different conditions for different biopsy sites. Data in parentheses are 90% confidence limits.

Data analysed	Biopsy site	Correlation	Slope (%/%)	Error of the estimate <sup>d</sup>		
				Percent units	Standardized <sup>c</sup>	Magnitude
<b>Prediction of single concentrations</b>						
Two conditions <sup>a</sup>	Contra	0.17	0.42	71	5.6	Unclear
		(-0.20, 0.49)	(-0.49, 1.32)	(45, 89)	(-22, 26)	
	Mid	0.32	0.73	68	2.9	Very large to extremely large
		(-0.05, 0.59)	(0.06, 1.59)	(41, 85)	(1.0, 12)	
Four conditions <sup>b</sup>	Contra	0.10	0.23	62	9.0	Unclear
		(-0.18, 0.37)	(-0.35, 0.73)	(39, 79)	(-42, 44)	
	Mid	0.20	0.43	61	4.5	Unclear
		(-0.08, 0.46)	(-0.14, 1.04)	(31, 77)	(-19, 26)	
Six conditions <sup>c</sup>	Contra	0.17	0.40	56	5.3	Unclear
		(-0.08, 0.40)	(-0.07, 0.88)	(35, 73)	(-12, 28)	
	Mid	0.26	0.57	54	3.3	Very large to extremely large
		(0.02, 0.48)	(0.06, 1.12)	(33, 70)	(1.3, 14)	
<b>Prediction of changes in concentration</b>						
Two conditions <sup>a</sup>	Contra	0.22	0.42	71	5.6	Unclear
		(-0.14, 0.53)	(-0.22, 1.46)	(50, 89)	(-21, 25)	
	Mid	0.42	0.73	68	2.9	Very large to extremely large
		(0.08, 0.67)	(0.23, 1.87)	(45, 87)	(1.2, 9.8)	
Four conditions <sup>b</sup>	Contra	0.24	0.24	62	8.7	Unclear
		(-0.04, 0.49)	(-0.32, 1.26)	(44, 72)	(-30, 31)	
	Mid	0.46	0.48	59	4.0	Unclear
		(0.20, 0.65)	(-0.02, 1.59)	(39, 70)	(-8.1, 21)	
Six conditions <sup>c</sup>	Contra	0.31	0.40	56	5.3	Extremely large
		(0.07, 0.51)	(0.09, 1.38)	(40, 67)	(2.0, 18)	
	Mid	0.48	0.57	54	3.3	Very large to extremely large
		(0.26, 0.65)	(0.21, 1.65)	(37, 65)	(1.3, 8.5)	

<sup>a</sup>Baseline, glycogen depleted.

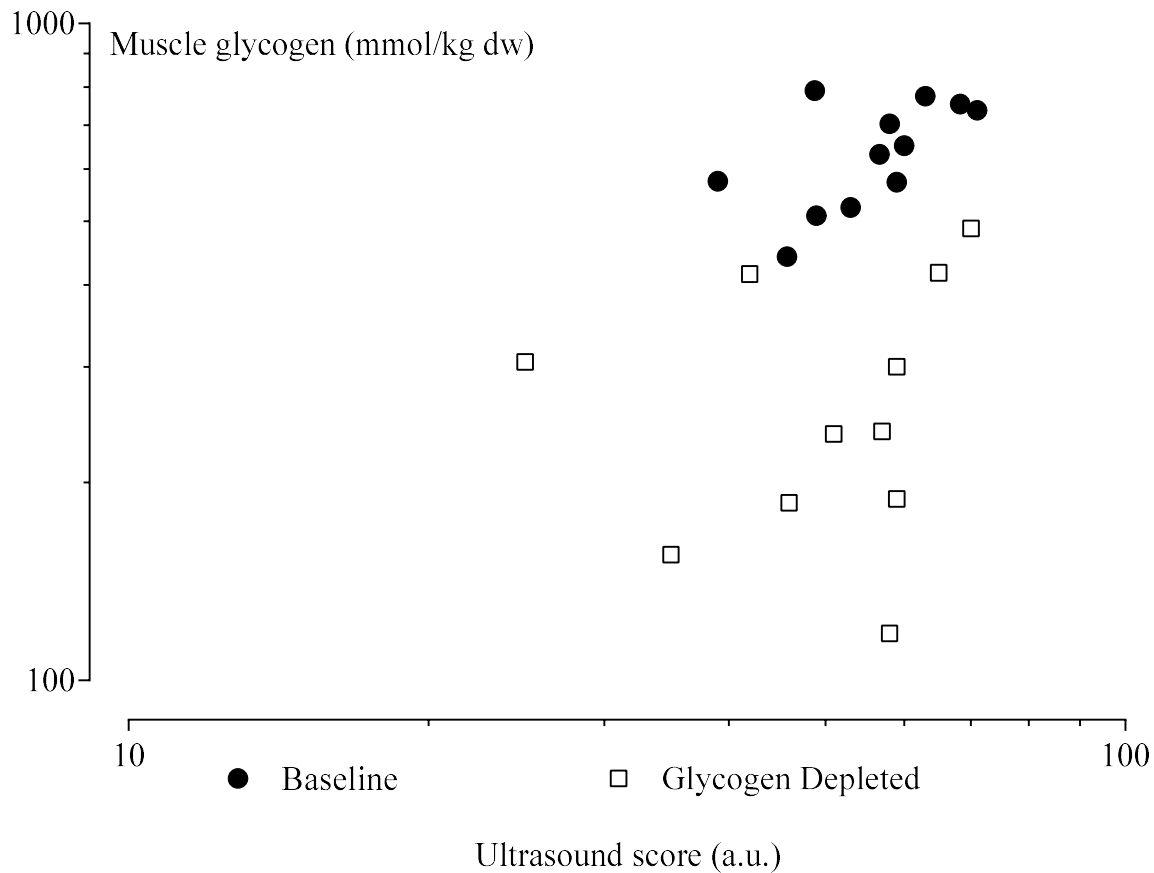
<sup>b</sup>Baseline, glycogen depleted, placebo, glycogen-loaded.

<sup>c</sup>Baseline, glycogen depleted, placebo, glycogen -loaded, creatine-loaded, glycogen – creatine loaded.

<sup>d</sup>Free of error of measurement in glycogen concentration.

<sup>e</sup>Thresholds for small, moderate, large, very large, and extremely large errors: 0.1, 0.3, 0.6, 1.0, and 2.0 respectively.

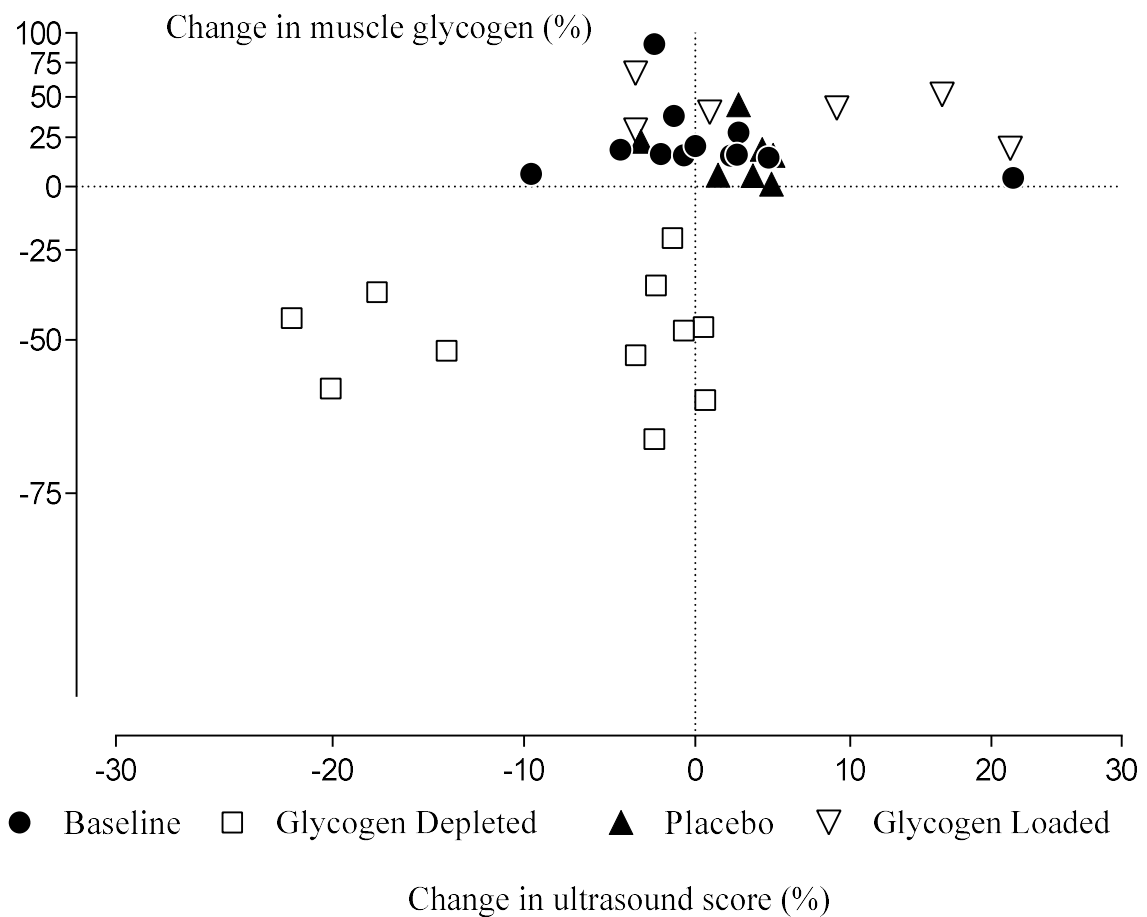
Contra, images captured at the site contralateral to the biopsy; mid, images captured at the midpoint of the thigh



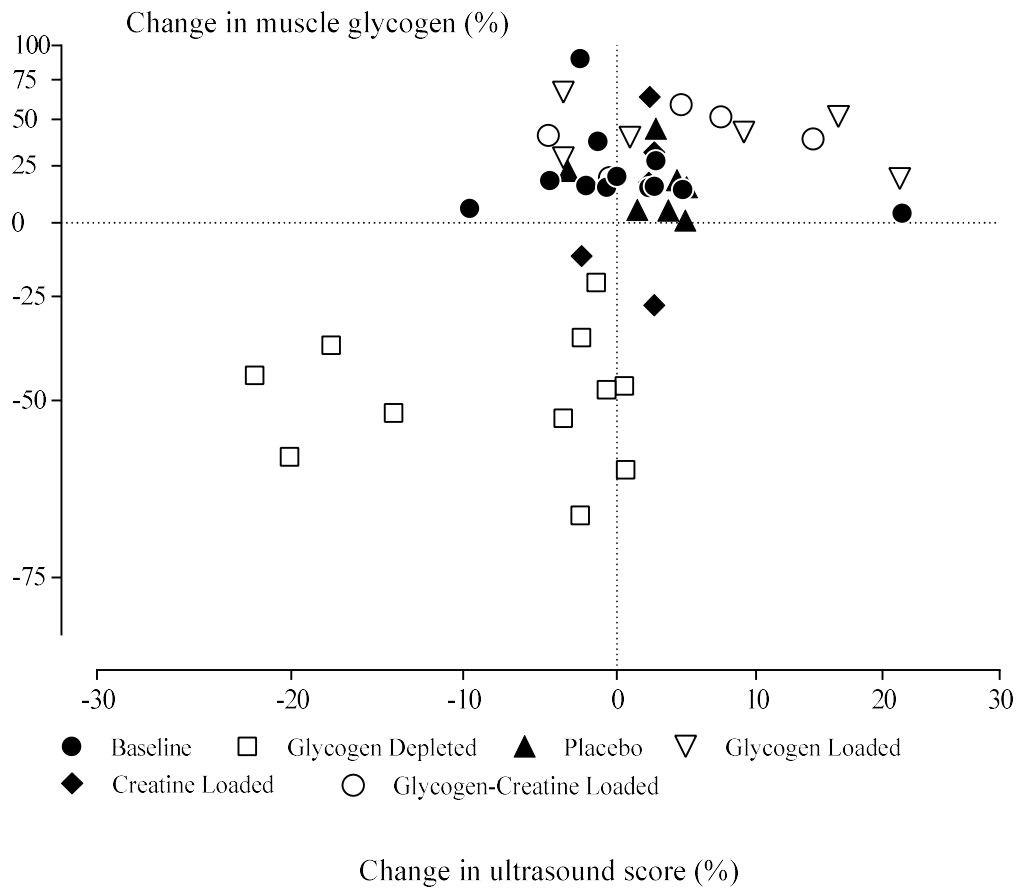
**Figure 7.1.** Cross-sectional relationship between biopsy muscle glycogen and ultrasound score measured at the mid-thigh site on the contralateral leg. Data shown are combined from the baseline and depleted conditions. Axis are log scales.

**Figure 7.2** shows a moderate relationship between the observed changes in biopsy derived muscle glycogen compared with the ultrasound score for all non creatine treatment conditions at the Mid ultrasound site. The magnitude of the error for predicting changes in muscle glycogen from the ultrasound showed similar magnitude of errors as the single measurements across all sites (**Table 7.3**).

The inclusion of the Creatine Loaded and Glycogen–Creatine Loaded conditions into the linear and mixed models did not change the relationship between the biopsy measurement and the ultrasound score (**Figure 7.3**). The magnitude of the prediction errors also remained the same (**Table 7.3**).



**Figure 7.2** Comparison of change in glycogen status between measured glycogen concentration and ultrasound score from the mid-thigh on the contralateral leg for baseline, glycogen depleted, placebo and glycogen loaded treatment conditions. Participants' means have been rescaled to means of zero. Axis are log scales.



**Figure 7.3.** Comparison of the change in glycogen status between biopsy glycogen concentration and ultrasound score from the mid-thigh site on the contralateral leg for all treatment conditions. Participants' means have been rescaled to means of zero. Axis are log scales.

The correlation between the updated Muscle Fuel Estimate and the biopsy glycogen was greater for both sites for the four and six conditions compared to original ultrasound scores (**Table 7.4**). However, the error in the prediction remained either Unclear or ranged from Very Large to Extremely Large. (**Table 7.4**). The error in the prediction also remained either unclear or very large to extremely large when the updated muscle fuel estimate predicted the change in muscle glycogen levels between the conditions. The scatterplots showing the relationships have been provided in the supplementary data appendix (Chapter 12).

**Table 7.4.** Linear relationships for prediction of single glycogen concentrations and changes in muscle fuel estimates derived with data from different conditions for different biopsy sites. Data in parentheses are 90% confidence limits.

Data analyzed	Biopsy site	Magnitude	Correlation	Slope (%/%)	Error of the estimate <sup>d</sup>		
					Percent Units	Standardized <sup>e</sup>	Magnitude
Prediction of single concentrations							
Two conditions <sup>a</sup>	Contra	Unclear	0.16 (-0.20, 0.48)	0.30 (-0.37, 0.96)	71.5 (42, 89)	5.8 (-21.7, 25.9)	Unclear
	Mid	Very large to extremely large	0.22 (-0.14, 0.53)	0.21 (-0.13, 0.55)	70.3 (42, 88)	4.2 (-4.6, 19.8)	Unclear
Four conditions <sup>b</sup>	Contra	Unclear	0.24 (-0.04, 0.49)	0.43 (-0.08, 0.93)	59.8 (35, 77)	3.7 (-13.5, 19.5)	Unclear
	Mid	Unclear	0.31 (0.03, 0.54)	0.31 (0.15, 0.54)	58 (33, 75)	2.8 (1.4, 6.8)	Very large to extremely large
Six conditions <sup>c</sup>	Contra	Unclear	0.28 (0.03, 0.48)	0.48 (0.06, 0.90)	53.8 (32, 71)	3.2 (-4.5, 15.8)	Unclear
	Mid	Very large to extremely large	0.36 (0.12, 0.55)	0.35 (0.12, 0.58)	51.5 (29, 58)	2.4 (1.2, 4.7)	Very large to extremely large
Prediction in changes in concentration							
Two conditions <sup>a</sup>	Contra	Unclear	0.26 (-0.11, 0.56)	0.30 (-0.37, 0.96)	71.5 (47, 89)	5.8 (-22.5, 29.3)	Unclear
	Mid	Very large to extremely large	0.38 (0.02, 0.64)	0.21 (-0.13, 0.55)	70.3 (46, 89)	4.2 (1.5, 17.2)	Very large to extremely large
Four conditions <sup>b</sup>	Contra	Unclear	0.31 (0.03, 0.54)	0.43 (-0.06, 0.93)	59.4 (40, 72)	3.7 (-5.6, 15.9)	Unclear
	Mid	Unclear	0.43 (0.17, 0.61)	0.32 (0.05, 0.59)	56.3 (34, 70)	2.7 (1.2, 5.8)	Very large to extremely large
Six conditions <sup>c</sup>	Contra	Extremely large	0.34 (0.10, 0.54)	0.48 (0.06, 0.90)	53.8 (37, 66)	3.2 (1.4, 12.1)	Very large to extremely large
	Mid	Very large to extremely large	0.47 (0.25, 0.64)	0.36 (0.13, 0.59)	50.8 (31, 64)	2.3 (1.1, 3.9)	Very large to extremely large

<sup>a</sup>Baseline, glycogen depleted.

<sup>b</sup>Baseline, glycogen depleted, placebo, glycogen-loaded.

<sup>c</sup>Baseline, glycogen depleted, placebo, glycogen -loaded, creatine-loaded, glycogen – creatine loaded.

<sup>d</sup>Free of error of measurement in glycogen concentration.

<sup>e</sup>Thresholds for small, moderate, large, very large, and extremely large errors: 0.1, 0.3, 0.6, 1.0, and 2.0 respectively.

Contra, images captured at the site contralateral to the biopsy; mid, images captured at the midpoint of the thigh

## 7.5 Discussion

This is the first independent study to investigate the validity of ultrasound technology (MuscleSound®) to assess muscle glycogen content with the added novelty of undertaking the investigation over a large range of variations in muscle solute and water content. We found poor correlation between the criterion (biopsy derived) assessments of muscle glycogen and the ultrasound derived estimates, both for absolute glycogen concentrations and for changes due to glycogen loading or exercise depletion. Our results confirmed the hypothesis that large changes in muscle glycogen and/or change in muscle water or solutes unrelated to muscle glycogen would challenge the ability of MuscleSound® to track muscle glycogen over the ranges that occur in sports nutrition practice. The poor correlations seen in our study may reflect the failure of the presently available algorithms, derived from studies of exercise-associated glycogen depletion, to have relevance to the wider manipulation of the muscle content of glycogen, water and creatine achieved in the present study. It may also reflect some limitations of the present study design or execution. However, the principle that muscle glycogen concentrations can be estimated from changes in the grey scale of ultrasound images as a surrogate for muscle water content is also challenged, particularly when muscle water content is changed by practices that are unrelated to glycogen stores.

The ultrasound technique for assessment of muscle glycogen investigated in this study is a commercially available product that involves the capture of a high-frequency ultrasound image which is then processed using proprietary cloud-based software to produce a glycogen score in arbitrary units (a.u.) (Hill & Millan, 2014; Nieman et al., 2015). The image greyscale produced on an ultrasound scan is based on the intensity of the ultrasound “echo” or reflection of an ultrasound beam, with the beam being both produced and detected by the transducer forming the ultrasound image (Aldrich, 2007). Ultrasound beams are reflected at the boundary between two materials with different acoustic impedances with strong reflections showing as white on the ultrasound image and weaker echoes being grey (Aldrich, 2007).



The boundary between soft tissue and water has an echo or reflection of 0.2%, while the boundary between muscle and fat is 1.08%. The boundary between soft tissue and air reflects 99% of the beam (Aldrich, 2007). Proprietary information within the MuscleSound® software aligns a darker image with greater glycogen stores using the principal that greater glycogen and its associated water content in the muscle fibre should reflect the lower echo intensity between soft tissue and water (Aldrich, 2007; Nieman et al., 2015). Conversely, in instances when glycogen is low and there is less fluid, the echo intensity is greater due to increased visibility of other tissue boundaries resulting in a brighter image (Aldrich, 2007; Nieman et al., 2015).

This process of identifying glycogen based on its bound fluid assumes that the relationship between muscle glycogen and bound water is constant. Although it is well accepted that fluid is stored when glycogen is formed, the persistence of the ratio over a range of glycogen concentrations has been questioned (McBride et al., 1941; Sherman et al., 1982). A previous investigation using rat livers described a ratio of 1:2.7 for grams of glycogen to water when there is no change to non-glycogen solids (McBride et al., 1941). Meanwhile, Sherman et al. (Sherman et al., 1982) reported no gram to gram ratio could be calculated between glycogen and water in rat skeletal muscle. A human study that measured total body water and muscle glycogen following carbohydrate loading found a glycogen to water ratio ranging from 1:3 to 1:5 (Olsson & Saltin, 1970). Furthermore, a study in which cyclists were deliberately dehydrated by ~5% BM or 8% body water during 15 h of refuelling after cycling exercise found a lower muscle water content associated with glycogen storage compared with a trial involving euhydrated recovery (Neufer et al., 1991). Most recently, Shiose et al. (Shiose et al., 2016) assessed total body water by deuterium dilution and reported a ratio of ~1:4 following 72 h of carbohydrate loading. Thus, it is unlikely that a tight ratio between muscle water and glycogen actually exists, with uncertainty in the ratio being attributed to differences in non-glycogen solids, such as fat, protein or connective tissue (McBride et al.,

1941; Sherman et al., 1982) as well as alterations in body water content that are commonly seen in sport (Neufer et al., 1991).

Our findings contradict two previous validation studies (Hill & Millan, 2014; Nieman et al., 2015) that found strong correlations between values for pre-exercise, post-exercise and change scores in muscle glycogen between biopsy-derived and MuscleSound® measurement techniques (Hill & Millan, 2014; Nieman et al., 2015). Nieman et al (Nieman et al., 2015) reported a significant (0.92) correlation between these two measurement techniques for the change in muscle glycogen in the vastus lateralis following a 75 km cycling ergometer protocol lasting 2.8 h. We estimated the absolute decrease in muscle glycogen seen in that investigation study (~ 307 mmol/kg dry weight (d.w.) (based on a conversion factor of 4.28 between wet and dry weight measurements (Hawley et al., 1997) ), was slightly smaller than the change seen in the current study (330 mmol/l kg d.w.). Significant correlations between the two measurements techniques for pre (0.92,  $p<0.001$ ) and post (0.90,  $p<0.001$ ) exercise glycogen concentrations were also reported by Nieman and colleagues. Although the absolute pre- and post-exercise muscle glycogen concentrations were not provided, they were estimated from graphical representations to be ~397 and 90 mmol/kg d.w., respectively (Nieman et al., 2015).

Meanwhile, in the second study, Hill et al. reported a correlation of 0.81 ( $p<0.0001$ ) between change scores of biopsy-derived and ultrasound measures of glycogen concentration of the rectus femoris following 90 min of laboratory-based cycling (Hill & Millan, 2014). They reported pre and post exercise muscle glycogen values of 418 and 268 mmol/kg d.w, with significant correlations of 0.93 ( $p<0.0001$ ) and 0.94 ( $p<0.0001$ ) between techniques for each respective time point. Their glycogen loading protocol instructed participants to consume a diet providing a carbohydrate intake of 8 g/kg BM/d for three days prior to the exercise session (Hill & Millan, 2014), achieving a mean pre-exercise glycogen concentration that was ~ 150 mmol/kg d.w. less than our baseline values. Furthermore, the pre- to post-

exercise decrease of 149 mmol/kg d.w. was smaller than that seen in both the current study and that of Nieman and colleagues (Nieman et al., 2015), and corresponded to a 20 a.u. decrease in the ultrasound glycogen score (Hill & Millan, 2014). Hill and Millan (Hill & Millan, 2014) stated that the attenuated glycogen depletion was because of the high fitness levels of their athletes; however, we note also the shorter duration of the cycling bout of 1.5 h.

The contradictory results of our studies might be attributed to a number of factors. Our study manipulated absolute values of muscle glycogen over a wider range than seen in the previous studies (from ~155 mmol/kg d.w. following exercise depletion to 843mmol/kg d.w. following glycogen loading) as well as incorporating another dietary strategy known to alter muscle solute and water content. The MuscleSound<sup>®</sup> technique appeared to poorly track the decrease in muscle glycogen with exercise depletion, showing mean changes of -2.2 and -5.0 a.u for contra and mid legs respectively. Indeed, since the unit of scale for the MuscleSound<sup>®</sup> ultrasound score was 5 a.u., this technique appeared to offer a blunt technique to monitor the decrease in muscle glycogen content achieved in this study. Furthermore, the change in muscle glycogen from “normalised” to “loading” was clearly evident from the direct (biopsy-derived) measures of glycogen, but failed to show a substantial change in MuscleSound<sup>®</sup> scores. Only in the glycogen loading trial with prior creatine supplementation trial, did the MuscleSound<sup>®</sup> technique show a trivial and likely possible increase in the ultrasound score.

It is well documented that creatine supplementation acutely increases body mass (~1 kg) with the change being attributed mostly to water weight (Dalbo et al., 2008; Deminice et al., 2016; Powers et al., 2003). Indeed, as an osmotic solute, creatine pulls water into the muscle cell increasing intracellular fluid, and subsequently, total body water (Dalbo et al., 2008). Results from a separate part of this study observed that our creatine loading protocol increased muscle creatine by 6%; this is a smaller relative change than the 22% changes in muscle glycogen due to carbohydrate loading seen in the same study (Bone et al., 2017).

Changes in total body water and intracellular water of 1.3 and 1.4% (Creatine Loaded), and 2.3 and 2.2% (Glycogen Loaded) were also detected (Bone et al., 2017). As such, based on the theory underpinning the use of ultrasound to track muscle glycogen changes, creatine loading should also cause the echo intensity to decrease and produce a darker image (Aldrich, 2007; Nieman et al., 2015). Therefore, such changes in non-glycogen solutes and their effect on muscle water might need to be separately included in the glycogen score algorithm to allow it to become a valid surrogate measure of muscle glycogen in a greater number of scenarios. Accumulation of additional data, such as that collected in the present study might assist in the evolution of a more robust algorithm that is able to account for a greater range of perturbations in muscle solute and water content as achieved in this study and seen in everyday sports nutrition practice.

As in all studies, there is a requirement to consider limitations in the study design, methodology and researcher expertise in explaining results that are incongruent with the current literature. Therefore the possibility that our protocols failed to achieved the desired manipulation of muscle glycogen need to be addressed. The close supervision of the exercise task to achieve glycogen depletion, and the provision of tailored pre-packaged meals to achieve the various levels of glycogen repletion is regarded as best practice in achieving dietary control (Jeacocke & Burke, 2010). Indeed, the data reported in this paper were collected using standard techniques by individuals who were familiar with the protocols required. Furthermore, the results from these protocols fit the expected changes of water/glycogen increase with glycogen loading, and water/glycogen decrease with prolonged exercise. We acknowledge that, due to the design of the larger study, the biopsy capturing exercise-derived glycogen depletion was not taken directly after exercise, but was instead taken the following morning. However, we minimised the potential for glycogen storage by providing participants with a low carbohydrate diet ( $<1$  g/kg body mass) for the intervening period, and the biopsy derived measurement of vastus lateralis glycogen showed values that

were substantially lower than pre-exercise concentrations. Nevertheless, there is the possibility that other changes occurred in the muscle during this recovery period that were not present in the previous studies; for example the hepatic resynthesis of glucose and glycogen from the lactate produced during exercise (Hermansen & Vaage, 1977).

In addition, we note the limitations of deriving correlations between two measured variables to provide a validation of one of these measures. Although it is a criterion method, biopsy derived measures of glycogen concentration can be variable as noted by the technical error of measurement of 22%. Incorporating the biopsy variability into the statistical analysis standardized the data and reduced the error in the prediction of muscle glycogen from the MuscleSound score. Furthermore, the confidence limits for the single measurements are trustworthy only to the extent that the sample of values is reasonably representative of the values that would be obtained if single measurements were taken from a random sample of athletes. The confidence limits for the analysis of changes in glycogen concentration are more trustworthy, because the repeated measurements have been properly accounted for.

Finally, we note the possibility that despite the training and practice undertaken by our research team, that we did not master the techniques needed to optimise the use of the MuscleSound® technology. However, we note that the protocol has been developed for practical application in the sports environment in which a range of nutrition, medical or fitness practitioners might undertake the ultrasound images under field conditions. Therefore, we undertook a greater degree of standardization of the training of technicians and the protocol of image capture than might be expected in the real life conditions to which the MuscleSound® technology is at least partly directed.

In conclusion, we acknowledge the exciting potential and value of having a relatively inexpensive, portable and non-invasive method to measure muscle glycogen in sports nutrition research and practice. However, our study failed to find accurate estimations of

muscle glycogen concentrations, in absolute concentrations or to note change to either increase or deplete muscle stores, when an ultrasound-based technique (MuscleSound<sup>®</sup>) was compared to biopsy derived measures of glycogen. This may be both a problem of the underlying principles of the technique as well as the failure of currently available algorithms to cover a larger range of changes in muscle glycogen, or other manipulations of muscle solutes and water, than are often seen in sports nutrition practice. It may also be a fault of the current study design and execution, although we followed similar principles to other investigators who have undertaken such validation studies.

## Preface to Chapter 8

The results of Chapter 7 (*Study 4*) indicate that estimates of muscle glycogen concentration by ultrasound are not valid. However, in Chapter 6 (*Study 3*), changes in TBW mirrored the changes observed in muscle glycogen concentration and DXA-derived measurements of LM. Therefore, it may be possible to use changes in TBW as a tool to indicate when DXA artefacts of LM caused by changes in muscle glycogen and fluid have occurred.

It may be possible to “correct” for this artefact by applying a transformation factor, to DXA-derived measurements of LM in known situations where exercise or dietary intake has likely to have caused a change in intramuscular glycogen and water.

The real-life implications of the error in body composition assessment induced by manipulations of intra-cellular glycogen and fluid on the interpretation of relative REE have yet to be explored. One such scenario in which this could occur is in the athlete who has adopted a ketogenic low carbohydrate high fat (LCHF) diet. A large study on the effects of LCHF diet on the endurance performance of male race walkers provided a convenience sample to explore these concerns. Thus, the following chapter uses the results and outcomes from the previous chapters of this thesis to investigate not only if adaptation to a ketogenic LCHF diet affects REE but also whether the artefact in DXA estimates of LM due to glycogen and fluid affects the interpretation of results of REE in elite male athletes.

## **8.0 Study Five: Measurement of resting energy expenditure following a low carbohydrate high fat diet is affected by artefacts in DXA measurement of lean mass**

This chapter is comprised of the following manuscript under preparation to be submitted to *PLoS One*:

Bone, JL, Ross, ML, Welvaert, M, Burke, LM

Measurement of resting energy expenditure following a low carbohydrate high fat diet is affected by artefacts in DXA measurement of lean mass.

This study was funded by a grant from the Australian Catholic University Research Fund awarded to Prof Louise M Burke.



## 8.1 Abstract

### *Background:*

Variations in muscle glycogen concentration caused by exercise and/or changes in dietary intake can influence dual energy x-ray absorptiometry (DXA) estimates of lean mass (LM) and the related measure of fat free mass (FFM). Since resting energy expenditure (REE) is typically assessed relative to FFM, artefacts in measurements of LM could potentially affect the interpretation of such assessments. Low carbohydrate high fat diets (LCHF) which are associated with chronic reductions in muscle glycogen have remerged as a putative performance aid for endurance and ultra-endurance sports. The aim of this study was to investigate if adaptation to such a diet affects REE in elite athletes, and whether artefacts in DXA-derived measures of FFM interfere with the investigation of this issue.

### *Methods:*

Nineteen elite male race walkers completed a 21 d dietary intervention in conjunction with intensified training. Athletes were grouped into either LCHF intervention (n=9) or an energy-matched control (CHO) diet (n=10) for the duration of the study. Body composition by DXA, total body water (TBW) and REE were measured at Baseline and Post-Intervention. Estimates of FFM were corrected with a transformation factor in individuals who showed meaningful changes in TBW consistent with glycogen depletion. Linear mixed modelling was used to assess if there were changes in REE at baseline and post-intervention, both with and without the correction factor for FFM.

### *Results:*

There were significant decreases in mean FFM (-1.4; 95% CI -2.0, -0.80 kg) and TBW (-2.9; -3.9, 0.5 L) between baseline and non-corrected post intervention values in the LCHF group. No changes were observed in FFM when corrected for loss of muscle glycogen and fluid. There was no change in REE in the CHO group using both the corrected and

uncorrected FFM values. In the LCHF group, there was a significant decrease in REE compared to baseline with the corrected values; however, this was not apparent when baseline values were compared with uncorrected post-intervention values.

*Conclusion:*

A reduction in relative REE was observed following a 21 d adaptation to a LCHF diet in elite racewalkers while REE remained stable in a control group of athlete. This effect was only detected when a correction factor was applied to DXA estimates of FFM which were affected by an artefact associated with reduced glycogen stores. Failure to account for this artefact would have implications for the detection and management of suppressed metabolic rate and low energy availability in such a scenario.

## 8.2 Introduction

Low energy availability (LEA), defined as a failure of energy intake to cover the energy cost of supporting a wide range of body functions once the energy cost of exercise is taken into account, is associated with impairment of health and athletic performance (Mountjoy et al., 2014). The causes of this energy mismatch in athletes include disordered eating, overzealous weight loss programs, and inadequate knowledge or ability to consume sufficient food to cover the cost of a high volume training/competition regimen. LEA is associated with, and can be identified via a reduction in metabolic rate, as long as there is confidence in the reliability of measurement of resting energy expenditure (REE) and the absence of artefacts that could alter the interpretation of results. Considering LEA and REE are assessed relative to fat free mass (FFM), acute changes in muscle glycogen and associated water storage, achieved through exercise and glycogen loading, is one such artefact that impacts estimates of lean mass (LM) by dual x-ray absorptiometry (DXA) (Bone et al., 2017) and subsequently effects interpretation of LEA and/or relative-REE. This DXA artefact has also been observed in healthy untrained populations (Rouillier et al., 2015; Toomey et al., 2017). Another scenario where this DXA artefact effects LEA or relative-REE may occur would be in athletes who follow a ketogenic low carbohydrate high fat (LCHF) diet. The re-emergence of claims that a low carbohydrate high fat (LCHF) diet can enhance endurance/ultra-endurance performance (Noakes et al., 2014; Phinney, 2004; Volek et al., 2016) has brought it back into consideration in sports nutrition practice. Notwithstanding evidence to refute these claims in terms of endurance performance {Burke, 2016 #531}, there is a need to explore its effects on other health issues and body function. Whether such extreme changes in macronutrient intake within a controlled energy intake have an effect on REE is one such issue.

Measuring REE in subjects who follow a LCHF diet presents a potential methodological challenge as well as a physiological interest. Indeed, there is reason to expect

that chronic intake of a LCHF, which results in a decrease in muscle glycogen stores (Phinney et al., 1983) similar to the decrease observed through acute glycogen depletion (Bone et al., 2017), may cause an artefact in the estimation of the lean mass (LM) component of FFM. A reduction in the estimates of FFM due to the artefact of reduced muscle glycogen and fluid could potentially interfere with the interpretation of relative REE, and identification of LEA in populations who follow a ketogenic LCHF diet. Accordingly, the aim of the present study was to measure REE in athletes in response to a 21 day adaptation to a LCHF diet and intensified training program, using standardised practices that have been shown to enhance the reliability of REE assessment. To assist in the interpretation of results, we intended to incorporate the results of our previous work which identified the magnitude of the artefact caused by the reduction of muscle glycogen and associated muscle water.

### 8.3 Methods

*Participants:*

A convenience sample of 19 elite male race walkers provided written informed consent prior to study commencement. This study was approved by the Australian Institute of Sport Human Resource Ethics Committee. Participant characteristics are presented in **Table 8.1**

**8.1**

**Table 8.1.** Participant descriptive characteristics presented as mean  $\pm$  standard deviation (SD).

	All (n=19)	LCHF (n=9)	CHO (n=10)
Age (y)	26.9	28.8	25.5
Height (cm)	179.7	180.6	179.0
Body mass (kg)	68.3	68.4	68.2

LCHF: Low carbohydrate high fat diet group; CHO: carbohydrate diet group (control)

### *Study design:*

This study implemented a non-randomised parallel group design to investigate the effect of dietary interventions during a four week training camp. There were nine participants in the intervention group and eleven controls. All participants underwent the same training schedule and physical testing.

### *Dietary intervention:*

The two groups followed a 21 d dietary intervention in which all meals and snacks were provided to participants and consumed under supervision. The ketogenic LCHF diet followed by the intervention group was modelled on the dietary principles of Volek & Phinney (2012), with fat providing 75-80% of energy intake, carbohydrate intake being restricted to <50 g/d, and protein intake being limited to 2.2 g/kg BM (~16% of energy). The control group followed an energy- and protein-matched diet in which 65% of energy was provided by carbohydrate. Details of the logistics of achieving and monitoring the diets are provided elsewhere (Mirtschin et al. *in review*).

### *Indirect calorimetry protocol*

Resting metabolic rate was measured using indirect calorimetry using an outpatient protocol (Bone & Burke, 2017). All measures were completed between 5:30-8:30am following an overnight 8 h fast and overnight rest from exercise. Upon arrival, participants lay supine for a 25 min rest period. Ten minutes into the rest, participants familiarised themselves with breathing through a mouthpiece. At the end of the rest period, participants put on a nose clip, a Douglas Bag was connected, and expirate was collected for 10 min periods in duplicate.

### *Determining REE*

The gas fractions for oxygen and carbon dioxide were determined using Ametek oxygen and carbon dioxide analysers (Ametek Inc, PA, USA). The Haldane transformation (Haldane, 1918) was used to determine oxygen consumption and carbon dioxide production. Laboratory temperature, pressure and humidity were recorded. Volume was measured using a 350 L Tissot spirometer (Warren Collins, Braintree, MA, USA). The Weir equation (Weir, 1949) was used to convert gas fractions to energy expenditure in kJ/min and extrapolated to 24 h to provide REE. The mean REE from the duplicate bags was calculated and divided by FFM to get relative REE (kJ/kg FFM/d).

### *Body composition:*

Body composition was assessed via DXA on the same morning as the REE measurement, following standardised protocols of subject presentation and placement on the scanning bed (Nana et al., 2012a). All scans were conducted on a GE Lunar iDXA (Madison, WI) and one technician operated the DXA and analysed all images using encore software (V16, GE, Madison, WI) (Nana et al., 2015). The technical error of measurement for the DXA was 0.1% for total mass, 0.4% for lean mass, 1.6% for fat mass and 0.4% for bone mass.

### *Total body water and hydration status:*

Total body water (TBW) was measured using bioelectrical impedance spectroscopy (BIS) (IMP SFB7, ImpediMed Limited, Queensland, Australia) and analysed using BioImp Analysis 5.4.0 Software (ImpediMed Limited, Queensland, Australia) as previously described (Bone et al., 2017). Briefly, participants lay supine for 15 min prior to triplicate measures being recorded. The mean of the measures were calculated and used for analysis. Hydration

status was monitored on the morning of the DXA scan by measurement of urine specific gravity (USG) from a first void mid-stream urine sample collected upon waking (UG-a, Atago Refractometer, Japan).

### *Data analysis*

Changes in TBW were used as a proxy for increases or decreases in muscle glycogen stores, based on previous work from our group in which we showed acute diet/exercise induced manipulations in muscle glycogen content was associated with changes in TBW and artefacts in DXA-derived estimates of LM (Bone et al., 2017). A transformation factor to correct for this DXA artefact was calculated using these data. Specifically, we noted that the proportion of the decrease in body mass attributed to LM following glycogen depletion was 0.80 (95% CI; 0.63, 0.99) whereas, the proportion of the increase in body mass attributed to LM was 0.71 (0.42, 1.01). The threshold where a change in LM was detected as meaningful corresponded to a decrease in TBW of 0.91 L and an increase of 1.07 L respectively.

The transformation factor was applied to Post-Intervention values based on the changes in TBW and BM compared to Baseline. A “negative” transformation factor to correct for the “decrease” of FFM due to muscle glycogen depletion was applied to all individuals who had a decrease in TBW greater than 0.91 L. Participants who had increases in TBW greater than 1.07 L had the “positive” transformation factor applied to correct for “increases” in FFM due to increased muscle glycogen. For the “negative” transformation the difference in BM was multiplied by 0.8 and added to the Post-Intervention DXA estimated FFM. For the “positive” transformation the difference in body mass was multiplied by 0.71 and subtracted that from the Post-Intervention DXA estimated FFM. No transformation factor was applied to participants whose change in TBW did not meet these thresholds. All data were pooled in their respective intervention groups and labelled Post-Intervention-Corrected<sub>threshold</sub> (Post-

IC<sub>threshold</sub>). This was then compared to Baseline and original Post-Intervention values for REE and FFM. To minimise bias, an additional group where the "positive" transformation factor was applied to all participants in the CHO group and the 'negative transformation factor' was applied to all participants in the LCHF group was included (Post-Intervention-Corrected<sub>all</sub> or Post-IC<sub>all</sub>). The Post-IC<sub>all</sub> FFM and REE were then compared to Baseline, Post-Intervention and Post- IC<sub>threshold</sub> values.

### *Statistics*

Normality for all data was visually assessed by Q-Q plots of the variable residuals and no significant deviations were detected. Linear mixed models, using an unstructured covariance matrix, were used to assess if there were difference between groups (diet), within groups over time (Baseline to Post-Intervention) and the diet-time interaction on all variables for FM, TBW, RER, FFM, absolute REE (kJ/d) and relative REE (kJ/kg FFM/d). Relative REE and FFM had additional comparisons against Post-IC<sub>threshold</sub> and Post-IC<sub>all</sub> values. Data presented are estimated means and 95% confidence intervals (CI) unless otherwise stated. All analysis was completed using SPSS (Version 23 for Windows, SPSS Inc, Chicago, IL)

## **8.4 Results**

Baseline and post intervention FM, TBW, USG, RER and absolute REE are presented in **Table 8.2**. With the exception of RER, there was no significant difference in any of these variables between the groups at baseline. There was no change in USG across time in either of the groups. Similarly, there was no difference in TBW in the CHO group over time. However, TBW was significantly lower by 2.2 L (95% CI -3.92, -0.47) in the LCHF group Post-Intervention compared to Baseline. RER was significantly lower in the LCHF group following the dietary intervention compared to baseline and against the CHO group. FM was



reduced by a small but significant amount in both groups over the 21 d. There were no significant effects of adaptation to either dietary intervention on absolute REE (kJ/d).

**Table 8.2.** Fat mass, total body water (TBW), urinary specific gravity (USG), respiratory exchange ratio (RER) and resting energy expenditure (REE) pre and post 21 day adaptation to LCHF or high CHO availability diets (estimated mean; 95% confidence interval).

	CHO		LCHF	
	Baseline	Post-Intervention	Baseline	Post-Intervention
TBW (L)	43.1 (39.8, 46.3)	43.1(40.1, 46.0)	42.7 (39.3, 46.2)	40.5 (37.4, 43.6) <sup>‡</sup>
USG (g/dL)	1.021 (1.018, 1.025)	1.020 (1.015, 1.024)	1.022 (1.018, 1.026)	1.023 (1.018, 1.026)
RER	0.88 (0.86, 0.91)	0.89 (0.87, 0.92)	0.83 (0.80, 0.86)*	0.80 (0.77, 0.82) <sup>†‡</sup>
FM (kg)	8.4 (7.3, 9.5)	7.3 (6.5, 8.1) <sup>‡</sup>	8.3 (7.2, 9.5)	7.3 (6.3, 8.2) <sup>‡</sup>
REE (kJ / d)	7450 (6897, 8003)	7349 (6855, 7842)	7488 (6905, 8071)	7233 (6712, 7753)

\*p<0.05 between groups at baseline; † p<0.05 between groups post-intervention ‡ p<0.05 compared to baseline.

**Table 8.3** shows the post intervention values for FFM before and after applying the transformation factor within each group. There was no effect of dietary adaptation on FFM in the control group. This did not change when the correction factor was applied to those individuals who met the threshold for correction (Post-IC<sub>threshold</sub>) or when transformation was applied to the whole CHO group (Post-IC<sub>all</sub>). In the LCHF group, FFM was significantly lower by 1.4 kg (95% CI -2.0, -0.80) following the intervention and prior to applying the transformation factor. When the transformation factor was applied to all participants or to only those participants whose TBW change met the threshold, the difference was no longer significant compared to baseline.

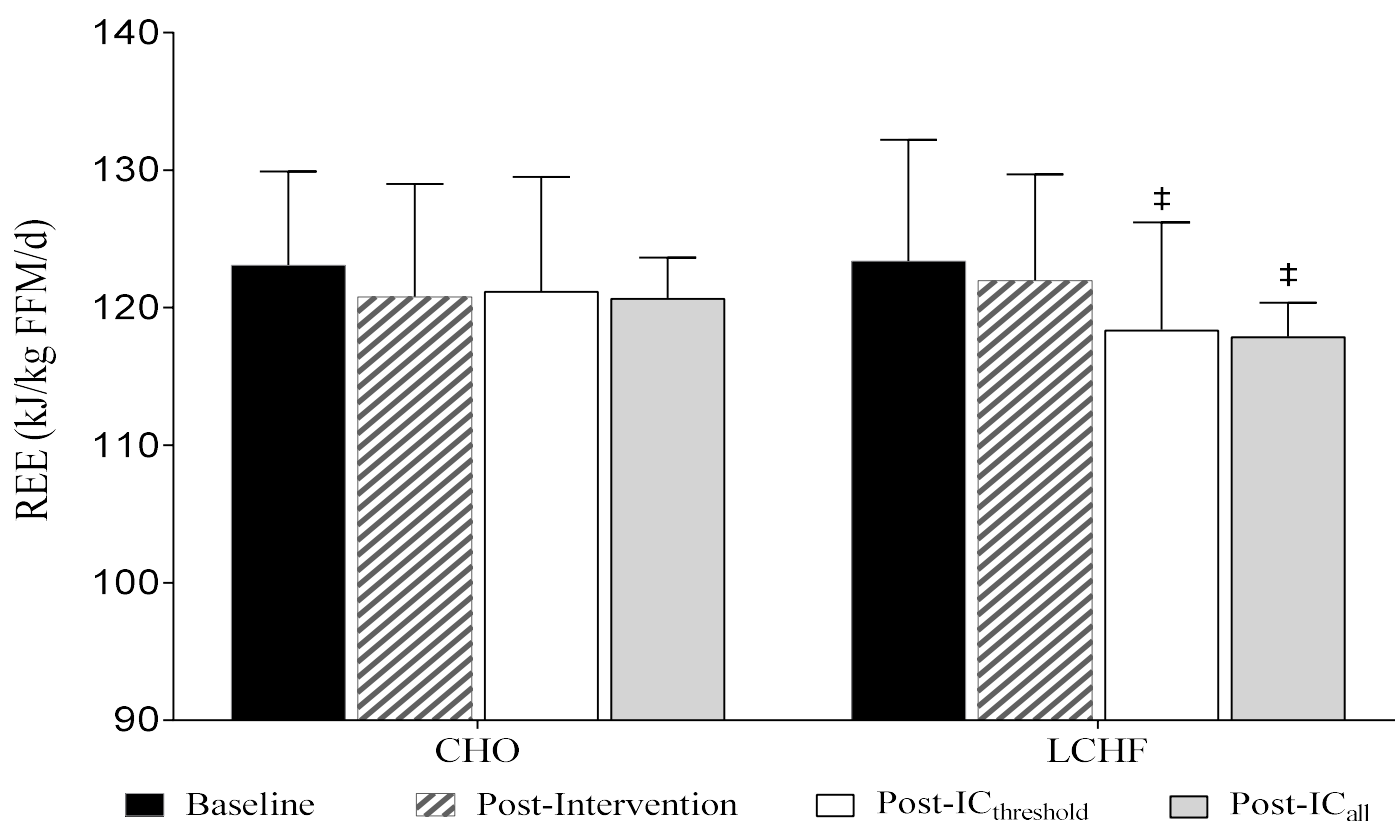
**Table 8.3.** Fat free mass (FFM) pre and post 21 day adaptation to LCHF or high CHO availability diets (estimated mean; 95% confidence interval).

	CHO	LCHF
Baseline	60.84 (57.25, 64.43)	60.76 (57.0, 64.5)
Post-Intervention	60.84 (57.33, 64.35) <sup>‡</sup>	59.35 (55.65, 63.05) <sup>‡</sup>
Post-IC <sub>threshold</sub>	60.64 (56.95, 64.34)	61.20 (57.30, 65.09)
Post-IC <sub>all</sub>	60.89 (57.42, 64.35)	61.48 (57.82, 65.13)

Post-IC<sub>threshold</sub>: Post-Intervention-Corrected<sub>threshold</sub>; Post-IC<sub>all</sub>: Post-Intervention-Corrected<sub>all</sub> or Post-IC<sub>all</sub>; <sup>‡</sup> p<0.05 compared to baseline LCHF

Relative REEs for the LCHF and CHO groups are presented in **Figure 8.1**. There was no difference in relative REE at Baseline between the LCHF and CHO groups (123.4 and 123.2 kJ/kg FFM/d respectively, p=0.955). There was no change in relative REE across the dietary adaptation periods in either group, based on uncorrected values.

Eight participants in the LCHF group and five participants in the CHO group had a change in TBW that met the threshold to apply the transformation factor to the post intervention value (Post-IC<sub>threshold</sub>). When this was applied in the LCHF group the estimated mean relative REE decreased to 118.4 kJ/kg FFM/d. When the correction factor was applied to all participants in LCHF (Post-IC<sub>all</sub>) the estimated mean decreased to 117.9 kJ/kg FFM/d. Both Post-IC<sub>threshold</sub> and Post-IC<sub>all</sub> were significantly different from Baseline in the LCHF group (-4.9 kJ/kg FFM/d; 95% CI -0.8, -9.1; and -5.5 kJ/kg FFM/d; -1.0, -10.0 respectively). In the CHO group, there was no difference between Baseline and Post-intervention relative REE when the transformation factor was applied to selected participants or all participants.



**Figure 8.1.** Relative REE (kJ/kg FFM/d) at Baseline, Post-Intervention, Post-Intervention-Corrected<sub>threshold</sub> (Post-IC<sub>threshold</sub>) and Post-Intervention-Corrected<sub>all</sub> (Post-IC<sub>all</sub>) pre and post 21 day adaptation to LCHF or high CHO availability diets. Data presented are raw means and standard deviations. Post-IC<sub>threshold</sub>; only values that met the threshold were transformed in each group. Post-IC<sub>all</sub>, all values were transformed in each group. ‡ p<0.05 compared to baseline LCHF.

## 8.5 Discussion

The findings from this investigation indicate that the artefact in the DXA-derived measurement of LM associated with depletion of muscle glycogen and water is important as it appears to alter the interpretation of a measurement of REE relative to FFM. This study is the first to show that a seemingly small error in the measurement of body composition by DXA can have a substantial carryover effect when this information is used to interpret nutritional status relative to biologically active tissue. In this case, the failure to correct for the artefact in FFM would lead to the failure to detect a reduction in relative-REE associated with a dietary change to a ketogenic LCHF diet. This could lead to the failure to recognise to a potentially important side-effect of this diet at the population level, while at the individual level, it could

prevent the assessment of LEA and delay the implementation of strategies to correct this potentially harmful condition. Both issues merit further investigation.

Previous work from our centre showed that acute increases in muscle glycogen and TBW, achieved via 48 h of carbohydrate loading, were detected by the DXA as a gain of LM (Bone et al., 2017). In contrast, decreases in muscle glycogen and TBW were detected by the DXA as a loss of LM. Depletion was achieved via a 120 km cycling protocol completed on the day prior to the “depleted” DXA measurement. The period between the exercise session and the DXA scan allowed for the restoration of sweat losses incurred by the exercise (time and fluid intake), such that the decrease in TBW was achieved via changes in intramuscular water. All DXA measurements in this study were completed in the morning following an overnight fast and rest. This protocol was based on prior research that indicated that dehydration and plasma volume shifts which occur as a result of exercise also create an artefact in DXA measures of LM (Nana et al., 2013); this is why Best Practice Protocols recommends overnight fasting and confirmation of euhydration via USG (Nana et al., 2015). Importantly, our work not only confirmed the presence of the artefact in measurements of LM associated with glycogen and bound water, but also developed a crude transformation factor to account for this.

An example of applying the correction factor is presented in **Table 8.4**. In this case, absolute REE decreased as did relative-REE however the Post-intervention score was above the 126 kJ/kg FFM/d threshold for LEA, so although a reduction has occurred it could be interpreted that there is minimal need for concern, nutrition support or intervention.

**Table 8.4** Application of correction factor on an individual participant.

Baseline	Baseline	Baseline	Post-	Post Intervention		
REE	FFM	REE (kJ/ kg	Intervention		FFM (kg)	REE (kJ/ kg
(kJ/d)		FFM/d	REE (kJ/d)			FFM/d)
7612	55.9	136.2	7101	Uncorrected	54.8	129.5
				Corrected	56.6	125.5

Change in TBW from Baseline exceeded 0.9 L. thereby difference in BM was multiplied by 0.8 and this result was added to Post-Intervention Bone and LM mass measures to calculate the corrected FFM.

When the correction factor is not applied, the lack of change in relative REE fails to reflect the reduction in absolute REE and the physiological changes that may have potentially occurred (Bielohuby et al., 2011; Fraser et al., 2000; Mountjoy et al., 2014). These physiological changes could include alterations to regulators of anabolism and energy, given there is evidence of reduced growth hormone and IGF-1 activity and decreased free triiodothyronine (fT3) in rats and clinical human populations (Bielohuby et al., 2011; Fraser et al., 2000). Once the correction factor is applied, relative REE is now below the threshold and in combination with the reduction in absolute REE would justify a path of further investigation of LEA (such as blood biochemistry markers), and potential interventions. This example highlights the potential importance of the use of the correction factor; however, it should be noted that measurements of REE should be used as part of a spectrum of diagnostic markers, which include biochemistry and performance at training, to assess energy availability in athletes to best gauge the meaningfulness of the change (Mountjoy et al., 2014).

The present study, embedded into a larger investigation of the effects of the ketogenic LCHF in endurance athletes, offered the dual opportunities of examining the effect of the diet on REE as well as investigating the real-world significance of the error in measuring LM by DXA when there is a chronic change in muscle glycogen and its bound water. Due to the

involvement of elite athletes as participants, it was impractical to directly measure changes in muscle glycogen stores as a result of the 21 d of dietary interventions. However, results of the larger study showed indirect evidence of changes in glycogen associated with diets of high CHO availability and restricted CHO intake, respectively. Specially, we observed a small increase in rates of whole body CHO oxidation, as measured by indirect calorimetry, during a prolonged exercise session as a result of a diet providing high CHO availability (Burke et al, *in review*). This is consistent with the literature which shows that increases in muscle glycogen stores are associated with high CHO oxidation rates (Hawley & Leckey, 2015). Therefore, the increases in TBW in some of the CHO group participants in the current study could be the result of an increase in muscle glycogen content above baseline values. More importantly, there was a pronounced decrease in rates of CHO utilisation over a range of exercise intensities in the LCHF group (Burke et al, *in review*), providing indirect evidence of a significant reduction in muscle glycogen stores. Indeed, a study of a 4 week intervention with a similar ketogenic LCHF diet in well-trained cyclists has also reported a 51% decrease in resting muscle glycogen stores. This decrease in muscle glycogen is similar to the 57% decrease in muscle glycogen achieved by acute exercise-induced depletion in our recent study (Bone et al., 2017).

The current study measured USG as a crude marker of extracellular hydration, and found no differences between baseline and post-intervention hydration status in either diet group. This provides indirect evidence that the reduction in TBW following adaptation to LCHF was associated with a reduction in intracellular water, particularly that bound to glycogen. We note that there was a small loss of BM and FM over the course of the 21 d of dietary adaptation and intensified training. However, the groups received an energy matched diet and showed equal FM losses over this period, so there is no rationale to expect a greater loss of “real” LM (e.g. muscle mass) over this period in the LCHF group. Therefore, we feel justified in explaining this apparent change of ~ 1.4 kg in the DXA-derived measurements of

LM in the LCHF as an artefact, and accepting that “real” LM was maintained in this group. We also note that calculations of absolute REE from equations based on  $\text{VO}_2$ ,  $\text{VCO}_2$  and RER may be affected by the contribution of the oxidation of ketone bodies on these measurements. Indeed, Frayn (Frayn, 1983), calculates that in the storage and utilisation of beta-hydroxybutyrate 4.5 mol of  $\text{O}_2$  are consumed and 4 mol of  $\text{CO}_2$  produced resulting in an RER of 0.89. However, we are unable to apply correction factors to the current calculations to allow for the contribution of ketone bodies to fasting metabolism

To the authors’ knowledge, there are no other investigations of the effect of a ketogenic LCHF diet on REE in athletes, however sparse literature involving mixed and overweight populations is available. Of most relevance are the findings of Urbain et al. (2017), who reported a decrease in absolute REE of  $\sim 389 \text{ kJ/d}$  in a healthy population, including both sedentary and very active people, after six weeks of adaptation to a ketogenic diet ( $>75\%$  energy from fats). Crude estimates of relative REE, derived from the information provided in the results section of this paper showed a decrease of  $7.5 \text{ kJ/kg FFM/d}$  between baseline and post intervention values. These results, even uncorrected for potential errors in the measurement of FFM used in this study (air displacement plethysmography), are not dissimilar to the change seen in our study when the period of adaptation to the diets is taken into account. In contrast, Alessandro et al. (2015) concluded there was no change in absolute REE in healthy overweight individuals following 20 d of adaptation to an energy restricted low carbohydrate ( $<30\text{g/d}$ ) diet. However, this finding was only reported qualitatively rather than providing quantitative data as evidence for this statement. Meanwhile, results from overweight adults who have lost weight indicate that consuming a low carbohydrate diet (10% of energy from carbohydrate, 60% from fat) to maintain weight loss, attenuates the decrease in REE that occurs during weight loss (Ebbeling et al., 2012). All of these studies involved free living subjects who were instructed on how to follow a ketogenic LCHF or low carbohydrate diet via the provision of menus or meal plans with permitted and non-permitted

foods. Dietary compliance, monitored via food diaries/diet recalls or daily urinary ketones, was likely to be less than that achieved in the present study, where all food was provided to participants, and weighed food records were maintained (Jeacocke & Burke, 2010).

The cause and outcomes of the apparent reduction in relative-REE with the ketogenic LCHF diet in the present study are currently unexplored and require further confirmation and investigation. Possible causes include decreases in T3 a regulator of energy metabolism or decrease in anabolic activity due to reductions in IGF-1 or increases in cortisol resulting in decreased testosterone (Fraser et al., 2000; Lane et al., 2010; Loucks & Heath, 1994; Loucks et al., 2011). In an athlete population, a reduction in relative REE could be associated with LEA syndrome, with its range of impairments of health, body composition maintenance and training adaptations (Loucks et al., 2011; Melin et al., 2015; Mountjoy et al., 2014; Vanheest et al., 2014). Given the significant effect of these issues on illness, injury and performance outcomes, more work is warranted. If the finding can be confirmed, the secondary issue is the accuracy of diagnosis of the problem in athletes who chronically follow a LCHF diet or have reduced glycogen stores due to other causes (e.g. energy restriction). In these populations, based on the current study, there is a likelihood that changes in relative REE over time or in comparison to a threshold for diagnosis of LEA could potentially be masked by the artefact in measurement of FFM, at least by DXA. This should be factored into a practitioners' interpretations of RMR assessments of such athletes, either by changing the thresholds used to denote problematic measures of REE or by applying a transformation factor, such as the one used in this study, to correct the estimates of REE.

Several limitations of the methodology and application of the current study are acknowledged. Due to the constraints of the larger investigation in which the present study was undertaken, muscle glycogen could not be measured by muscle biopsy at Baseline or Post-intervention to confirm the presence (and magnitude) of this potential source of error in measurements of LM. Future investigations of both this error/artefact in calculation of



relative REE and the chronic effects of ketogenic LCHF on metabolic rate would benefit from including direct measurements of muscle glycogen before and after the dietary intervention using nuclear magnetic resonance, since undertaking muscle biopsies on elite athletes poses a practical and ethical challenge. However, this solution is only likely to be applicable to further research on the topic, since it would be impractical for athlete servicing needs. Following the application of the correction factor, it appears as though FFM increased by 400 g ( $PI_{\text{threshold}}$ ) and 700g ( $PI_{\text{All}}$ ) respectively after 21 d adaptation of the ketogenic LCHF diet. Although the increases were not statistically significant, it indicates that further work may be needed to refine and validate the correction factor, as well as to confirm the utility and value of including measurements of TBW with DXA-measurements of LM as a proxy measure of changes in muscle (and liver) concentrations. The lack of inclusion of female athletes is another limitation of the current investigation. However, conducting this study in females would bring challenges with regards to standardising measures of REE around the menstrual cycle. Nevertheless, such work is encouraged.

In conclusion, adaptation to a ketogenic LCHF diet produces an artefact in the DXA estimate of FFM due to decreases in glycogen concentration and water storage. This resulted in an apparent reduction in relative-REE, once the artefact was corrected with a crude transformation factor. This may have implications when relative-REE is used as one of a range of markers to detect or manage LEA in athletes. However, as there was no statistical change in absolute REE, further work is required to determine the reproducibility of these results. Further refinement of the correction factor is warranted in scenarios where reduced glycogen concentration is suspected or confirmed.

## **9.0 Summary and future directions**

### **9.1 Summary**

The primary aim of this thesis was to investigate the reliability of protocols used to measure resting energy expenditure in athletes. The body of work presented investigated and identified key variables that influence either the measurement or interpretation of REE in this population. The findings from these studies have implications for how low energy availability is screened, managed and monitored when using REE as the diagnostic tool, within the high performance sports environment where the problem of Low Energy Availability is becoming increasingly recognised as a major risk factor for injury, impaired health, and sub-optimal performance. This has obvious currency for systems such as Australia's Winning Edge program, which seeks to achieve international sporting success via the adoption of standardised Best Practice Protocols for athlete preparation and support across a network of sports medicine/science providers. However, it may also leave a legacy for improved care and education of sub-elite athletes and committed exercisers who exist outside such networks, but in whom similar problems may occur. In this case, benefits may translate to better community health (reduced healthcare costs, fewer sick days etc.), and greater participation and enjoyment of sport and exercise programs.

Low energy availability (LEA) is associated with, and can be identified via a reduction in REE, as long as there is confidence in the reliability of measurement and an absence of artefacts that could alter the interpretation of results. The first step in this project was to review the literature (Chapter 2) to clarify the terminology used to describe metabolic rate and to highlight the differences in the definitions and measurement/calculation of BMR, RMR, BEE and REE. Factors or protocols likely to influence the measurement of REE were identified, with particular interest in those which had the lowest levels of investigation and/or the highest likelihood of causing variability or artefacts in outcomes. These were combined

with practical questions or challenges that had been previously encountered in the measurement of REE in the athlete daily training environment to identify the following factors that were considered of primary importance to investigate in the target population:

- Daily variation in REE under normal living conditions
- Effect of the testing environment and patient preparation (inpatient vs outpatient measurements) on measurement of REE
- Effect of duration of the overnight fast on REE measurements
- Effect of acute exercise (i.e. exercise on the day(s) prior to measurement) on REE measurements
- Effect of common artefacts in measurement of body composition on calculations of REE
- Effect of extreme macronutrient changes on REE measurements

There are multiple consequences of using protocols with poor reliability and validity in the measurement of REE. When a single measurement is used to screen for LEA there is a risk of failing to diagnose a problem that needs management (false negative) as well as wasting resources on an athlete who has been incorrectly diagnosed (false positive). When using REE to monitor changes over time, measurement errors could lead to a failure to observe a problem that is developing at an early stage or to identify activities or behaviours that are problematic. Equally, it will interfere with the assessment of a planned intervention or therapeutic activity to manage LEA to determine if it has been effective in causing a change in REE. This outcome could interfere with the clinical management of an individual athlete as well as a judgement of the efficacy or harm of the intervention per se.

Chapter 4 (*Study 1*) addressed the first three variables by comparing the absolute values for REE, and the reliability of measurement of REE, under inpatient (bedside) or outpatient (laboratory) protocols. It could be argued that we effectively compared

measurements of BEE (inpatient) and REE (outpatient). However, as an 8 h overnight fast was used for both conditions, both protocols were technically REE measurements as per the definitions provided in Chapter 2. Ideally, the methodology of *Study 1* would have included a comparison between an 8- and 12 h fast for both inpatient and outpatient protocols. However, given the impractical nature of a 12 h fast in athletes in the daily training environment and the minimal thermic effect of a meal on metabolic rate after 8 h, this time frame was used as a compromise between athlete compliance and scientific rigor (Fullmer et al., 2015; Kinabo & Durnin, 1990).

The results from *Study 1* indicate that there is no difference in measurements of REE conducted with the use of inpatient or outpatient protocols. There was large variation in the REE results for *Study 1*, particularly for the outpatient protocol, suggesting that ideal practice for interpreting single or serial measures of REE would include the capture of multiple measures of an individual athlete to determine a baseline typical error. However, it was also concluded that this is not always probable in a high performance environment and that the typical errors determined from *Study 1* could be used when interpreting longitudinal measures of REE. Furthermore, the outpatient protocol had a bigger typical error, meaning it has less sensitivity in detecting changes in REE and would require a bigger difference between measurements to be considered a real change. Therefore, although both protocols have good day to day reliability, they should not be used interchangeably for longitudinal monitoring of REE.

*Study 2* (Chapter 5) was designed to investigate if a standardised rest period from exercise is required prior to measurement of REE. Previous studies in endurance athletes have reported that the acute effects of exercise on metabolic rate are no longer present after 39- and 72 h (Herring et al., 1992; Tremblay et al., 1988). However, given that the majority of elite athletes undertake training at least once a day, six to seven days a week, enforcing a rest period up to 72 h may not always be feasible. Therefore, *Study 2* investigated the effect of

shorter recovery periods on metabolic rate, measuring REE following exercise at ~ 12 h intervals up to a total rest period of 48 h. This provides additional information to the current literature in which REE was measured at 24 h intervals (Herring et al., 1992) or only at baseline and 72 h later (Tremblay et al., 1988). To enhance the external validity of the methodological design, and to extend the results to a range of sports and training protocols, this study was purposefully not restricted to endurance athletes, nor were the training sessions prescribed in terms of mode, duration or intensity of exercise.

The results from *Study 2* indicate that the acute effect of exercise had subsided ~48 h after exercise. However, the differences were within the typical error for REE when measured using the outpatient protocol and of lower magnitudes than what has been previously reported in the literature. Possible explanations for the smaller magnitude of decrease in REE are: insufficient sample size; heterogeneous population and variability in the exercise stimulus between participants; and lack of a sufficient change in energy flux between training and rest days. The sample size is most likely the biggest factor in preventing a meaningful difference in REE from being detected across 12 h time points. However, as reported in Chapter 5, this study was hampered by the difficulty in recruiting well-trained individuals, let alone elite athletes; the resistance to refraining from exercise for up to 48 h proved to be surprisingly difficult to overcome. Indeed, only 10 of the 16 participants who showed any interest in the study by completing the screening questionnaire actually went on to commence the study. Possible explanations for the lack of interest in the study and/or willingness to refrain from training include: fear of not maximising adaptation during the training block; habituation to a daily training routine, and fear of disruption to energy balance goals.

The lack of a clear change of energy flux in the current study may have also contributed to the REE results. Typically, a high energy flux (high energy intake and expenditure) through the metabolic system contributes to the increase in REE following

exercise (Bullough et al., 1995) and a change to low energy flux (low energy intake and expenditure) may be part of the REE decay. Although there was a significant decrease in estimated total energy expenditure between training and rest days among the subjects who completed the current study, there was no difference in their total (self-reported) energy intake between the days. Therefore, the energy intake on the training days may not have been high enough to elicit a state of increased REE via high energy flux. This concept provides another reason for needing to study elite athletes who periodise energy intake on training days and non-training days and achieve distinct changes in energy flux. However, the challenges of achieving compliance from high level athletes (and their coaches) to disturb their training programs and include significant rest periods remain a prominent barrier to overcome.

The first two studies of this thesis aimed to standardise what happens either before or during REE assessment protocols. *Study 3* (Chapter 6) focused on the application of the results of these protocols by examining the reliability of the measurement of FFM which is included in the calculation and interpretation of REE assessments. Previous work on the reliability and validity of DXA measurement of body composition has shown that acute activities such as a recent bout of exercise or intake of food or fluid increase the error in the DXA estimate of LM. This occurs principally because changes in total body water (TBW) are perceived by DXA as a change in LM. Thus, the best practice protocol promoted for DXA assessments of body composition (Nana et al., 2015), and commonly used throughout Australian's Winning Edge network involves the presentation of the athlete in an overnight fasted and rested state. However, the effects of other and often longer-term changes in body water content on DXA-estimates of body composition remain unknown, although practitioners have been aware of apparent anomalies in body composition assessments which include unexpected increases and decreases of small but meaningful amount of LM between assessments. *Study 3* explored the hypothesis that changes in muscle solutes (e.g. glycogen

and creatine), and the fluid bound with them, might create an artefact in DXA measurements of LM. This is important since athletes are likely to experience wide variations in their muscle glycogen concentrations due to training and or periodisation of their carbohydrate availability to enhance metabolic adaptations to training (Marquet et al., 2016). Use of supplemental forms of creatine to increase muscle creatine content provides another potential source of change in muscle solute and water.

*Study 3* included the use of bioelectrical impedance spectroscopy (BIS) to measure changes in TBW with various manipulations of muscle solute. This technique has been validated in male athletes (Kerr et al., 2015) and offered practical advantages over the gold standard technique of deuterium dilution due to its ease of use and low participant and researcher burden. A series of exercise and diet manipulations to increase or reduce muscle glycogen were implemented in the face of normal or loaded muscle creatine stores. A glycogen depleted-creatine loaded measurement was the only combination that was not able to be achieved within the design of *Study 3*. However, given that creatine loading alone had a trivial effect on LM estimates, it is reasonable to conclude that there would have been an unclear change on total body LM under this treatment condition.

The results of *Study 3* indicate that glycogen depletion produces a trivial decrease in DXA estimates of total LM, however the threshold for reaching a substantial change was 1.4% and there was a 1.3% change observed under this condition. Furthermore, a possible substantial decrease in leg LM was observed following the Glycogen-Depletion protocol. As anticipated, glycogen loading, alone and when combined with creatine supplementation, was associated with substantial increases in estimates of LM of 2.1 and 3.0% respectively. The changes in TBW mirrored the changes in estimates of LM with substantial increases following glycogen loading, creatine loading and glycogen and creatine loading, and exercise induced depletion resulting in a substantial decrease in TBW.

*Study 3* indicated that DXA estimates of LM can be acutely influenced by changes in intramuscular fluid and substrates, when no real change in muscle protein content is likely to have occurred. Therefore, when interpreting the results of DXA measurements of body composition, it is important to consider whether recent changes in dietary intake or training have occurred, and whether this may have caused substantial changes in muscle glycogen/water content. Factoring such events into the interpretations may be important, both for identifying whether real changes in LM have occurred and for using absolute measures of components of body composition in assessments of nutritional status.

Investigation of the potential for an accessible and non-invasive technique to estimate muscle glycogen at the time of a DXA scan was the catalyst behind *Study 4* (Chapter 7). The novel MuscleSound® method uses ultrasound and proprietary software to estimate muscle glycogen based on the premise that water bound to stored glycogen and released during exercise changes the brightness of the ultrasound image. Although validation studies had been undertaken, using a cycling bout to achieve simple changes in glycogen stores, we identified that the design of *Study 3* (Chapter 6) provided an excellent opportunity to compare the estimates of muscle glycogen from MuscleSound® against the biopsy criterion method, under a more extreme glycogen-depleting protocol as well as glycogen loading protocols that were not included in the previous validation studies. The inclusion of creatine supplementation in the study design provided an additional challenge to the ability of the MuscleSound® to track changes in muscle glycogen when changes in intramuscular fluid were not directly related to glycogen.

The results of *Study 4* found that MuscleSound® estimates of glycogen did not correlate well with the clear changes in biopsy-derived muscle glycogen concentrations resulting from the depleting and loading protocols. Therefore, we determined, in contrast to the previously conducted validation studies, that the MuscleSound® technique does not have the precision to detect changes in muscle glycogen that are commonly seen in athletes.



Discrepancies between the results of *Study 4* and the previous publications could be due to the greater extremes in muscle glycogen achieved by our depletion and loading protocols, as well as the separation of glycogen depletion and dehydration in the post-exercise measurements. Specifically, our protocol involved the collection of the “depletion” measurements on the morning after the glycogen depleting cycling task, to coincide with the DXA measurements of body composition according to our Best Practice Protocol. However, a secondary, and useful, feature of this methodological piece was the opportunity to allow participants to rehydrate during the ensuing (~ 14-16 h) recovery period while minimising glycogen recovery via the provision of a diet providing minimal carbohydrate content. This is in contrast to the conditions used in the other validation studies of the MuscleSound® technique whereby post-exercise assessments were conducted in the likely co-existence of exercise-induced dehydration. Although the results of *Study 3* indicated that the MuscleSound® technique was unable to provide a valid and reliable estimate of total muscle glycogen content or changes in glycogen due to a variety of manipulations, we found changes in total body water measured by BIS reflected the changes observed in biopsy derived muscle glycogen. Therefore, we noted that the concept of tracking TBW in otherwise euhydrated individuals as a proxy for changes in muscle glycogen content appears to be valid, and the BIS should be considered as a tool to monitor changes in muscle glycogen under these conditions.

The final study of this thesis (Chapter 8) investigated the significance of the artefact in DXA estimates of LM due to changes in muscle glycogen and water content, when interpreting the assessment of relative REE in situations when a change in muscle glycogen is induced through extreme dietary practices. Indeed, *Study 5* had two aims: to investigate whether adaptation to a ketogenic low carbohydrate high fat (LCHF) diet in elite male athletes is associated with a change in relative REE, and to test the significance of the small but robust clear in the DXA measurement of LM in glycogen-depleted individuals on the calculation and interpretation of relative REE. This is relevant due to the re-emergence of claims LCHF diet

can enhance endurance/ultra-endurance performance and appears to be practiced by some elite athletes.

A limitation of *Study 5* was that muscle biopsies were not taken at baseline and post-intervention to confirm any changes in muscle glycogen stores with either diet. However, other researchers have reported that the decrease in resting muscle glycogen stores achieved by a 4 week adaptation to a LCHF diet (Phinney et al., 1983) is similar in magnitude to the acute depletion in muscle glycogen observed in our previous investigation of the DXA artefact in LM assessment (*Study 3*). Based on the results of *Study 3*, estimates of TBW by BIS in rested and euhydrated individuals were deemed to be a suitable indicator of presence of a meaningful change in intramuscular water to which a correction factor could be applied to DXA estimates of LM to provide a more valid estimate of LM (either for over or under estimation). It could also be argued that using a four compartment model of body composition when measuring and interpreting REE would be more appropriate. However, this requires extensive resources and time, and would not be practical in an athlete servicing scenario. Therefore, we restricted our investigation to methodologies that would be more practical and accessible in a real world environment.

The results from *Study 5* indicate that it is important to correct estimates of FFM for the artefact in DXA measurement of LM in individuals with depleted muscle glycogen and bound water stores. Indeed, we noted a mean LM “loss” of ~ 1.4 kg in the LCHF group following 3 weeks of a CHO-restricted diet that was likely to reflect changes in intramuscular solutes and water rather than a true change in muscle mass or other body tissues. Although this may seem a small error, has the potential to alter the measurement and interpretation of relative REE – the measurement of energy expenditure at rest from the traditionally identified metabolically active tissues. Specifically, we noted that there was no change in estimates of relative REE in either group as a result of the 3 week diet-exercise intervention. However, when a transformation factor was used to correct estimates of FFM in the LCHF group, either

for all individuals or only those who showed a substantial change in TBW measured by BIS, the reduction in relative REE was now deemed to be statistically significant. To ensure that this finding was not biased by applying the correction factor to the individuals in the LCHF intervention group, a similar transformation was applied to the control group to account for increases in post-intervention LM that should be more correctly attributed to increases in muscle solutes/water.

*Study 5* provides an appropriate conclusion to this body of work by providing evidence of the importance of establishing good reliability and validity in measurements of body composition and energy expenditure in special populations such as elite athletes. Indeed, it leaves an interesting legacy in challenging further investigations of the effect of ketogenic LCHF diets on resting metabolism in athletes. More importantly, it underscores the potential risks of undertaking measurements of nutritional status in athletes without adequate standardisation of measurement or insight into interpreting the results. In the current case, poor measurement technique and lack of insight would have been associated with a type II error – failure to detect a real effect. Given the significant issues associated with reduced metabolic rate and low energy availability in athletes, this is would be an unfortunate oversight.

## **9.2 Limitations**

Although limitations of individual studies were addressed in the relevant chapters, some elements appeared as common themes throughout this thesis and should be addressed.

### **9.2.1 Statistics**

Different statistical methods were used to analyse the results of *Studies 1, 2 and 5* compared with *Studies 3 and 4*. With the exception of *Study 4*, the statistical methods used in each respective study were chosen to make them compatible with the current literature, and in

particular, the series of publications from our research group on this topic. The magnitude based inference approach has previously been used in studies investigating DXA reliability by our centre and it was considered appropriate to continue with this method. Conversely, for *Study 4* it was decided that the statistics used in the previous two validation studies did not provide sufficient clarification with regards to the errors of the prediction equation and therefore linear mix modelling was used.

Consistency with our work regarding REE was attempted by ensuring that all studies on this topic (*Studies 1, 2 and 5*) used the same statistical model of null hypothesis testing, and the use of statistical significance based on p-values, with further insights gained from confidence limits. Furthermore, linear mixed modelling was used in the group of studies in which parallel group designs were used in the investigation of different treatments.

It is important to note that all studies used parametric statistical analysis, with the differences primarily residing in the methods used to make inferences around the results. At no point was an interpretative method chosen because it provided a “better outcome”.

### **9.2.2 Generalisability**

With the exception of *Study 1*, the studies in this thesis did not include female participants. The rationale for the exclusion of female athletes in *Studies 2-5* include the challenging logistics of controlling for the menstrual cycle and its likely effects on several main variables under investigation (e.g. changes in resting metabolism, shifts in body fluids). Although we were unable to control for the phase of the menstrual cycle in female subjects who participated in *Study 1*, the intervention period involved a very short time frame (2-3 days) in which we did not anticipate changes to occur. Therefore, the generalisability of the results of our studies to female athletes should be treated with caution and in conjunction with the available literature (if any) for those specific variables on that particular intervention.

Although the goal of this thesis was to produce best practice guidelines for the measurement of REE in high performance athletes, only two studies contained participants from this target population. *Study 1* contained a mixture of elite (national representatives) and sub-elite (state representative) athletes, while *Study 5* involved elite and highly trained male race-walkers. *Studies 3* and *4* recruited participants who were well trained as evidenced by their  $\text{VO}_2\text{max}$  ( $65.2 \pm 7.1$  ml/kg/min), but came mainly from an endurance background. However, like the race-walkers they were endurance trained. Therefore, the applicability to the results in this collection of work to resistance and team-sport athletes requires further investigation.

The correction factor in *Study 5* was developed on endurance trained male athletes. Furthermore, it was developed on a cohort of 18 participants. As a tool it is still relatively crude and potentially limited by the bluntness of the BIS ability to detect smaller change in TBW. In addition, changes in muscle glycogen concentrations are not the only cause for changes in TBW; indeed a separate correction factor would be needed for female athletes at various stages across the menstrual cycle or for use with athletes who are following a low residue diet. Thus further development of the correction factor is required before it is applied to other sporting populations.

### **9.3 Future directions**

More investigations are required to address the gaps in the literature concerning REE protocols in female and non-endurance athlete groups. Furthermore, studies involving female athletes should address the potential differences in metabolic rate between and among athletes who have natural menstrual cycles, those who are on the contraceptive pill or other long term forms of contraception such as an intra-uterine device, and those who have menstrual disturbances. It is possible that daily variability in measurements of REE, and the effects of acute exercise or extreme macronutrient changes on REE may differ from male athletes, as

well as between these groups. Effects of absolute and fluctuating concentrations of reproductive hormone may also affect total body water and its impact on measures of DXA LM, thus requiring separate investigations of the utility of BIS to track changes of interest in TBW, as well as determine when and what correction factor should be applied to DXA estimates of LM.

Further development of the correction factor and using changes in TBW to account for changes in muscle glycogen is required to determine its sensitivity, reliability and applicability to female athletes and other sports. Whether the correction factor is still valid in sports where extreme acute changes in body mass have occurred (such as when making weight for combat sports) is also of interest.

Future studies on the acute effect of exercise on measures of REE require robust strategies to recruit elite athletes and increase sample sizes. Such strategies could include the staging of camps with incentives for attendees to refrain from training for multiple days (such as education activities or medical screenings); other options would be to measure REE during a taper, or following a major event after which it is likely that many athletes would take a scheduled break from training (e.g. a marathon).

Finally, more evidence is required to support the energy flux concept and whether or not this theory is influenced by acute changes of the macronutrient content of the evening meal on the night prior to REE measurement. This is of particular relevance to current nutrition practices in which athletes may chronically follow a LCHF diet or integrate periods of training with low CHO availability before or after a session.

## **9.4 Conclusion**

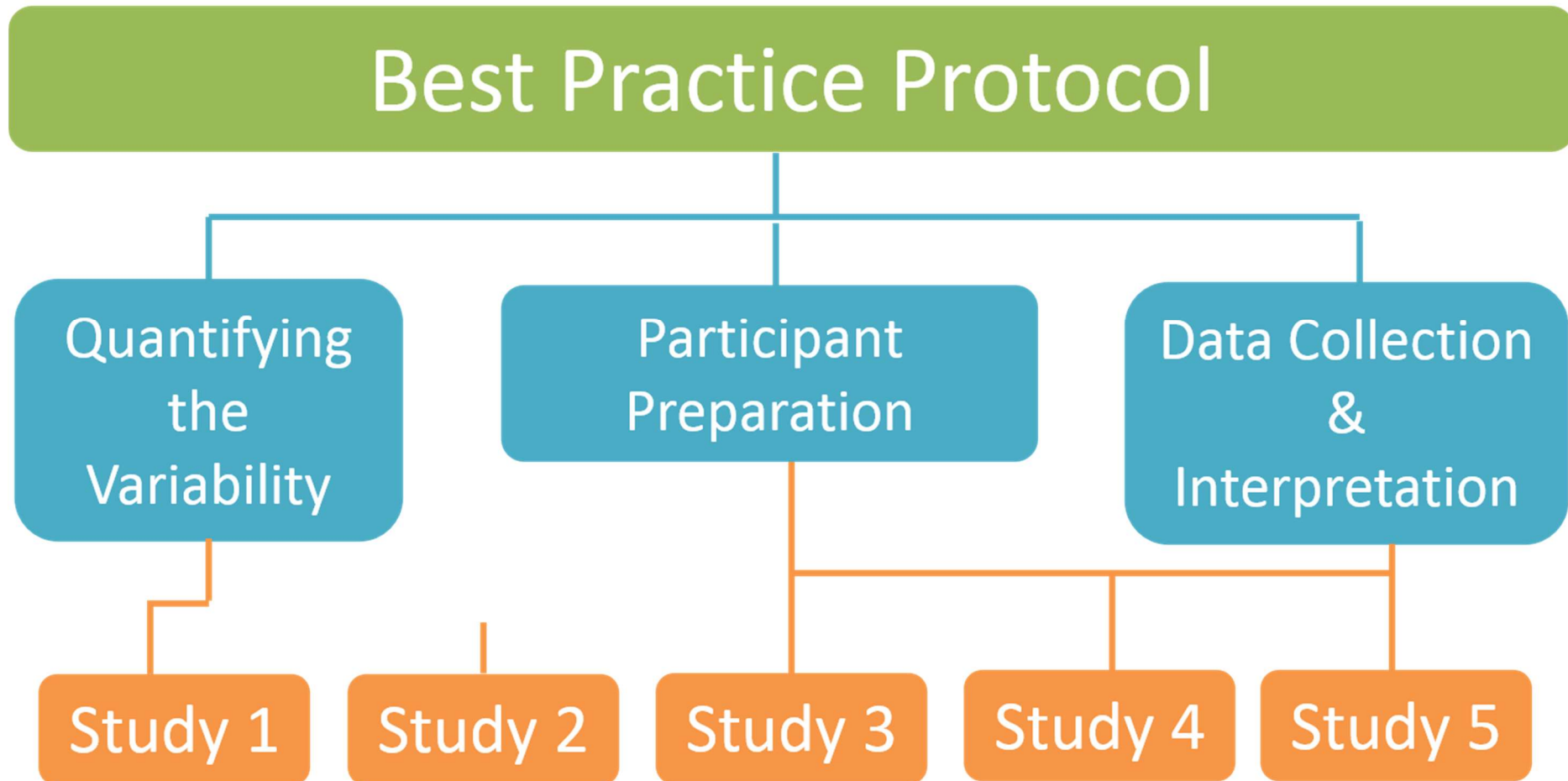
This thesis investigated three variables that influence the measurement or interpretation of REE in athletes, as well as studying the use of two novel techniques to help

correct for potential errors/artefacts in measurements. The outcomes of the completed body of work include

- Demonstration of the reliability of REE measurements following an 8 h overnight fasting protocol, with identification of a typical error to assist practitioners to determine when a real change in REE is likely to have occurred.
- Suggestion that the acute effect of exercise on metabolic rate in a heterogeneous athlete population may take a period of 48 h of rest to return REE to baseline values.
- Identification of an artefact in the DXA estimate of LM when there is manipulation of the muscle content of glycogen and creatine (and associated water).
- Failure to validate the use of commercial ultrasound technology and proprietary software to provide a non-invasive assessment of muscle glycogen content or its changes.
- Demonstration of the utility of using a transformation factor based on Bioelectrical Impedance Spectroscopy-derived changes in TBW to reduce the artefact in DXA-estimates of LM in athletes who have major changes in muscle glycogen content.
- Identification of a potential reduction in relative REE following adaptation to a ketogenic LCHF diet, which may have important implications in the consideration of Low Energy Availability in athletes

Together, the body of work presented in this thesis provides evidence that can be used to update protocols for measuring and interpreting REE in and how they could affect the identification and management of low energy availability in athletes (**Figure 9.1**).

**Figure 9.1.** Contribution of thesis studies to RMR Best Practice Protocol in athletes.





## 9.5 Personal Reflections

This thesis aimed to marry scientific rigor with practical application within a high performance environment. The process of completing a Confirmation of Candidature allows the development of the PhD road map with clear study questions, outcome goals, and consideration of resources, skill sets and timeframes. However, the completion of the journey requires an ability to embrace good luck, unforeseen circumstances, and changes in direction. This series of doctoral studies included examples of both good and bad fortune. Study 2 provided the greatest need for resilience in the face of setbacks. Recruitment and testing for this project was actually completed twice. Round 1 produced a worrying number of anomalous measurements of REE within the data set, despite careful collection protocols, equipment checks and advice from experienced practitioners and technicians. Persistence in checking equipment eventually determined the source of the problem: the degrading of some of the O-rings on mouthpieces and connections to the metabolic cart. The hard lesson was that failure of the most random and minor pieces of equipment can necessitate the cancellation of a whole data set/study. However, the positive outcome of this lesson was an appreciation of my developing expertise and increased confidence in my ability to identify and interpret the quality of data. Study 2 was also originally intended to be a fully powered investigation of an elite population. However, the difficulties in recruiting high calibre athletes (both round 1 and 2) to fulfil the requirements and the acknowledgement that we would need to move away from our target population to obtain an adequate sample size, made me rethink the use of my time and resources and to reclassify it as a pilot study.

The silver lining of redoing Study 2 meant that I had to extend my candidature which coincided with an opportunity to better use those resources in Study 5. A large dietary intervention and training study was being planned within the AIS Sports Nutrition Research group and it provided a perfect opportunity to test out how one element of my work

(identification of an artefact in DXA-estimates of LM) would affect interpretations of REE in a group of world class athletes when muscle glycogen was manipulated by a LCHF diet. This example was just one of many I encountered throughout my PhD, where being in an environment which undertakes both high performance research and athlete preparation provided an unforeseen opportunity to contribute to other multidisciplinary activities while cross-fertilising my own work. And although the final journey was somewhat different to the path that was originally planned, it meant discovering a persistence and confidence in my expertise, as well as an appreciation that serendipitous chances can lead to clinically impacting and meaningful outcomes.

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## 11.0 Research Portfolio Appendix

### a) Publications and Statement of Contribution of Others

- 1) **Bone, J L**, Ross, M L, Tomcik, K A, Jeacocke, N A, Hopkins, W G, & Burke, L M. (2017). Manipulation of muscle creatine and glycogen changes dual x-ray absorptiometry estimates of body composition. *Medicine and Science in Sports and Exercise*, 49, 1029-1035.

Accepted 21 November 2016: Available online 30 November 2016;

*Contribution statement:* JLB was primarily responsible for, data collection, data analysis, writing and submitting the manuscript. MLR was involved with data collection and reviewing the manuscript. KT was involved with data collection and analysis of muscle tissue. NJ was involved in dietary design and implementation. WGH was involved with statistical analysis. LMB was involved in study design, and reviewing the manuscript.

*Approximate percentage contributions:* J. L. Bone 65%; M. L. Ross 10%; K. Tomcik 5%; N. Jeacocke 5%; W. G. Hopkins 5%; L. M. Burke 10%

I acknowledge that my contribution to the above paper is 65%.

J.L. Bone:



Date: 16<sup>th</sup> June 2017

As principal supervisor of this project, I certify that the above contributions are true and correct:

L.M. Burke



Date: 16<sup>th</sup> June 2017



- 2) **Bone, J L, & Burke, L M.** (2017). No difference in young adult athletes resting energy expenditure when measured under inpatient or outpatient conditions. *International Journal of Sport Nutrition and Exercise Metabolism*, In press., 1-15.

Accepted by on the 27 Oct 2017

*Contribution statement:* JLB was primarily responsible for study design, obtaining ethical approval, data collection, data analysis, writing and submitting the manuscript. LMB was involved in study design, and reviewing the manuscript.

*Approximate percentage contributions:* J. L. Bone 75%; L. M. Burke 25%

I acknowledge that my contribution to the above paper is 75%.

J.L. Bone:



Date: 16<sup>th</sup> June 2017

As principal supervisor of this project, I certify that the above contributions are true and correct:



L.M. Burke

Date: 16<sup>th</sup> June 2017

- 3) **Bone, J L**, Ross, M L, Hopkins, G, Tomcik, K A, Jeacocke, N A, & Burke, L M. Ultrasound technology fails to provide indirect estimate of muscle glycogen concentration.

In preparation, to be submitted to *Nutrients*

*Contribution statement:* JLB was primarily responsible for, data collection, data analysis, writing and submitting the manuscript. MLR was involved with data collection and reviewing the manuscript. KT was involved with data collection and analysis of muscle tissue. NJ was involved in dietary design and implementation. WGH was involved with statistical analysis. LMB was involved in study design, and reviewing the manuscript.

*Approximate percentage contributions:* J. L. Bone 65%; M. L. Ross 10%; K. Tomcik 5%; N. Jeacocke 5%; W. G. Hopkins 5%; L. M. Burke 10%

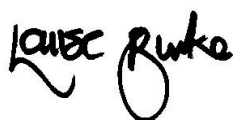
I acknowledge that my contribution to the above paper is 65%.

J.L. Bone:



Date: 16<sup>th</sup> June 2017

As principal supervisor of this project, I certify that the above contributions are true and correct:



L.M. Burke

Date: 16<sup>th</sup> June 2017

- 4) **Bone, J L**, Ross, M L, Welvaert, M, & Burke, L M. Measurement of resting energy expenditure following a low carbohydrate high fat diet is affected by artefacts in DXA measurement of lean mass.

In preparation, to be submitted to *Plos One*.

*Contribution statement:* JLB was primarily responsible for, data collection, data analysis, writing and submitting the manuscript. MLR was involved with reviewing the manuscript. SF was involved in dietary design and implementation. MW was involved with statistical analysis. LMB was involved in study design, and reviewing the manuscript.

*Approximate percentage contributions:* J. L. Bone 65%; M. L. Ross 10%; M. Welvaert 10%; L. M. Burke 15%

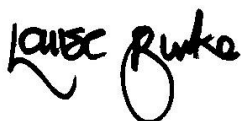
I acknowledge that my contribution to the above paper is 65%.

J.L. Bone:



Date: 16<sup>th</sup> June 2017

As principal supervisor of this project, I certify that the above contributions are true and correct:



L.M. Burke

Date: 16<sup>th</sup> June 2017

## **b) Additional Publications**

- 5) Bone, J, & Burke, L M. (2016). DXA estimates of body composition and carbohydrate loading. *Annals of Nutrition and Metabolism*, 68, 228-229. doi: 10.1159/000445510  
Accepted 16 March 2016; Published 14 April 2016

*Contribution statement:* JLB wrote the first draft of the letter. LMB was involved in final proofing.

*Approximate percentage contributions:* J. L. Bone 75%; L. M. Burke 25%

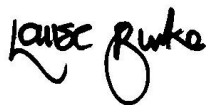
I acknowledge that my contribution to the above paper is 75%.

J.L. Bone:



Date: 16<sup>th</sup> June 2017

As principal supervisor of this project, I certify that the above contributions are true and correct:



L.M. Burke

Date: 16<sup>th</sup> June 2017

### c) Conference presentations

1. **Bone, J L**, Ross, M L, Hopkins, G, Tomcik, K A, Jeacocke, N A, & Burke, L M. Ultrasound technology fails to provide indirect estimate of muscle glycogen concentration, *American College of Sports Medicine*, Boston, June 4, 2016. [Poster presentation]

*Contribution Statement:* This presentation was based on the work from Publication 4 (see above for author contribution). The presentation was designed and delivered by JLB. LB reviewed the poster and provided feedback.

2. **Bone, J L**, Ross, M L, Tomcik, K A, Jeacocke, N A, Hopkins, W G, & Burke, L M. (2016). Manipulation of muscle creatine and glycogen changes DXA estimates of body composition, *European Congress of Sport Science*, Vienna, July 6 2016. [Mini Oral presentation]

*Contribution Statement:* This presentation was based on the work from Publication 3 (see above for author contribution). The presentation was designed and delivered by JLB. LB reviewed the presentation and provided feedback.

## **d) Individual papers**

### **11.1 Published paper which forms the basis of Chapter 4**

**Bone, J L, & Burke, L M.** (2017). No difference in young adult athletes resting energy expenditure when measured under inpatient or outpatient conditions. *Int J Sport Nutr Exerc Metab, In press.*, 1-15.

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<https://doi.org/10.1123/ijsnem.2016-0315>

### **11.2 Published paper which forms the basis of Chapter 6**

**Bone, J L, Ross, M L, Tomcik, K A, Jeacocke, N A, Hopkins, W G, & Burke, L M.** (2017). Manipulation of muscle creatine and glycogen changes dual x-ray absorptiometry estimates of body composition. *Medicine and Science in Sports and Exercise*, 49, 1029-1035.

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### **e) Ethics approvals, letters to participants and consent forms**

The following approval notices cover all five studies for the work completed during this thesis. Amendments to the original ethics application were made for studies 2, 3 and 4, these amendments and approvals have been attached.

**11.2 Study 1: No difference in young adult athletes resting energy expenditure when measured under inpatient or outpatient conditions.**

**A) Notice of Approval**



**MINUTE**

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TO: Julia Bone CC:  
FROM: Ms Helene Rushby  
SUBJECT: Approval from AIS Ethics Committee DATE: 15<sup>th</sup> April 2015

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On the 14<sup>th</sup> April 2015, the AIS Ethics Committee gave consideration to your submission titled ***"Standardisation of RMR in athlete population – comparison of bedside and outpatient measurements"***. The Committee saw no ethical reason why your project should not proceed.

The approval number for this project: 201504002

It is a requirement of the AIS Ethics Committee that the Principal Researcher (you) advise all researchers involved in the study of Ethics Committee approval and any conditions of that approval. You are also required to advise the Ethics Committee immediately (via the Secretary) of:

Any proposed changes to the research design,  
Any adverse events that may occur,

Researchers are required to submit **annual status reports** and **final reports** to the secretary of the AIS Ethics Committee. Details of status report requirements are contained in the "Guidelines" for ethics submissions.

Please note the approval for this submission expires on the 30<sup>th</sup> June 2017 after which time an extension will need to be sought.

If you have any questions regarding this matter, please don't hesitate to contact me on (02) 6214 1577

A handwritten signature in blue ink, appearing to read 'Helene Rushby', is written over the typed name and title.

Sincerely  
Helene Rushby  
Secretary, AIS EC



## *B) Information to Participant*

Page 1 of 3

### INFORMATION TO PARTICIPANTS

**Research Title:** Standardisation of RMR in an athlete population – Comparison of bedside and outpatient measurements

**Principal Researcher:** Julia Bone  
Email: [Julia.bone@ausport.gov.au](mailto:Julia.bone@ausport.gov.au)  
Tel: 02 6214 1641

**Co Researcher:** Prof Louise Burke  
Email: [Louise.burke@ausport.gov.au](mailto:Louise.burke@ausport.gov.au)  
Tel: 02 6214 1351

We would like to invite you to participate in this original postgraduate research project. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

#### **Aim:**

The primary aim of this project is to quantify the difference in resting metabolic rate (RMR) when it is measured at your bedside compared to when it is measured in a simulated laboratory setting (outpatient).

#### **Benefits:**

This study will allow us to determine a reliable and valid protocol for measuring RMR in elite athletes. You will get an accurate measure of your resting metabolic rate as well as of your body composition. The results of this study will go towards refining a best practice protocol for measuring RMR in elite athletes.

#### **What is involved?**

Your RMR is the measurement of how much energy your body uses to function when at rest. When you have a low RMR it may impair some body systems such as bone formation, immune function and protein synthesis which puts you at risk of soft tissue injury and may increase days lost to training. Therefore, it is important to have a reliable and practical protocol to measure RMR in athletes.

You will be required to stay overnight at AIS Residences for the duration of the study.

RMR is measured first thing in the morning after you've woken up, but before you are fully alert. RMR is measured by indirect calorimetry, which is when we collect the air you breathe out into large foil bags (Douglas Bag). For this study 3 RMR measures are required. Therefore you will need to stay overnight at the AIS for a minimum of 3 nights. If you currently live at the AIS we will require access to your room for those 3 mornings. On the first and second morning your RMR will be done either as a bedside measure or simulated lab measure.

#### **Bedside Measure:**

We will wake you up and give you a mouthpiece, while you lie in bed. The mouthpiece is similar to what you would use during a VO<sub>2</sub>max test. After 10 min of familiarising yourself with the mouthpiece you will put on a nose-clip and we will attach the first of two Douglas bags. Each bag will be attached for 10 min.

#### **Outpatient Measure:**

You will be woken up and asked to walk approximately 500m (to simulate walking to the physiology lab). After you return from your walk, you will have to return to a "resting" state. You will lie back down in your bed for a total of 25 min before we attach the first Douglas bag. In this instance we provide you with the mouthpiece after the first 10 min of your 25min "resting" time. The collection time of 10 min per bag remains the same.

It is important to breathe normally and remain relaxed during the collection periods. The concentrations of oxygen and carbon dioxide and the volume of air you breathed into each bag are then measured to calculate your metabolic rate.

Please note NS refers to the NHMRC 'National Statement' for the ethical conduct of research. For further information please go to <http://www.nhmrc.gov.au> and follow the links



You will be randomised the order of which you do the measures for mornings one and two. On morning three you will repeat either the bedside or SL measure.

Your RMR measurement is done after an 8 hour overnight fast, before you have eaten or have had something to drink and prior to exercise.

You will need to complete a food and activity diary during the study and wear an activity monitor during the day and night prior to each measurement to monitor dietary intake, exercise and other activity patterns to account for the potential confounding of RMR measurements due to these factors. RMR will not be measured following "rest" days from exercise.

The amount of fat free mass you have is an important factor when interpreting your RMR results. We use DXA to determine your fat free mass. Your DXA scan will be done on the morning of your bedside measure. You will need to lie still on the DXA scanning bed for 5-6 min while wearing minimal clothing (underwear for males and underwear and crop top with no underwire for females). You will need to provide a urine sample on the morning of your DXA scan.

#### **Total and daily time commitment:**

If you currently live or are staying at the AIS we anticipate that measurements will take an average of 1 hour each morning for a total of 3 hours for the duration of the study. If you are not currently staying at the AIS, you will be expected to stay overnight for 3-4 nights (approximately 10-12 hours per night). This would be a total time commitment of 36-48 hours. Breakfast, lunch and dinner will be provided during your stay if required.

#### **Who we are recruiting?**

We are recruiting male and female National and State level representative athletes for this study. You must be aged between 16-40 years old and are using the AIS as part of your daily training environment or for attending a camp. We will be excluding athletes who have a thyroid disorder or take medications that affect metabolism (ie Prednisone, anti-depressants), carrying an illness, pregnant or lactating and if they have recently been exposed to other sources of radiation or who are required to receive other exposure in the near future (eg X-ray, other medical contrast investigations). You will be required to complete a screening questionnaire and Pre-DXA questionnaire to assess your eligibility for the study.

#### **Adverse Effects and Withdrawal:**

The study has been deemed to be very low risk. You may feel some discomfort at having to wear a mouth piece. You are able to see clearly out of the hood and breathe normally. The study involves having a DXA scan which emits a small amount of radiation. The amount of radiation you will receive from the DXA is considerably less than what you would receive during a flight from Sydney to Perth. Participation in this study is completely voluntary. You are not under any obligation to participate. If you agree to participate, you can withdraw yourself and your data from the study at any time without adverse consequences.

#### **Confidentiality:**

Any data collected in the duration of the experiment will be stored electronically at the AIS. The data will be non-identifiable. All electronic data will be password protected, only accessible by the AIS Nutrition research team, and any paper records will be stored under lock and key.

Any information that you provide as well as your test results will be kept confidential. No data from any other individual participant will be provided to you by the researchers. The only people, other than yourself, who will have access to the information will be those who are directly involved with conducting the study. If you are a member of a National Sporting Organisation your identifiable results may also be made shared with coaching and support staff (e.g. sport dietitians, physiologist and doctors) to assist with servicing and competition preparation. By being involved in this study you agree to give us your permission to share your results with your coach and servicing support staff. The raw data will be retained by the principal investigator for five years in a secure cabinet at the Australian Institute of Sport. At the conclusion of this five year period all material containing confidential information will be destroyed. Any published data, oral presentations or written reports will exclude the names and other identifying personal traits of participants.

#### **Ethics Approval:**

Please note NS refers to the NHMRC 'National Statement' for the ethical conduct of research. For further information please go to <http://www.nhmrc.gov.au> and follow the links

This study has been approved by the Australian Institute of Sport ethics committee. If you have any concerns please feel free to contact the secretary of the AIS Ethics Committee on 02 6214 1577.

**Further information:**

Please contact Julia Bone or Prof. Louise Burke if you have any questions, concerns, or require further information in regards to any aspect of participating in this study.



### C) Informed Consent Form for Participants

Page 1 of 1

#### 'INFORMED CONSENT' FORM (Adult)

Project Title: Standardisation of RMR in athlete population – Comparison of bedside and outpatient measurements.

Principal Researchers: Julia Bone  
Co Researcher: Prof Louise Burke,

This is to certify that I, \_\_\_\_\_ hereby agree to participate as a volunteer in a scientific investigation as an authorised part of the research program of the Australian Sports Commission under the supervision of Louise Burke and Julia Bone.

The investigation and my part in the investigation have been defined and fully explained to me by Louise Burke or Julia Bone and I understand the explanation. A copy of the procedures of this investigation and a description of any risks and discomforts has been provided to me and has been discussed in detail with me.

- I have been given an opportunity to ask whatever questions I may have had and all such questions and inquiries have been answered to my satisfaction.
- I understand that I am free to deny any answers to specific items or questions in interviews or questionnaires.
- I understand that I am free to withdraw consent and to discontinue participation in the project or activity at any time, without disadvantage to myself.
- I understand that I am free to withdraw my data from analysis without disadvantage to myself.
- I understand that any data or answers to questions will remain confidential with regard to my identity.
- I certify to the best of my knowledge and belief, I have no physical or mental illness or weakness that would increase the risk to me of participating in this investigation.
- I am participating in this project of my (his/her) own free will and I have not been coerced in any way to participate.

*Privacy Statement: The information submitted will be managed in accordance with the ASC Privacy Policy.*

☐ I consent to the ASC keeping my personal information.

Signature of Subject: \_\_\_\_\_ Date: \_\_\_\_/\_\_\_\_/\_\_\_\_

I, the undersigned, was present when the study was explained to the subject/s in detail and to the best of my knowledge and belief it was understood.

Signature of Researcher: \_\_\_\_\_ Date: \_\_\_\_/\_\_\_\_/\_\_\_\_

Please note NS refers to the NHMRC 'National Statement' for the ethical conduct of research. For further information please go to <http://www.nhmrc.gov.au> and follow the links

### **'INFORMED CONSENT' FORM (Minor)**

**Project Title:** Standardisation of RMR in athlete population – Comparison of bedside and outpatient measurements.

Principal Researchers: Julia Bone  
Co-Researcher: Prof Louise Burke

This is to certify that I, \_\_\_\_\_ hereby agree to give permission to have my child participate as a volunteer in a scientific investigation as an authorised part of the research program of the Australian Sports Commission under the supervision of Louise Burke and Julia Bone.

The investigation and my child's part in the investigation have been defined and fully explained to me by Louise Burke or Julia Bone and I understand the explanation. A copy of the procedures of this investigation and a description of any risks and discomforts has been provided to me and has been discussed in detail with me.

- I have been given an opportunity to ask whatever questions my child or myself may have had and all such questions and inquiries have been answered to my satisfaction.
- I understand that my child is free to deny any answers to specific items or questions in interviews or questionnaires.
- I understand that my child is free to withdraw consent and to discontinue participation in the project or activity at any time, without disadvantage.
- I understand that my child is free to withdraw his/her data from analysis without disadvantage.
- I understand that any data or answers to questions will remain confidential with regard to my child's identity.
- I certify to the best of my knowledge and belief, my child has no physical or mental illness or weakness that would increase the risk to me (him/her) of participating in this investigation.
- My child is participating in this project of my (his/her) own free will and My child has) not been coerced in any way to participate.

*Privacy Statement: The information submitted will be managed in accordance with the ASC Privacy Policy.*

☐ I consent to the ASC keeping my personal information.

Signature of Participant: \_\_\_\_\_ Date: \_\_\_\_/\_\_\_\_/\_\_\_\_

Signature of Parent or  
Guardian of minor: (under 18 years) \_\_\_\_\_ Date: \_\_\_\_/\_\_\_\_/\_\_\_\_

I, the undersigned, was present when the study was explained to the subject/s in detail and to the best of my knowledge and belief it was understood.

Signature of Researcher: \_\_\_\_\_ Date: \_\_\_\_/\_\_\_\_/\_\_\_\_

Please note NS refers to the NHMRC 'National Statement' for the ethical conduct of research. For further information please go to <http://www.nhmrc.gov.au> and follow the links



### ***11.3 Study 2: The effect of prior exercise on measures of resting metabolic rate in trained men.***

#### ***A) Notice of Approval***

**Julia Bone**

---

**From:** Kylie Pashley <Kylie.Pashley@acu.edu.au> on behalf of Res Ethics <Res.Ethics@acu.edu.au>  
**Sent:** Thursday, 17 September 2015 11:56 AM  
**To:** Louise Burke; Louise Burke; Julia Bone  
**Cc:** Res Ethics  
**Subject:** 2015-133H Ethics application approved!

**Follow Up Flag:** FollowUp  
**Flag Status:** Completed

Dear Applicant,

Principal Investigator: Dr Louise Burke

Student Researcher: Julia Bone (HDR student) Ethics Register Number: 2015-133H Project Title: Standardisation of the measurement of Resting Metabolic Rate (RMR) in an athletic population- Effect of environment and prior exercise Risk Level: Low Risk Date Approved: 17/09/2015 Ethics Clearance End Date: 30/09/2016

This email is to advise that your application has been reviewed by the Australian Catholic University's Human Research Ethics Committee and confirmed as meeting the requirements of the National Statement on Ethical Conduct in Human Research.

The data collection of your project has received ethical clearance but the decision and authority to commence may be dependent on factors beyond the remit of the ethics review process and approval is subject to ratification at the next available Committee meeting. The Chief Investigator is responsible for ensuring that outstanding permission letters are obtained, interview/survey questions, if relevant, and a copy forwarded to ACU HREC before any data collection can occur. Failure to provide outstanding documents to the ACU HREC before data collection commences is in breach of the National Statement on Ethical Conduct in Human Research and the Australian Code for the Responsible Conduct of Research. Further, this approval is only valid as long as approved procedures are followed.

If your project is a Clinical Trial, you are required to register it in a publicly accessible trials registry prior to enrolment of the first participant (e.g. Australian New Zealand Clinical Trials Registry <http://www.anzctr.org.au/>) as a condition of ethics approval.

If you require a formal approval certificate, please respond via reply email and one will be issued.

Researchers who fail to submit a progress report may have their ethical clearance revoked and/or the ethical clearances of other projects suspended. When your project has been completed a progress/final report form must be submitted. The information researchers provide on the security of records, compliance with approval consent procedures and documentation and responses to special conditions is reported to the NHMRC on an annual basis. In accordance with NHMRC the ACU HREC may undertake annual audits of any projects considered to be of more than low risk.

It is the Principal Investigators / Supervisors responsibility to ensure that:

1. All serious and unexpected adverse events should be reported to the HREC with 72 hours.
2. Any changes to the protocol must be reviewed by the HREC by submitting a Modification/Change to Protocol Form prior to the research commencing or continuing. <http://research.acu.edu.au/researcher-support/integrity-and-ethics/>
3. Progress reports are to be submitted on an annual basis. <http://research.acu.edu.au/researcher-support/integrity-and-ethics/>

4. All research participants are to be provided with a Participant Information Letter and consent form, unless otherwise agreed by the Committee.
5. Protocols can be extended for a maximum of five (5) years after which a new application must be submitted. (The five year limit on renewal of approvals allows the Committee to fully re-review research in an environment where legislation, guidelines and requirements are continually changing, for example, new child protection and privacy laws).

Researchers must immediately report to HREC any matter that might affect the ethical acceptability of the protocol eg: changes to protocols or unforeseen circumstances or adverse effects on participants.

Please do not hesitate to contact the office if you have any queries.

Kind regards,

Kylie Pashley  
on behalf of ACU HREC Chair, Dr Nadia Crittenden

Ethics Officer | Research Services  
Office of the Deputy Vice Chancellor (Research) Australian Catholic University

THIS IS AN AUTOMATICALLY GENERATED RESEARCHMASTER EMAIL

## B) Initial Letter to Participant



### INFORMATION TO PARTICIPANTS – ALL CONDITIONS

**Project Title:** Standardisation of the measurement of Resting Metabolic Rate (RMR) in an athletic population. The effect of Environmental Factors and Exercise on the Measurement of RMR

**PRINCIPAL INVESTIGATOR:** Prof Louise Burke

**STUDENT RESEARCHER:** Julia Bone

**STUDENT'S DEGREE:** PhD

We would like to invite you to participate in this original postgraduate research project. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

Your RMR is the measurement of how much energy your body uses to function when at rest. When you have a low RMR it may impair some body systems such as bone formation, immune function and protein synthesis which puts you at risk of soft tissue injury and may increase days lost to training. Therefore, it is important to have a reliable and practical protocol to measure RMR in athletes.

#### **Aim:**

There are three aims to this study:

1. To see if RMR is higher when you are exposed to noise, cooler temperature or different lying position
2. To compare RMR following 1-3 days of exercise compared to 1-3 days of no exercise
3. To determine the minimum practical rest period from exercise prior to an RMR measurement

#### **Benefit:**

This study will allow us to refine our current protocols for measuring resting metabolic rate in athletes so that the measurement is accurate. You will see how different environments and how exercise can acutely change your RMR. You will also get an accurate measure of your RMR and body composition.

#### **What is involved?**

RMR is measured first thing in the morning after you've woken up, but before you are fully alert. RMR is measured by indirect calorimetry, which is when we collect the air you breathe out into large foil bags (Douglas Bag).

If you are staying at the AIS you will need to walk slowly from Residences to the Physiology lab. If you are coming from your house please drive or be driven to the AIS and walk slowly to the entrance of the physiology lab (Building 19).

Upon entering the lab you will lie down for a 25 minute rest period before we begin measuring your RMR. We will give you a mouthpiece or facemask 10 min into the rest period for you to familiarise yourself with it. Once the 25min rest period is finished we will attach the first of three Douglas bags. Each bag will be attached for 10 min. It is important to breathe normally and remain relaxed during the collection periods. The concentrations of oxygen and carbon dioxide and the volume of air you breathed into each bag are then measured to calculate your metabolic rate.



This study involves three different conditions. We are asking you participate in one of three of these conditions.

**Condition A: - The effect of Environmental Factors on the Measurement of RMR**

You will be required to have your RMR measured under four different settings.

**Setting 1:** Standard RMR – This is your baseline RMR measurement. You're RMR will be measured in a quiet room under temperate conditions (22-24°C) while lying still on your back.

**Setting 2:** Noise condition – you will be required to listen to music/or sounds during your RMR measurement using headphones, while lying on your back in a room set to 22-24°C.

**Setting 3:** Positioning - you will be reclined using pillows behind your back so that you are reclining but not sitting up right or you will be given the option to move on to your side during the measurement. The room will remain quiet and set to a temperature of 22-24°C.

**Setting 4:** Your RMR will be measured in a room set to a temperature of 15°C. Skin temperature will be measured using an activity monitor and skin temperature. You will be required to lie on your back in a quiet room.

The order of the setting you have your RMR measured under will be randomised. On the morning of your first RMR you will have your body composition assessed by a whole body DXA scan.

**Condition B & C – The acute effect of exercise on the measurement of RMR in an athletic population**

Part 1: 1-3 days training vs 1-3 days no training

**Non-training Protocol:** You will have three RMR measurements done over three consecutive mornings. During this time you will not be permitted to do any exercise. The first of three RMRs will be measured after not having exercised for one day prior to measurement. Your body composition will be assessed by DXA scan after your 2<sup>nd</sup> RMR measurement.

**Training Protocol:** You will undertake a self-selected resistance or aerobic exercise session (Post-ex day 0). The following morning you will have your RMR measured (Post-ex day 1). You will not be allowed to exercise for the remainder of day. You will have your RMR measured again the next morning (Post-ex day 2). Following the day 2 RMR, you will complete another exercise session and have your RMR measured again the following morning (Post-ex day 3). . Your body composition will be assessed by DXA scan after your 2<sup>nd</sup> RMR measurement on Post-ex day 2.

Part 2: Afternoon training vs morning training

**Morning protocol:** You will be allocated to do either a self-selected hard resistance session or a cycling aerobic session to be completed prior to 12 pm. You will then rest and perform no further exercise or training prior to your RMR measure using the following morning. Depending on the results of part A, 24-72 h later you will rest in the morning and replicate the same training session, in the afternoon (to be finished by 6 pm) prior to your RMR measure the next day. Your body composition will be assessed by DXA scan after your 2<sup>nd</sup> RMR measurement.

**Pre-RMR Preparation**

For all conditions you will need to set an alarm to wake up. Upon waking please go to the toilet and collect a mid-stream urine sample. For the RMR to be accurate you will not be allowed to eat or drink until after your RMR test is finished for the morning. Breakfast will be provided once you have finished your RMR measurement for the morning.

You will be required to wear the same type of clothing throughout the study. Track pants and a t-shirt with thin cotton socks only. As we are looking at the effect of different temperatures it is important that you don't compensate when it is cooler. Please do not wear any tights under your track pants or thick socks, no gloves or beanie's or Ugg boots/slippers. You may wear a jacket to the lab however this will have to be removed for the rest and testing periods.

**Biochemical, Urinary and Physiological Measures.**

For each RMR condition, you will be weighed each morning and you will be required to provide a mid-stream first void urine sample to assess hydration status and urinary ketones. In addition, we will measure your blood pressure, fasting blood glucose and cholesterol once your RMR measures are finished but before you get off the bed.

**Food and Activity Monitoring**

You will be required to complete a food and activity diary during the study and wear a SenseWear activity monitor during the day prior to each measurement. This is to monitor your dietary intake, exercise and other activity patterns which may affect your RMR measurement. You will be required to wear the activity monitor overnight as this will provide us with your energy expenditure while you sleep as well as monitor your sleep duration and sleep quality.

**Body Composition**

The amount of fat free mass you have is an important factor when interpreting your RMR results. We use DXA to determine your fat free mass. Your DXA scan will be done on the morning of your bedside measure. You will need to lie still on the DXA scanning bed for 5-6 min while wearing minimal clothing (underwear for males and underwear and crop top with no underwire for females). You will need to provide a urine sample on the morning of your DXA scan.

**Summary of your commitment**

We anticipate that measurements will take an average of 1.5 hour each morning. For condition A this would total 6 hours in total over 4 mornings. For Condition B: 9 hours in total plus your commitment to not exercise for 3 days and do either resistance or cycling aerobic exercise for 3 days. For Condition C this would be 3 hours in total over 2 mornings plus committing to the morning and afternoon training sessions

**Who we are recruiting?**

We are recruiting well-trained competitive male and female athletes living locally or visiting the AIS, who are aged between 16-40 years old and who train for a minimum of 4 sessions or 20 hours per week for their sport, have a training history of at least 2 year or be representative for their sport at national and international level.

Participants will be excluded if they identify as having a thyroid or other metabolic disorders and are taking medications that affect metabolism (i.e. Prednisone, anti-depressants), have a current illness,



injury or a pregnant or lactating. Participants who have recently been exposed to other sources of radiation or who have a requirement to receive other exposure in the near future (e.g. conventional X-rays, other medical contrast investigations) will also be excluded. Amenorrheic athletes will be included if they report no current injuries or a current or previous diagnosis of hyper/hypo thyroidism.

You will be required to complete a screening questionnaire and Pre-DXA questionnaire to assess your eligibility for the study. They ask questions about the inclusion and exclusion criteria above. In addition female athletes will also be asked questions regarding their menstrual cycle and history due to difference in RMR over the menstrual cycle and if they are taking the oral contraceptive pill.

You will be required to sign a written informed consent form for either Condition A (Environment), Condition B-1 (3 days exercise/no exercise) or Part B-2(AM vs PM exercise). You will only be required to partake in one aspect of the study; however you can be involved in the entire study if they wish to.

#### **Adverse Effects and Withdrawal:**

The study has been deemed to be very low risk. You may feel some discomfort at having to wear a mouth piece or facemask. The study involves having a DXA scan which emits a small amount of radiation. The amount of radiation you will receive from the DXA is considerably less than what you would receive during a flight from Sydney to Perth. Participation in this study is completely voluntary. You are not under any obligation to participate. If you agree to participate, you can withdraw yourself and your data from the study at any time without adverse consequences.

#### **Confidentiality:**

Any data collected in the duration of the experiment will be stored electronically at the AIS. The data will be non-identifiable. All electronic data will be password protected, only accessible by the AIS Nutrition research team, and any paper records will be stored under lock and key.

Any information that you provide as well as your test results will be kept confidential. No data from any other individual participant will be provided to you by the researchers. The only people, other than yourself, who will have access to the information will be those who are directly involved with conducting the study. If you are a member of a National Sporting Organisation or a dAIS athlete your identifiable results may also be shared with coaching and support staff (e.g. sport dietitians, physiologist and doctors) to assist with servicing and competition preparation. By being involved in this study you give us your permission to share your results with your coach and servicing support staff. The raw data will be retained by the principal investigator in a secure cabinet and server network at the Australian Institute of Sport. Any published data, oral presentations or written reports will exclude the names and other identifying personal traits of participants.

#### **Ethics Approval:**

This study has been approved by Human Research Ethics Committee at the Australian Catholic University (review number 2015 XXX) and the Australian Institute of Sport Ethics Committee (approval number).

#### **What if I have a complaint or any concerns?**

If you have any complaints or concerns about the conduct of the project, you may write to the Manager of the Human Research Ethics Committee care of the Office of the Deputy Vice Chancellor (Research) or Helene Rushby, Research Coordinator at the AIS.



Manager, Ethics  
c/o Office of the Deputy Vice Chancellor (Research)  
Australian Catholic University  
North Sydney Campus  
PO Box 968  
NORTH SYDNEY, NSW 2059  
Ph.: 02 9739 2519  
Fax: 02 9739 2870  
Email: [res.ethics@acu.edu.au](mailto:res.ethics@acu.edu.au)



Helene Rushby  
**Research Coordinator**  
Performance Research Centre  
**Australian Institute of Sport**  
PO BOX 176 Belconnen ACT 2616  
Ph: 02 62 14 1577  
Email: [helene.rushby@ausport.gov.au](mailto:helene.rushby@ausport.gov.au)

Any complaint or concern will be treated in confidence and fully investigated. You will be informed of the outcome.

**I want to participate! How do I sign up?**

If you wish to participate in this study please email your interest to [Julia.bone@ausport.gov.au](mailto:Julia.bone@ausport.gov.au). We will then contact you to discuss what the study involves and provide written consent forms. The Consent form, Pre-screening questionnaire and the Pre-DXA Questionnaire must be signed and completed. Completed consent forms may be returned by email or in person.

Yours sincerely,

Julia Bone

Louise Burke



*C) Informed Consent Form for Participants*

**'INFORMED CONSENT' FORM (Adult)**

**Project Title:** Standardisation of the measurement of Resting Metabolic Rate (RMR) in an athletic population - The acute effect of exercise on the measurement of RMR

**Researchers:** Julia Bone & Prof Louise Burke

This is to certify that I, [REDACTED] hereby agree to participate as a volunteer in a scientific investigation as an authorised part of the research program of the Australian Catholic University and the Australian Sports Commission under the supervision of Louise Burke and Julia Bone.

The investigation and my part in the investigation have been defined and fully explained to me by Louise Burke or Julia Bone and I understand the explanation. A copy of the procedures of this investigation and a description of any risks and discomforts has been provided to me and has been discussed in detail with me.

- I have been given an opportunity to ask whatever questions I may have had and all such questions and inquiries have been answered to my satisfaction.
- I understand that I am free to deny any answers to specific items or questions in interviews or questionnaires.
- I understand that I am free to withdraw consent and to discontinue participation in the project or activity at any time, without disadvantage to myself.
- I understand that I am free to withdraw my data from analysis without disadvantage to myself.
- I understand that any data or answers to questions will remain confidential with regard to my identity.
- I certify to the best of my knowledge and belief, I have no physical or mental illness or weakness that would increase the risk to me of participating in this investigation.
- I am participating in this project of my (his/her) own free will and I have not been coerced in any way to participate.

***Privacy Statement:** The information submitted will be managed in accordance with the ASC Privacy Policy.*

☐ *I consent to the ASC keeping my personal information.*

Signature of Subject: \_\_\_\_\_ Date: \_\_\_\_/\_\_\_\_/\_\_\_\_

I, the undersigned, was present when the study was explained to the subject/s in detail and to the best of my knowledge and belief it was understood.

Signature of Researcher: \_\_\_\_\_ Date: \_\_\_\_/\_\_\_\_/\_\_\_\_

## *D) Project Amendment Approval*

**Julia Bone**

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**From:** Ms Pratigya Pozniak <pratigya.pozniak@acu.edu.au>  
**Sent:** Friday, 15 January 2016 9:26 AM  
**To:** Dr Louise Burke; Julia Bone  
**Cc:** Ms Pratigya Pozniak  
**Subject:** 2015-133H Modification approved

Dear Louise,

Ethics Register Number : 2015-133H

Project Title : Standardisation of the measurement of Resting Metabolic Rate (RMR) in an athletic population- Effect of environment and prior exercise End Date : 30/09/2016

Thank you for submitting the request to modify form for the above project.

The Chair of the Human Research Ethics Committee has approved the following modification(s):

- Combine Parts B and C of the project. Participants would 2 RMR measurements per week over 3 weeks.

We wish you well in this ongoing research project.

Kind regards,  
Ms Pratigya Pozniak

Ethics Officer | Research Services  
Office of the Deputy Vice Chancellor (Research)

'''  
T: F:

THIS IS AN AUTOMATICALLY GENERATED RESEARCHMASTER EMAIL

*E) Updated Letter to Participant*



**INFORMATION TO PARTICIPANTS – ALL CONDITIONS**

**Project Title:** The effect of noise, temperature, body position and exercise on the measurement of resting metabolic rate.

**PRINCIPAL INVESTIGATOR:** Prof Louise Burke

**STUDENT RESEARCHER:** Julia Bone

**STUDENT'S DEGREE:** PhD

We would like to invite you to participate in this original postgraduate research project. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear or if you would like more information.

Your resting metabolic rate (RMR) is the measurement of how much energy your body uses to function when at rest. When you have a low RMR it may impair some body systems such as bone formation, immune function and protein synthesis which puts you at risk of soft tissue injury and may increase days lost to training. Therefore, it is important to have a reliable and practical protocol to measure RMR in athletes.

**Aim:**

There are two aims to this study:

1. To see if RMR is higher when you are exposed to noise, cooler temperature or different lying positions
1. To compare RMR when measured 12, 24, 36 and 48 hours post exercise.

**Benefit:**

This study will allow us to refine our current protocols for measuring resting metabolic rate in athletes so that the measurement is accurate. You will see how different environments and how exercise can acutely change your RMR. You will also get an accurate measure of your RMR and body composition.

**What is involved?**

RMR is measured first thing in the morning after you've woken up, but before you are fully alert. RMR is measured by indirect calorimetry, which is when we collect the air you breathe out into large foil bags (Douglas Bag).

If you are staying at the Australian Institute of Sport (AIS) you will need to walk slowly from Residences to the Physiology lab. If you are coming from your house please drive or be driven to the AIS and walk slowly to the entrance of the physiology lab (Building 19).

Upon entering the lab you will lie down for a 25 minute rest period before we begin measuring your RMR. We will give you a mouthpiece or facemask 10 min into the rest period for you to familiarise yourself with it. Once the 25min rest period is finished we will attach the first of three Douglas bags. Each bag will be attached for 10 min. It is important to breathe normally and remain relaxed during the collection periods. The concentrations of oxygen and carbon dioxide and the volume of air you breathed into each bag are then measured to calculate your metabolic rate.

This study involves two different conditions. We are asking you participate in one of two of these conditions.



**Condition A: - The effect of Environmental Factors on the Measurement of RMR**

You will be required to have your RMR measured under four different settings.

**Setting 1:** Standard RMR – This is your baseline RMR measurement. You're RMR will be measured in a quiet room under temperate conditions (22-24°C) while lying still on your back.

**Setting 2:** Noise condition – you will be required to listen to music/or sounds during your RMR measurement using headphones, while lying on your back in a room set to 22-24°C.

**Setting 3:** Positioning - you will be reclined using pillows behind your back so that you are reclining but not sitting up right or you will be given the option to move on to your side during the measurement. The room will remain quiet and set to a temperature of 22-24°C.

**Setting 4:** Your RMR will be measured in a room set to a temperature of 15°C. Skin temperature will be measured using an activity monitor and skin temperature. You will be required to lie on your back in a quiet room.

The order of the setting you have your RMR measured under will be randomised. On the morning of your first RMR you will have your body composition assessed by a whole body DXA scan.

**Condition B – The acute effect of exercise on the measurement of RMR in a trained population**

You will have your RMR measured over two consecutive mornings over 3 different weeks. Your RMR will be measured under one of the following exercise conditions.

- 1) You will complete your standard training session in the morning (finishing prior to 10am) and then not train the rest of the day (Day 0). Early the next morning (Day 1) you will have your RMR. Following your RMR you will not be permitted to train or do any exercise for the rest of the day. You will then come back on the morning of Day 2 and have your RMR measured a final time. You are then free to train as usual.
- 2) You will complete your standard training session in the afternoon (between 12pm – 4pm) and then not train the rest of the day (Day 0). Early the next morning (Day 1) you will have your RMR measured. Following your RMR you will not be permitted to train or do any exercise for the rest of the day (Day 1). You will then come back on the morning of Day 2 and have your RMR measured a final time. You are then free to train as usual.

You will have to come in a third time to repeat part 1. The order of the conditions will be randomised. The total time commitment for Condition B is 6 RMR testing sessions, (each session is ~ 1 hour long). Your Body composition will be assessed by DXA each week after your Day 1 RMR measurement.

**Pre-RMR Preparation**

For all conditions you will need to set an alarm to wake up. Upon waking please go to the toilet and collect a mid-stream urine sample. For the RMR to be accurate you will not be allowed to eat or



drink until after your RMR test is finished for the morning. Breakfast will be provided once you have finished your RMR measurement for the morning.

You will be required to wear the same type of clothing throughout the study. Track pants and a t-shirt with thin cotton socks only. As we are looking at the effect of different temperatures it is important that you don't compensate when it is cooler. Please do not wear any tights under your track pants or thick socks, no gloves or beanie's or Ugg boots/slippers. You may wear a jacket to the lab however this will have to be removed for the rest and testing periods.

### **Biochemical, Urinary and Physiological Measures.**

For each RMR condition, you will be weighed each morning and you will be required to provide a mid-stream first void urine sample to assess hydration status and urinary ketones. In addition, we will measure your blood pressure, fasting blood glucose and cholesterol once your RMR measures are finished but before you get off the bed. Your glucose and cholesterol will be measured by taking a finger prick blood sample on your left or right hand.

### **Food and Activity Monitoring**

You will be required to complete a food and activity diary during the study and wear a SenseWear activity monitor during the day prior to each measurement. This is to monitor your dietary intake, exercise and other activity patterns which may affect your RMR measurement. You will be required to wear the activity monitor overnight as this will provide us with your energy expenditure while you sleep as well as monitor your sleep duration and sleep quality.

### **Body Composition**

The amount of fat free mass (mass of your muscles, bones, organs and fluid) you have is an important factor when interpreting your RMR results. We use DXA to determine your fat free mass. Your DXA scan will be done on the morning of your bedside measure. You will need to lie still on the DXA scanning bed for 5-6 min while wearing minimal clothing (underwear for males and underwear and crop top with no underwire for females). You will need to provide a urine sample on the morning of your DXA scan.

### **Summary of your commitment**

We anticipate that measurements will take an average of 1.5 hour each morning. For condition A this would total 6 hours in total over 4 mornings. For Condition B: 6 hours of testing in total plus your commitment to not exercise for the 2 or 1.5 day period and to keep the food and activity diary each week.

### **Who we are recruiting?**

We are recruiting well-trained competitive male athletes living locally or visiting and staying at the AIS, who are aged between 16-40 years old and who train for a minimum of 4 sessions or 10 hours per week for their sport, have a training history of at least 2 year or be representative for their sport at national and international level.

Participants will be excluded if they identify as having a thyroid or other metabolic disorders and are taking medications that affect metabolism (i.e. Prednisone, anti-depressants), have a current illness, injury or are pregnant or lactating. Participants who have recently been exposed to other sources of radiation or who have a requirement to receive other exposure in the near future (e.g. conventional

X-rays, other medical contrast investigations) will also be excluded. Amenorrheic athletes will be included if they report no current injuries or a current or previous diagnosis of hyper/hypo thyroidism.

You will be required to complete a screening questionnaire and Pre-DXA questionnaire to assess your eligibility for the study. They ask questions about the inclusion and exclusion criteria above.

You will be required to sign a written informed consent form for either Condition A (Environment) or Condition B Exercise). You will only be required to partake in one aspect of the study; however you can be involved in the entire study if you wish to.

#### **RMR Familiarisation Visit**

If you meet our eligibility criteria, we ask that you briefly visit the AIS prior to starting the study so that you can familiarise yourself with the mouthpiece the protocol we will use. This visit should take no longer than 30 min.

#### **Adverse Effects and Withdrawal:**

The study has been deemed to be very low risk. You may feel some discomfort at having to wear a mouth piece or facemask. The study involves having a DXA scan which emits a small amount of radiation. The amount of radiation you will receive from the DXA is considerably less than what you would receive during a flight from Sydney to Perth. Participation in this study is completely voluntary. You are not under any obligation to participate. If you agree to participate, you can withdraw yourself and your data from the study at any time without adverse consequences.

#### **Confidentiality and Data Storage:**

Any data collected during this study will be stored electronically at the AIS. The data will be non-identifiable. All electronic data will be password protected, only accessible by the AIS Nutrition research team, and any paper records will be stored under lock and key.

The non-identifiable data may be kept for future studies. By signing the Consent Form you agree that your non-identifiable data can be analysed for future research.

Any information that you provide as well as your test results will be kept confidential. No data from any other individual participant will be provided to you by the researchers. The only people, other than yourself, who will have access to the information will be those who are directly involved with conducting the study.

The raw data will be retained by the principal investigator in a secure cabinet and server network at the AIS as per the Australian Privacy Principles (APP) for 5 years. If you are an NSO or dAIS sponsored or scholarship athlete any scientific data will be obtained for 25 years and medical information will be retained for 75 years. For further information please read the APP which our researchers can provide with the informed consent form.

Any published data, oral presentations or written reports will exclude the names and other identifying personal traits of participants.

#### **NSO and dAIS Athletes**



If you are a member of a National Sporting Organisation (NSO) or a dAIS sponsored athlete and have been recruited to the study through your sport, coach, dietitian, doctor, strength & conditioning trainer or physiologist your identifiable results may also be shared with coaching and support staff. This is to assist with sport servicing and your competition preparation. For example, you may see the dietitian for nutrition monitoring and your coach or exercise physiologist may make changes to your training load. By being involved in this study you give us your permission to share your results with your coaching and support staff team.

**Ethics Approval:**

This study has been approved by Human Research Ethics Committee at the Australian Catholic University (Ethics Register Number 2015-133H) and the Australian Institute of Sport Ethics Committee.

**What if I have a complaint or any concerns?**

If you have any complaints or concerns about the conduct of the project, you may write to the Manager of the Human Research Ethics Committee care of the Office of the Deputy Vice Chancellor (Research) or Helene Rushby, Research Coordinator at the AIS.

Manager, Ethics  
c/o Office of the Deputy Vice Chancellor (Research)  
Australian Catholic University  
North Sydney Campus  
PO Box 968  
NORTH SYDNEY, NSW 2059  
Ph.: 02 9739 2519  
Fax: 02 9739 2870  
Email: [res.ethics@acu.edu.au](mailto:res.ethics@acu.edu.au)

Helene Rushby  
**Research Coordinator**  
Performance Research Centre  
**Australian Institute of Sport**  
PO BOX 176 Belconnen ACT 2616  
Ph: 02 62 14 1577  
Email: [helene.rushby@ausport.gov.au](mailto:helene.rushby@ausport.gov.au)

Any complaint or concern will be treated in confidence and fully investigated. You will be informed of the outcome.

**I want to participate! How do I sign up?**

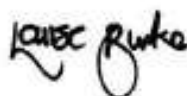
If you wish to participate in this study please email your interest to [Julia.bone@ausport.gov.au](mailto:Julia.bone@ausport.gov.au). We will then contact you to discuss what the study involves and provide written consent forms. Please advise if you would also like a copy of the Australian Privacy Principles. The Consent form, Pre-screening questionnaire and the Pre-DXA Questionnaire must be signed and completed. Completed consent forms may be returned by email or in person.

Yours sincerely,

Julia Bone



Louise Burke



**11.4 Study 3: Manipulation of muscle creatine & glycogen changes DXA estimates of body composition and Study 4: Ultrasound Technology Fails To Provide Indirect Estimate of Muscle Glycogen Concentration**

*A) Notice of Approval*



**MINUTE**

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TO:	Dr Louise Burke	CC:
FROM:	Ms Helene Rushby	
SUBJECT:	Approval from AIS Ethics Committee	DATE: 23 <sup>rd</sup> May 2014

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On the 24<sup>th</sup> April 2014, the AIS Ethics Committee gave consideration out of session to your submission titled **"Interactive effect of manipulating muscle creatine and glycogen stores on endurance performance and DXA measurements of lean mass"**. The Committee saw no ethical reason why your project should not proceed.

The approval number for this project: 20140612

It is a requirement of the AIS Ethics Committee that the Principal Researcher (you) advise all researchers involved in the study of Ethics Committee approval and any conditions of that approval. You are also required to advise the Ethics Committee immediately (via the Secretary) of:

Any proposed changes to the research design,  
Any adverse events that may occur,

Researchers are required to submit **annual status reports** and **final reports** to the secretary of the AIS Ethics Committee. Details of status report requirements are contained in the "Guidelines" for ethics submissions.

Please note the approval for this submission expires on the 30<sup>th</sup> June 2016 after which time an extension will need to be sought.

If you have any questions regarding this matter, please don't hesitate to contact me on (02) 6214 1577

A handwritten signature in black ink, appearing to be 'Helene Rushby', written over a horizontal line.

Sincerely  
Helene Rushby  
Secretary, AIS EC

## *B) Initial Letter to Participant*

### INFORMATION TO PARTICIPANTS

**Research Title** Interactive effect of manipulating muscle creatine and glycogen stores on endurance performance and DXA measurements of lean mass

**Principal Researcher:**

**Prof. Louise Burke (Australian Institute of Sport)**

- Tel: 02 6214 1351 0422 635 869
- Email: [louise.burke@ausport.gov.au](mailto:louise.burke@ausport.gov.au)

**Co-Researchers:**

**Mr. Kristyen Tomcik (Australian Catholic University)**

- Tel: 0405 903 080
- Email: [kktomc001@myacu.edu.au](mailto:kktomc001@myacu.edu.au)

**Prof. John Hawley (Australian Catholic University)**

- Tel: 03 995335523 0408 089 959
- Email: [john.hawley@acu.edu.au](mailto:john.hawley@acu.edu.au)

**Julia Bone (Australian Institute of Sport)**

- Tel 02 6214 1641 0487 780 843
- Email: [Julia.bone@ausport.gov.au](mailto:Julia.bone@ausport.gov.au)

We would like to invite you to participate in this original research project, a collaboration between the AIS and ACU. You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. We realise this information sheet is lengthy, but please take time to read it carefully and discuss it with others if you wish. Ask us if you would like more information.

#### Background

The performance of endurance sports is reliant on muscle stores of glycogen (carbohydrate). Indeed, strategies that increase this muscle fuel (carbohydrate loading) are able to enhance race outcomes by extending the time that optimal pacing strategies can be maintained. Some studies have shown that glycogen stores can be further increased if the muscle has been previously loaded with creatine. Creatine supplementation protocols, which increase this muscle fuel associated with brief high-intensity exercise, are familiar to athletes who undertake repeated sprint events and resistance training. However, if they can also enhance glycogen stores, they may provide an ergogenic aid for endurance athletes such as road cyclists, marathon runners and cyclists. To date the performance benefits of increased levels of carbohydrate loading in endurance events haven't been investigated. One particular issue that might need to be explored is the effect of the extra body mass associated with both creatine and carbohydrate loading that occurs due to the extra fuel stored in the muscle and the water bound to it.

While the primary aim of manipulating muscle glycogen and creatine stores is to enhance performance, a secondary outcome of changing muscle fuel and water content is to change the results of a DXA scan of lean mass of the region. This might explain why we observe that DXA measurements of what we interpret as "muscle" can suddenly fluctuate by 0.5-1 kg without reflecting a real change. Since muscle size and strength are important in sports performance, we are always searching for ways to either improve our ability to measure them reliably or to better interpret the results of DXA scans.

The current study has been designed to achieve two separate outcomes.

Please note NS refers to the NHMRC 'National Statement' for the ethical conduct of research. For further information please go to <http://www.nhmrc.gov.au> and follow the links

**Aim:**

1. The **primary aim** is to investigate whether creatine loading increases the glycogen storage achieved by a carbohydrate loading program, and whether this translates into better performance of a prolonged cycling protocol designed to mimic elements of real-life racing
2. A secondary **specific aim**, is to investigate the size of the alterations in estimates of lean mass, **measured** using DXA, due to creatine loading and the depletion and supercompensation of muscle glycogen stores.

**Benefits:**

We are aiming for two separate benefits from undertaking this study of glycogen and creatine loading.

- The first theme focuses on the potential application of creatine supplementation to the performance of endurance athletes using a protocol that simulates some important aspects of real-life sport. One particular element of interest is the effect of any increase in body mass on performance of a weight-sensitive sporting protocol
- The second theme addresses the possibility that alterations in these muscle fuels introduce an artefact into DXA estimates of body composition in athletes. Knowing this can help us to either create a more reliable set of standardised conditions for measuring lean mass, or to better interpret the results of measurements under certain conditions.

Both aspects of this study are novel and will help with the preparation of athletes.

**What is involved?**

- Your first task will be to undertake testing of your aerobic capacity (VO<sub>2</sub>max) and a chance to practice part of the performance protocol we have chosen for this study. The performance trial is described in more detail more below, but on this testing day, you will only do half of the Veloiron ride (50 km including sprints) followed by riding your own bike on a treadmill which is at an 8% incline to simulate riding up a hill.
- You will then undertake a second practice day within the next week to make sure you are familiar with the best pacing strategies and technical skills needed to perform well in the real trials
- Performance trials will be undertaken three times, each a week apart from the other. In each case, you will be provided with a packaged diet for 48 hours beforehand, which you must follow faithfully, and you will undertake a standardised training program for the last 24 hours.
  - All participants will receive the same dietary program before Performance Trial 1 – a menu providing a carbohydrate intake of 6 g per kg of your body mass, which should allow you to recover normal stores of muscle glycogen.
  - At the end of Performance Trial 1, the group will be divided into two – with one half receiving a creatine loading and maintenance supplementation protocol for the following 2 weeks, while the other half will receive placebo (dummy) supplements. This supplementation program will require you to undertake 5 days in which you will consume a number of capsules of a creatine or placebo powder at 4 separate times of the day (breakfast, lunch, after training and dinner). For the remainder of the two week period you will only need to consume a single dose of capsules with your breakfast meal. This activity will mean that Trials 2 and 3 will allow a comparison between a Creatine Group and a Placebo Group
  - Before Performance Trials 2 and 3, you will receive each of two different diets in an order that we will keep secret from you until the end of the study. One menu will provide the same amount of carbohydrate as in Trial 1, while the other will contain twice as much carbohydrate to allow you to carbohydrate load. Half of each of the two groups (Creatine or Placebo) will receive the carbohydrate loading one first while the other half will do the opposite pattern. This part of the study will investigate the effects of carbohydrate loading with or without prior creatine loading
- Each Performance Trial day will follow the same procedures
  - You will arrive at the Lab fasted (drinking only water since ~ 9 pm the night before the trial) on the morning of the trial). The first activity will be to

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undertake a whole body DXA scan which requires you to lie still on the DXA scanning bed for ~ 5 minutes. A separate booklet explaining the DXA procedure will be provided to you.

- A measurement of total body water using a BIS machine will be undertaken while you remain lying down. This scan will require us to place 4 electrodes on your wrists and ankles. No radiation is involved.
- Following the scans, we will perform a biopsy to take a small amount of muscle from your quadriceps. This will be performed by an experienced sports physician, under local anaesthetic and following the AIS Best Practice Protocol that is described in a separate booklet.
- You will then consume a standard breakfast simulating a pre-race meal and wait for 2 hours before commencing your Performance Trial.
- The Performance trial will start with a 100 km cycling protocol undertaken on the Velotron ergometer which has been set up to mimic your bike geometry. You will undertake most of this at a set workload, determined by the results of your initial testing. However at prescribed segments, you will be asked to undertake a 1 km or 4 km interval as fast as possible where we will measure your performance time (10-14, 20-21, 30-34, 40-41, 50-54, 60-61, 70-74, 80-81, 90-94 and 99-100 km). This represents some of the challenges of road cycling on a flat course where your weight is supported. You will be aware of the elapsed distance during the 100 km protocol, but not the time or your power outputs during the sprint phases.
- At the finish of this segment, you will switch to riding your own bike on a treadmill set to an incline of 8% to mimic an uphill finish to a road race. You will be asked to ride for as long as you can at a speed that is equivalent to 88% of your Peak Power Output. Again, your elapsed and final finishing time will be kept hidden from you until the end of the study.
- During each Performance Trial you will consume carbohydrate and fluid according to a standardised race nutrition plan
- To encourage the best performance possible, we will organise a prize bank of \$15,000 which will be offered to the top 5 riders (\$5000, 4000, 3000, 2000 and 1000) based on a formula in which the mean performance over the three rides (sprint times and hill ride to exhaustion) is calculated and normalised to baseline physiological test results so that each rider has an equal chance of performing well
- At the conclusion of Performance trial 1, you will be provided with a diet that is low in carbohydrate that you must consume for the rest of the day. The aim of this diet is to keep your "race" depleted glycogen stores low until the next morning, when we will repeat a DXA scan and muscle biopsy under our AIS Best Practice Protocols. This "depletion" measure will only occur once during the study. At the completion of Performance Trials 2 and 3 you will be allowed to consume a menu of your choice for the next couple of days while you recover and complete your training program until the next pre-Trial preparation period. Overall, we will work with you to negotiate your training program over the ~ 21 days of your participation in the study so that it remains constant/standardised

#### **Summary of your commitment**

- We anticipate that your participation in this study will span ~ 35 hours, over the course of 6 visits to the laboratory
  - Preliminary testing
  - Practice day,
  - Performance Trial 1 (DXA and BIS scan, muscle biopsy and Riding protocol),
  - Depletion testing day (DXA and BIS scan, muscle biopsy
  - Performance Trial 2(DXA and BIS scan, muscle biopsy and Riding protocol),
  - Performance Trial 3 (DXA and BIS scan, muscle biopsy and Riding protocol)
- We will provide you with an honorarium of \$800 to compensate you for travel costs, food costs outside the packaged diets, and lost work time during your participation in the trial

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- You will undertake 4 periods of dietary control: 3 x 48 hours prior to each Performance Trial, and 1 x 12-18 hours post Performance Trial 1. Diets will be prepared to meet individual preferences and intolerances.

A total of 4 biopsies and 4 DXA/BIS scans will be undertaken

#### **Who we are recruiting?**

- Participants who are endurance trained, sub-elite cyclists or triathletes, aged 18-40 y who are currently completing a weekly cycling training load of > 250 km/week and have a > 2 y history of competing in road races or triathlons.
- Exclusion criteria:
- History of abnormal bleeding/clotting or needle phobia that would prevent you from having a muscle biopsy
- Recent exposure to radiation (e.g. medical imaging techniques) that would prevent you from having 4 DXA scans)

#### **Adverse Effects and Withdrawal:**

- As explained in more detail in our booklet on the procedures in our trial, it is common for subjects to experience some mild muscle soreness during the initial 48 hrs of recovery after a muscle biopsy. However this should not restrict movement or function. In some rare cases mild bruising has occurred but these symptoms disappear within a week. The principal researcher will monitor the status of subjects in the days after an experimental trial. In addition, although the possibility of infection and significant bruising is small, subjects are informed to contact the principal researcher if by chance this should eventuate. The principal researcher will then refer any individuals with these symptoms to the project doctor.
- Participation in this clinical trial is voluntary and participants are free to withdraw from the study at any point if they deem they can no longer adhere or complete the requirements of the trials.

#### **Confidentiality:**

- You will be provided with a full copy of your own results on the completion of the study, as well as the general findings of the study. No data from any other individual participant will be provided to you by the researchers.
- Confidential information will be kept by the principal investigator in a lockable filing cabinet and in a personal computer protected by password. In addition, the participants will be identified by number only. All address lists will be kept securely in the primary investigator's care and only be available to the primary investigator and appropriate co-researchers. The presentation of results will not make reference to individual participants when the data are published. The raw data will be retained by the principal investigator for five years in a secure cabinet at the Australian Institute of Sport. At the conclusion of this five year period all material containing confidential information will be destroyed.

#### **Ethics Approval:**

- The project set out in the attached application, including the adequacy of its research design and compliance with recognised ethical standards, has the approval of the Australian Institute of Sport (AIS). If you have any concerns, please contact the secretary of the AIS Ethics Committee on 02 6214 1577.

#### **Further information:**

- Please contact Principal Researcher Prof. Louise Burke (AIS) or Co-Researchers Julia Bone (AIS) Prof. John Hawley (ACU) and Mr. Kristy Tomcik (ACU) if you have any questions, concerns, or require further information in regards to any aspect of participating in this study.

Please note NS refers to the NHMRC 'National Statement' for the ethical conduct of research. For further information please go to <http://www.nhmrc.gov.au> and follow the links



C) *Informed Consent Form for Participants*

**'INFORMED CONSENT' FORM (Adult)**

**Project Title: Interactive effect of manipulating muscle creatine and glycogen stores on endurance performance and DXA measurements of lean mass**

**Principal Researchers: Prof. Louise Burke**

This is to certify that I, [REDACTED] hereby agree to participate as a volunteer in a scientific investigation as an authorised part of the research program of the Australian Sports Commission under the supervision of Prof. Louise Burke, Mr. Kristylen Tomcik, Miss Julia Bone and Prof. John Hawley..

The investigation and my part in the investigation have been defined and fully explained to me by Prof. Louise Burke/Miss Julia Bone and I understand the explanation. A copy of the procedures of this investigation and a description of any risks and discomforts has been provided to me and has been discussed in detail with me.

- I have been given an opportunity to ask whatever questions I may have had and all such questions and inquiries have been answered to my satisfaction.
- I understand that I am free to deny any answers to specific items or questions in interviews or questionnaires.
- I understand that I am free to withdraw consent and to discontinue participation in the project or activity at any time, without disadvantage to myself.
- I understand that I am free to withdraw my data from analysis without disadvantage to myself.
- I understand that any data or answers to questions will remain confidential with regard to my identity.
- I certify to the best of my knowledge and belief, I have no physical or mental illness or weakness that would increase the risk to me of participating in this investigation.
- I am participating in this project of my (his/her) own free will and I have not been coerced in any way to participate.

*Privacy Statement: The information submitted will be managed in accordance with the ASC Privacy Policy.*

☐ *I consent to the ASC keeping my personal information.*

Signature of Subject: \_\_\_\_\_ Date: \_\_\_\_/\_\_\_\_/\_\_\_\_

I, the undersigned, was present when the study was explained to the subject/s in detail and to the best of my knowledge and belief it was understood.

Signature of Researcher: \_\_\_\_\_ Date: \_\_\_\_/\_\_\_\_/\_\_\_\_

*D) Project Amendment Approval*



**MINUTE**

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**TO:** Ms Helene Rushby **CC:** Louise Burke  
**FROM:** Dr Meg Ross, ACU Postdoctoral Fellow, AIS Sports Nutrition  
**SUBJECT:** Ethics – Minor Variation (R2)  
**DATE:** 20 Jun 2014

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**Minor Variation (R2) to project # 20140612**

**Interactive effect of manipulating muscle creatine and glycogen stores on endurance performance and DXA measurements of lean mass (Creatine-Glycogen study)**

We are seeking approval from the AIS Ethics Committee to include two minor variations to the current research study design, which would further improve the rigour and outcomes of the study. Please view the amended version (changes amended by highlighted text) of our submission to AIS Ethics, that we would like the committee to consider. To serve as a reminder:

**The original protocol has been approved for the following minor variation:**

- the Addition of MuscleSound ® (ultrasound) measurement of muscle glycogen content at time points at which a biopsy has taken place to allow correlation study of muscle glycogen estimates via direct and indirect techniques

**This version (R2) adds further variation:**

- The extension of the TT protocol from 100 km to 120 km to ensure that it is a glycogen limiting exercise protocol
- The addition of capillary blood samples (finger-prick) to measure blood lactate concentrations on completion of each sprint (n=12) and the hill climb (n=1; total n= 13)

It is anticipated these two variations will add only minor discomfort to the research subject, in having a finger-pick by a lancet, for the purpose of sampling capillary blood— a regularly-performed routine procedure in our laboratory, and cycling an additional 20 km—an increase in total distance that is well within the capability of subjects that will be recruited for this study. The estimated time commitment for each subject will remain the same.

Sincere thanks to the members of the committee for considering these changes.

Sincerely,

Signed:

Principle Researcher and Head of Department



## MINUTE

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TO: Ms Julia Bone CC: Louise Burke  
FROM: Ms Helene Rushby  
SUBJECT: Approval from AIS Ethics Committee DATE: 30<sup>th</sup> June 2014

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On the 10<sup>th</sup> June 2014, the AIS Ethics Committee gave consideration to the minor variation in your submission titled **"Interactive effect of manipulating muscle creatine and glycogen stores on endurance performance and DXA measurements of lean mass"**. The Committee saw no ethical reason why your project should not proceed.

The approval number for this project: 20140612

It is a requirement of the AIS Ethics Committee that the Principal Researcher (you) advise all researchers involved in the study of Ethics Committee approval and any conditions of that approval. You are also required to advise the Ethics Committee immediately (via the Secretary) of:

Any proposed changes to the research design,  
Any adverse events that may occur,

Researchers are required to submit **annual status reports** and **final reports** to the secretary of the AIS Ethics Committee. Details of status report requirements are contained in the "Guidelines" for ethics submissions.

Please note the approval for this submission expires on the 30<sup>th</sup> June 2016 after which time an extension will need to be sought.

If you have any questions regarding this matter, please don't hesitate to contact me on (02) 6214 1577

A handwritten signature in black ink, appearing to read 'Helene Rushby', is written over a horizontal line.

Sincerely  
Helene Rushby  
Secretary, AIS EC



## INFORMATION TO PARTICIPANTS



**Research Title** Interactive effect of manipulating muscle creatine and glycogen stores on endurance performance and DXA measurements of lean mass

**Principal Researcher:**

**Prof. Louise Burke (Australian Institute of Sport)**

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**Dr Meg Ross (Australian Institute of Sport)**

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We would like to invite you to participate in this original research project, a collaboration between the AIS and Australian Catholic University (ACU). You should only participate if you want to; choosing not to take part will not disadvantage you in any way. Before you decide whether you want to take part, it is important for you to understand why the research is being done and what your participation will involve. We realise this information sheet is lengthy, but please take time to read it carefully and discuss it with others if you wish. Ask us if you would like more information.

### Background

The performance of endurance sports is reliant on muscle stores of glycogen (carbohydrate). Indeed, strategies that increase this muscle fuel (carbohydrate loading) are able to enhance race outcomes by extending the time that optimal pacing strategies can be maintained. Some studies have shown that glycogen stores can be further increased if the muscle has been previously loaded with creatine. Creatine supplementation protocols, which increase this muscle fuel associated with brief high-intensity exercise, are familiar to athletes who undertake repeated sprint events and resistance training. However, if they can also enhance glycogen stores, they may provide an ergogenic aid for endurance athletes such as road cyclists and marathon runners. To date the performance benefits of increased levels of carbohydrate loading in endurance events haven't been investigated. One particular issue that might need to be explored is the effect of the extra body mass associated with both creatine and carbohydrate loading that occurs due to the extra fuel stored in the muscle and the water bound to it.

While the primary aim of manipulating muscle glycogen and creatine stores is to enhance performance, a secondary outcome of changing muscle fuel and water content is to change the results of a DXA scan of lean mass of the region. This might explain why we observe that DXA measurements of what we interpret as "muscle" can suddenly fluctuate by 0.5-1 kg without reflecting a real change. Since muscle size and strength are important in sports

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performance, we are always searching for ways to either improve our ability to measure them reliably or to better interpret the results of DXA scans.

The current study has been designed to achieve three separate outcomes.

**Aim:**

1. The primary aim is to investigate whether creatine loading increases the glycogen storage achieved by a carbohydrate loading program, and whether this translates into better performance of a prolonged cycling protocol designed to mimic elements of real-life racing
2. A secondary specific aim, is to investigate the size of the alterations in estimates of lean mass, measured using DXA, due to creatine loading and the depletion and supercompensation of muscle glycogen stores.
3. The study will also be used to validate a non-invasive technique of measuring muscle glycogen content using ultrasound technology

**Benefits:**

We are aiming for two separate benefits from undertaking this study of glycogen and creatine loading.

- The first theme focuses on the potential application of creatine supplementation to the performance of endurance athletes using a protocol that simulates some important aspects of real-life sport. One particular element of interest is the effect of any increase in body mass on performance of a weight-sensitive sporting protocol
- The second theme addresses the possibility that alterations in these muscle fuels introduce an artefact into DXA estimates of body composition in athletes. Knowing this can help us to either create a more reliable set of standardised conditions for measuring lean mass, or to better interpret the results of measurements under certain conditions.

Both aspects of this study are novel and will help with the preparation of athletes.

**What is involved?**

- Your first task will be to undertake testing of your aerobic capacity (VO<sub>2</sub>max) and a chance to practice part of the performance protocol we have chosen for this study. The performance trial is described in more detail more below, but on this testing day, you will only do half of the Velotron ride (60 km including sprints) followed by riding your own bike on a treadmill which is at an 8% incline to simulate riding up a hill.
- You will then undertake a second practice day within the next week to make sure you are familiar with the best pacing strategies and technical skills needed to perform well in the real trials
- Performance trials will be undertaken three times, each a week apart from the other. In each case, you will be provided with a packaged diet for 48 hours beforehand, which you must follow faithfully. During this time you will also undertake a standardised training program but you are not required to attend the AIS for those sessions.
  - All participants will receive the same dietary program before Performance Trial 1 – a menu providing a carbohydrate intake of 6 g per kg of your body mass, which should allow you to recover normal stores of muscle glycogen.
  - At the end of Performance Trial 1, the group will be divided into two – with one half receiving a creatine loading and maintenance supplementation protocol for the following 2 weeks, while the other half will receive placebo (no active ingredients) supplements. This supplementation program will require you to undertake 5 days in which you will consume a number of capsules of a creatine or placebo powder at 4 separate times of the day (breakfast, lunch, after training and dinner). For the remainder of the two week period you will only need to consume a single dose of capsules with your breakfast meal. This activity will mean that Trials 2 and 3 will allow a comparison between a Creatine Group and a Placebo Group
  - Before Performance Trials 2 and 3, you will receive each of two different diets in an order that we will keep secret from you until the end of the study. One menu will provide the same amount of carbohydrate as in Trial 1, while the other will contain twice as much carbohydrate to allow you to carbohydrate

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load. Half of each of the two groups (Creatine or Placebo) will receive the carbohydrate loading one first while the other half will do the opposite pattern. This part of the study will investigate the effects of carbohydrate loading with or without prior creatine loading

- Each Performance Trial day will follow the same procedures
  - You will arrive at the Lab fasted (drinking only water since ~ 9 pm the night before the trial) on the morning of the trial). The first activity will be to undertake a whole body DXA scan which requires you to lie still on the DXA scanning bed for ~ 5 minutes. A separate booklet explaining the DXA procedure will be provided to you.
  - A measurement of total body water using a BIS machine will be undertaken while you remain lying down. This scan will require us to place 4 electrodes on your wrists and ankles. No radiation is involved.
  - Following the scans, we will take an ultrasound image and preform a biopsy to take a small amount of muscle from your quadriceps. The biopsy will be performed by an experienced sports physician, under local anaesthetic and following the AIS Best Practice Protocol that is described in a separate booklet.
  - You will then consume a standard breakfast simulating a pre-race meal and wait for 2 hours before commencing your Performance Trial.
  - The Performance trial will start with a 120 km cycling protocol undertaken on the Velotron ergometer which has been set up to mimic your bike geometry. You will undertake most of this at a set workload, determined by the results of your initial testing. However at prescribed segments, you will be asked to undertake a 1 km or 4 km interval as fast as possible where we will measure your performance time (10-14, 20-21, 30-34, 40-41, 50-54, 60-61, 70-74, 80-81, 90-94 and 99-100 km). This represents some of the challenges of road cycling on a flat course where your weight is supported. You will be aware of the elapsed distance during the 120 km protocol, but not the time or your power outputs during the sprint phases.
  - At the finish of this segment, you will switch to riding your own bike on a treadmill set to an incline of 8% to mimic an uphill finish to a road race. You will be asked to ride for as long as you can at a speed that is equivalent to 88% of your Peak Power Output. Again, your elapsed and final finishing time will be kept hidden from you until the end of the study.
  - During each Performance Trial you will consume carbohydrate and fluid according to a standardised race nutrition plan, and we will also take a drop of blood from your finger-tip or earlobe at various distances throughout the ride, in order to measure blood lactate.
  - To encourage the best performance possible, we will organise a prize bank of \$10,000 based on a formula in which the mean performance over the three rides (120km, sprint times and hill ride to exhaustion) is calculated and normalised to baseline physiological test results so that each rider has an equal chance of performing well
- At the conclusion of Performance trial 1, you will undergo another ultrasound of your quadriceps. No biopsy will be taken at this time. You will then be provided with a diet that is low in carbohydrate that you must consume for the rest of the day. The aim of this diet is to keep your "race" depleted glycogen stores low until the next morning, when we will repeat a DXA scan, BIS, ultrasound and muscle biopsy under our AIS Best Practice Protocols. This "depletion" measure will only occur once during the study. At the completion of Performance Trials 2 and 3 you will be allowed to consume a menu of your choice for the next couple of days while you recover and complete your training program until the next pre-Trial preparation period. Overall, we will work with you to negotiate your training program over the ~ 21 days of your participation in the study so that it remains constant/standardised

#### Summary of your commitment

- We anticipate that your participation in this study will span ~ 35 hours, over the course of 6 visits to the laboratory
  - Preliminary testing
  - Practice day,

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- Performance Trial 1 (DXA, BIS scan, ultrasound, muscle biopsy and Riding protocol),
- Depletion testing day (DXA, BIS scan, ultrasound and muscle biopsy
- Performance Trial 2(DXA, BIS scan, ultrasound, muscle biopsy and Riding protocol),
- Performance Trial 3 (DXA, BIS scan, ultrasound, muscle biopsy and Riding protocol)
- We will provide you with an honorarium of \$600 to compensate you for travel costs, food costs outside the packaged diets, and lost work time during your participation in the trial
- You will undertake 4 periods of dietary control: 3 x 48 hours prior to each Performance Trial, and 1 x 12-18 hours post Performance Trial 1. Diets will be prepared to meet individual preferences and intolerances.

A total of 4 biopsies, 8 ultrasounds and 4 DXA/BIS scans will be undertaken

**Who we are recruiting?**

- Participants who are endurance trained, sub-elite cyclists or triathletes, aged 18-40 y who are currently completing a weekly cycling training load of > 250 km/week and have a > 2 y history of competing in road races or triathlons.
- Exclusion criteria:
- History of abnormal bleeding/clotting or needle phobia that would prevent you from having a muscle biopsy
- Taken a creatine supplement within 6 weeks of first performance trial
- Recent exposure to radiation (e.g. medical imaging techniques) that would prevent you from having 4 DXA scans)

**Adverse Effects and Withdrawal:**

- As explained in more detail in our booklet on the procedures in our trial, it is common for subjects to experience some mild muscle soreness during the initial 48 hrs of recovery after a muscle biopsy. However this should not restrict movement or function. In some rare cases mild bruising has occurred but these symptoms disappear within a week. The principal researcher will monitor the status of subjects in the days after an experimental trial. In addition, although the possibility of infection and significant bruising is small, subjects are informed to contact the principal researcher if by chance this should eventuate. The principal researcher will then refer any individuals with these symptoms to the project doctor.
- Participation in this clinical trial is voluntary and participants are free to withdraw from the study at any point if they deem they can no longer adhere or complete the requirements of the trials.

**Confidentiality:**

- You will be provided with a full copy of your own results on the completion of the study, as well as the general findings of the study. No data from any other individual participant will be provided to you by the researchers.
- Confidential information will be kept by the principal investigator in a lockable filing cabinet and in a personal computer protected by password. In addition, the participants will be identified by number only. All address lists will be kept securely in the primary investigator's care and only be available to the primary investigator and appropriate co-researchers. The presentation of results will not make reference to individual participants when the data are published. The raw data will be retained by the principal investigator for five years in a secure cabinet at the Australian Institute of Sport. At the conclusion of this five year period all material containing confidential information will be destroyed.

**Ethics Approval:**

- The project set out in the attached application, including the adequacy of its research design and compliance with recognised ethical standards, has the approval of the Australian Institute of Sport (AIS). If you have any concerns, please contact the secretary of the AIS Ethics Committee Helene Rushby on 02 6214 1577.

- Please contact Julia Bone on [Julia.bone@ausport.gov.au](mailto:Julia.bone@ausport.gov.au) or 02 6214 1641 if you have any questions, concerns, or require further information in regards to any aspect of participating in this study.

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*f) Muscle Biopsy Questionnaire*

**MUSCLE BIOPSY QUESTIONNAIRE**

**NAME:** \_\_\_\_\_

**DATE:** \_\_\_\_\_ **D.O.B:** \_\_\_\_\_ **Age:** \_\_\_\_\_

1. Have you or your family suffered from any tendency to bleed excessively? (eg. Haemophilia) or bruise easily?

Yes No Don't know

If yes, please elaborate \_\_\_\_\_

2. Are you allergic to local anaesthetic?

Yes No Don't know

If yes, please elaborate \_\_\_\_\_

3. Do you have any skin allergies?

Yes No Don't know

If yes, please elaborate \_\_\_\_\_

4. Have you any allergies that should be made known?

Yes No Don't know

If yes, please elaborate \_\_\_\_\_

5. Are you currently on any medication?

Yes No Don't know

If yes, please elaborate \_\_\_\_\_

6. Do you take Aspirin, non-steroidal analgesics or Warfarin?

Yes No Don't know

If yes, please elaborate \_\_\_\_\_

7. Do you have any other medical problem that should be made known?

Yes No Don't know

If yes, please elaborate \_\_\_\_\_

8. Do you suffer from low blood pressure or postural hypotension?

Yes No

9. Have you ever had problems when donating blood?

Yes No

To the best of my knowledge, the above questionnaire has been completed accurately and truthfully.

**Signature:** \_\_\_\_\_ **Date:** \_\_\_\_\_



**11.5 Study 5: Measurement of resting energy expenditure following a low carbohydrate high fat diet is affected by artefacts in DXA measurement of lean mass**

**A) Notice of Approval**



**MINUTE**

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TO: Prof. Louise Burke CC:  
FROM: Ms Helene Rushby  
SUBJECT: Approval from AIS Ethics Committee DATE: 12.12.16

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On the 6<sup>th</sup> of December 2016, the AIS Ethics Committee gave consideration out of session to your submission titled ***"Supernova 2: Dietary periodisation and de-adaptation to support training outcomes in elite race walkers"***. The Committee saw no ethical reason why your project should not proceed.

The approval number for this project is: 20161201

It is a requirement of the AIS Ethics Committee that the Principal Researcher (you) advise all researchers involved in the study of Ethics Committee approval and any conditions of that approval. You are also required to advise the Ethics Committee immediately (via the Secretary) of:

Any proposed changes to the research design,  
Any adverse events that may occur,

Researchers are required to submit **annual status reports** and **final reports** to the secretary of the AIS Ethics Committee. Details of status report requirements are contained in the "Guidelines" for ethics submissions.

Please note the approval for this submission expires on the 30<sup>th</sup> December 2018 after which time an extension will need to be sought.

If you have any questions regarding this matter, please don't hesitate to contact me on (02) 6214 1577

Sincerely

A handwritten signature in blue ink, appearing to read 'HR', is written over a circular stamp.

Helène Rushby  
Secretary, AIS EC

## **Supernova 2**

### **Dietary periodisation and de-adaptation for race performance in elite race walkers**

A project of AIS Sports Nutrition and Mary MacKillop Institute, Australian Catholic University

Supported by Athletics Australia

Principal organiser: Louise Burke, Head of Sports Nutrition, Australian Institute of Sport, PO Box 176, Belconnen ACT 2616, 0422 635 869, Louise.burke@ausport.gov.au

## **Overview**

We are planning to stage Supernova 2 Training camp in January-February 2017 for elite Australian and international race walkers. This document provides

- Key highlights of the findings of Supernova 1
- a brief outline of Supernova camp and its logistics and requirements

## **Background**

Supernova 1 was conducted in November 2015 and January 2016 with the participation of 21 elite/well-trained race walkers for a total of 29 “camp experiences”.

The camps created a dynamic environment which produced high quality training experiences and knowledge sharing, and left a legacy of great friendships, performance breakthroughs at the following championships (and beyond), and some innovative research findings.

We examined the effects of three different diets on the success of an intensified training block in terms of performance, training capacity and quality of life, and various measures of health and metabolism

1. High Carb - the traditional sports nutrition guidelines of high carbohydrate eating around every training session
2. LCHF - the heavily promoted Low Carb High Fat diet
3. Periodised Carb – a mix and match of high carbohydrate and low carbohydrate support around specific training sessions

A series of research papers was produced by the study, which are in the process of being published and presented at conferences.

- The key paper is planned for publication in a high impact science journal - Journal of Physiology – in November 2016
- The study will provide the basis of a symposium at the American College of Sports Medicine annual conference in May 2017.
- The full results of the study are currently embargoed until journal publication, but will be made available to all Supernovans on the day of publication.
- Other opportunities are being investigated to allow interactive sharing of the findings with Australian coaches and sports scientists
- There is great interest and respect for the research outcomes of Supernova 1, with social media already picking up on the preliminary information that has been released at conferences over the past 4 months

The goal of Supernova 2 is to build on the research findings from Supernova 1 to provide new information that can be built into the preparation of endurance athletes. Most



importantly, our goal is to use the resources created by Supernova 2 to provide Australian race walkers and coaches with an opportunity for high quality training and knowledge sharing.

### What were the key findings of Supernova 1?

- The 3 week training camp produced a significant and equal improvement in  $\text{VO}_{2\text{max}}$  for all groups – mean improvements of  $\sim 4 \text{ ml/kg/min}$
- The LCHF group struggled to complete their training sessions: reporting fatigue and a greater perception of effort. There was a particular impairment of speed/endurance in the higher intensity sessions (hill session and rep session). In many, but not all cases, there was a gradual improvement in these symptoms over the 3 weeks. The LCHF diet caused a range of other side effects including a rash in 3 of 10 subjects
- The LCHF group adapted to the diet by increasing their ability to burn fat as an exercise fuel – in fact, some of the rates of fat oxidation seen in our walkers were the highest ever reported! **This finding supports the claims made about the LCHF diet**
- Theoretically, burning fat to produce muscle power requires extra oxygen compared to the oxygen cost of burning carbohydrate. This is exactly what occurred during the treadmill economy tests and the 25 km walk session.
  - After 3 weeks of training, the High carb and Periodised Carb group used the same amount of oxygen to walk at each of the 4 different treadmill speeds. But because training improved  $\text{VO}_{2\text{max}}$ , this translated to a reduced percentage of aerobic capacity to produce the same speed. For example at the second speed ( $\sim 50 \text{ km race pace}$ ), the average oxygen cost of walking was  $\sim 75\% \text{ VO}_{2\text{max}}$  before the training camp and  $\sim 70\% \text{ VO}_{2\text{max}}$  afterwards. At the fourth speed ( $\sim 20 \text{ km race pace}$ ), the average oxygen cost of walking was  $\sim 90\% \text{ VO}_{2\text{max}}$  before the training camp and  $\sim 86\% \text{ VO}_{2\text{max}}$  afterwards. The same outcome was seen over the duration of the 25 km walk. **In other words, 3 weeks of training allowed the two Carb groups to improve their walking economy at two key speeds.**
  - The LCHF did not improve their walking economy. The oxygen cost of walking was increased to cover the increased use of fat, and cancelled out the benefits of having an increased  $\text{VO}_{2\text{max}}$ . For example, it took  $75\%$  of  $\text{VO}_{2\text{max}}$  to walk at the second speed before the training camp, but it still took  $75\% \text{ VO}_{2\text{max}}$  to walk at this speed after 3 weeks of training.
- The High Carb and Periodised Carb groups improved their 10 km race performances by an equal amount
  - 6.6% improvement for High carb
  - 5.3% improvement for Periodised Carb
- The LCHF group did not improve their 10 km race performance (mean change = 1.6% slower time)
- Interpretation = LCHF is not suitable as a chronic diet for athletes who need to compete at intensities  $>70\%$  of  $\text{VO}_{2\text{max}}$ . Even though the muscle can be trained to burn more fat, this requires a greater oxygen supply and results in a lower economy (a greater % of  $\text{VO}_{2\text{max}}$  is needed to produce the same power or speed). This may not be a problem at moderate exercise intensities, but when the event is carried out at intensities around the lactate threshold, there is no capacity to meet the extra oxygen demand. Therefore, maximum sustained speed is reduced and capacity for higher intensity bursts is compromised. **The finding of impaired performance contradicts the claims made about the LCHF diet**

## Unanswered questions to follow up in Supernova 2

Although the immediate effect of the LCHF diet was an impairment of training capacity and race performance, exit interviews and observations of performance over the next couple of months raised the possibility that it might produce a longer term benefit.

Suggestions for a possible carryover effect were:

1. Like altitude training, performance under the LCHF diet is impaired, but the effects of the diet persist for a couple of weeks. This would allow the athlete to have a “sweet spot” where they go into the race with the benefits of having carbs to burn economically, but a legacy of greater capacity for fat oxidation or more mitochondria in the muscle (the muscle furnace which turns fat and carbs into power). **This hypothesis suggests that the LCHF creates muscle retooling that persists for at least 3 weeks, leaving at least short term metabolic advantages**
2. **The effect of the LCHF diet was to “reset RPE”.** Because the slog of training with the LCHF diet was so great, the athlete’s brain changes its perception of what “fatigue”, “pain” and “effort” are. Some people suggest that after female athletes have a baby, they return to sport with greater tolerance for pain/fatigue because the pain of childbirth has “hardened them up”. **This hypothesis suggests there is no metabolic carryover from the LCHF, but short term performance could be improved because the athlete is tougher and more able to handle pain/fatigue during exercise**

## An overview of Supernova 2

### Who we are recruiting?

We are inviting Australian male and female race walkers who meet the following criteria

- Have met the appropriate **IAAF walks standards international level participation**
- Are in training for 20 or 50 km race walking competition and able to handle the study training load
- Will be racing at 20 km National Championships in Adelaide on Feb 19 2017

**Note:**

- **Priority given to Athletics Australia NASS members and other Senior Athletes**
- **Competitive international race walkers will also be invited to increase the subject pool and to provide an elite training group**

### Benefits

Your involvement in the project offers the following support:

- Ground transport once you have arrived in Canberra, Australia
- **Full accommodation, meals and training support for a 6 week period, at AIS Canberra**
- Access to Recovery, weekly massage and necessary medical treatment
- Full reports of your individual results and first access to the results of the research project
- Prize money as incentive for performance testing

### What is required from you?

- Availability to participate in a 6 week high quality training camp



- Adherence to the compulsory sessions of a standardised training program (but able to set own program for other sessions)
- Adherence to one of three dietary programs
  - **High carb** (high carb availability for each training session)
  - **Low-carbohydrate, high-fat (LCHF)– ketogenic diet**
  - **Periodised carb** (targeted mix of high and low availability for different sessions)
- Willingness to undertake **performance testing** (walking economy test, 10 km (track) walking race)
- Willingness to undertake some **minimally invasive tests of health, training capacity and metabolism** (see below for details).
- Being in good shape at the start of the study on Jan 1, and able to race in near or peak form at the 20 km national championships in Adelaide on Feb 19 2017
- Ideally, having a recent 20 km race performance result (including December 50 km Nationals)

#### Notes of special opportunities for Australian race walkers:

- You will be guaranteed the diet that you would like to try (we will use the international athletes to balance out the rest of the dietary intervention groups)
- We are also interested to hear from coaches of training groups who would like to collaborate or have input/involvement
- If you are unable to undertake the full study, we can still offer you an opportunity to join some of the training experience. You can arrange your own accommodation in Canberra or we can arrange it for you at the AIS at your cost. NASS athletes should discuss with their APAs how travel and accommodation for this activity might be supported.

### What are the scientific aims of Supernova 2?

The aim of Supernova 2 is to continue the investigation of different approaches to training nutrition to prepare for optimal performance in an endurance event. The study will be broken into 3 phases

Phase	Protocols	Issues of interest
1. Adaptation  Pre and post testing around 3 weeks of training/diet	Walkers will be divided into 3 different groups for supervised diet and training <ul style="list-style-type: none"> <li>- High Carb availability</li> <li>- Periodised Carb availability</li> <li>- LCHF</li> </ul>	Pre- and post testing will look at <ul style="list-style-type: none"> <li>- Performance at IAAF sanctioned 10 km race</li> <li>- VO<sub>2</sub>max</li> <li>- Walking economy at different intensities</li> <li>- Walking economy and fuel use in 20-25 km Long Walk</li> <li>- Training capacity and well being</li> <li>- Health issues</li> <li>- Iron metabolism</li> <li>- Bone metabolism</li> <li>- Perception of effort during training</li> <li>- Mood and mental functioning</li> </ul>

2. De-adaptation  Testing within 2 weeks of return to high CHO diet	All walkers will return to a diet with High Carb availability  Diet and training will continue to be supervised and monitored	Testing during this period will focus on <ul style="list-style-type: none"> <li>o changes in fuel use and walking economy as the LCHF group return to Carb intake in their everyday diet and during training sessions</li> <li>o Training capacity</li> <li>o Perception of effort during training</li> <li>o Mood and mental functioning</li> </ul>
3. Race Taper and 20 km National Race Walking championships (19/2/17)	Walkers will be permitted to undertake their own diet and training programs for the week prior to the race, within the philosophy of training taper and competition with high CHO availability	Walkers will be required to record their training and diet during this week  Race monitoring by researchers will focus on <ul style="list-style-type: none"> <li>o Fluid and Carb intake during the race</li> <li>o 20 km performance</li> <li>o RPE and pacing</li> </ul>

A calendar of events is provided as a general summary of the study (Figure 1) and an overview of how the dietary treatments could be implemented over a typical training week is provided in Figure 2

### What will we be testing?

The following table summarises the tests we will be conducting in the study, with colour coding showing the days on which they are collected

Grey = throughout the study; Other colours note a single day where multiple tests will come from the same sampling opportunities

Table 1 Summary of study variables collected in the study			
Issue	Metric	Timing of test	Comments
Training capacity	Daily training and life quality	Throughout 6 week training block	Athletes will be required to fill out daily training logs on Google Docs noting sessions completed, RPE and outcomes, sleep quality and DALDA
	Training capacity	2 sessions per week throughout 6 week training block	Training outcomes at interval and hill session – Google docs and researcher-captured metrics
Performance	VO <sub>2</sub> max and Walking economy test  (substrate utilisation at various walking intensities)	Pre- and post 3 w dietary periodisation intervention	Treadmill test with 4 steps at increasing speed and a ramp test to measure VO <sub>2</sub> max. Blood lactate and ketone concentrations will be measured pre and post test
	10 km race on AIS track	Pre- and post 3 w dietary periodisation intervention	Participants will undertake a 10 km race on the AIS track under competition conditions with the race being sanctioned by IAAF



			and open to all competitors
	20 km race	National championships (Adelaide) – Feb 19	Times will be compared with predicted 20 km time extrapolated from Pre-treatment 10 km race time
Body composition  Note that a DXA scan involves exposure to tiny amounts of radiation, which are well below safety limits	DXA measurement of whole body composition	Pre- and post 3 w dietary periodisation intervention	Lean mass, fat mass
Resting metabolic rate and hormone health	Measurement of RMR and hormones in Fasted, rested conditions	Pre- and post 3 w dietary periodisation intervention	RMR is measured from expired gases when lying down at rest  A venepuncture will collect blood to test: Free testosterone, IGF-1, T3
Insulin sensitivity	Oral Glucose Tolerance test done as response to CHO-rich breakfast	Pre- and post 3 w dietary periodisation intervention, and after return to HCHO diet (long walks 1,3,4)	Blood samples will be taken from a cannula every 30 min for 2 h to measure the glucose, insulin response to a standard CHO rich meal
Carbohydrate utilisation	Substrate utilisation during graded exercise test (fasted)	Part of walking economy test – pre and post 3 week diet intervention	Rates of total muscle use of CHO and fat at different exercise intensities will be measured from gases collected by the AIS metabolic cart
	Total muscle carbohydrate use during Long walk	At 0, 6, 12, 18 and 24 km of long walk undertaken at beginning and end of training block under high acute CHO availability (long walks 1, 3, 4)	Rates of total muscle use of CHO and fat at steady state training intensity will be measured from gases collected by the AIS metabolic cart
		At 0, 12 and 24 km of long walk undertaken at end of training block under dietary intervention (long walk 2)	Rates of total muscle use of CHO and fat at steady state training intensity will be measured from gases collected by the AIS metabolic cart
	Muscle use of CHO consumed during exercise during CHO-fed long walk  Note that this protocol will involve the addition of minute amount of a	At 0, 6, 12, 18 and 24 km of long walk undertaken at beginning and end of training block under high acute CHO availability (long walks 1, 3, 4)	Rates of CHO oxidation coming from a glucose drink consumed during session will be measured by capturing expired breath and measuring how much “label” is present  Gut comfort

	radioactive form of glucose to the drink, so that it can be detected in the breath as it is burned by the muscle. The total amount of radiation involved in this study is well below safety limits		
Lipid status	Blood Lipid profile	Pre- and post 3 w dietary periodisation intervention	The blood sample taken by the venepuncture will measure blood Cholesterol (total and sub-fractions), triglycerides
Immune system	Illness logs	Throughout 6 week training block	Google docs
	Blood immune markers – resting and response to exercise	Pre and 3 h post Long Walks 1,2 - pre- and post 3 w dietary periodisation intervention	On arrival in the morning and at the end of Long Walks 1 and 2 we will collect a blood sample by venepuncture straight after the walk, and after 3 hours of recovery to measure a range of cytokines and immune cells
Inflammation	Chronic inflammation, soreness, injuries	Throughout 3 week training block	Google docs
	Blood markers of inflammation – resting and response to exercise	Pre and 3 h post Long Walks 1,2 - pre- and post 3 w dietary periodisation intervention	The blood samples taken at the beginning and the end of Long Walks 1 and 2 will also measure inflammatory cells
Iron status	Iron studies	Pre- and post 3 w dietary periodisation intervention	The blood sample taken from the venepuncture will also measure Hb, ferritin, transferrin saturation, sTfR
	Hepcidin response to training session	Pre and 3 h post Long Walks 1,2 - pre- and post 3 w dietary periodisation intervention	The blood samples taken at the beginning and the end of Long Walks 1 and 2 will also measure a hormone that affects iron metabolism (hepcidin)
Bone markers	Bone mineral density Via DXA	Baseline measure	Hip and Spine BMD
	Response of bone markers to training session	Pre and 3 h post Long Walks 1,2 - pre- and post 3 w dietary periodisation intervention	The blood samples taken at the beginning and the end of Long Walks 1 and 2 will also measure characteristics that reflect bone metabolism (PTH, CTX1, PINP)
Mood and mental function	Mood	At end of each week	Validated questionnaire on mood
	Mental function	Pre and post hill training session	Stroop Word colour test



### **Adverse Effects and Withdrawal:**

- This study is built around a standard intensified training block, supplemented with routine testing of metabolism and performance. We do not expect subjects to experience excessive amounts of hardship or long-term impairments of training capacity, even though (as experienced in the case of altitude training), the interaction of training and diet in some sessions will be considered “hard” and requiring “adaptation”. Experienced coaches and sports scientists will be supervising the program to distinguish between temporary training stress and larger concerns.
- Nevertheless, participation in this trial is voluntary and participants are free to withdraw from the study at any point if they deem they can no longer adhere or complete the requirements of the trials.

### **Confidentiality:**

- You will be provided with a full copy of your own results on the completion of the study, as well as the general findings of the study. No data from any other individual participant will be provided to you by the researchers. We will endeavour to get the results of each test back to you as they are completed; however, some analyses will take place on the completion of the study. Lay reports and published papers derived from this study will be made available via the Facebook Private Group site
- We will also provide a report, with your permission to your coach. This will include group mean results and your individual results
- Confidential information will be kept by the principal investigator in a lockable filing cabinet and in a personal computer protected by password. In addition, the participants will be identified by number only. All address lists will be kept securely in the primary investigator’s care and only be available to the primary investigator and appropriate co-researchers. The presentation of results will not make reference to individual participants when the data are published. The raw data will be retained by the principal investigator for five years in a secure cabinet at the Australian Institute of Sport. At the conclusion of this five year period all material containing confidential information will be destroyed.

### **Ethics Approval:**

This study has been approved by the Australian Institute of Sport ethics committee. If you have any concerns, you should contact the secretary of the AIS Ethics Committee on 02 6214 1577.

### **Further information:**

Please contact me if you have any further questions or interest in this study

- **AIS Sports Nutrition, PO Box 176, Belconnen ACT 2616**  
0422 635 869  
[Louise.burke@ausport.gov.au](mailto:Louise.burke@ausport.gov.au)

### *C) Informed Consent Form for Participants*

#### **'INFORMED CONSENT' FORM**

Project Title: Dietary periodisation to support training outcomes in elite race walkers – Supernova 2

Principal Researchers: Louise Burke, Meg Ross, Laura Garvican-Lewis, Ida Heikura, Jill Leckey, John Hawley, Avish Sharma, David Pyne, Alannah McKay, Brent Vallance, Amelia Carr, Peter Peeling, Marijke Welvaert, Stephan Praet

This is to certify that I, \_\_\_\_\_ hereby agree to participate as a volunteer in a scientific investigation as an authorised part of the research program of the Australian Sports Commission under the supervision of Louise Burke.

The investigation and my part in the investigation have been defined and fully explained to me by Louise Burke or Meg Ross and I understand the explanation. A copy of the procedures of this investigation and a description of any risks and discomforts has been provided to me and has been discussed in detail with me.

- I have been given an opportunity to ask whatever questions I may have had and all such questions and inquiries have been answered to my satisfaction.
- I understand that I am free to deny any answers to specific items or questions in interviews or questionnaires.
- I understand that I am free to withdraw consent and to discontinue participation in the project or activity at any time, without disadvantage to myself.
- I understand that I am free to withdraw my data from analysis without disadvantage to myself.
- I understand that any data or answers to questions will remain confidential with regard to my identity.
- I certify to the best of my knowledge and belief, I have no physical or mental illness or weakness that would increase the risk to me of participating in this investigation.
- I am participating in this project of my (his/her) own free will and I have not been coerced in any way to participate.

**Privacy Statement:** *The information submitted will be managed in accordance with the ASC Privacy Policy.*

☐ *I consent to the ASC keeping my personal information.*

Signature of Subject: \_\_\_\_\_ Date: \_\_\_\_/\_\_\_\_/\_\_\_\_

I, the undersigned, was present when the study was explained to the subject/s in detail and to the best of my knowledge and belief it was understood.

Signature of Researcher: \_\_\_\_\_ Date: \_\_\_\_/\_\_\_\_/\_\_\_\_

## 11.6 Participant information and resources for studies 1, 2, 3 and 5

### A) Pre-DXA Questionnaire

PRE-DXA QUESTIONNAIRE		Date ____/____/____
Title _____ Name _____		
Date of birth ____ / ____ / ____ Sport _____		
Height _____ cm Weight _____ kg USG _____		
Have you had any x-ray in the past 12 months? (e.g. CT, PET, X-ray, DXA)		
<input type="checkbox"/> Yes / <input type="checkbox"/> No		
→ If Yes, what was it? _____		
→ When was it? _____		
Please tell us about your previous or current injury (e.g. surgery, scoliosis, fractures)		
_____		
Do you have orthopaedic pins, prosthesis or implants? <input type="checkbox"/> Yes / <input type="checkbox"/> No		
→ If Yes, what and where is it? _____		
Do you have a pace maker? <input type="checkbox"/> Yes / <input type="checkbox"/> No		
Do you feel comfortable lying still on your back for 5-10mins? <input type="checkbox"/> Yes / <input type="checkbox"/> No		
Do you have any body piercings? <input type="checkbox"/> Yes / <input type="checkbox"/> No		
→ If Yes, please take them out before a scan		
Do you have any upcoming procedures you are preparing for? (e.g. colonoscopy, gastroscopy etc.) <input type="checkbox"/> Yes / <input type="checkbox"/> No If yes, what? _____		
<b>For FEMALES only:</b>		
Are you pregnant or at high risk of becoming pregnant? <input type="checkbox"/> Yes / <input type="checkbox"/> No		
Are you currently breastfeeding? <input type="checkbox"/> Yes / <input type="checkbox"/> No		
Do you know the date of the start of your most recent menstrual cycle? <input type="checkbox"/> Yes / <input type="checkbox"/> No		
If Yes _____ (please write date)		
If No please tick one of the following: <input type="checkbox"/> 0-6 days, <input type="checkbox"/> Within last month,		
<input type="checkbox"/> 2-3 months ago, <input type="checkbox"/> 4-5 months ago <input type="checkbox"/> 6 months or more, <input type="checkbox"/> unknown		
What is your typical cycle length?		
<input type="checkbox"/> <20 days <input type="checkbox"/> 20-25 days <input type="checkbox"/> 26-30 days <input type="checkbox"/> 31-35 days <input type="checkbox"/> 36-40 days <input type="checkbox"/> >40 days		
<input type="checkbox"/> Irregular <input type="checkbox"/> Unknown		
<b>THANK YOU!</b>		



## What to DO and WEAR for DXA



- NO** compression garments (2XU, Skins)
- NO** tight lycra shorts / cycling knicks
- NO** shorts with thick waist band
- NO** shorts with reflective pipings or trims
- NO** zip, buckles, belts and buttons
- NO** body piercings. You **MUST** take them out
- NO** jewellery (earrings, ring, glasses, necklace, watch, hair clips etc)

Step 1	Wear appropriate clothing
Step 2	Overnight fasted (no food <u>OR</u> drink)
Step 3	Collect 1 <sup>st</sup> urine for UsG analysis
Step 4	Bladder voided just before scan

C) Food diary and Training Log

Day 1 :am

Date:

Meal/Training	Food/Drink	Quantity	Comments e.g. Where you ate	Hunger Rating 1-10
Before Training Time :				
Training Time:				Training RPE
Breakfast Time:				
Snack Time:				
Lunch Time:				

**\*\*HUNGER RATING: (1 = No Appetite – 10 = Extremely Hungry)**

Day 1 :pm

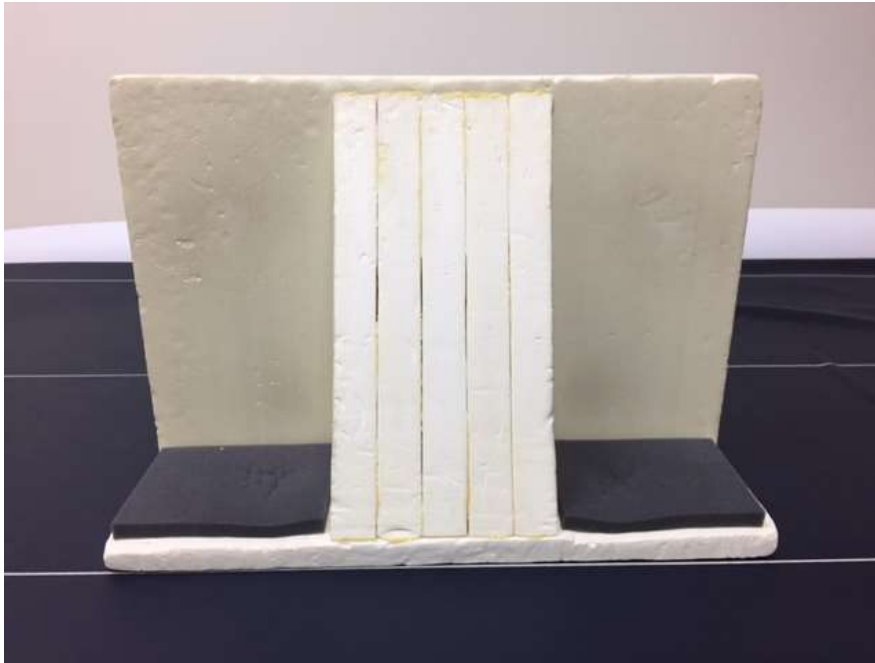
Date:

Meal/Training	Food/Drink	Quantity	Comments e.g. Where you ate	Hunger Rating 1-10
Snack  Time :				
Training  Time:				Training RPE
Dinner  Time:				
Snack  Time:				
Supplement (Brand & Dose)  Time:				

**\*\*HUNGER RATING: (1 = No Appetite – 10 = Extremely Hungry)**

### ***11.7 DXA Positioning Aids***

A) Feet positioning aid



B) Hand positioning aids

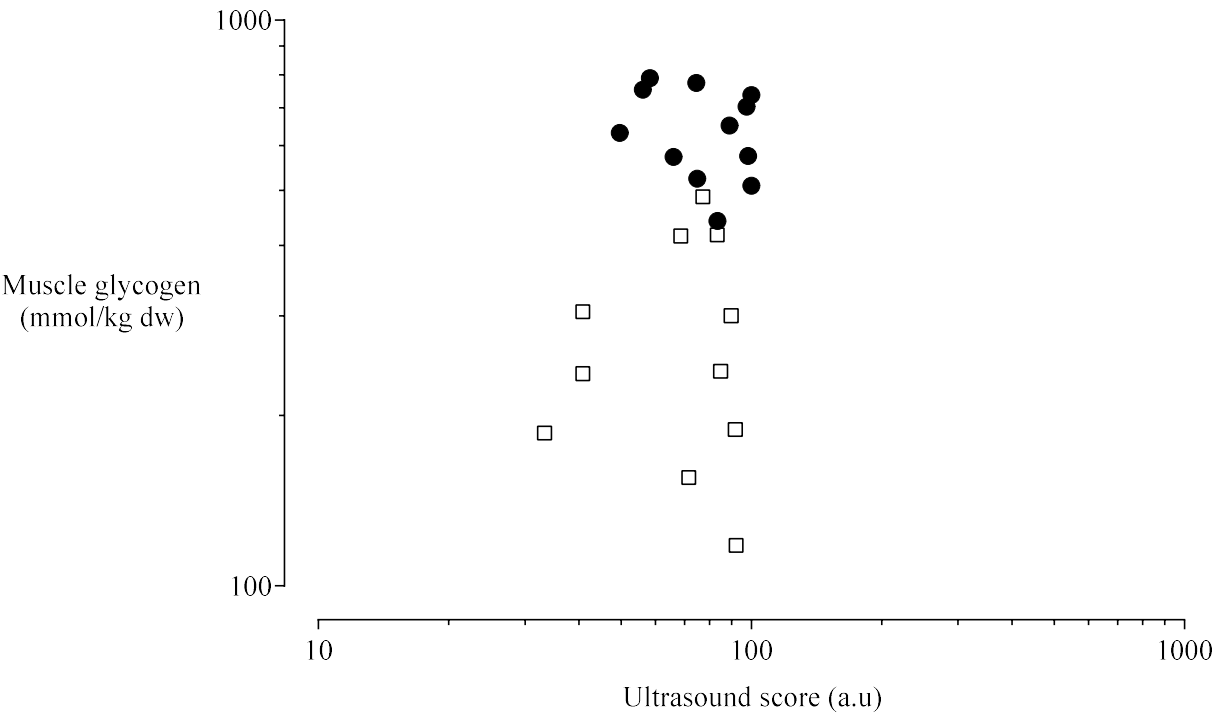


# 12.0 Supplementary Data Appendix

The following graphs have been submitted as supplementary data as part of *Study 4*'s manuscript submission to *Nutrients*.

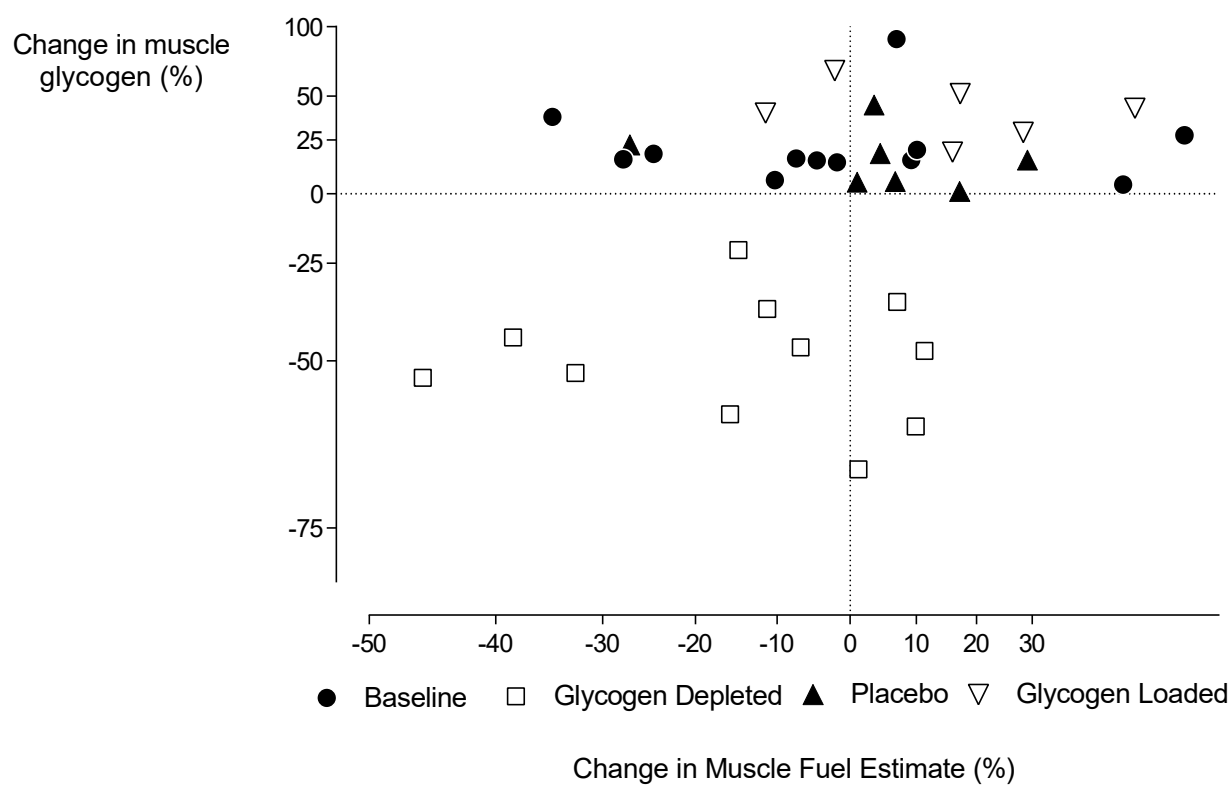
## Supplementary Data

Muscle Fuel Estimates plotted against Biopsy Muscle Glycogen concentrations

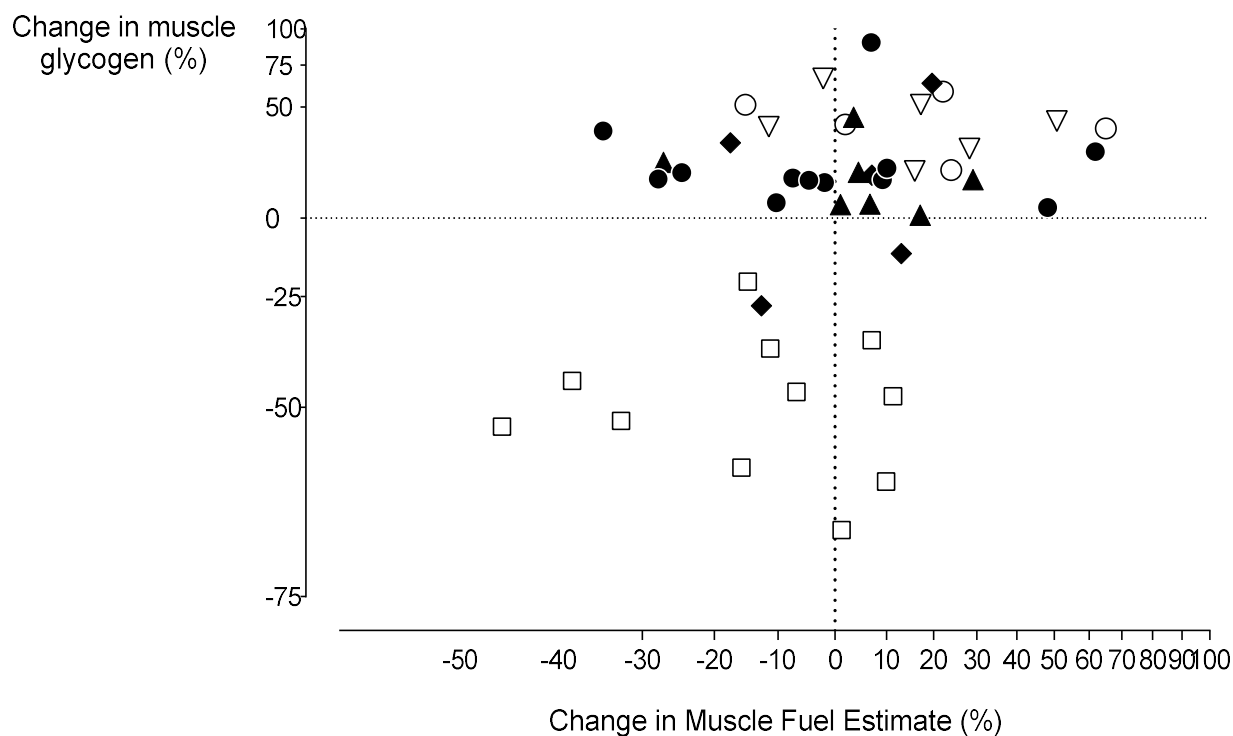


*Figure S1:* Cross-sectional relationship between biopsy muscle glycogen and Muscle Fuel Estimate measured at the mid thigh site on the contralateral leg. Data show are combined from the baseline and depleted conditions. Axis are log scales. ● Baseline , □ Glycogen Depleted





*Figure S2:* Comparison of change in glycogen status between measured glycogen concentration and Muscle Fuel Estimate from the mid thigh on the contralateral leg for baseline, glycogen depleted, placebo and glycogen loaded treatment conditions. Participants means rescaled to means of zero. Axis are log scales.



*Figure S3:* Comparison of the change in glycogen status between biopsy glycogen concentration and Muscle Fuel Estimate from the mid thigh site on the contralateral leg for all treatment conditions. Participants means rescaled to means of zero. Axis are log scales. ● Baseline, □ Glycogen Depleted, ▲ Placebo, ▽ Glycogen Loaded, ◆ Creatine Loaded, ○ Glycogen-Creatine Loaded