

**Amino Acid Metabolism and Protein Requirements in Active, Trained  
Adult Males Using the Indicator Amino Acid Oxidation (IAAO) Technique**

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Distributed Date: September 4, 2015  
Defense Date: September 23, 2015

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Amino acid metabolism and protein requirements in active, trained adult males using the Indicator Amino Acid Oxidation Technique, MSc 2015, Jeffrey Packer, Department of Exercise Science, University of Toronto

**Abstract:**

The present study utilized the minimally invasive Indicator Amino Acid Oxidation (IAAO) technique to evaluate the impact of variable intensity exercise on protein requirements in trained, young adult males. Six trained males partook in 1-10 metabolic trials each consisting of a variable intensity exercise protocol followed by 8 hourly meals providing a variable amount of protein (0.2-2.6g/kg/d), 6g/kg of carbohydrate, and sufficient energy. Protein was provided as crystalline amino acids with the exception of tyrosine (40mg/kg/d) and phenylalanine (30.5mg/kg/d with 5.46 mg/kg over 4h as L-[<sup>13</sup>C]phenylalanine). The estimated average requirement (EAR) was determined from the breakpoint of the <sup>13</sup>CO<sub>2</sub> excretion after application of bi-phase linear regression. Analysis for the correlation between protein intake and <sup>13</sup>CO<sub>2</sub> excretion revealed the EAR to be 1.35 g/kg/d ( $r^2= 0.64$ ), and the safe intake encompassing the upper 95%CI to be 1.64 g/kg/d.

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## **List of Abbreviations**

BCAA – Branch-Chain Amino Acids  
BCOAD – Branched-Chain Oxo-Acid Dehydrogenase Enzyme  
BIA – Bioelectrical Impedance Analysis  
DAAO – Direct Amino Acid Oxidation  
DEXA – Dual-Energy X-Ray Absorptiometry  
EAA – Essential Amino Acids  
EAR – Estimated Average Requirement  
FAO – Food and Agriculture Organization of the United Nations  
FFM – Fat-Free Mass  
FM – Fat Mass  
IAAO – Indicator Amino Acid Oxidation (Technique)  
IPAQ – International Physical Activity Questionnaire  
LIST – Loughborough Intermittent Shuttle Test  
NBAL – Nitrogen Balance (Technique)  
NEAA – Non-Essential Amino Acids  
PA – Physical Activity  
PAR-Q+ – Physical Activity Readiness Questionnaire  
RDA – Recommended Dietary Allowance  
REE – Resting Energy Expenditure  
WBPS – Whole-Body Protein Synthesis  
WHO – World Health Organization

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## **Chapter 1. Introduction:**

The adequate ingestion of dietary protein is a critical factor in ensuring the healthy growth and development of lean body mass in individuals of all ages. Protein requirements are not universal across demographic groups as differences in age, physical activity levels, and sex may result in changes to optimal protein requirements (FAO, WHO 2007). Global initiatives seek to increase physical activity in individuals of all ages in order to enhance their general health and well-being, yet a major knowledge gap exists as the impact of chronic and acute physical activity on protein requirements is not well understood. Research suggests that methods such as Nitrogen Balance (NBAL) often used to estimate protein requirements may not be accurate, and can potentially produce underestimates of true requirements (Huymayun et al., 2007). It is therefore essential to utilize novel, alternative techniques to evaluate protein requirements in the presence of exercise in order to provide optimal nutritional guidance to active populations. Thus, the present study utilized the gold standard minimally invasive IAAO technique to evaluate the effects of an acute bout of variable intensity exercise on protein requirements in active, trained young adult males. It was hypothesized that protein requirements would be: 1) greater than the current requirements established on the basis of NBAL, and; 2) greater than those requirements previously determined by the IAAO technique in non-active populations. This project represents a comprehensive examination of the effect of variable intensity exercise on protein requirements in active, trained young adult males. It demonstrates the feasibility of application of the IAAO technique in active individuals, while also providing updated guidelines for protein consumption in active populations.

## **Chapter 2 - Review of Literature:**

### **2.1 Introduction**

The subsequent literature review will first provide a scientific background of the metabolic fates of ingested protein, in addition to establishing the current recommended dietary allowance (RDA) (Section 2.2). An explanation of the various techniques often used to develop protein requirements, including NBAL and infusion protocols will then be summarized (Section 2.3), followed by a description of the development and practicality of the minimally-invasive Indicator Amino Acid Oxidation (IAAO) technique (Section 2.4). Lastly, the effects of both endurance and resistance exercise on protein metabolism will be provided (Section 2.5). A focus on young, adult males will be emphasized throughout the review. It was hypothesized that protein requirements would be: 1) greater than the current requirements established on the basis of NBAL, and; 2) greater than those requirements previously determined by the IAAO technique in non-active populations.

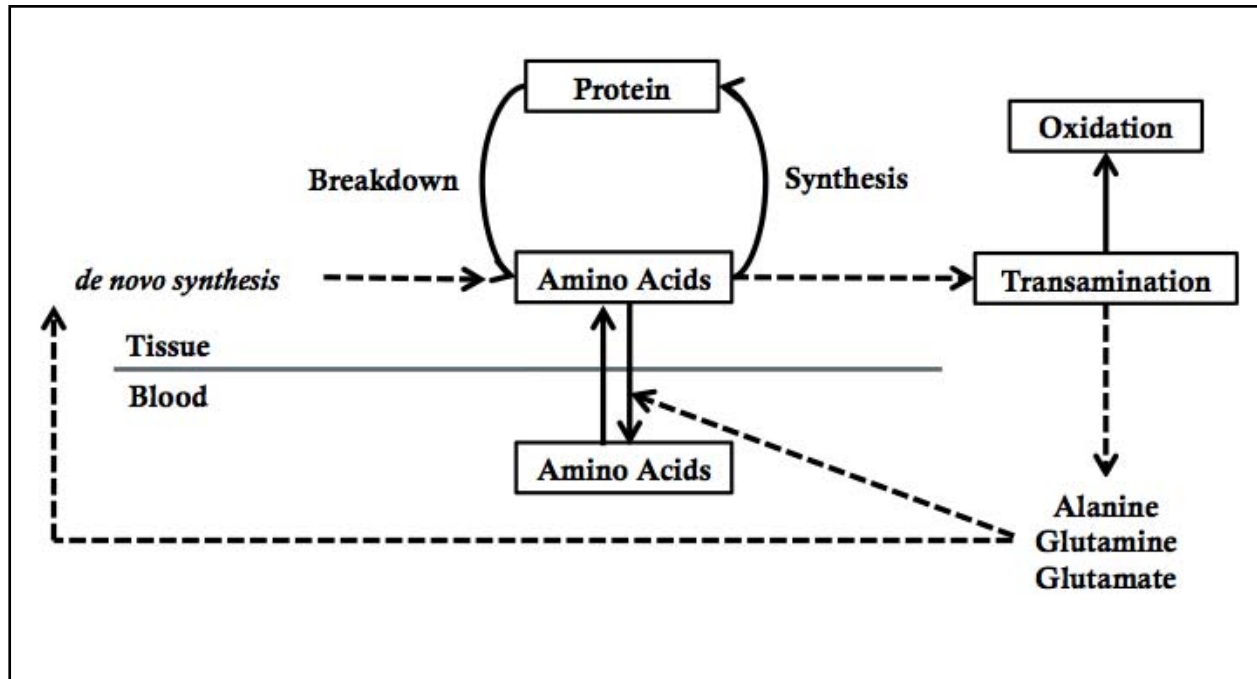
## 2.2 Metabolic Fates of Protein

### 2.2.1 Overview of Protein Metabolism

Protein is a macronutrient that serves many regulatory and structural purposes within the human body, including being an integral component of enzymes, antibodies, cell receptors, hormones, as well as muscle and bone. A single protein is comprised of a combination of amino acids, all of which contain an amino group (-NH<sub>2</sub>), a carboxylic acid group (-COOH), and an R-group that varies depending on the amino acid. Protein is the only dietary source of nitrogen, and therefore it must be consumed in adequate quantities to ensure optimal health. There exist 20 amino acids, of which 9 are considered essential amino acids (EAA) that must be obtained through one's diet (histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine). The remaining 11 non-essential amino acids (NEAA) can be synthesized to varying degrees within the human body, and under most conditions do not need to be consumed in large quantities.

Within the body, protein is in a continuous state of metabolic flux, commonly referred to as protein turnover, which is characterized by concurrent protein synthesis and degradation (breakdown) (M. Tarnopolsky, 2004) (**Figure 1**). Protein turnover can be influenced by various external stimuli including exercise, dietary composition, and energy intake. The content of whole-body protein is based on the algebraic difference between the rate of protein synthesis from free amino acids and the rate of protein degradation to amino acids. Thus, the free amino acid pool is constantly being recycled, as it serves not only to provide amino acids for protein synthesis, but is also continuously being replenished by the

breakdown of endogenous protein. The following sections will outline the major fates of protein (synthesis, degradation, and oxidation).



**Figure 1.** Protein turnover and amino acid flux. Intracellular and blood-based amino acids comprise the amino acid pool. Branch-chain amino acids can be preferentially transaminated and oxidized within the muscle, while other amino acids must be transported to the liver or kidneys to be transaminated and oxidized.

Adapted From: Phillips, S. M. (2004). Protein requirements and supplementation in strength sports. *Nutrition*, 20(7-8), 689-695.

### 2.2.2 Protein Synthesis

Protein synthesis is a highly regulated two-step process consisting of transcription and translation. Transcription occurs in the nucleus of a cell, where a signal induces the expression of DNA encoding for a specific protein or group of proteins by generating a complementary mRNA template. Translation then turns the mRNA template into a functional protein through the stringing together of several amino acids via peptide bond formation. For the most part, the acute regulation of protein synthesis occurs at the level of mRNA translation.

A 'fed-state' may be accomplished through the dietary consumption of protein or through the ingestion or infusion of exogenous amino acids (primarily EAA) (Burd, Tang, Moore, & Phillips, 2009). Upon oral ingestion, protein is broken down into its individual amino acid components and small peptide fragments by the digestive system and absorbed via the small intestines and transported to the liver by the portal vein. These amino acids and peptide fragments may be sequestered within the intestines or liver (collectively referred to as the splanchnic bed) for essential tissue function (e.g. constitutive and/or export protein synthesis) in a process called splanchnic extraction prior to being released into circulation for metabolism in other central and peripheral tissues. Exogenous amino acids that are administered via ingestion or infusion are also absorbed into the blood. The amino acid composition in the blood and extracellular and intracellular fluid is commonly known as the free amino acid pool, which serves as the primary facilitator of whole body protein synthesis (M. Tarnopolsky, 2004). Amino acids within the cell or those circulating in the blood stream can be used for the synthesis of protein. Thus, whole body protein synthesis is augmented in a fed-state mainly due to an enhanced amino acid pool available for synthesis (Bohe, Low, Wolfe, & Rennie, 2003; Svanberg, 1998; Tipton & Wolfe, 1998).

Free amino acids are provided to the amino acid pool via protein degradation in a fasted state, of which some amino acids will be recycled to synthesize protein (M. Tarnopolsky, 2004). However, free amino acids may also be used to provide energy via oxidation (See Section 2.2.4), which ultimately removes them from the free amino acid pools, and renders them unavailable to support protein synthetic rates during fasting.

### ***2.2.3 Protein Degradation***

Within the human body, protein degradation is accomplished by proteases. Three major systems serve to degrade protein, which include the ubiquitin-proteasome pathway, the lysosomal systems, and the calpain systems (Belcastro, Shewchuk, & Raj, 1998; Lecker, Solomon, Mitch, & Goldberg, 1999). Proteases function by hydrolyzing the peptide bonds that hold amino acids together, ultimately cleaving individual amino acids from the protein molecule. Once cleaved from protein, amino acids enter the amino acid pool in the blood or extracellular fluid. Upon entering the amino acid pool, amino acids can be re-synthesized into structural or regulatory proteins and/or oxidized for the purpose of generating energy, the latter of which results in hepatic urea synthesis and ultimately nitrogen excretion (Belcastro et al., 1998; Lecker et al., 1999).

### ***2.2.4 Amino Acid Oxidation***

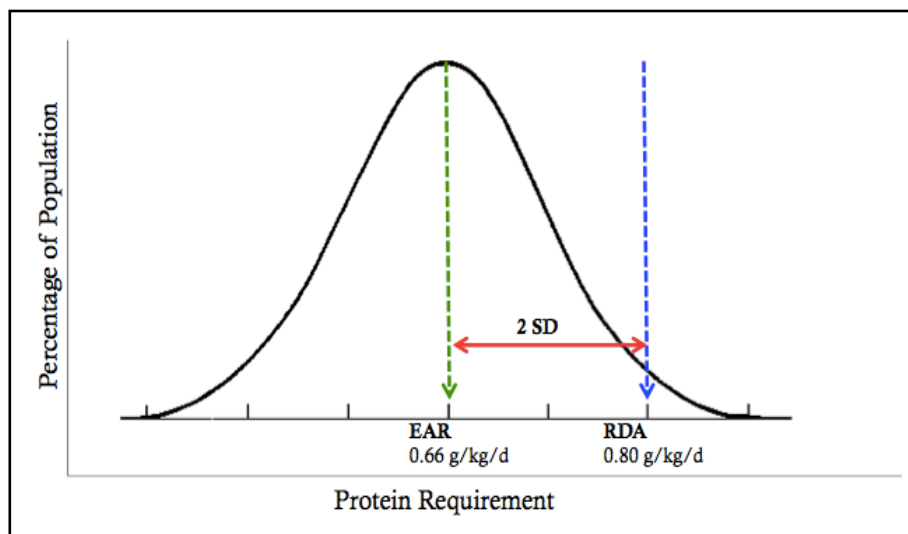
The oxidation of amino acids is a process that can use protein as a fuel source for the production of ATP (Millward, 1998). Additionally, in contrast to fat and carbohydrate metabolism, amino acids cannot be stored to a great extent within the human body and therefore any excess amino acids (i.e. those that cannot be incorporated into any protein pools) are removed via oxidative pathways (Millward, 1998). During amino acid oxidation, amino acids are required to lose their amino group either through transamination or deamination. Transamination involves the transfer of the amino group from an amino acid to another molecule (usually  $\alpha$ -ketoglutarate). The transamination process differs depending on the type of amino acid. Branch-chain amino acids (leucine, isoleucine, and valine) are predominantly oxidized within muscle tissue, while other amino acids, such as

phenylalanine, must first be processed by the liver before transamination occurs (M. Tarnopolsky, 2004). Phenylalanine oxidation is also unique, as it must first be hydroxylated into tyrosine prior to transamination (Shiman & Gray, 1998). Regardless, transamination leaves behind a carbon-skeleton of the amino acid that may be reassembled into Citric Acid Cycle intermediates (e.g. acetyl CoA) and be consumed within the mitochondria as a substrate for the production of ATP. Deamination is a process that takes place primarily in the liver, and serves to remove the amino group from an amino acid, which ultimately produces ammonia. Ammonia can then be excreted directly by the kidneys, or converted to urea within the liver via the addition of CO<sub>2</sub> prior to its subsequent urinary excretion; collectively, these processes represent the major route of nitrogen loss in humans. The remaining carbon-skeleton of the amino acid can then be used to generate ATP.

Several circumstances may cause amino acids to undergo oxidation. In states of excessive amino acid consumption, any surplus amino acids will be deaminated and excreted, as any additional amino acid consumption above the requirement will not be used for protein synthesis (Millward, 1998). Additionally, during prolonged periods of fasting and/or endurance exercise, skeletal muscle protein may be degraded into its amino acids components, which may then be transaminated with their carbon-skeletons being utilized as a source of energy (McKenzie et al., 2000; Smith & Rennie, 1996). Carbohydrate availability may also influence amino acid oxidation during exercise, as studies have determined that amino acid oxidation is heightened in response to low dietary carbohydrate intakes (Howarth et al., 2010). In summary, any condition (i.e. exercise, fasting) that increases amino acid oxidation may ostensibly increase protein requirements.

### 2.2.5 Estimated Average Requirement & Recommended Dietary Allowance

The recycling of amino acids in the human body is not fully efficient (FAO, WHO 2007). During normal processes, proteins that have been degraded into amino acids may not immediately be re-synthesized (FAO, WHO 2007). Since free amino acids cannot be stored, deamination results in daily losses of nitrogen via the excretion of urea. Additionally, miscellaneous routes of nitrogen loss occur via hair, nail, skin, digestive system, and fecal losses. As a result, humans must consume a daily estimated average requirement (EAR) of 0.66g/kg/day (FAO, WHO 2007). The EAR represents the minimum level of protein to offset daily losses of nitrogen in 50% of the population (**Figure 2**).



**Figure 2.** The EAR and RDA. The EAR corresponds to the average requirement of 50% of the population, while the RDA encompasses the level of protein that ensures 97.5% of the population is not deficient.

However, the EAR may not sufficiently define adequate protein intake for all individuals within a population (FAO, WHO 2007). Therefore, a recommended dietary allowance (RDA) has been established as the EAR plus two standard deviations to cover 97.5% of the population, and is currently set at 0.8g/kg/day (FAO, WHO 2007). It embodies the amount

of dietary protein sufficient to support the synthesis and oxidation of protein, while also accounting for the fundamental inefficiency inherent to the recycling of protein and amino acids, and the daily obligatory losses of nitrogen.

## **2.3 Methods Determining Protein Requirements**

### ***2.3.1 Nitrogen Balance***

Nitrogen Balance (NBAL) has been the most commonly used method to determine protein requirements since the 20<sup>th</sup> century (Rand, Pellett, & Young, 2003). NBAL involves the measurement of the total nitrogen intake ( $N_{IN}$ ) via dietary consumption or infusion, and the total nitrogen excretion ( $N_{OUT}$ ), the latter of which primarily occurs through feces, urine, and sweat losses (Rand et al., 2003; Young, Bier, & Pellett, 1989). Nitrogen equilibrium (nitrogen balance, net zero balance) is obtained when the sum of all avenues for nitrogen intake is equal to that of the sum of nitrogen losses (Net Zero Balance:  $N_{IN} = N_{OUT}$ ). NBAL is positive when  $N_{IN}$  exceeds  $N_{OUT}$ , providing for anabolism or a net gain of protein.

Conversely, NBAL is negative when  $N_{OUT}$  is greater than  $N_{IN}$ , leading to a catabolic state, or loss of protein (M. Tarnopolsky, 2004). The estimated protein requirement is determined from the NBAL technique using strict dietary controls that provide subjects with varying levels of protein intakes for ~7 days with the subsequent direct measurement and/or estimated excretion of nitrogen. This permits the calculation of NBAL at each level of dietary intake (M. Tarnopolsky, 2004). A plot of nitrogen intake (x-axis) vs. nitrogen balance (y-axis) is generated, and linear regression analysis can then be used to determine the x-intercept, which corresponds to the EAR, or zero net-balance. A buffer of two standard deviations is added as a 'safety factor' to the EAR to account for variability

between individuals resulting in an RDA that encompasses approximately 97.5% of the population (Rand et al., 2003; M. Tarnopolsky, 2004; Young et al., 1989). The current EAR and RDA provided by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) are based on a collection of studies that have predominantly employed NBAL to determine protein requirements (FAO, WHO 2007).

A meta-analytical review by Rand et al. sought to examine studies that employed NBAL as a means to determine protein requirements (EAR and RDA) in healthy adults (Rand et al., 2003). The meta-analysis gathered the results from a total of 19 studies, accounting for 235 individual subjects. Each of the 19 studies incorporated into the meta-analytical review were required to have provided subjects with a minimum of three different nitrogen intakes ranging from deficient (resulting in negative NBAL) to excessive (resulting in positive NBAL) intake. Additionally, the studies utilized similar protocols, which required subjects to consume a test diet for 10-14 days. A 10-14 day buffer period is required for the participants' metabolism to adjust to the altered dietary intake and to ensure consistent nitrogen equilibrium is obtained (Rand et al., 2003). Nitrogen intake ( $N_{IN}$ ) could therefore be readily determined and regulated. Urinary and fecal nitrogen samples, which typically account for 90-95% of total excretion (M. A. Tarnopolsky et al., 1988), are used to assess the metabolic response to the varying dietary intakes to allow for an estimation of the total nitrogen excretion. Only 12 of the 19 studies accounted for miscellaneous losses of nitrogen (such as hair, dermal, skin, and exhaled losses of nitrogen). The relationship between nitrogen intake and nitrogen balance was computed for each of the 235 subjects, providing for an understanding of the estimated protein requirements for each of the 19 studies. The

meta-analysis established an EAR and RDA to be 0.65 and 0.83 g/kg/d respectively. These are similar to current EAR and RDA values proposed by the FAO and WHO, corresponding to 0.66 g/kg/day and 0.80 g/kg/day respectively.

### ***2.3.2 Strengths of NBAL***

There exist several advantages inherent to the application of the NBAL technique for its use in determining protein requirements. First, NBAL is a non-invasive means of determining protein requirements, as intravenous infusions and muscle biopsies are not required as part of the application of the NBAL technique. Thus, NBAL presents very minimal risk to participants, and is therefore in theory practical in all demographic groups. Additionally, NBAL represents a relatively straightforward means of determining protein requirements as the independent variable (dietary nitrogen consumption) can be easily controlled for. Miscellaneous losses of nitrogen (i.e. sweat, hair, exhalation losses) although difficult to measure directly, are often estimated based on pre-existing data that may be generalizable to the population. Thus, NBAL is arguably a practical method of determining protein requirements that generally presents minimal risk to healthy participants.

### ***2.3.3 Limitations of NBAL***

Although NBAL has been the method most frequently used to establish the current EAR and RDA, it has several limitations that may bring in to question its ability to obtain accurate estimates of protein requirements. Several studies suggest that NBAL often underestimates the true value of nitrogen excretion ( $N_{OUT}$ ), as it is difficult to measure miscellaneous losses of nitrogen (sweat, hair, exhalation, etc) (Forbes, 1973; Humayun, Elango, Ball, & Pencharz,

2007). The impact of this error in miscellaneous losses results in an underestimation of  $N_{OUT}$ , which subsequently results in an underestimation of nitrogen excretion and an erroneously high nitrogen balance. This may ultimately lead to an underestimation of true protein requirements (Humayun et al., 2007). This may especially be the case in athletes, who have exercise-induced increases in metabolism and sweat loss (Consolazio F., Neslon A., Matoush L., Harding R., & Canham J, 1963). NBAL studies also require subjects to consume a regulated study diet for an extended period of time, usually lasting one to three weeks in an attempt to control for nitrogen consumption ( $N_{IN}$ ), and to allow subjects to adapt to the new protein consumption (Rand et al., 2003; FAO, WHO 2007). Subject adherence to the prescribed study diet is therefore essential, and any deviation from the study diet could result in inaccurate protein requirements. This is especially problematic when utilizing NBAL to determine protein requirements in children, as their dieting regimen is more sporadic than adults. NBAL also provides very little information regarding the dynamic process of whole-body protein turnover and amino acid flux (M. Tarnopolsky, 2004). NBAL fails to provide information pertaining to the adaptive changes in whole-body protein synthesis and oxidation in response to prolonged dietary or exercise stimuli. Lastly, the work of Humayan et al. has highlighted a potential statistical limitation with respect to the utilization of NBAL to determine protein requirements (Humayun et al., 2007). The current EAR and RDA for protein consumption has been estimated through the use of a meta-analysis that employed single linear regression on several studies that employed NBAL. The EAR was determined to be the point where the regression line intersected with zero balance. However, the use of single linear regression analysis to determine the EAR on such comprehensive NBAL data may underestimate true protein requirements. The results

concluded that the current EAR (0.66g/kg/day) and RDA (0.80 g/kg/day) determined by single linear regression were underestimated by 29% and 33% respectively.

#### ***2.3.4 Overview of Infusion Techniques***

Some of the limitations associated with NBAL have provided the impetus for researchers to develop alternative methods that could potentially be used to determine protein requirements. Infusion techniques involve the provision of stable isotopes of amino acids that often have a  $^{13}\text{C}$  rather than the normal  $^{12}\text{C}$ -carbon on one of the atoms of primarily an EAA (FAO, WHO 2007). Subjects are required to partake in a dietary adaptation period prior to engaging in the infusion protocol. However, this adaptation period is often shorter in length than that of NBAL (Elango, Humayun, Ball, & Pencharz, 2009). Generally, a test (or indicator) amino acid is infused intravenously for a period usually ranging from 4 to 24 hours where a subject can be in a fasted or fed state. When the EAA is oxidized, the  $^{13}\text{C}$  is cleaved from the molecule in the first step of oxidation when it is labeled on the first carbon (e.g. L- $^{13}\text{C}$ ]leucine or L- $^{13}\text{C}$ ]phenylalanine). Breath (for  $^{13}\text{CO}_2$  enrichment) and urine or blood samples (for estimates of precursor enrichment) can then be collected and analyzed for their respective enrichments. These data can then be modeled (typically with single-pool steady state kinetics) to determine the turnover and, more importantly, rate of oxidation of the infused amino acid, which collectively provide an understanding of its metabolism within the human subject and resultantly their protein requirement (Wagenmakers, 1998).

As with any method, there exist both strengths and limitations inherent to infusion protocols. In contrast to NBAL, infusion protocols are able to provide important information pertaining to the dynamic process of whole-body protein turnover and amino acid flux (M. Tarnopolsky, 2004). Additionally, infusion protocols may not require the dietary adaptation period to be as lengthy as those analogous adaptation periods associated with NBAL. This may enhance the practicality of infusion protocols (FAO, WHO 2007). However, infusion protocols are somewhat invasive in nature, as they require participants to partake in intravenous infusions that can last from 4-24 hours. Additionally, the costs associated with infusion protocols often exceed that of NBAL, representing what could be perceived as a limitation.

### ***2.3.5 Conclusions***

Although NBAL has been valued as the gold standard when determining protein requirements, there are several flaws inherent to its methodology. Also, despite being a formidable alternative to the utilization of NBAL for determining protein requirements, infusion studies are rather invasive. As a result, the use of alternative methodologies, such as the non-invasive Indicator Amino Acid Oxidation (IAAO) technique, has been developed to determine protein requirements in humans.

## **2.4 The Minimally Invasive Indicator Amino Acid Oxidation (IAAO) Technique**

### ***2.4.1 History of the Minimally Invasive IAAO Technique***

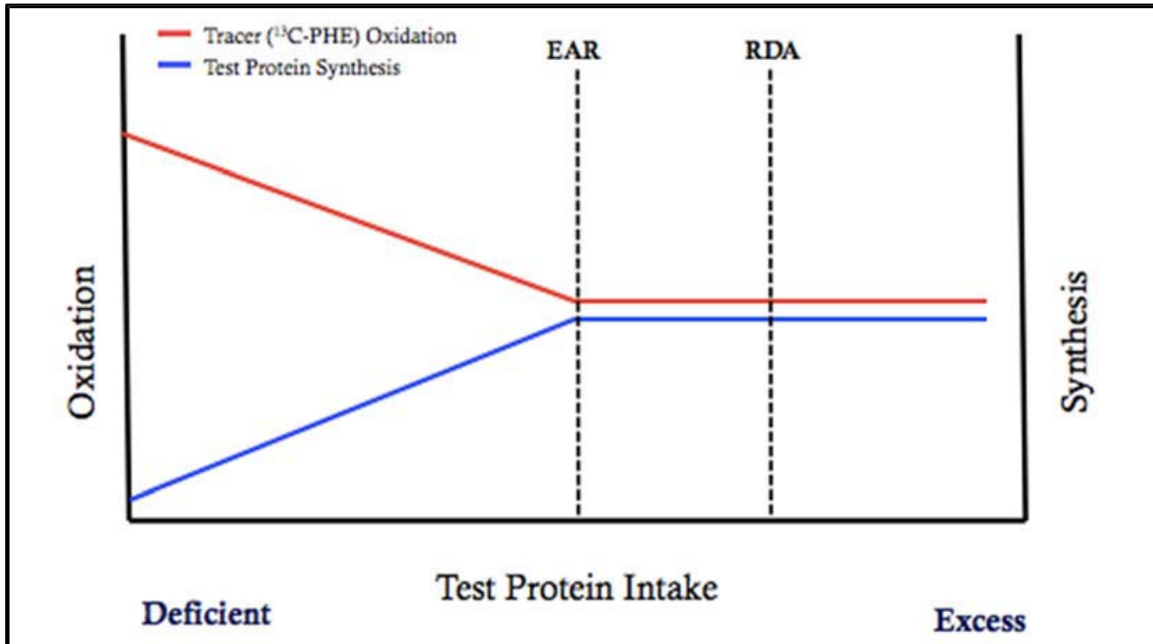
Until the late 1980's, nearly all of the studies that sought to determine protein requirements in humans used the NBAL technique (Pencharz & Ball, 2003). However, once the limitations with NBAL were realized, alternative methods that rely on stable isotopes to determine protein requirements were developed (Pencharz & Ball, 2003); one of these alternative methods was the indicator amino acid oxidation technique. Initial studies performed by Kim et al. (Kim, McMillan, & Bayley, 1983), and Ball & Bayley (Ball & Bayley, 1984) in the early 80's first implemented the IAAO technique in young pigs in order to determine their amino acid requirements for feeding. In 1986, a series of direct amino acid oxidation (DAAO) studies reported significantly higher amino acid requirements for leucine, valine, and lysine for humans than those that were being prescribed by the Food and Agriculture Organization of the United Nations (FAO), and the World Health Organization (WHO) (Pencharz & Ball, 2003). The DAAO studies required participants to consume crystalline amino acid mixtures sufficient to maintain NBAL. The <sup>13</sup>C-labelled test amino acids (leucine, valine, and lysine) were then infused at varying intakes to determine their individual requirements (Meguid et al., 1986; Meredith, Wen, Bier, Matthews, & Young, 1986). Due to the results of these studies, there was a shift towards developing a stable, non-invasive isotope protocol that could be utilized for the obtainment of accurate protein requirements in humans. The first published report of the minimally invasive IAAO protocol for human subjects was performed by Pencharz and Ball in 1993 (Pencharz & Ball, 2003). The results from this study supported the previous DAAO studies, suggesting that

protein requirements may exceed those values typically reported in NBAL studies (Zello, Pencharz, & Ball, 1990; Zello, Pencharz, & Ball, 1993). The minimally invasive IAAO technique has subsequently been refined and utilized in several protein requirement studies in a variety of populations ranging from young adult males (Humayun et al., 2007) to elderly females (Rafii et al., 2015).

#### ***2.4.2 Methodological Application of the Minimally-Invasive IAAO Technique***

The minimally invasive IAAO technique utilizes stable isotopes (most commonly  $^{13}\text{C}$ ) to specifically label a single 'indicator' amino acid (Pencharz & Ball, 2003). The minimally invasive IAAO technique dictates that an indicator amino acid is always supplemented in excess of the protein requirement, while the remaining intake of amino acids will range from being deficient to surfeit of the protein requirement through the consumption of crystalline amino acids based on the amino acid composition of egg protein (FAO, WHO 2007). When a single amino acid (often an EAA) from the diet is limiting (below the requirement) then the other amino acids (including the indicator) cannot be optimally utilized for protein synthesis and are subsequently directed towards oxidation (FAO, WHO 2007) (Zello, Wykes, Ball, & Pencharz, 1995). Accordingly, in a situation where the indicator amino acid is consumed in excess, while the remaining test protein consumption is deficient, any amount of the indicator amino acid in excess of the test protein will be directed towards oxidation. In this scenario, one would expect the levels of  $^{13}\text{C}$  from the oxidized indicator amino acid to be high and overall protein synthesis to be compromised. As the consumption of the limiting amino acid(s) in the test protein is increased, a lesser proportion of the indicator amino acid will be oxidized; this would result in a

corresponding decrease in the appearance of the  $^{13}\text{C}$  label from the indicator amino acid appearing in breath as a greater proportion of the ingested amino acids are utilized for protein synthesis (**Figure 3**) (Zello et al., 1995). However, once the protein requirement for the test amino acid(s) is reached, any additional intake of the test protein above the protein requirement should not result in a further decrease in the indicator amino acid oxidation as this amino acid will always be consumed in excess of its requirement (Zello et al., 1995). Additionally, there should be no further increases in protein synthesis once the test protein intake exceeds that of the protein requirement. The point where no further decreases in indicator amino acid oxidation (as measured via  $^{13}\text{CO}_2$  excretion) are seen despite increases in the test protein intake is termed the 'breakpoint' and is modeled by bi-phase linear regression (Figure 5) (Zello et al., 1995). It is this point that determines the EAR of the test protein, essentially allowing for protein requirements to be established from the minimally invasive IAAO technique. The minimum requirement can then be determined by adding two standard deviations to the EAR (breakpoint).



**Figure 3.** Trends in amino acid (AA) synthesis and oxidation during IAAO technique. The break-point (EAR) has been denoted with the dashed line. The minimum requirement is determined by adding two standard deviations to the EAR.

The isotopically labeled [1-<sup>13</sup>C]-phenylalanine is the most commonly used indicator amino acid when administering the minimally invasive IAAO technique (Zello et al., 1995). Subjects are required to consume the indicator amino acid, along with several different test protein intakes throughout the minimally invasive IAAO protocol. Subjects partake in roughly 7 isolated trials whereby the test protein consumption will range from deficient to excessive protein intake (Zello et al., 1995). The relationship between the test protein intake and the indicator amino acid oxidation is determined during each trial, allowing for a breakpoint (EAR) to be established, and minimum protein requirements to be elucidated.

### ***2.4.3 The Minimally Invasive IAAO Technique & Protein Requirements***

Several studies have implemented the minimally invasive IAAO technique for the purpose of establishing protein requirements since it the first published report in humans was introduced in 1993. A study by Humayun et al. in 2007 used the technique to estimate protein requirements for the first time in healthy, young adult men (Humayun et al., 2007). A total of eight healthy young adult men of different ethnicities were recruited for the study. In accordance with most studies that have used the minimally invasive IAAO technique, L-[1-<sup>13</sup>C]-phenylalanine was used as the indicator amino acid. Subjects received one of 7 dietary test protein intakes ranging from deficient (0.10 g/kg/d) to excess (1.8 g/kg/d) over the 7 trial days. Upon analysis, the estimated EAR and RDA corresponded to 0.93 g/kg/d and 1.2 g/kg/d respectively. Both of these values are greater than the analogous values often reported from NBAL techniques (EAR: 0.66 g/kg/d; RDA: 0.80g/kg/d), suggesting that the current EAR and RDA may be underestimates of true protein requirements. These values also corresponded well with the reanalysis of historical NBAL data using bi-phase linear and linear analysis that included NBAL studies conducted at high protein intakes (Humayun et al., 2007).

Although the minimally invasive IAAO technique is a novel non-invasive method to determine protein requirements, there exist some limitations to its practicability. Firstly, the nature of the minimally invasive IAAO protocol requires participants to engage in several (~7) metabolic trial days lasting at least 6-8 hours each. There are therefore issues related to participant withdrawal from the study before its completion. Additionally, participants are required to consume hourly liquid meals on trial days. Although these

meals provide a sufficient energy, they may not adequately mimic a typical dietary regimen, which primarily consist of three substantial meals and unbalanced protein distribution (de Castro, Bellisle, Feunekes, Dalix, & De Graaf, 1997).

#### ***2.4.4 Conclusions***

The recent utilization of the minimally invasive Indicator Amino Acid Oxidation technique has consistently determined that protein requirements exceed the analogous values previously determined by studies that have implemented NBAL methodologies. However, any effect that physical activity elicits on protein requirements has yet to be determined using the minimally invasive IAAO technique. Therefore, given the potential underestimation of protein requirements by NBAL and the potential for enhanced requirements in active populations, it is important to apply the minimally invasive IAAO technique to determine if exercise is capable of impacting protein requirements.

### **2.5 Effect of Exercise on Protein Metabolism**

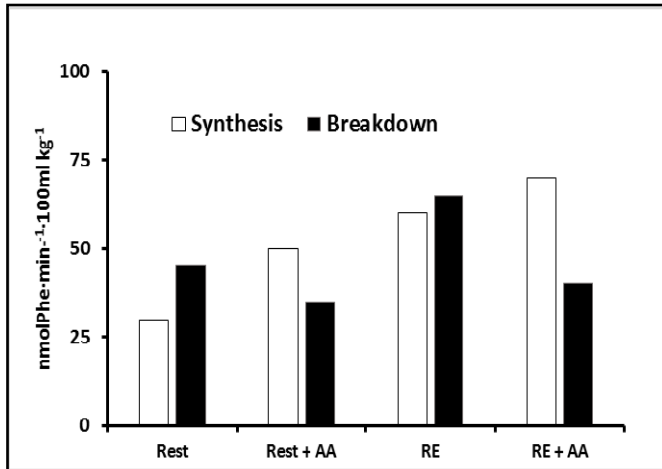
#### ***2.5.1 General Overview***

Protein synthesis, degradation, and oxidation are highly regulated processes that must accommodate the rapidly changing requirements of the muscle cell during physical activity (Burd, Tang, Moore, & Phillips, 2009). Exercise is capable of eliciting an effect on both muscle protein synthesis and oxidation, which would affect muscle and potentially whole-body protein balance (i.e. synthesis & breakdown) when compared to resting conditions (Phillips, 2004; M. Tarnopolsky, 2004). Since exercise is incorporated into training regimes

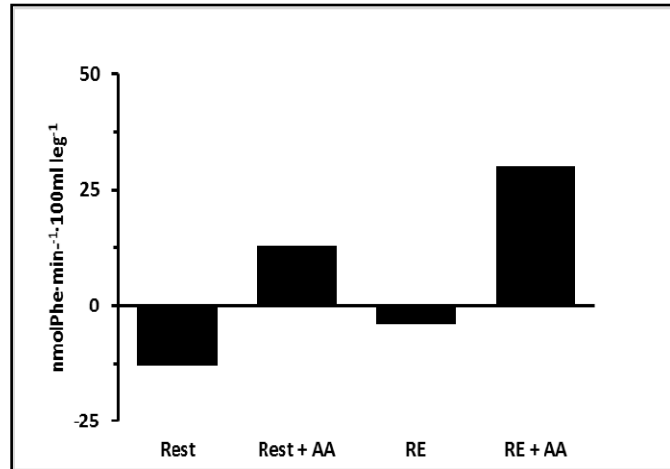
for athletes and is a vital component of a healthy lifestyle, the impact that exercise has on current minimum requirement must be examined in order to ensure adequate protein consumption for healthy, active populations. The following sections will outline the impact of resistance and endurance exercise on protein metabolism and the RDA by providing a review of important literature.

### ***2.5.2 Effect of Resistance Exercise on Protein Metabolism, RDA***

Resistance exercise is well documented to elicit changes in protein metabolism, primarily within the muscle (Burd et al., 2009). Various studies have provided evidence that a single bout of resistance exercise significantly enhances muscle protein synthesis when compared to baseline values, shifting muscle protein balance to a more positive state for up to 48 hours following resistance exercise (**Figure 4**) (Phillips, Tipton, Aarsland, Wolf, & Wolfe, 1997; Biolo, Maggi, Williams, Tipton, & Wolfe, 1995; Chesley, MacDougall, Tarnopolsky, Atkinson, & Smith, 1992; Yarasheski, Zachwieja, & Bier, 1993). However, despite the augmented state of muscle protein synthesis, net protein balance remains negative in the fasted state (i.e. in the absence of dietary protein or amino acids) as muscle protein breakdown is also increased in response to a single bout of resistance exercise to provide a source of amino acids to support protein synthesis (Biolo et al., 1995; Phillips, Tipton, Aarsland, Wolf, & Wolfe, 1997; Phillips, Tipton, Ferrando, & Wolfe, 1999).



**Figure 4.** The impact of resistance exercise, and amino acid consumption on protein synthesis and breakdown. AA = Amino Acids, RE = Resistance Exercise. Adapted From: Phillips, S. M. (2004). Protein requirements and supplementation in strength sports. *Nutrition*, 20(7-8), 689-695.



**Figure 5.** The impact of resistance exercise, and amino acid consumption on protein balance. AA = Amino Acids, RE = Resistance Exercise. Adapted From: Phillips, S. M. (2004). Protein requirements and supplementation in strength sports. *Nutrition*, 20(7-8), 689-695.

Muscle protein balance remains negative following resistance exercise unless amino acids are either administered via infusion or through oral consumption (**Figure 5**) (Biolo et al., 1995; Borsheim, Tipton, Wolf, & Wolfe, 2002; Rasmussen, Tipton, Miller, Wolf, & Wolfe, 2000). Thus, resistance exercise and amino acid consumption elicit a synergistic effect on protein synthesis (Borsheim et al., 2002; Miller, Tipton, Chinkes, Wolf, & Wolfe, 2003), whereby net protein balance is sufficiently positive to support muscle hypertrophy.

The changes in muscle protein metabolism with resistance exercise and the associated muscle growth with training have prompted questions regarding the sufficiency of the current RDA (0.8 g/kg/d) for individuals partaking in such forms of exercise (Gibala et al., 2000; M. A. Tarnopolsky et al., 1992). Some evidence suggests that individuals that regularly partake in resistance exercise may require protein consumption in excess of the current RDA (as discussed below) (Gibala et al., 2000; M. A. Tarnopolsky et al., 1992). It is hypothesized that this additional protein would be required to support the enhanced levels

of protein synthesis required for muscle hypertrophy while also serving to replenish any additional catabolic losses of protein in response to resistance training (Gibala, MacDougall, Tarnopolsky, Stauber, & Elorriaga, 1995; M. A. Tarnopolsky et al., 1992).

Conversely, various studies have suggested that regular exercise (and potentially resistance exercise) may improve the efficiency of protein use (Butterfield & Calloway, 1984; Torun, Scrimshaw, & Young, 1977). The seminal work performed by Butterfield and Calloway in 1984 revealed that an improved efficiency of nitrogen utilization occurred as a result of physical activity (Butterfield & Calloway, 1984). Six healthy, young adult males were required to consume the FAO/WHO safe protein intake of 0.57 g/kg/d in an initial dietary adaptation period. Upon adaptation to the study diet, participants then engaged in light treadmill based exercise consisting of 3 20-minute brisk walks. Toward the latter portion of the study an additional exercise stimulus was added to increase the subjects' energy output, which consisted of two 30-minute phases of bicycling. Upon analysis of urinary, fecal, and sweat nitrogen values throughout the protocol, results concluded that the implemented physical activity resulted in an improvement in the over-all nitrogen economy of the exercising subjects, effectively sparing nitrogen (Butterfield & Calloway, 1984). If a similar metabolic adaptation occurs as a result of resistance exercise, it is conceivable that the current RDA may be sufficient for individuals that regularly partake in resistance exercise.

Several studies have demonstrated that protein requirements are enhanced in response to resistance exercise. A study by Lemon et al. found evidence of a greater protein

requirement associated with strength training in novice athletes (Lemon, Tarnopolsky, MacDougall, & Atkinson, 1992). Twelve young, adult males were required to consume a protein intake of 1.35 g/kg/d or 2.62 g/kg/d during the initial stages of an intensive bodybuilding regimen. NBAL was used to determine both zero NBAL and protein requirements. Despite consuming 1.35 g/kg/d, which is ~69% greater than the current RDA, participants were in a net negative nitrogen balance. The results established a natural NBAL was achieved on a diet providing 1.4-1.5 g protein/kg/d, which resulted in an estimated protein requirement of 1.6-1.7 g/kg/d. This finding suggests that protein requirements during the initial stages of a bodybuilding regimen are two times higher than the current RDA of 0.8 g/kg/day (Lemon et al., 1992). A study performed by Tarnopolsky et al. (M. A. Tarnopolsky, MacDougall, & Atkinson, 1988) sought to evaluate protein requirements in young adult males of different training statuses. Three groups of six males were recruited corresponding to bodybuilders, endurance athletes, and sedentary controls. Each group participated in an initial experiment designed to evaluate the protein requirements using NBAL while the participants consumed their habitual dietary intake. A supplemental experiment performed by Tarnopolsky et al. employed a study diet that altered the protein intake for each group of participants, while also using NBAL to determine protein requirements (M. A. Tarnopolsky et al., 1988). All participants were required to maintain their habitual training routines throughout both experiments. The results from the NBAL data revealed that the protein requirements of bodybuilders were 1.12 times greater than those required by sedentary controls (M. A. Tarnopolsky et al., 1988). Similar results of elevated protein requirements have also been demonstrated in elite weight lifters and power lifters (Dohm, Williams, Kasperek, & van Rij, 1982).

Additionally, in a subsequent study (M. A. Tarnopolsky et al., 1992), thirteen healthy, young adult males were recruited, of which seven were categorized as strength-trained athletes (SA), and six as sedentary controls (S). SA subjects were required to have performed regular exercise to increase their strength for at least two months prior to the study. Subjects partook in three experiments where they were randomly assigned to consume one of three levels of protein intake: a low protein diet (LP) of 0.86 g/kg/d, a moderate protein diet (MP) of 1.40 g/kg/d, and a high protein diet (HP) corresponding to 2.40 g/kg/d. Strength-trained athletes were required to maintain their habitual activity during all the experiments, which based on the inclusion of team sport athletes incorporated both anaerobic and aerobic components (i.e. circuit weights, rugby drills, etc). Protein requirements were assessed using NBAL. Results indicated that zero NBAL for the strength-trained athlete group far exceeded that of the sedentary group (SA: 1.41 g/kg/d vs. S: 0.69 g/kg/d). Additionally, the recommended dietary intake for the strength-trained athlete group exceeded the current RDA of 0.8 g/kg/d (SA: 1.76 g/kg/d).

Although some evidence has suggested a heightened protein requirement in response to resistance training (Lemon et al., 1992; M. A. Tarnopolsky et al., 1988; M. A. Tarnopolsky et al., 1992), several studies have demonstrated that the current RDA may in fact be adequate for those who partake in regular resistance exercise (Campbell, Crim, Young, Joseph, & Evans, 1995; Phillips et al., 1997; Phillips, 2004; Torun et al., 1977). There exists evidence that the current RDA is adequate for resistance-trained individuals, as regular resistance exercise can result in protein being used more efficiently by the human body (Campbell et al., 1995; Phillips et al., 1999; Phillips et al., 2002). A study by Phillips et al. (Phillips et al.,

2002) supports a more efficient use of dietary protein in healthy, resistance-trained males. For example, subjects performed an acute bout of single-leg resistance exercise before and following an 8-week resistance-training program. Following the exercise stimulus, subjects were fed and received an infusion of [d<sub>5</sub>]- and [<sup>15</sup>N]-phenylalanine to determine muscle protein synthesis and muscle protein breakdown. Upon comparison of the results pre-to-post training, it appeared that resistance-exercise increased resting muscle protein synthesis and breakdown without impacting protein balance, while also attenuating the acute response to an acute bout of resistance-exercise. This provides evidence of a more efficient use of protein in response to regular resistance exercise. Studies published by Moore et al. (Moore et al., 2007), and Hartman et al. (Hartman, Moore, & Phillips, 2006) have reported greater whole body protein anabolism when consuming a moderate (i.e. 1.2-1.4g/kg/d) protein diet after as compared to before 12-wk of resistance training. The results from both studies provided evidence of a more efficient whole body utilization of amino acids in response to the resistance training.

In summary, it appears there is little consensus as to the effect of resistance exercise on whole body protein requirements given the dichotomy between studies that suggest the RDA is sufficient (Campbell, Crim, Young, Joseph, & Evans, 1995; Phillips et al., 1997; Phillips, 2004; Torun et al., 1977), whereas others do not (Lemon et al., 1992; M. A. Tarnopolsky et al., 1988; M. A. Tarnopolsky et al., 1992). Although there is a general consensus that the use of protein becomes more efficient with regular resistance training, it is still controversial as to whether this enhanced efficiency of protein use is able to offset

the increased protein requirement necessary for enhanced levels of protein synthesis and potential catabolic losses of protein in response to bouts of resistance training.

### ***2.5.3 Effect of Endurance Exercise on Protein Metabolism, RDA***

Endurance exercise has been documented to elicit changes in whole body and muscle protein metabolism. For example, studies have demonstrated that there are intensity-dependent increases in muscle and mitochondrial protein synthesis and potentially corresponding increases muscle protein breakdown as well after endurance exercise (Carraro et al., 1990; Carraro, Stuart, Hartl, Rosenblatt, & Wolfe, 1990; Di Donato et al., 2014; Wilkinson et al., 2008). Specifically, increases in the synthesis of the enzyme citrate synthase, and electron transport chain proteins integral to complexes I-III result from endurance exercise (McKenzie et al., 2000). Additionally, endurance training has been shown to elicit physiological changes that can improve one's aerobic capacity, including increased quantities of hemoglobin, myoglobin, and capillaries. Some scientists advocate that a heightened RDA is required to support these physiological changes associated with enhanced protein synthesis following endurance exercise (Friedman & Lemon, 1989; Lamont, Patel, & Kalhan, 1990; Meredith, Zackin, Frontera, & Evans, 1989).

Endurance exercise is also generally characterized by high (and sometimes sustained) levels of energy expenditure. Although carbohydrates and fats are the predominant macronutrients used to provide energy during endurance exercise, amino acids may contribute 1-6% of the total energy to the active skeletal muscle (McKenzie et al., 2000; Phillips, Atkinson, Tarnopolsky, & MacDougall, 1993; L. J. Tarnopolsky, MacDougall,

Atkinson, Tarnopolsky, & Sutton, 1990). Muscle is capable of oxidizing certain amino acids for the generation of ATP to help fuel endurance exercise (McKenzie et al., 2000). The branch-chain amino acids (BCAA) isoleucine, leucine, and valine are preferably oxidized in the muscle by the rate-limiting enzyme branch-chain oxo-acid dehydrogenase (BCOAD) (Boyer & Odessey, 1991; Lamont, McCullough, & Kalhan, 1999; Smith & Rennie, 1996). Studies have demonstrated that there is an increase in the capacity for BCOAD activity following endurance training (Lamont et al., 1999; McKenzie et al., 2000). Furthermore, studies have shown increases in the oxidation of the BCAA leucine and the EAA lysine during endurance exercise and that these oxidative losses may be related to total oxygen consumption (Lamont et al., 1990; Lamont et al., 1999; McKenzie et al., 2000; Phillips et al., 1993).

Both muscle protein synthesis and amino acid oxidation are enhanced in response to endurance exercise (Moore, Camera, Areta, & Hawley, 2014). However, despite these changes in protein metabolism, there exists some debate as to whether dietary protein requirements are altered in response to such an exercise stimulus (M. Tarnopolsky, 2004). It appears that protein requirements differ depending on the sex, training status, and specifically the nature (frequency, intensity, & duration) of the endurance exercise (M. Tarnopolsky, 2004). For this reason, studies have sought to determine requirements associated with one of three demographic groups: sedentary to recreationally active individuals (low-moderate intensity endurance exercise) (el-Khoury et al., 1997; Forslund et al., 1999), moderately to well-trained endurance athletes (training 4-5d/week and  $\geq 60$

min/d) (Lamont et al., 1990; Meredith et al., 1989; Phillips et al., 1993), and elite endurance athletes (~12h/week) (Brouns et al., 1989; M. A. Tarnopolsky et al., 1988).

Forslund et al. performed well-controlled and comprehensive study evaluating the effects of both protein intake and moderate endurance activity on 24-h leucine turnover and macronutrient utilization (Forslund et al., 1999). Fourteen healthy young adult males were split into either a normal protein intake group consuming 1.0 g/kg/d, or a high protein group consuming 2.5 g/kg/d for 6 days. Subjects were required to perform 90-min cycling exercise twice per day at 45-50%  $\text{VO}_2\text{-max}$  each study day. The subjects were then infused with L-[1- $^{13}\text{C}$ ]-leucine for 24 hours on day 7 to evaluate protein oxidation. Protein balance was determined to be slightly negative in the normal protein group, yet was significantly positive in the high protein group. This was accompanied by a greater leucine oxidation in the high protein group, which would suggest an excess consumption of this macronutrient. Additionally, the high protein group saw a far greater contribution of protein for energy than that of the low protein group. These results suggest that a dietary protein requirement of 1.0 g/kg/d should be sufficient for individuals partaking in recreational moderate intensity endurance exercise (el-Khoury et al., 1997; Forslund et al., 1999).

Several studies have also sought to determine if protein requirements are elevated in moderately to well-trained endurance athletes using NBAL (Lamont et al., 1990; Meredith et al., 1989; Phillips et al., 1993). Phillips et al. employed both NBAL and leucine infusion to determine if the protein requirement of 0.86 g/kg/d was adequate for endurance trained individuals (Phillips et al., 1993). It was demonstrated that leucine oxidation was greater in

male as compared to female subjects, which suggests that males are more reliant on the utilization of protein for energy during endurance exercise than females. However, both sexes were found to be in negative nitrogen balance, suggesting that the protein requirement of 0.86 g/kg/d was insufficient for endurance athletes. This was supported by a study published by Meredith et al. (Meredith et al., 1989), as young and middle-aged male endurance athletes were recorded to have a safe protein intake of 1.26g/kg/d.

Protein requirements for elite endurance athletes (training ~12h/week,  $VO_2$ -max ~70mlO<sub>2</sub>/kg/min) have also been determined using NBAL (Brouns et al., 1989; M. A. Tarnopolsky et al., 1988). Tarnopolsky et al. conducted well-controlled and comprehensive study evaluating protein requirements in elite endurance athletes (M. A. Tarnopolsky et al., 1988). Six top sport runner and Nordic ski endurance athletes (Body Fat %: 7.1 +/- 0.8; Training Load > 125 km/week) and six sedentary controls were recruited. Participants were required to consume their habitual dietary intake while maintaining their habitual physical activity levels. An additional experiment employed a study diet that altered the protein intake for each group of participants in order to determine if varying the consumption of protein results in a change in nitrogen balance (M. A. Tarnopolsky et al., 1988). The results suggested that the elite endurance athletes' protein requirement (1.6 g/kg/g) far exceeded the analogous requirement for the sedentary controls (0.86 g/kg/d) (M. A. Tarnopolsky et al., 1988). Studies by Meredith et al. (Meredith, Zackin, Frontera, & Evans, 1989) and Friedman and Lemon (Friedman & Lemon, 1989) have also reported an increased protein requirement in elite endurance athletes (Training Volume: 12-16km running per day). Meredith et al. recruited 6 young and 6 middle aged men who consumed

0.6, 0.9, and 1.2 g/kg/d of high-quality protein over three separate 10-day periods while maintaining their habitual training regimen (Meredith et al., 1989). The results suggested that the participants' protein requirements (0.94 g/kg/d) exceeded the current RDA (0.80g/kg/d). In a study conducted by Friedman & Lemon in 1989, five well-trained endurance runners ( $\text{VO}_2\text{-max} \sim 70$ ) consumed either the RDA, or 1.7 times the RDA (high-protein trial) on two separate occasions for 6 days while maintaining their typical training regimen (Friedman & Lemon, 1989). Results suggested that the RDA of 0.80 g/kg/d was inadequate for the endurance athletes as nitrogen retention was significantly reduced, while nitrogen retention remained positive during the high-protein trial. In summary, there appears to be a heightened protein requirement for highly trained endurance athletes.

There appears to be no significant increase in protein requirements for individuals that recreationally engage in endurance activity, yet there is considerable evidence of a heightened protein requirement for endurance-trained individuals and especially elite endurance athletes. The RDA for these populations may be ~20-100% greater than the current RDA depending on the nature of the endurance exercise stimulus, the training status, and sex of the individual.

#### ***2.5.4 Effect of Variable Intensity Exercise on Protein Metabolism, RDA***

Although a wealth of studies have examined the individual impacts of either resistance or endurance training (exercise) on protein metabolism and requirements, no study has sought to determine how a combination of both training types can impact dietary protein requirements. Since athletic training for most team sports often resembles that of variable

intensity exercise that incorporates both aerobic (e.g. increased oxygen consumption) and resistive (e.g. high force stop-and-go) components, it could be argued that no previous study has elucidated how this type of exercise modality alters protein requirements; this would be important for the optimization nutrition in these unique athletes.

A study performed by Coffey et al., in 2010 sought to determine the impact of cycling sprint interval training on cell signaling and protein synthesis (Coffey et al., 2011). Results suggested enhanced myofibrillar and mitochondrial protein synthetic rates following the sprint interval training. Like variable intensity exercise, sprint interval training is capable of activating both aerobic and anaerobic energy systems. As such, it is plausible that a variable intensity exercise stimulus mimicking that of sport could result in enhancements to myofibrillar and mitochondrial protein synthesis, potentially increasing protein requirements in individuals who regularly partake in such an exercise routine (i.e. team-sport athletes) (M. A. Tarnopolsky et al., 1992).

## ***2.6 Conclusions, Gaps in Literature***

The impacts that both resistance and endurance exercise have on protein requirements have been extensively studied but with limited consensus reached. The utilization of the NBAL technique suggests that protein requirements may be enhanced in individuals who regularly perform such exercise. Nevertheless, no study to date has sought to determine if variable intensity weight-bearing exercise, such as that performed during team sports such as soccer, rugby, and to some extent hockey, is capable of changing the dietary protein requirement. Variable intensity exercise resembles that of organized sports and training as

it is capable of stimulating both the anaerobic energy systems associated with resistance training, and the aerobic systems that provide energy for endurance training (Bangsbo, Mohr, & Krstrup, 2006). Moreover, weight bearing sports such as soccer or football are also associated with high force muscle contractions and acceleration / deceleration events, which might be a stimulus for muscle growth and/or induce mild levels of muscle damage (Ascensao et al., 2008; Magalhaes, Rebelo, Oliveira, Silva, Marques, & Ascensao, 2010a). Therefore, this exercise modality with elements of both resistive and aerobic exercise is unique from those previously studied (i.e. resistance vs. endurance), which primarily focused on opposite ends of the strength-endurance continuum.

The utilization of the minimally invasive Indicator Amino Acid Oxidation technique has consistently demonstrated that protein requirements may exceed those values determined using the NBAL technique. However, the minimally invasive IAAO technique has never been utilized to determine if physical activity elicits an effect on protein requirements. Thus, it is essential to utilize the minimally invasive IAAO technique to determine protein requirements in response to a variable intensity exercise stimulus. It is hypothesized that the results from the present study will yield protein requirements that are higher than the current EAR and RDA as established through NBAL, in addition to those requirements established using the IAAO technique in untrained populations.

## **Chapter 3 - Research Proposal:**

### **3.1 Introduction**

Based on the previously described knowledge base, the research study was divided into a total of 3 sequential research phases, encompassing 3-10 laboratory visits depending on the participant. The research proposal section will serve as a general outline of the purpose and objectives associated with each research phase. Additionally, a study timeline for the participants will be presented in the form of a schematic of the overall study. Lastly, a timeline of the course of the project will be presented. A more in-depth review of the materials and methodology will be provided in Chapter 4.

### **3.2 Phases of Research**

A total of 7 active, trained young adult male participants were recruited for the present study. Participants partook in a range of 3-10 trials encompassing the three phases of the research study. The three phases of the proposed study are described below.

#### *Phase I – Introductory Session (1 x 1.5h):*

Objectives: Phase I consisted of a single laboratory visit lasting approximately 1.5 hours, and had two primary objectives. First, it served to provide the participants with a comprehensive oral introduction to the study protocol in order to ensure that they were properly informed before being asked to provide consent. Once participants were provided with a detailed overview of the study protocol, they were given the opportunity to ask questions, and subsequently signed the required consent document indicating their participation in the study. The first phase of research was also used to obtain background information with respect to the participants' general health and habitual activity levels.

#### *Phase II – Body Composition Analysis, Fitness Assessments (1 x 2h):*

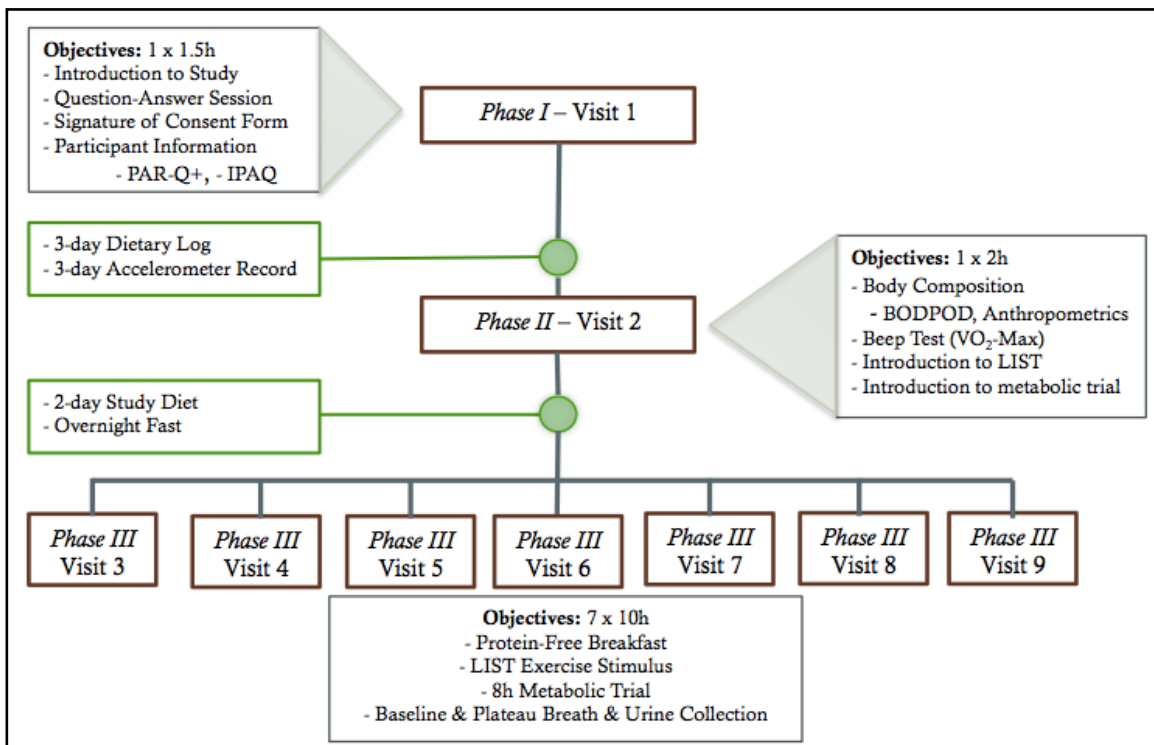
Objectives: Phase II was comprised of a single laboratory visit of approximately 2 hours, and had three main objectives. A detailed analysis of body composition was first performed, utilizing the BodPod (Air Displacement Plethysmography, COSMED USA, Inc., Concord, California), and basic anthropometric measurements such as height (cm) and weight (kg). Upon completion of the various body composition measurements, participants were required to perform the beep test, which was used to characterize the participants' fitness. Participants were then given an introduction to the exercise stimulus and metabolic

trials that were performed on the remaining metabolic trial days. Additionally, participants were required to consume a 2-day study diet prior to each metabolic trial day.

*Phase III – Metabolic Trials (7 x 10h):*

Objectives: Phase III required participants to visit the laboratory for approximately 10 hours on 1-10 different occasions depending on the participant. Each metabolic trial followed the same protocol. Subjects were first required to complete an exercise stimulus followed by an 8-hour metabolic trial where they consumed the study diet. An in-depth review of the metabolic trial methodology will be provided in Chapter 4.

**3.3 Research Schematic**



\* Participants engaged in a range of 1-10 Phase III metabolic trials

### 3.4 Project Timeline

<b>Element</b>	<b>Start Date</b>		<b>End Date</b>	
<i>Proposal Defense</i>	September 2014	With completed literature review	September 2014	
<i>IAAO Training</i>	October 2014	In collaboration with Sick Kids Hospital	October 2014	
<i>Pilot Data Collection</i>	October 2014	In collaboration with Sick Kids Hospital	January 2015	
<i>Data Collection (Phases I-III)</i>	February 2015	In collaboration with Sick Kids Hospital	June 2015	Analysis concurrent with data collection
<i>Writing</i>	July 2015	Thesis, journal article submissions	August 2015	Plan for all degree requirements met by August 2015

\* Participants can be in different phases of the study at any given time-point

## **Chapter 4 – Methodology:**

### **4.1 Introduction**

This section will provide a detailed description of the progression from Phase I to Phase III of the present study. The study design, methodology, and materials inherent to each phase will be clarified. Moreover, a brief explanation of the statistical analyses will be provided.

## **4.2 Participants & General Study Design**

7 active, trained males ( $23 \pm 1$  years;  $177.5 \pm 6.2$  cm;  $83.3 \pm 6.3$  kg;  $13.5\% \pm 5.1\%$  BF;  $52.3 \pm 5.4$  mlO<sub>2</sub>/kg/min; mean  $\pm$  95% CI) were recruited from various varsity sports teams at the University of Toronto. The Physical Activity Readiness Questionnaire (PAR-Q+) was used to assess any health risks prior to participant enrollment in the study. The International Physical Activity Questionnaire (IPAQ) and VO<sub>2</sub>-max test (Beep Test) confirmed that the participants' fitness levels met the requirements of the study (moderate-vigorous activity  $\geq$  5d/week, predicted VO<sub>2</sub>-max  $\sim$  50mlO<sub>2</sub>/kg/min respectively). All participants partook in a minimum of one metabolic trial (Phase III) (range: 1 to 10 trials) in addition to completing the independent trials associated with Phase I and Phase II (details below).

## **4.3 Phase I, Introductory Session**

Phase I consisted of a single laboratory visit lasting approximately 1.5 hours. This visit served as an introductory session that allowed for the participants to become familiar with the study design. Participants were first given a comprehensive oral introduction to the study design in addition to a clear explanation of the objectives of the study. All participants were then given an opportunity to ask questions related to the study as they reviewed the consent document. This ensured that they understood what their commitment would entail before providing informed consent. Upon completion and signature of the consent document, background information was obtained pertaining to the participants' general health and habitual fitness levels using the Physical Activity Readiness Questionnaire (PAR-Q+) and the IPAQ respectively. Once participants satisfied the criteria pertaining to age, health status, and fitness levels presented in the aforementioned

questionnaires, they were then instructed to record a 3-day dietary log, and to wear the Sensewear Body Media Armband Accelerometer for three days prior to the Phase II laboratory visit. These methods provided the investigators with an understanding of the participants' free-living energy expenditure, which helped to design the participants' individual feeding protocols to be administered during the metabolic trials of Phase III.

#### **4.4 Phase II, Body Composition Analysis & Fitness Assessment**

Phase II consisted of a single laboratory visit lasting approximately 2 hours. All participants arrived to the laboratory having worn the Sensewear Body Media Armband Accelerometer for 3 days, and having completed a 3-day dietary log. Participants were required to have abstained from consuming solid food or liquid, and from engaging in physical activity for 2 hours prior to Phase II. Air Displacement Plethysmography (BodPod) (COSMED USA, Inc., Model 2007A, Concord, California) was used to determine baseline whole body mass (BW) (kg), fat mass (FM), percent body fat (%BF) and fat-free mass (FFM) of each participant both prior to and after the completion of the metabolic trials. An averaged weight (kg) and FFM (kg) at the two time-points were used for all subsequent calculations for outcome variables **(Table 1) (See Section 4.6)**. There was an average change pre-to-post corresponding to -0.74% BF, -0.077kg FFM, and -0.83kg BW. Resting energy expenditure (REE) was estimated using the BodPod for each participant, and was compared to REE data recorded by the Sensewear Body Media Armband Accelerometer during sleep **(See Section 4.5)**. The Sensewear Body Media Armband Accelerometer has been validated for its use in determining REE and free-living daily energy expenditure in adults (Malavolti et al., 2007; St-Onge, Mignault, Allison, & Rabasa-Lhoret, 2007). Upon completion of all measurements,

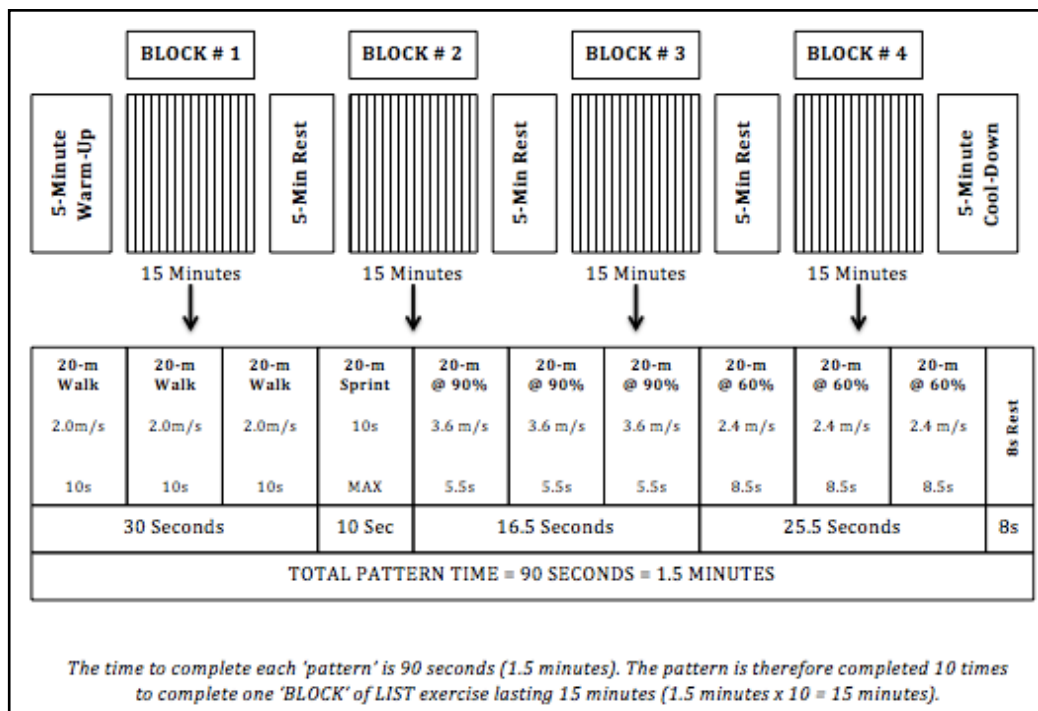
participants were familiarized with the aerobic fitness assessment protocol to ensure that they were comfortable with all equipment (polar-HR monitor, Sensewear Body Media Armband Accelerometer). The Beep Test was used to determine each participant's predicted maximal oxygen consumption ( $VO_2$ -max). This test is well established in adults, and consists of a 10-15 minute running test with a graded increase in work-output, until participants reach volitional fatigue, or an age-predicted max-HR (Leger & Lambert, 1982). Participants were given 15 minutes to rest, and were provided with an explanation of the protocol for the subsequent trial days. Participants then partook in an introductory modified Loughborough Intermittent Shuttle Test (LIST) (Nicholas, Nuttall, & Williams, 2000). This ensured that participants were able to complete the modified LIST. Additionally, it provided an estimate of their exercise energy expenditure that was determined by converting the energy expenditure of four pilot participants during the modified LIST stimulus to an average value in kcal/kg/minute. This information ensured that the metabolic diet in each of the metabolic trials of Phase III provided sufficient energy to offset what was expended during the modified LIST stimulus (See Section 4.5).

#### **4.5 Phase III, Metabolic Trials**

*Pre-Trial:* Phase III consisted 1-10 laboratory visits (depending on the participant). Each laboratory visit lasted approximately 10 hours and consisted of two components: the modified LIST exercise stimulus and a subsequent metabolic trial. Two days prior to each of the seven metabolic trials, participants were required to adhere to a predetermined study diet providing 1.2 g/kg/d of protein and sufficient energy to cover the habitual energy expenditure as previously measured during the 3-d accelerometer record (see

below). Additionally, subjects were required perform an overnight fast before each metabolic trial. Upon reporting to the laboratory, participants ingested a liquid-based, protein-free breakfast providing 1g/kg of carbohydrates (0.5 g/kg Polycose, and 0.5 g/kg Orange Gatorade Powder) to ensure that they were not exercising in the fasted state.

*Modified LIST:* Participants partook in a modified LIST stimulus. The LIST is a variable intensity exercise test consisting of repeated 20-m shuttle runs resembling play in organized sports such as rugby and soccer (Armstrong & Welsman, 2006; Castagna, D'Ottavio, & Abt, 2003; Stroyer, Hansen, & Klausen, 2004). The modified LIST was comprised of 4 segments of 15 minutes of variable intensity exercise including sprinting, running (90% VO<sub>2</sub>-max speed), jogging (60% VO<sub>2</sub>-max speed) and walking paces (**Figure 6**). The modified LIST integrated components of both resistance and endurance exercise sufficient to stimulate enhanced muscle and mitochondrial protein turnover.



**Figure 6.** The modified Loughborough Intermittent Shuttle Test (LIST).

The modified LIST intensities were previously determined from the speed obtained at a level 12 on the beep test (4.0 m/s). The total time commitment for the LIST exercise stimulus was approximately 75 minutes (4 x 15 minute blocks of variable intensity exercise + 3 x 5 minutes of rest between blocks).

*Study Diet:* After completing the LIST test in its entirety, participants returned to the laboratory where they were required to immediately consume the first of 8 hourly meals comprising the study diet. Each metabolic trial study diet provided a variable amount of protein, 6g/kg of carbohydrate (according to carbohydrate consumption guidelines for general training needs in moderate-highly active individuals established by Burke et al.) (Mountjoy et al., 2011) and sufficient energy to ensure that participants were in a net positive energy balance on trial day (see below for details).

#### *Study Diet Energy Intake*

The study day diet consisted of eight liquid beverages containing an assigned protein intake and sixteen protein-free cookies. The cookies ensured that participants consumed some solid food on trial day, thus reducing the likelihood of attrition or participant withdrawal **(See IAAO Manual in Appendix for Recipe and Nutritional Information)**.

The total energy intake was calculated using the equation:

$$\text{Total Energy Intake} = (\text{REE} \times 1.5) + [(0.1425_{(\text{kcal/kg/min})} \times \text{Weight}_{(\text{kg})} \times 75_{(\text{min})}) \times 1.1]$$

Where REE = Resting Energy Expenditure During Sleep (kcal) (recorded using the Sensewear Body Media Armband Accelerometer) (Malavolti et al., 2007); 1.5 = Activity Factor (to account for the active nature of the participants and to be consistent with previous IAAO studies in rested adults) (Humayun et al., 2007); 0.1425 = Average Energy Expenditure During LIST Exercise Stimulus (kcal/kg/min); 75 Minutes = Duration of the modified LIST Exercise Stimulus; and 1.1 = 10% Buffer for Energy Expended During the LIST Exercise Stimulus (to ensure participants were in a surplus of energy and to account for individual differences in energy expenditure).

Protein was provided as crystalline amino acids modeled on the basis of egg protein with the exception of excess tyrosine (40mg/kg/d) and the indicator amino acid phenylalanine (30.5mg/kg/d with 5.46 mg/kg as L-[<sup>13</sup>C]phenylalanine) (Humayun et al., 2007).

Phenylalanine metabolism occurs in the liver, such that it is first hydroxylated to form tyrosine. Tyrosine can then either be used as a substrate for protein synthesis, or be transaminated or deaminated, which releases CO<sub>2</sub> containing the carboxyl carbon from phenylalanine. When extracellular tyrosine is low and plasma concentrations of phenylalanine are normal, tyrosine will be preferentially used in protein synthesis as opposed to being degraded or exported. Thus, tyrosine was provided in excess to ensure the carboxyl carbon of phenylalanine would appear in breath through its incorporation into tyrosine and subsequent transamination or deamination (Shiman & Gray, 1998). All participants were randomly assigned to consume 1-10 different test protein intakes in a random order on each of the metabolic trial days associated with Phase III. The test protein intakes were designed to cover deficient to excessive intake (0.2-2.6 g/kg/d), according to

previous studies implementing IAAO and NBAL protocols in adults (Humayun et al., 2007). A test protein intake range of 0.2-2.6 g/kg/d was utilized in consideration of the Canadian Society for Exercise Physiology and American College of Sports Medicine's recommendations for protein consumption in trained populations of (1.2 to 1.7 g/kg/d) (American Dietetic Association et al., 2009), which are based primarily on NBAL studies. Therefore, an upper level of 2.6g/kg/d was selected given the potential that NBAL underestimates true requirements (Humayun et al., 2007) and to ensure a plateau in  $F^{13}CO_2$  was achieved, the latter of which is a pre-requisite for robust bi-phase linear modeling and breakpoint analysis.

The first 4 hourly meals consumed directly after the modified LIST exercise stimulus contained the test protein, unlabeled phenylalanine, and tyrosine as tracer ingestion began on the 5<sup>th</sup> hourly meal. We had previously confirmed through pilot studies that background  $^{13}CO_2$  and carbon dioxide production was stable over this period while consuming the test diet (see Figures 4 and 5 in Appendix), which ensured a stable background enrichment and metabolism was achieved prior to tracer ingestion. Prior to the 5<sup>th</sup> meal, 4 breath samples were taken at 15-minute intervals, and 3 urine samples were collected at 30-minute intervals in order to establish baseline  $^{13}CO_2$  (via continuous-flow isotope ratio mass spectrometry) and L-[ $^{13}C$ ]phenylalanine enrichment (via liquid chromatography tandem mass spectrometry) respectively (Humayun et al., 2007). A priming dose of  $NaH^{13}CO_3$  (0.176mg/kg) and L-[ $^{13}C$ ]-phenylalanine (1.86mg/kg) were ingested on the 5<sup>th</sup> hourly meal. The rate of  $CO_2$  production was then measured over a 20-25 minute period following the 5<sup>th</sup> hourly meal, but before the 7<sup>th</sup> hourly meal using indirect calorimetry (MOXUS

Metabolic Cart, AEI Technologies) to determine steady state metabolism. 1.2mg/kg of L-[<sup>13</sup>C]-phenylalanine replaced an equivalent amount of unlabeled phenylalanine and was ingested in the remaining three hourly meals to induce isotopic steady state. Eight plateau breath and five plateau urine samples were collected at 15 and 30-minute intervals, respectively, commencing 2 hours after the onset of tracer ingestion (Humayun et al., 2007).

#### **4.6 Data Organization**

An initial set of 42 test protein intakes ranging from 0.20 to 2.25 g/kg/d were separated into 7 different protein intake ranges (i.e. 0.20 – 0.45 g/kg/d; 0.50 – 0.75 g/kg/d; 0.80 – 1.05 g/kg/d; 1.10 – 1.35 g/kg/d, 1.40 – 1.65 g/kg/d, 1.70 – 1.95 g/kg/d, and 2.00 – 2.25 g/kg/d). Each protein intake range encompassed 6 protein intake levels separated by 0.05 g/kg/d increments. For example, protein intakes corresponding to 0.20, 0.25, 0.30, 0.35, 0.40, and 0.45 g/kg/d represented the 6 protein intake levels within the range of 0.20 – 0.45 g/kg/d. Each participant was randomly assigned to consume one protein intake level within each of the seven protein intake ranges over the seven metabolic trials.

A total of 40 of the initial 42 test protein intakes were completed, as one participant only completed 5 of the 7 trials. As a result, an additional 5 trials were performed at test protein intakes corresponding to 0.225, 2.45, 2.50, 2.55, and 2.60 g/kg/d. A total of 45 metabolic trials completed by 7 participants were conducted, exceeding the previous minimum trials (i.e. 35) performed with the minimally invasive IAAO protocol (Elango et al., 2011).

Atoms Percent Excess (A.P.E.) for each metabolic trial was determined by subtracting the averaged baseline breath  $^{13}\text{CO}_2$  enrichment from the averaged plateau breath  $^{13}\text{CO}_2$  enrichment.  $^{13}\text{CO}_2$  excretion ( $F^{13}\text{CO}_2$ ) was then calculated using the formula below.

$$F^{13}\text{CO}_2 = \frac{(\text{FCO}_2)(\text{ECO}_2)(44.6)(60)}{(W)(0.82)(100)}$$

$\text{FCO}_2$  =  $\text{CO}_2$  Production Rate (mL/min)

$\text{ECO}_2$  =  $^{13}\text{CO}_2$  Enrichment (A.P.E)

44.6 ( $\mu\text{mol/mL}$ ) & 60 (min/hour) = Used to convert  $\text{FCO}_2$  to micromoles/hour

W = Mass of participant (either in kg body weight or kg fat-free mass, as appropriate)

0.82 = Correction factor for  $\text{CO}_2$  retained in the body because of bicarbonate fixation (Elango et al., 2011)

100 = Used to convert  $\text{ECO}_2$  into a fraction

$F^{13}\text{CO}_2$  (per kg body weight or per kg fat-free mass) served as the primary outcome variable, and was plotted as a function of test protein intake (relative to body mass) in order to establish the breakpoint (EAR) (See Section 5.2). Phenylalanine oxidation was determined by dividing  $F^{13}\text{CO}_2$  by  $^{13}\text{C}$ -phenylalanine enrichment in urine, and was expressed as a function of mass (kg) or FFM (kg) versus protein intake in order to establish the breakpoint (EAR) in oxidation data (See Section 5.2).

Breakpoint measurements utilizing  $F^{13}\text{CO}_2$  have been shown to be similar to breakpoint measurements for phenylalanine hydroxylation by using enrichments in apolipoprotein B-100, which is a hepatic export protein (Rafii et al., 2008). Therefore, breath measurements

are presumed to be representative of the intracellular enrichment of phenylalanine in the liver and are preferable to the rate of phenylalanine oxidation calculated from plasma or urine enrichments. Requirements for the protein intake were therefore determined by using  $F^{13}CO_2$  data, as they have been suggested to be more robust and appropriate for making recommendations (Rafii et al., 2008).

#### **4.7 Statistical Analyses**

All  $F^{13}CO_2$  and phenylalanine oxidation data was inputted to GraphPad Prism version 5.00 for Windows (GraphPad Software, San Diego California USA) to estimate whether a linear or bi-phase linear regression analysis explained more variability. GraphPad version 5.00 for Windows (GraphPad Software, San Diego California USA) was subsequently used for all statistical analyses. Biphasic linear regression analysis was used to determine the effect of protein intake on  $F^{13}CO_2$  and phenylalanine oxidation. Significance was established at  $P < 0.05$ . To determine the mean protein requirement, a bi-phase linear regression analysis was performed on  $F^{13}CO_2$  (as the primary outcome variable) and phenylalanine oxidation (as a secondary outcome variable).

## **Chapter 5 – Results:**

### **5.1 Subject Characteristics**

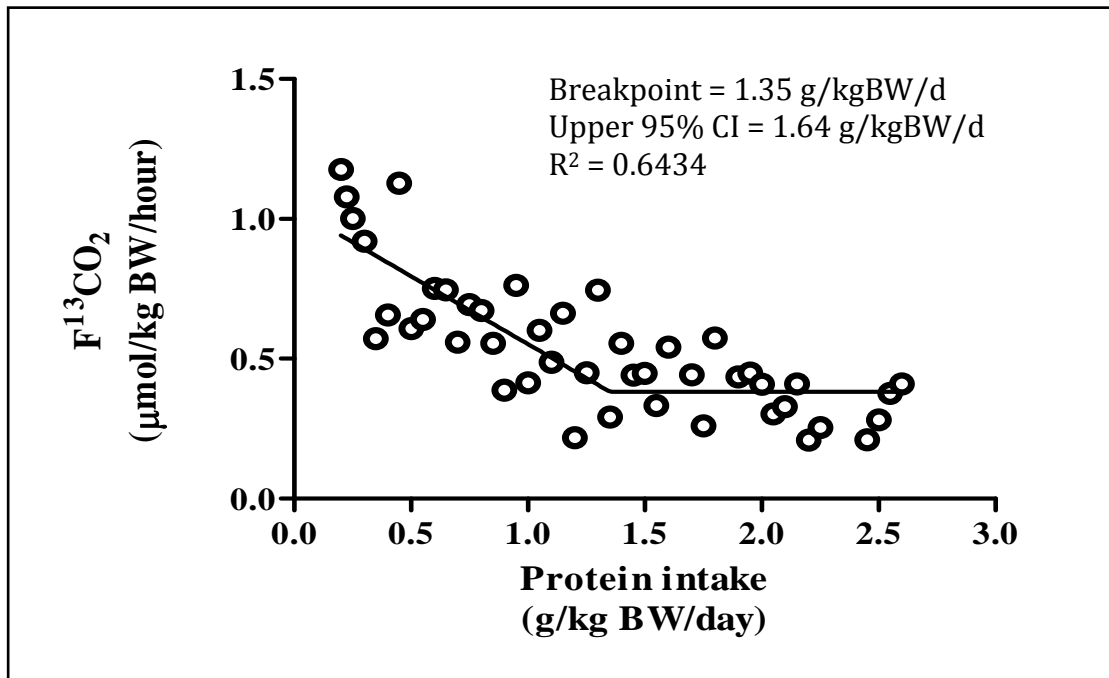
Seven young adult men ( $23 \pm 1y$ ) participated in the study. All participants were active and in a trained state, most having competed for a varsity sports team. All participants met the inclusion criteria pertaining to fitness having obtained a minimum level of 10 on the beep test (predicted  $VO_2\text{-max} \sim 50$ ) and having engaged in moderate-vigorous activity for at least 5d/week. Subject characteristics are presented in **Table 1**. Weight (kg), FFM (kg), and body fat (%BF) measures recorded before and upon completion of the study were averaged and used for all primary outcome calculations.

**Table 1** Subject characteristics and energy intakes of young adult men ( $n = 7$ ) who participated in the study

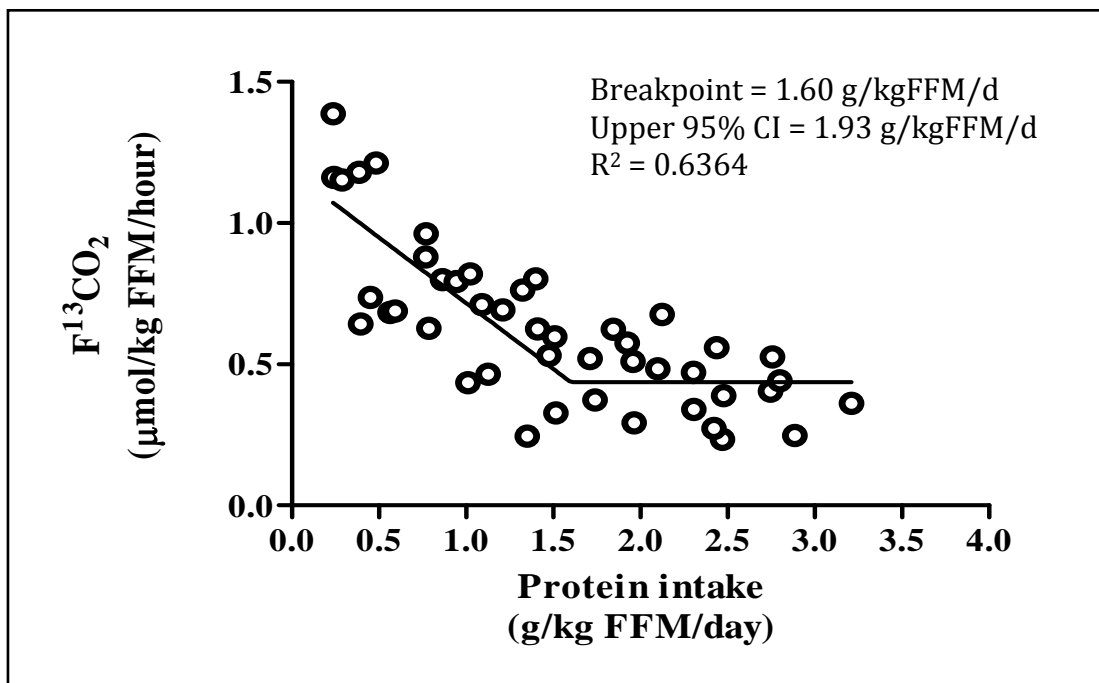
<b>Characteristic:</b>	<b>Value (mean <math>\pm</math> 95% CI):</b>
Age (y)	$22.9 \pm 0.8$
Height (cm)	$177.5 \pm 6.2$
Weight (kg)	$82.3 \pm 5.7$
FFM (kg)	$71.1 \pm 5.1$
Percent Body Fat (%)	$13.5 \pm 4.3$
Predicted $VO_2\text{-Max}$ (ml/kg/ $O_2$ /min)	$52.3 \pm 5.4$
Habitual Energy Expenditure (kcal/d)	$3596 \pm 446$
Resting Energy Expenditure (kcal/d)	$2221 \pm 135$
Study Day Energy Intake (kcal/d)	$2867 \pm 177$

## 5.2 F<sup>13</sup>CO<sub>2</sub> Excretion:

Bi-phase linear regression analysis explained more of the variability for protein intake versus F<sup>13</sup>CO<sub>2</sub> (per kg body mass) than simple linear regression ( $r^2$  Bi-Phase = 0.6434;  $r^2$  Linear = 0.5921;  $p = 0.0182$ ). The average participant VCO<sub>2</sub> expressed in both absolute and relative terms corresponded to  $276 \pm 16.9$  mL/min (mean  $\pm$  95% CI) and  $3.36 \pm 0.26$  mL/kg/min respectively. The rate of <sup>13</sup>CO<sub>2</sub> excretion (F<sup>13</sup>CO<sub>2</sub>) via the oxidation of L-[1-<sup>13</sup>C]-phenylalanine decreased in participants with increasing protein intakes up to 1.35 g/kg/d (**Figure 7**). Additional increases in protein intake did not result in further decreases in F<sup>13</sup>CO<sub>2</sub> values. This indicated that no additional excretion of F<sup>13</sup>CO<sub>2</sub> occurred after the protein intake of 1.35g/kg/d was reached suggesting non-oxidative disposal of phenylalanine (and by extension, potentially protein synthesis) was maximized. Bi-phase linear regression analysis of F<sup>13</sup>CO<sub>2</sub> versus protein intake corresponded to a breakpoint (estimated average requirement, EAR) of 1.35 g/kg/d ( $r^2 = 0.64$ ). The safe population intake estimated by the upper 95% confidence interval of the breakpoint was calculated to be 1.64 g/kg/d with a lower CI of 1.06 g/kg/d. Expressing the data as a function of FFM (kg) correlated to a breakpoint (EAR) of 1.60 g/kgFFM/d ( $r^2 = 0.64$ ), and a safe population intake of 1.93 g/kgFFM/d with a lower CI of 1.27 g/kgFFM/d (**Figure 8**).



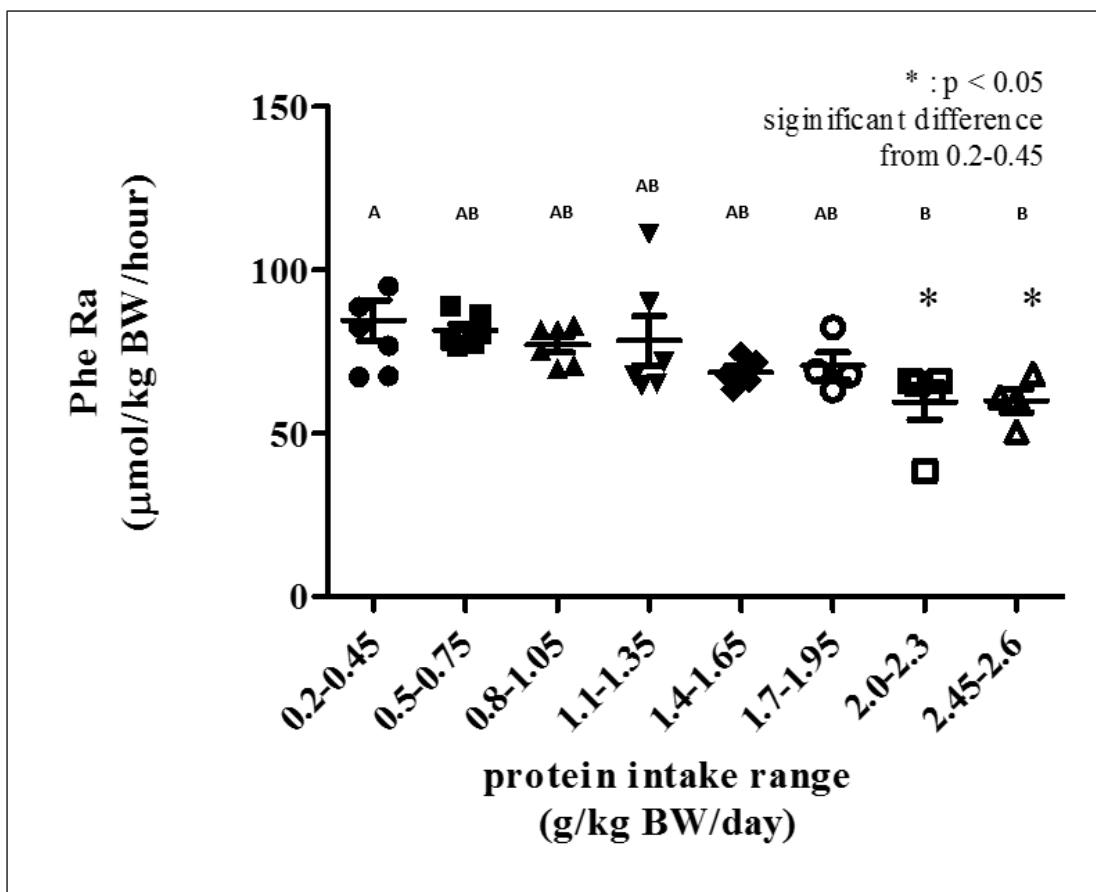
**Figure 7.** Influence of dietary protein intake on the production of  $^{13}\text{CO}_2$  from the oxidation of orally administered L-[1- $^{13}\text{C}$ ]-phenylalanine ( $\text{F}^{13}\text{CO}_2$ ) in active, young adult males. The breakpoint estimated the mean protein requirement, and corresponded to 1.35 g/kg/d, with the upper 95% CI (or population-safe intake) estimated to be 1.64 g/kg/d.



**Figure 8.** Influence of dietary protein intake on the production of  $^{13}\text{CO}_2$  from the oxidation of orally administered L-[1- $^{13}\text{C}$ ]-phenylalanine ( $\text{F}^{13}\text{CO}_2$ ) in active, young adult males. The breakpoint estimated the mean protein requirement, and corresponded to 1.60 g/kgFFM/d, with the upper 95% CI (or population-safe intake) estimated to be 1.93 g/kgFFM/d.

### 5.3 Phenylalanine Flux

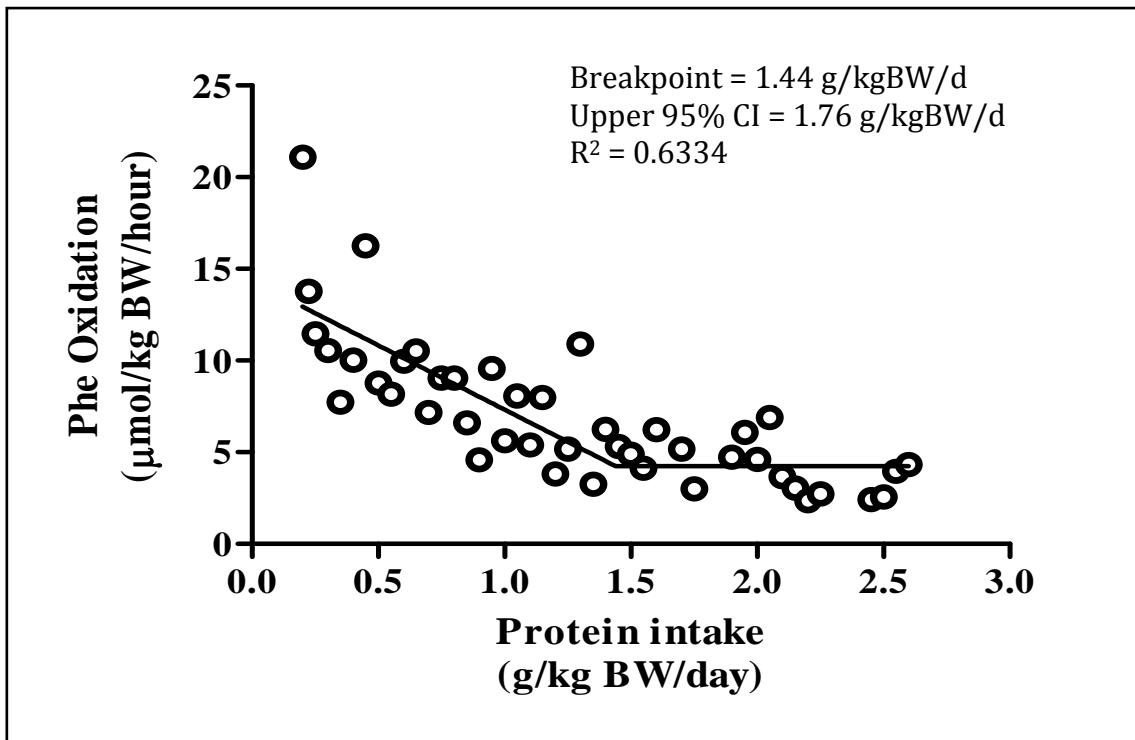
Phenylalanine flux measured from urine samples did not change significantly with different test protein intakes ranging from 0.5 g/kg/d to 2.6 g/kg/d (**Figure 9**). However, high test protein intakes (2.0 to 2.3 g/kg/d; 2.45 to 2.60 g/kg/d) were significantly different from the lowest test protein intake range of 0.2 g/kg/d to 0.45 g/kg/d ( $p < 0.05$ ) (**Figure 9**).



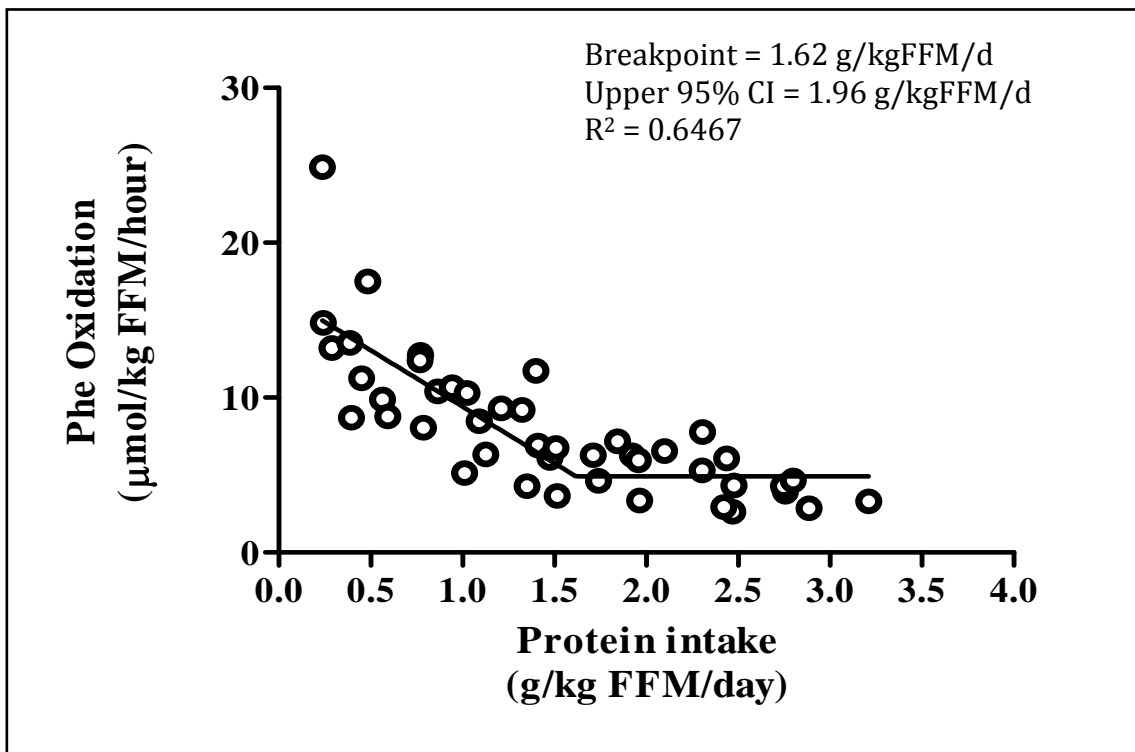
**Figure 9.** Phenylalanine flux corresponding to the eight test protein ranges. Flux did not change significantly with test protein intakes from 0.5 g/kg/d to 2.60 g/kg/d. Flux for test protein intakes of 2.0 to 2.30 g/kg/d and 2.45 to 2.60 g/kg/d were significantly different than the intake range of 0.20 to 0.45 g/kg/d.

#### 5.4 Phenylalanine Oxidation

Phenylalanine oxidation declined due to increasing protein intakes until 1.4 – 1.5 g/kg/d and 1.6 – 1.7 g/kg FFM/d (**Figures 10 & 11**), after which additional increases in protein intakes did not further impact phenylalanine oxidation. Bi-phase linear regression analysis identified a breakpoint and upper 95% CI for the relationship between protein intake and phenylalanine oxidation to be 1.44 and 1.76 g/kg/d, respectively, for body weight (kg) ( $r^2 = 0.63$ ), and 1.62 and 1.96 g/kg FFM/d, respectively, for fat-free mass ( $r^2 = 0.65$ ). A reanalysis of phenylalanine oxidation data was conducted using an average participant urinary phenylalanine enrichment value to estimate the potential bias that the negative relationship between protein intake and phenylalanine flux may have had our breakpoint analysis of phenylalanine oxidation. This reanalysis resulted in a breakpoint in the phenylalanine oxidation data of that was ~8% higher at 1.56 g/kg/d (**See Figure 1 in Appendix**); this suggests that the non-steady state phenylalanine flux (derived from the urinary phenylalanine enrichment) had only a minor effect on the breakpoint (i.e. EAR) estimated from phenylalanine oxidation (i.e. Figure 10)



**Figure 10.** Influence of dietary protein intake on Phe oxidation in active young adult males (n =45 observations). Bi-phase linear regression analysis revealed the breakpoint and upper 95% to be 1.44 and 1.76 g/kg/d respectively.



**Figure 11.** Influence of dietary protein intake on Phe oxidation in active young adult males (n =45 observations). Bi-phase linear regression analysis revealed the breakpoint and upper 95% to be 1.60 and 1.96 g/kgFFM/d respectively.

## **Chapter 6 – Discussion:**

### **6.1 Introduction**

The current dietary requirements for protein consumption are based primarily on NBAL data. Although numerous studies examine the individual impacts of exclusively resistance (Lemon et al., 1992; M. A. Tarnopolsky et al., 1988; M. A. Tarnopolsky et al., 1992) or endurance training (exercise) (Brouns et al., 1989; M. A. Tarnopolsky et al., 1988) on protein metabolism and requirements, no study has sought to directly determine how an exercise modality with elements of each (i.e. variable intensity exercise) can impact dietary protein requirements. The purpose of the present study was to utilize the minimally invasive IAAO technique for the first time in the presence of a variable intensity weight bearing exercise stimulus in active, trained young adult males. We report a breakpoint in  $F^{13}CO_2$  data of 1.35 g/kg/d (i.e. the EAR) and a minimum population intake estimated by the upper 95% confidence interval of the breakpoint (population-safe protein requirement) to be 1.64 g/kg/d, the latter of which would represent a daily intake that would be sufficient for >95% of this active population. The results from this study yielded protein requirements that are: i) in excess of the current EAR and RDA; ii) greater than those previously determined in inactive populations using the minimally-invasive IAAO method, and; iii) at the upper range (if not greater) than those previously suggested for athletes according to general consensus statements (American Dietetic Association et al., 2009). The following sections will contextualize our results in light of previous studies determining protein requirements by NBAL and IAAO. Potential avenues for future research will also be provided in light of a discussion of the strengths, limitations, and implications of the present study.

## 6.2 Results, Comparison to NBAL & IAAO Studies

This is the first study to-date that has employed the minimally invasive IAAO technique both in an active population and in the presence of an exercise stimulus. We observed that the breakpoint in  $F^{13}CO_2$  data was 1.35 g/kg/d (**Figure 9**), which would represent the EAR. The minimum population intake estimated by the upper 95% confidence interval of the breakpoint (population-safe protein requirement) was 1.64 g/kg/d, which would subsequently represent a daily intake that would be sufficient for >95% of this active population. These results are 105% higher than the current EAR and RDA, which are 0.66g/kg/d and 0.80g/kg/d respectively (FAO, WHO 2007). This distinction is also apparent when the protein requirements determined in the present study are compared to that of the most comprehensive meta-analysis of NBAL data to-date (Rand et al., 2003), which established an EAR of 0.65 g/kg/d and an RDA of 0.83 g/kg/d. Thus, our estimate of the protein requirement for our active population determined by IAAO is substantially greater than that previously determined in non-active individuals utilizing NBAL, which would be consistent with previous NBAL studies in active populations (Lemon et al., 1992; M. A. Tarnopolsky et al., 1988; M. A. Tarnopolsky et al., 1992) and could suggest that active individuals engaged in weight-bearing, variable intensity exercise require a greater protein intake than their non-active peers.

While it is possible that our protein requirements are elevated by our exercise stimulus, it should be considered that the greater requirement determined herein by the minimally invasive IAAO technique may be specific to our tracer model given the previously suggested underestimation of NBAL (Humayun et al., 2007). Thus, it may be more relevant

to compare our results in light of previous studies in non-active individuals that utilized similar methodology to increase the internal validity of our data. The dietary protein intakes established in the present study were also greater than the requirements in all published IAAO work on adults thus far. A study by Humayun et al. in 2007 utilized the IAAO technique in the most comparable demographic group to the present study: healthy, young adult males (Humayun et al., 2007). The study utilized an identical crystalline amino acid mixture while following the same 8-hour feeding and tracer ingestion protocol as the present study. The mean (EAR) and population-safe (RDA) protein requirements were found to be 0.93 and 1.2 g/kg/d respectively. These results are 31% and 27% lower than the EAR and population-safe protein requirement determined by the present study. Other studies that have employed the IAAO technique to determine protein requirements in less active populations of older adults have yielded similar results whereby the protein requirements established are lower than those found in the present study (Rafii et al., 2015; Tang et al., 2014). Thus, the hypotheses that the present study would yield protein requirements in excess of both the current EAR and RDA established on the basis of the NBAL technique and studies utilizing the minimally-invasive IAAO technique in less active individuals were supported.

### **6.3 Results Explained, Methodological Considerations**

A variety of methodological considerations can potentially explain the differences in protein requirements when comparing the present study to the existing NBAL and IAAO data. Several studies have suggested that NBAL can underestimate the value of nitrogen excretion, as miscellaneous losses of nitrogen (hair, sweat, exhalation, etc.) are often left

unaccounted for since they are difficult to measure accurately (Forbes, 1973; Humayun, Elango, Ball, & Pencharz, 2007). An underestimation of nitrogen excretion can result in an erroneously high nitrogen balance, and consequently an underestimation of true protein requirements. The suggestion that the current EAR and RDA may be underestimates of protein requirements was supported by the work of Humayun et al. in 2007 who reanalyzed historical NBAL data using bi-phase linear regression while including studies at high protein intakes that were previously deemed outliers (Humayun et al., 2007). Upon the application of bi-phase linear regression to historical NBAL data while including studies conducted at higher protein intakes, the EAR and RDA were increased (EAR – 0.91 g/kg/d; RDA – 1.00 g/kg/d). These requirements were comparable to those established using the minimally invasive IAAO technique in young, adult males (EAR – 0.93 g/kg/d; RDA – 1.20 g/kg/d) (Humayun et al., 2007). This suggests that simple linear regression for statistical analysis of NBAL data may be inappropriate for determining true protein requirements when all studies are included, as those previously deemed as outliers may have a pronounced effect on minimal estimates when they are included with the other NBAL data. However, the bi-phase linear regression model is not biased by over- or underestimates and is therefore arguably a more robust model to determine the EAR and potentially true protein requirements, provided the data conform to this model. In support of this, a greater proportion of the variance in our  $F^{13}CO_2$  data were explained by bi-phase compared to linear modeling when expressed to whole body ( $r^2 = 0.6434$  vs.  $0.5921$ , respectively;  $p = 0.0182$ ) and relative to FFM ( $r^2 = 0.6364$  vs.  $0.5679$ , respectively;  $p = 0.0075$ ).

The study performed by Humayun et al. in 2007 recruited eight healthy, young adult males and utilized an identical crystalline amino acid mixture while following the same 8-hour feeding and tracer ingestion protocol as the present study (Humayun et al., 2007). Each participant consumed seven protein intakes corresponding to 0.10, 0.30, 0.60, 0.90, 1.20, 1.50, and 1.80 g/kg/d. In contrast, the present study assigned one protein intake within each of the seven aforementioned protein intake ranges from 0.20 g/kg to 2.60g/kg to each participant (See Section 4.6). This allowed for a more comprehensive examination of the impact of protein intake on phenylalanine oxidation to be deduced as a total of 45 protein intakes were consumed compared to seven in the Humayun et al. study. The results from the present study (EAR = 1.35 g/kg/d; RDA 1.64 g/kg/d) were significantly greater than those established in the Humayun et al. study (EAR = 0.93 g/kg/d; RDA = 1.20 g/kg/d), and may be explained by the trained demographic group recruited in addition to the incorporation of an acute exercise stimulus into the minimally-invasive IAAO protocol.

#### **6.4 Physiological Considerations, & Implications for Active Individuals**

Humayun et al. (2007) and the present study employed the same components of the minimally invasive IAAO technique. As such, the differences in results may be attributed to the incorporation of the LIST exercise stimulus into the IAAO protocol of the present study. Numerous NBAL studies suggest that protein requirements are increased due to resistance (Lemon et al., 1992; M. A. Tarnopolsky et al., 1988; M. A. Tarnopolsky et al., 1992) and/or endurance exercise (Brouns et al., 1989; Friedman & Lemon, 1989; M. A. Tarnopolsky et al., 1988). The modified LIST exercise stimulus consisted of repeated 20-m shuttles at various intensities ranging from brisk walking to maximal sprints. The most comparable variable

intensity exercise modality to the modified LIST is that of sprint interval training. Coffey et al. sought determined the impact of cycling sprint interval training on cell signaling and protein synthesis (Coffey et al., 2011). Results suggested enhanced myofibrillar and mitochondrial protein synthetic rates following sprint interval training. Similar to that of sprint interval training, the stop-start nature of the test and repeated maximal sprints inherent to the modified LIST exercise stimulus were able to stimulate rapid myofibrillar contractions in addition activating the anaerobic energy systems (ATP-PCr & Anaerobic Glycolysis). As such, it is plausible that a modified LIST exercise stimulus used in the present study may have resulted in enhancements to myofibrillar and mitochondrial protein synthesis, resulting in the heightened protein requirement. Furthermore, a study performed by Magalhaes et al., also illustrated that the LIST is capable of inducing muscle damage similar to that caused by a soccer match (Magalhaes, Rebelo, Oliveira, Silva, Marques, & Ascensao, 2010). Additionally, the stop-start nature of the LIST exercise stimulus characterized by acceleration and deceleration events were capable of eliciting rapid and repeated eccentric contractions that have been demonstrated to enhance muscle protein breakdown (Fielding et al., 1991). As such, the modified LIST exercise stimulus stimulated many of the same metabolic and physiological pathways inherent to resistance exercise, which may have partially been responsible for the heightened protein requirement determined in the present study. Alternatively, if the potential exercise-induced muscle damage in the LIST resulted in a greater protein breakdown that was not reciprocated by an equal stimulation of protein synthesis, then a greater protein turnover induced by the LIST in the absence of enhanced intracellular reutilization of amino acids could have manifested in the greater protein requirement observed herein during

recovery. These possibilities would require future studies that incorporate parallel measures of tissue-specific protein turnover and net balance (e.g. muscle) in order to enhance our understanding of the physiological rationale for our heightened protein requirement.

The prolonged nature (~75 minutes) of the modified LIST exercise stimulus would be sufficient to stimulate the body's aerobic energy systems that are activated during prolonged bouts of endurance exercise. Despite the majority of the modified LIST exercise stimulus being of lower intensity (i.e. rest, walking, jogging), this exercise test was originally developed to mimic the physiological demands of a soccer match in which average energy expenditure is generally ~70%  $VO_2$ -max (Bangsbo, Mohr, & Krstrup, 2006; (Nicholas, Nuttall, & Williams, 2000). As such, it should be noted that the relatively long duration and high intensity of the modified LIST exercise stimulus (75 minutes) may have increased the breakdown and oxidation of amino acids for energy (van Loon, Greenhaff, Constantin-Teodosiu, Saris, & Wagenmakers, 2001). Although carbohydrates and to a certain extent fat would be the predominant macronutrients used to provide energy during endurance exercise of ~70% $VO_{2max}$ , amino acids may contribute 1-6% of the total energy to the active skeletal muscle in men (McKenzie et al., 2000; Phillips, Atkinson, Tarnopolsky, & MacDougall, 1993; L. J. Tarnopolsky, MacDougall, Atkinson, Tarnopolsky, & Sutton, 1990; van Loon et al., 2001). Moreover, carbohydrate availability can influence amino acid oxidation during exercise, as studies have determined that amino acid oxidation is heightened in response to low dietary carbohydrate intakes and limited glycogen availability (Howarth et al., 2010; Lemon & Mullin, 1980). Although participants were

provided with 1 g/kg of a carbohydrate beverage prior to engaging in the modified LIST stimulus, they were required to have fasted overnight, and as such were likely in a net negative energy balance towards the latter stages of the exercise stimulus. Given the likely relatively high average VO<sub>2</sub> during the LIST (~70%VO<sub>2</sub>max) that would have relied heavily on endogenous carbohydrate stores to fuel it (McKenzie et al., 2000; Phillips, Atkinson, Tarnopolsky, & MacDougall, 1993; L.; van Loon et al., 2001), the potentially limited muscle glycogen availability late in the LIST could have resulted in a relatively increased utilization of amino acids as a fuel source. Therefore, it is possible that participants in the present study oxidized a high proportion of amino acids (i.e. a minimum of 5-6%) for energy during the modified LIST stimulus, especially during the later stages of the exercise stimulus due to a glycogen-depleted state. Assuming an approximate total LIST energy expenditure of 800kcal (based off analysis of the Sensewear Body Media Armband Accelerometer) whereby 5% of energy contribution was provided via amino acid oxidation, a total of 40kcal of energy during the modified LIST would have been provided via amino acid oxidation. This would correspond to 10g of oxidized protein for energy, or approximately 0.13 g/kg/d of protein lost during the modified LIST for an 80kg participant. When comparing present study that of the Humayun et al., study, there exists a 0.44 g/kg/d difference in safe-level protein requirements. The direct oxidation of amino acids for energy during the modified LIST may account for approximately 30% of this difference. The remaining differences may be explained by the uniqueness of the modified LIST exercise stimulus, as it incorporated components of both resistance and endurance exercise that may have caused substantial metabolic stress sufficient to further increase protein requirements.

Although the FAO/WHO recognize exercise-induced increases in amino acid oxidation and nitrogen losses, they have yet to reach a consensus as to whether exercise is capable of increasing protein requirements (FAO, WHO 2007). However, the requirements determined in the present study (EAR = 1.35; upper 95% CI = 1.64) fall in-line with the Canadian Society for Exercise Physiology and American College of Sports Medicine general recommendations for endurance (1.2 – 1.4 g/kg/d) and resistance athletes (i.e. 1.2-1.7g/kg/d) (American Dietetic Association et al., 2009), and as stated previously are in accordance with a wealth of NBAL studies that have sought to examine protein requirements in habitually active individuals and athletes. These levels of protein intakes are significantly greater than the current RDA of 0.8 g/kg/d. However, the RDA encompasses a protein intake intended simply to prevent deficiency (Phillips, Moore, & Tang, 2007), and may therefore not be appropriate for such active individuals. Elite athletes often report consuming quantities of protein in excess of the current requirements (Burke et al., 2003), and are therefore likely to be more interested in optimizing their nutrition for enhanced sport performance than simply being in net protein balance. Thus, it remains to be seen whether the requirements established in the present study are a reflection of a ‘true requirement’ for active individuals, or serve as a guideline for optimal levels of protein intake required for enhancing sport performance.

### **6.5 Rate of Appearance and Phenylalanine Flux**

In comparison to the study conducted by Humayun et al. in 2007, the present study saw enhanced levels of phenylalanine flux at all protein intake levels, as the mean Ra for the present study was 77.02  $\mu\text{mol/kg BW/hour}$  compared to a mean Ra of 56.93  $\mu\text{mol/kg}$

BW/hour as determined in the Humayun et al. study (Humayun et al., 2007). These differences are likely explained by the incorporation of modified LIST exercise stimulus into the IAAO protocol, as research has suggested enhanced levels of whole body protein turnover in response to exercise (Rennie et al., 1981). A requirement for studies that utilize the IAAO technique is that phenylalanine flux is not impacted by protein intake. This provides evidence that the precursor pool for indicator oxidation does not change in size in response to the test protein intake (Humayun et al., 2007). Results from the present study suggest that there were decreases in phenylalanine flux (rate of appearance, Ra) in response to increasing test protein intakes, as the mean Ra in the two highest test protein intake ranges were significantly different from the mean Ra at the lowest protein intake range (**Figure 9**). Although no significant differences were seen in Ra in response to protein intake in the study conducted by Humayun et al., a similar trend of decreasing phenylalanine flux in response to increasing test protein intake was apparent (**See Figure 2 in Appendix**). The inverse relationship between phenylalanine flux and test protein consumption may be related to a dietary amino acid-induced suppression of endogenous (i.e. body) protein breakdown, which has been reported previously at the whole body protein levels (Churchward-Venne, Murphy, Longland, & Phillips, 2013). If this were the case in the present study, the protein-sparing effect of exogenous amino acids would have attenuated the appearance of unlabeled amino acids into plasma (and hence urine) at steady state, which would have manifested in a decrease in Ra following exercise.

To estimate the potential impact of the apparent reduction in phenylalanine flux with increasing protein intake, a reanalysis of phenylalanine oxidation data was conducted using an average participant urinary phenylalanine enrichment value to determine if the breakpoint would change under the circumstance where phenylalanine flux was constant throughout the range of test protein intakes. The reanalysis corresponded to a breakpoint in phenylalanine oxidation data of 1.56 g/kg/d (**See Figure 1 in Appendix**). This value is ~8% greater than the breakpoint of 1.44 g/kg/d reported in the present study from phenylalanine oxidation data (**Figure 10**) and could suggest that the results from the present study may represent a small underestimate of true protein requirements. While one of the benefits of the minimally invasive IAAO is not having to sample blood for to estimate the precursor enrichment, future studies may benefit from determining plasma and apolipoprotein B-100 enrichment as arguably more robust surrogates of hepatic intracellular enrichment to determine if the decrease in urinary phenylalanine enrichment with increasing protein intake observed herein is truly reflective of altered precursor enrichment. Future studies could also employ the IAAO technique in the presence of a variety of exercise stimuli, including the modified LIST, in order to determine if similar trends in phenylalanine flux are demonstrated in other populations post-exercise.

## **6.6 Strengths, Limitations, & Future Avenues of Research**

There exist several strengths inherent to the design of the present study. First, the IAAO technique was a minimally invasive means for determining protein requirements, deeming it practical in both healthy individuals and at-risk populations. Additionally, the utilization of the minimally invasive IAAO technique for determining protein requirements required

only a two-day dietary adaptation period prior to each metabolic trial (Elango et al., 2009). This allowed for a single participant to be tested over a range of deficient to excess protein intakes. In comparison, NBAL requires a 7-14 day dietary adaptation period, which often prevents participants from consuming more than three protein intake levels (Rand et al., 2003; FAO, WHO 2007). As a result, the present study was able to provide a comprehensive determination of protein requirements. The present study was the first to utilize the minimally invasive IAAO technique in the presence of an exercise stimulus that incorporated components of both resistance and endurance exercise resembling that of training and performance in organized team sports (Armstrong & Welsman, 2006; Castagna, D'Ottavio, & Abt, 2003; Stroyer, Hansen, & Klausen, 2004). Thus, the present study's protocol is applicable in a variety of athletic populations. Furthermore, the simplicity of the free-living, running-based modified LIST exercise stimulus deems the study design practicable in younger populations that engage in team sport variable intensity exercise; this may be as much as 30% of the general population who are recreationally active in team sports according to the 2015 Physical Activity Council Report.

The IAAO technique was a novel method for determining protein requirements in the present study, yet there exist limitations inherent to its practicability in future studies. Participants are required to consume hourly liquid meals on trial days. Although these meals provide a sufficient daily amount of total energy, they may not mimic a typical dietary regimen, which often consists primarily of three substantial meals and unbalanced protein intakes (de Castro et al., 1997). However, this feeding pattern may be ineffective at maximizing net protein balance, especially following exercise, as repeated ingestion of

moderate amounts of protein (~20g) at regular intervals have been shown to maximize protein synthesis and net balance (Areta et al., 2013; Moore et al., 2012).

Seeing as the present study was the first to utilize the IAAO technique to determine protein requirements in a trained demographic group and in the presence of an exercise stimulus, there are several potential avenues for future research. The present study's protocol can first be extended to include active, young adult females in an attempt to determine if there are sex-based differences in protein requirements for active individuals, given that differences in amino acid metabolism during exercise have been reported previously (Phillips, Atkinson, Tarnopolsky, & MacDougall, 1993). Additionally, the IAAO technique has never been applied to active populations of children or adolescents. Since the adequate ingestion of dietary protein is essential to support the optimal remodeling and deposition of lean body mass in active, growing children and adolescents, such a study would have major health and sport implications (Rodriguez, 2005). Beyond the application of the present study's protocol to alternative age groups, an interesting future avenue of research would be to apply the typical IAAO technique to more specialized athletic populations. For example, the IAAO technique could be applied to either endurance or resistance-trained athletes. Such research would require the modified LIST exercise stimulus to be replaced with an alternative exercise routine that is representative of the study population's typical training regimen. For resistance-trained athletes, a weight-lifting regimen could replace the modified LIST exercise stimulus, while a 20-km run could be used as an exercise stimulus in endurance-trained populations. The present study will therefore serve as a benchmark for which all subsequent IAAO studies in active populations could be compared to.

## **6.7 Conclusion**

The adequate ingestion of dietary amino acids is a critical factor in ensuring the development, and maintenance of lean body mass in individuals of all ages. Physical activity can alter protein and amino acid metabolism, and can increase protein requirements in highly active individuals (e.g. trained athletes). The purpose of the present study was to utilize, for the first time, the minimally invasive IAAO technique to evaluate the impact of a variable intensity exercise on protein requirements in active, trained young adult males. The hypotheses that the study's results would yield protein requirements in excess of the current requirements established using NBAL, in addition to those established in less active populations using the IAAO technique were validated. Future avenues of research will seek to improve upon the existing IAAO protocol, and to employ it in active females, children, adolescents, and in more specialized athletic populations.

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## **Appendices**

# Raw Data

All data in this appendix can be accessed via the excel document 'Master Data (August 20<sup>th</sup>)'

## Subject Characteristics:

<u>Participant #:</u>	<u>Age:</u>	<u>Height (cm):</u>	<u>Weight (kg):</u>	<u>% BF:</u>	<u>BodPod (FFM, kg):</u>	<u>BodPod RMR (kcal/d):</u>
1	23	178	88.18	13.15	76.58	2000
2	22	176.5	79.78	7.1	74.115	1948
3	23	183	87.09	11.1	77.4085	2036
4	22	174	87.72	22	68.4075	1835
6	24	185	84.82	15.2	71.914	1908
7	22	165	75.36	10.9	67.149	1771
5	24	181	73.41	15.1	62.344	1653

<u>Participant #:</u>	<u>Accelerometer RMR (J/min):</u>	<u>Accelerometer RMR (kcal/d):</u>	<u>Study Day E-Expenditure (kcal):</u>	<u>SD E-Intake (kcal):</u>
1	6.8834	2370	4592	3061
2	6.0865	2095	4080	2720
3	6.6316	2280	4444	2963
4	7.0298	2420	4661	3107
6	6.5149	2240	4357	2905
7	6.1158	2105	4043	2696
5	5.9278	2040	3923	2615

<u>Participant #:</u>	<u>Accelerometer E-Expenditure (kcal):</u>	<u>Beep Test Score:</u>	<u>VO2-Max:</u>	<u>Max-HR (bpm):</u>
1	3700	10.7	48.9	186
2	3500	12.7	55.7	198
3	4200	15	64.1	210
4	4200	10.6	48.6	180
6	3500	11.2	50.8	186
7	3100	10.2	47.4	162
5	2970	11	50.3	N/A

Subject #	Weight (kg)	FFM (kg)	PRO-Intake (g/kg)	A.P.E.	VCO2 (ml/min)	F13CO2	F13CO2 (FFM)
6	84.82	71.91	0.2	0.01085376	281.7111111	1.176408538	1.387609126
2	79.78	74.115	0.225	0.009403998	280.6956522	1.079758034	1.16228963
1	88.18	76.58	0.25	0.011219405	241.2650602	1.001766307	1.15350944
4	87.72	68.407	0.3	0.009076886	272.5128205	0.920232248	1.180036733
3	87.09	77.408	0.35	0.006278581	243.0444444	0.571809227	0.643329702
7	75.36	67.15	0.4	0.006264227	241.9662921	0.656377536	0.73662861
2	79.78	74.115	0.45	0.010333844	266.7471264	1.127560605	1.213746003
3	87.09	77.408	0.5	0.004580104	354.2459016	0.607972847	0.684016577
2	79.78	74.115	0.55	0.00584702	267.9078521	0.640764176	0.689741159
4	87.72	68.407	0.6	0.006556779	308.0041119	0.751312662	0.96342694
6	84.82	71.91	0.65	0.006258226	310.0375	0.746516634	0.880538741
7	75.36	67.15	0.7	0.004601528	280.6818182	0.559303698	0.627686176
1	88.18	76.58	0.75	0.00647773	289.8823529	0.694939197	0.800205515
6	84.82	71.91	0.8	0.0061841	282.7808219	0.672822356	0.793614132
4	87.72	68.407	0.85	0.004972644	300.0138889	0.555011996	0.711705707
7	75.36	67.15	0.9	0.003493907	256.8838428	0.388668585	0.436188601
2	79.78	74.115	0.95	0.007702033	242.04	0.762554216	0.820840253
3	87.09	77.408	1	0.003982914	277.2615385	0.413803797	0.465561346
1	88.18	76.58	1.05	0.006152891	264.3580081	0.601968652	0.693152204
4	87.72	68.407	1.1	0.00425865	308.0041119	0.487980059	0.625748984
1	88.18	76.58	1.15	0.007446627	240.4782609	0.662731418	0.763119045
3	87.09	77.408	1.2	0.002433046	239.0333333	0.217927819	0.245185688
6	84.82	71.91	1.25	0.004223053	277.3648649	0.450663058	0.531570582
2	79.78	74.115	1.3	0.006514465	279.9342105	0.745955559	0.802972873
7	75.36	67.15	1.35	0.002667755	252.7333333	0.291971112	0.327668548
2	79.78	74.115	1.4	0.004888827	277.6779661	0.55529574	0.597739919
6	84.82	71.91	1.45	0.003923703	292.4564367	0.441500635	0.52076323
4	87.72	68.407	1.5	0.003872376	311.3090909	0.448479892	0.575096936
7	75.36	67.15	1.55	0.003097448	248.6438356	0.333513299	0.374289832
1	88.18	76.58	1.6	0.005575369	262.5890411	0.541816707	0.623888707
3	87.09	77.408	1.65			0	0
1	88.18	76.58	1.7	0.004527471	264.3580081	0.442945548	0.510040982

7	75.36	67.15	1.75	0.002290812	262.9027778	0.260805094	0.29269206
6	84.82	71.91	1.8	0.00499188	298.8333333	0.573940834	0.676980413
3	87.09	77.408	1.85			0	0
4	87.72	68.407	1.9	0.003949421	296.7529412	0.436015672	0.559113757
2	79.78	74.115	1.95	0.004440971	247.5633803	0.449720337	0.484094832
1	88.18	76.58	2	0.0044141	250.9333333	0.409923412	0.472016799
3	87.09	77.408	2.05	0.003917727	206.3382353	0.302912952	0.34080055
6	84.82	71.91	2.1	0.002819339	304.010989	0.329769577	0.388973099
4	87.72	68.407	2.15	0.003072847	359.4318182	0.410895247	0.52690121
7	75.36	67.15	2.2	0.001897828	254.375	0.209056055	0.234615998
2	79.78	74.115	2.25	0.002211918	280.6966292	0.253971233	0.273383593
5	73.41	62.34	2.45	0.001700551	278.3404255	0.210418053	0.247782953
4	87.72	68.33	2.5	0.0024627	308.0041119	0.282190062	0.362267119
2	79.78	74.115	2.55	0.003440106	267.9078521	0.376994956	0.405810667
2	79.78	74.115	2.6	0.003742096	267.9078521	0.410089465	0.441434764

- **Highlighted trial indicates trial where participant became ill.**
- **Any trials where F13CO2 values = 0 were not conducted**

FIGURE 1. Phenylalanine Oxidation Re-Analysis

Breakpoint = 1.56g/kg/d

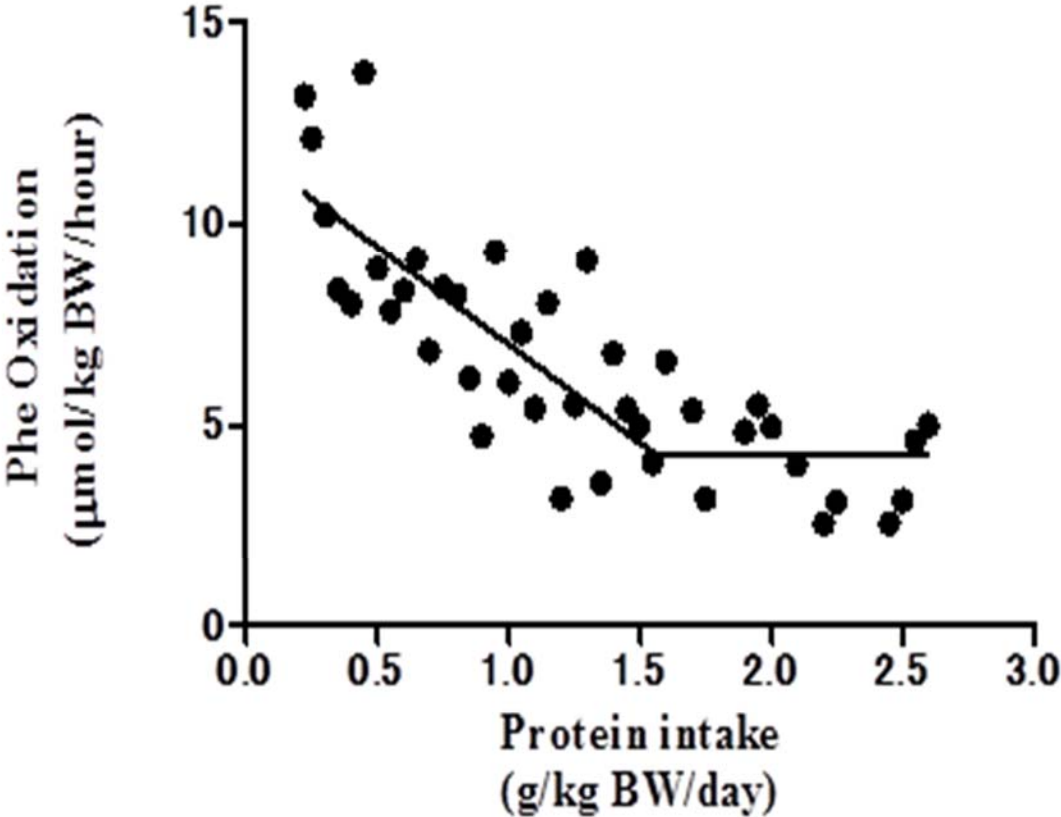
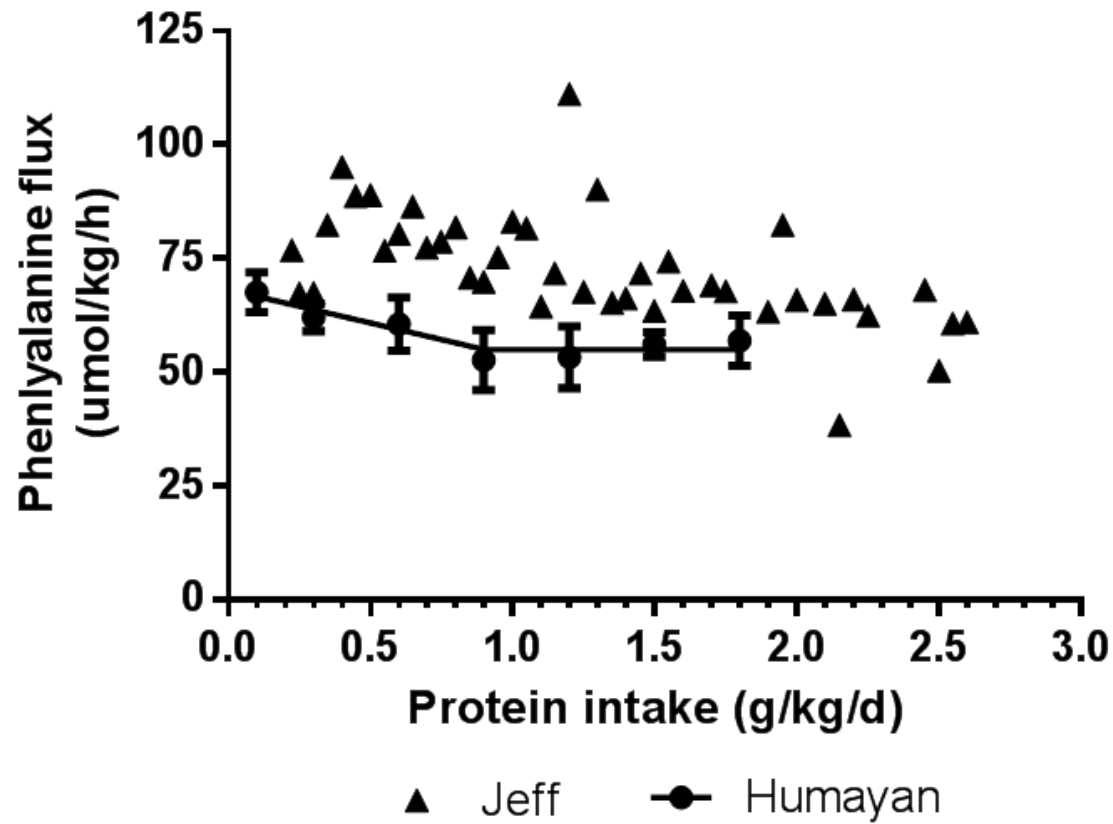


FIGURE 2. Comparison between Phenylalanine Flux Data and Protein Intake Including Humayan et al., 2007



1 Validation of the Indicator Amino Acid Oxidation Technique's Utilization In the Presence of a  
2 Variable Intensity Exercise Stimulus

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Jeffrey Packer

The University of Toronto

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13 Acknowledgements: Dan Moore, Denise Wooding, Hiro Kato, Michael Mazzulla, & Sidney  
14 Abou Sawan

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16 Key Words: IAAO, LIST

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18 Abstract Word Count: 198

19 Word Count: 1286

20 **ABSTRACT**

21           The purpose of the present study was to validate the Indicator Amino Acid Oxidation  
22 (IAAO) Technique for its use following a variable intensity running test through the analysis  
23 of background  $^{13}\text{CO}_2$  enrichment and resting  $\text{VCO}_2$  production. It was hypothesized that  
24  $^{13}\text{CO}_2$  enrichment and  $\text{VCO}_2$  production would reach a plateau within 2-4 hours following the  
25 variable intensity exercise stimulus that would be sustained throughout the remainder of the  
26 protocol, thus validating the IAAO Technique's use following such an exercise test. Three  
27 active adult males aged (22.7 +/- 1.5 years) partook in a single metabolic trial following a  
28 two-day adaptation period providing 1.2 g/kg/d of dietary protein. After fasting overnight,  
29 participants engaged in a modified version of the Loughborough Intermittent Shuttle Test  
30 (LIST) followed by an 8-hour metabolic trial consisting of 8 hourly isocaloric beverages each  
31 containing the equivalent of 1.2g/kg/d of complete protein. Breath samples were taken  
32 every 20-minutes throughout the 8-hour protocol using QuinTron breath collection bags.  
33  $\text{VCO}_2$  production was measured during the final 15-20 minutes of every hour of the 8-hour  
34 protocol using a MOXUS Metabolic Cart (AEI Technologies). Analysis of mass spectrometry  
35 and  $\text{VCO}_2$  data validated the use of the IAAO Technique following the LIST stimulus.

## 36 INTRODUCTION

37 The adequate ingestion of dietary amino acids (AA) is the most critical factor in  
38 ensuring the healthy growth and development of lean body mass in individuals of all ages.  
39 Current dietary recommendations for protein consumption are based predominantly on the  
40 nitrogen balance technique (NBAL). However, recent advances in the use of stable isotopes  
41 have provided for alternative methods that can be used to evaluate protein requirements  
42 such as the IAAO technique.

43 A study by Bross et al. sought to develop and validate the IAAO technique for its use in  
44 sedentary adults (1). Thirteen healthy adult females, and one healthy adult male received a  
45 4-h oral, primed, equal dose infusion of either L-[1-<sup>13</sup>C]phenylalanine or L-[1-<sup>13</sup>C]lysine after  
46 4 hours of steady-state feeding without the tracer. Isotopic plateau in <sup>13</sup>CO<sub>2</sub> was achieved  
47 within 120 minutes of phenylalanine or lysine infusion, suggesting that primed, equal dose,  
48 oral infusion produced steady-state conditions required for the implementation of the IAAO  
49 technique when determining protein requirements. The IAAO technique has since been  
50 implemented in several studies for the purpose of determining protein requirements (2, 5).  
51 One study by Humayun et al. utilized the IAAO technique in healthy, young adult men (2).  
52 The results from this study suggest that current protein requirements established using  
53 NBAL might underestimate true protein requirements.

54 Physical activity has been shown to potentially increase protein requirements in  
55 active individuals (3, 4), yet the IAAO technique has only been validated, and as such  
56 implemented in non-active populations (2). Thus, the objective of the present study is to  
57 validate the IAAO technique for its use in active populations by integrating the variable  
58 intensity LIST exercise stimulus into a typical IAAO protocol.

## 59 **METHODS**

60 Three healthy, young adult male subjects (age 22.7 +/- 1.5 years; height 181.3 +/- 3.5  
61 cm; weight 85.7 +/- 9.3 kg) were required to wear a Sensewear BodyMedia Armband  
62 Accelerometer for three days while engaging in their habitual exercise and dietary routine.  
63 Subjects were also required to complete a three-day dietary log. Habitual energy  
64 expenditures were averaged over the three-day accelerometer period, providing for an  
65 estimation of each subject's typical daily energy expenditure.

66 Subjects engaged in an overnight fast prior to the metabolic trial, and reported to  
67 laboratory in the rested state having fasted and abstained from alcohol and caffeine  
68 consumption for 24-hours. Upon arrival, subjects completed a 15-20 minute baseline VCO<sub>2</sub>  
69 collection. Subjects then consumed a protein-free breakfast providing 1g/kg of  
70 carbohydrates from Polycose (0.5g/kg) and Gatorade powder (0.5g/kg). Subjects then  
71 completed the LIST, which consisted of four 15-minute periods of variable intensity exercise  
72 (walking, sprinting, running, jogging) separated by 5 minutes of rest (**Figure 1**).

73 Upon completion of the LIST exercise stimulus, subjects consumed 8 isocaloric and  
74 isoproteinic hourly meals containing calories and protein equivalent to 2/3 of their daily  
75 energy expenditure as measured on the Sensewear Body Media Armband Accelerometer,  
76 and 2/3 of 1.2 g/kg/d of protein respectively. Breath samples were collected every 20 minutes  
77 throughout the 8-hour metabolic trial using QuinTron breath collection bags and vacuumed  
78 Sercon exertainers. VCO<sub>2</sub> was collected during the final 15-20 minutes of each hour  
79 throughout the 8-hour protocol using the MOXUS Metabolic Cart (AEI Technologies).

80 Breath samples were analyzed for <sup>13</sup>CO<sub>2</sub> enrichment using mass spectrometry. VCO<sub>2</sub>  
81 data for each time-point was exported to Microsoft Excel and averaged for analytic purposes.

## 82 RESULTS

83  $^{13}\text{CO}_2$  enrichment reached a plateau in all subjects within 3 hours of the first hourly  
84 meal's consumption. This plateau was sustained throughout the remainder of the protocol in  
85 all subjects (**Figure 2**). Baseline fasted and resting state  $^{13}\text{CO}_2$  enrichment was lower than  
86 the post-exercise, fed-state  $^{13}\text{CO}_2$  enrichment in all subjects.  $\text{VCO}_2$  data was consistent both  
87 within and between subjects throughout the 8-hour protocol (**Figure 2**).

## 88 DISCUSSION

89  $^{13}\text{CO}_2$  enrichment data followed a similar trend for all subjects, in that after  
90 approximately 3 hours of feeding following the LIST exercise stimulus, a plateau in  $^{13}\text{CO}_2$   
91 enrichment occurred. This plateau in  $^{13}\text{CO}_2$  enrichment was greater than baseline in all  
92 subjects, and was sustained throughout the remainder of the protocol (**Figure 2**). However,  
93 there were differences between subjects with respect to how  $^{13}\text{CO}_2$  enrichment at baseline  
94 compared to  $^{13}\text{CO}_2$  enrichment in the first one-to-two hours following the LIST exercise  
95 stimulus. One subject's baseline  $^{13}\text{CO}_2$  enrichment was greater than those analogous values  
96 corresponding to the first one-to-two hours of feeding following the LIST exercise stimulus  
97 (**Figure 3**). However, after three hours of feeding, this subject's  $^{13}\text{CO}_2$  enrichment had  
98 reached a plateau in excess of their baseline  $^{13}\text{CO}_2$  enrichment, similar to that of the other  
99 two subjects (**Figure 3**). These results could potentially be explained by subject-based  
100 differences in training status or habitual dietary consumption.

101  $\text{VCO}_2$  data was consistent in all subjects throughout the 8-hour protocol (**Figure 2**).  
102 However, two outliers were removed from the data. Since  $\text{VCO}_2$  is dependent on the energy  
103 expenditure of the participant, subject movement during  $\text{VCO}_2$  collection must be strictly

104 controlled in order to ensure consistency in the data. It is conceivable that the discrepancies  
105 in the outlier VCO<sub>2</sub> data may be attributed to a lack of consistency in monitoring of the  
106 subjects throughout the 20-minute VCO<sub>2</sub> collection. This represents a limitation of the  
107 present study despite the VCO<sub>2</sub> data being consistent once the outliers were removed.

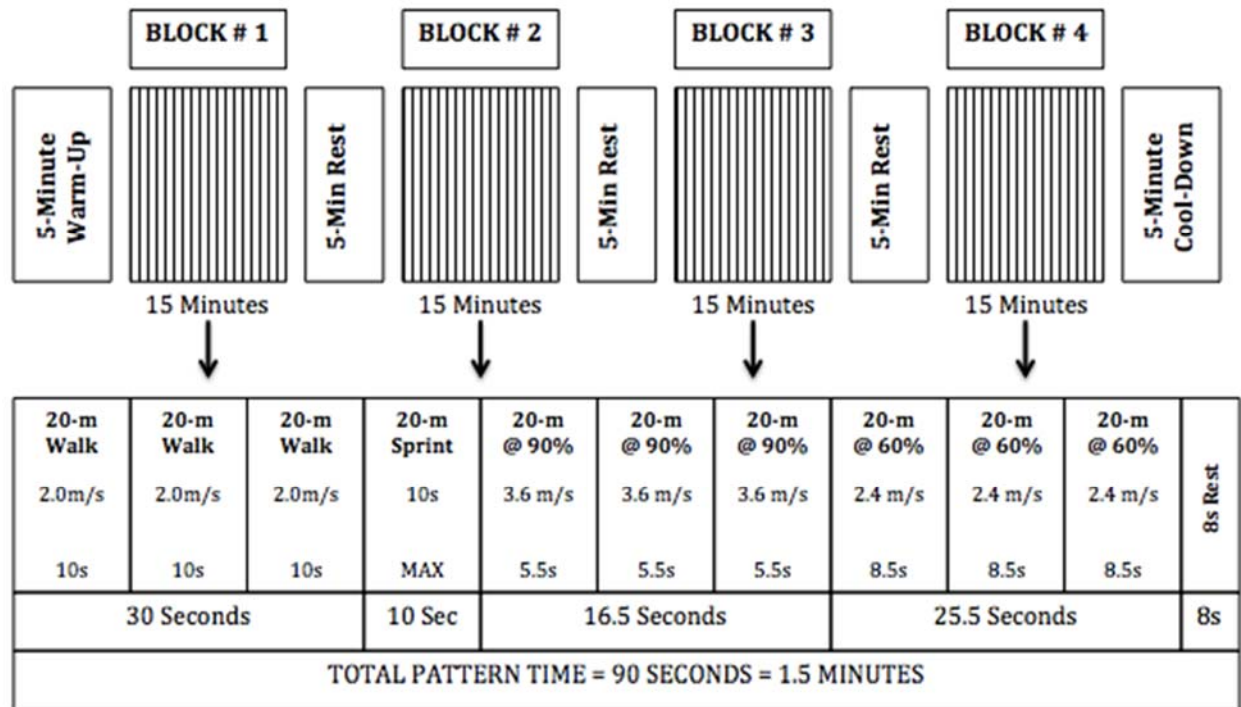
108         The sustained plateau in <sup>13</sup>CO<sub>2</sub> enrichment exhibited in all subjects in combination  
109 with the stable and consistent VCO<sub>2</sub> data justify that the IAAO Technique is a suitable method  
110 for determining protein requirements in the presence of the variable intensity LIST exercise  
111 stimulus. This has major research implications, as the IAAO Technique has yet to be used to  
112 determine protein requirements in active populations, and has never been used following  
113 any type of exercise stimulus. Since studies that have employed the IAAO Technique in non-  
114 active populations, and studies utilizing NBAL in active populations have both established  
115 that protein requirements may be greater than the current recommended dietary allowance  
116 (RDA) (2), it is plausible that future studies utilizing the IAAO Technique may yield protein  
117 requirements that are in excess of the present RDA.

## 118 **CONCLUSIONS**

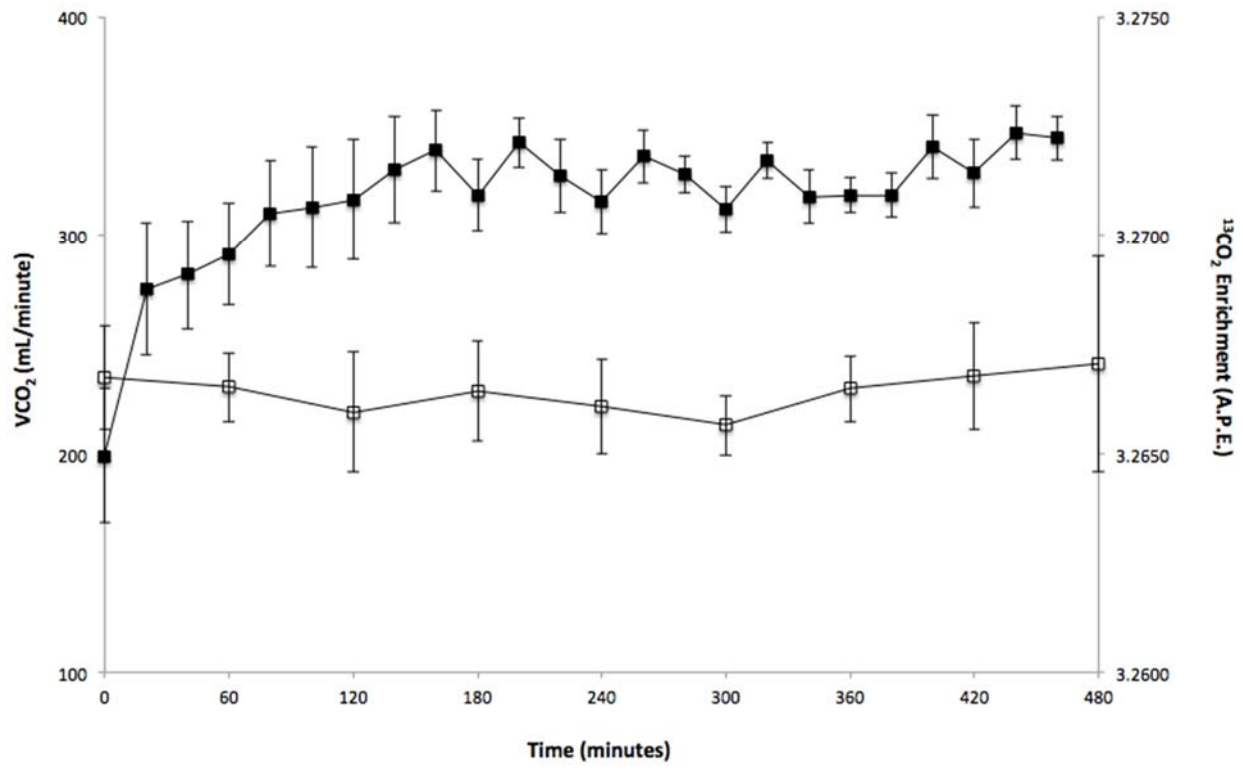
119         The outcomes of the present study validated the hypothesis that the IAAO Technique  
120 is suitable for its use in the presence of the variable intensity LIST exercise stimulus, as both  
121 <sup>13</sup>CO<sub>2</sub> enrichment and VCO<sub>2</sub> production reached a plateau within 3 hours following the  
122 variable intensity exercise stimulus that was sustained throughout the remainder of the  
123 protocol. Future avenues of research should seek to employ the IAAO Technique in athletic  
124 populations in order to establish optimal protein requirements for such individuals. Such  
125 studies will have major implications on both health and sport performance.

126 **REFERENCES**

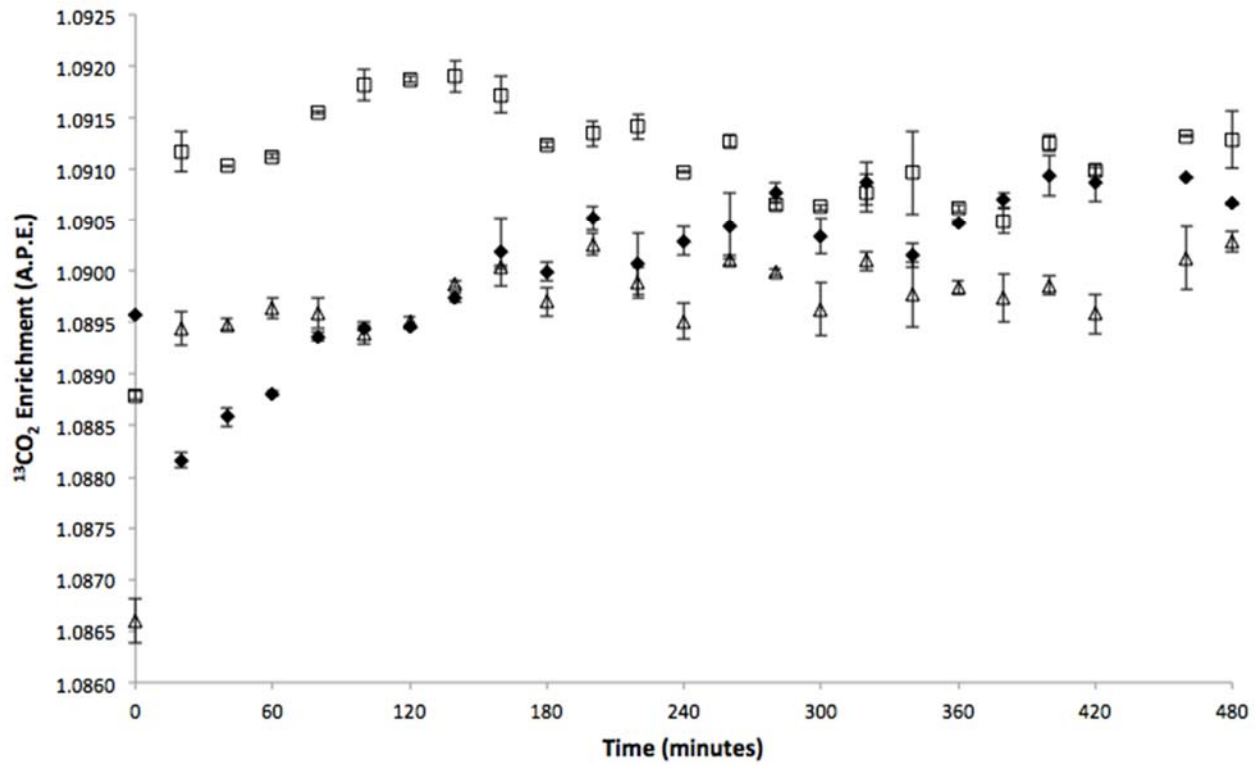
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**FIGURE 3.** The modified Loughborough Intermittent Shuttle Test (LIST). The LIST consists of four 15-minute periods of variable intensity exercise (walking, sprinting, running, jogging) separated by 5 minutes of rest. The time to complete each 'pattern' of the LIST is 90 seconds, which is repeated ten times to comprise one 15-minute block of variable intensity exercise.



**FIGURE 4.** The effect of experimental diet on <sup>13</sup>CO<sub>2</sub> enrichment expressed as atoms percent excess (A.P.E.; ■) and on the rate of CO<sub>2</sub> production (VCO<sub>2</sub>; □). A plateau in averaged <sup>13</sup>CO<sub>2</sub> enrichment and averaged VCO<sub>2</sub> was achieved for all subjects beginning at ~180 min and ~60 min respectively. Time of 0 equates to baseline <sup>13</sup>CO<sub>2</sub> enrichment and baseline VCO<sub>2</sub>.



**FIGURE 5.** The effect of experimental diet on  $^{13}\text{CO}_2$  enrichment expressed as atoms percent excess (A.P.E.) for each subject in the study (Subject 1 □; Subject 2 ◆; Subject 3 Δ). A plateau in  $^{13}\text{CO}_2$  production was achieved by ~180 minutes in all subjects. Subject 2 obtained a  $^{13}\text{CO}_2$  enrichment plateau at approximately 180 minutes despite initial post-exercise fed-state  $^{13}\text{CO}_2$  enrichment (0-180 minutes) being lower than baseline  $^{13}\text{CO}_2$  enrichment. Time of 0 equates to baseline  $^{13}\text{CO}_2$  enrichment.

# IAAO Study Lab Manual

Participant Name:  
Participant Number:

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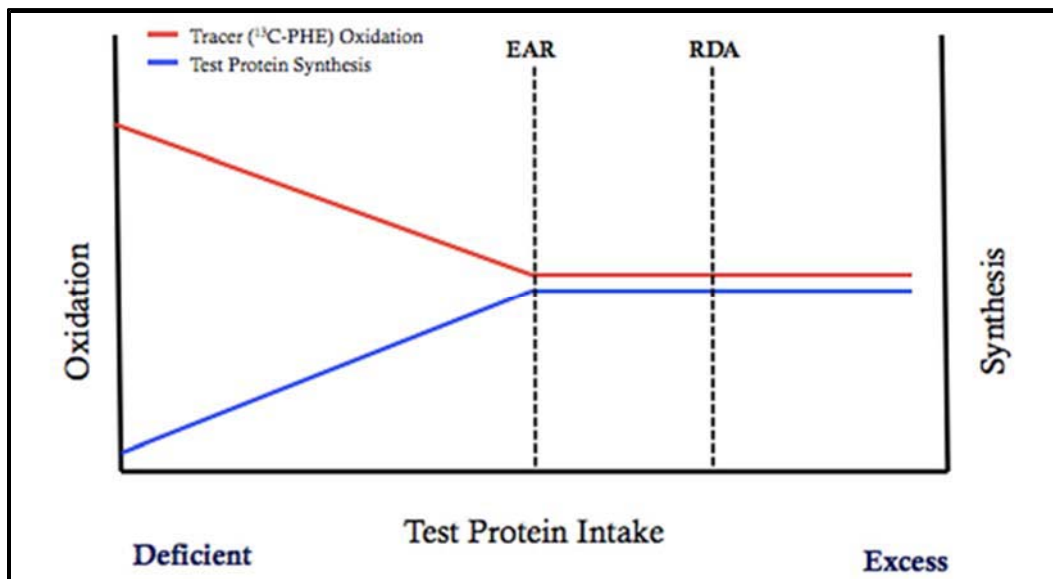
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## **Section 1. IAAO Study Overview:**

### **1.1 An Introduction to the IAAO Technique**

The IAAO technique utilizes stable isotopes ( $^{13}\text{C}$ ) to label a single 'indicator' amino acid. The IAAO technique dictates that an indicator amino acid is always supplemented in excess of the protein requirement, while the remaining intake of amino acids will range from being deficient to in excess of the protein requirement through the consumption of crystalline amino acids based on the amino acid pattern of egg protein. When a single essential amino acid from the diet is limiting (below the requirement), the other amino acids (including the indicator) cannot be optimally utilized for protein synthesis, and are directed towards oxidation. In a situation where the indicator amino acid is consumed in excess, while the remaining test protein consumption is deficient, any amount of the indicator amino acid in excess of the test protein will be directed towards oxidation. In this scenario, one would expect the levels of  $^{13}\text{C}$  from the oxidized indicator amino acid to be high, and overall protein synthesis to be compromised. As the consumption of the limiting amino acids in the test protein is increased, a lesser proportion of the indicator amino acid will be oxidized; this would result in a decrease in breath and urinary  $^{13}\text{C}$  from the indicator amino acid, as a greater proportion of the ingested amino acids are utilized for protein synthesis (See Figure Below). Once the protein requirement for the test amino acid is reached, any additional intake of the test protein above the protein requirement should not result in a further decrease in the indicator amino acid oxidation as this amino acid will always be consumed in excess of its requirement. Additionally, there should be no further increases in protein synthesis once the test protein intake exceeds that of the protein requirement. The point where no further decreases in indicator amino acid oxidation (as measured via  $^{13}\text{CO}_2$  production) are seen despite increases in the test protein intake is termed the 'breakpoint' (See Figure Below). It is this point that determines the Estimated Average Requirement (EAR) of the test protein, essentially allowing for protein requirements to be established from the IAAO technique. The Recommended Dietary Allowance (RDA) can be determined by adding two standard deviations to the EAR as determined by the breakpoint.



Isotopically labeled [1- $^{13}\text{C}$ ]-phenylalanine is the most commonly used indicator amino acid when administering the IAAO technique. In general, subjects are required to consume the indicator amino acid, along with several different test protein intakes throughout the IAAO protocol. Subjects partake in 7 isolated trials whereby the test protein consumption will range from deficient to excess. The relationship between the test protein intake and the indicator amino acid oxidation is determined during each trial, allowing for the breakpoint (EAR), and RDA to be established.

### **1.2 Purpose of the Present Study**

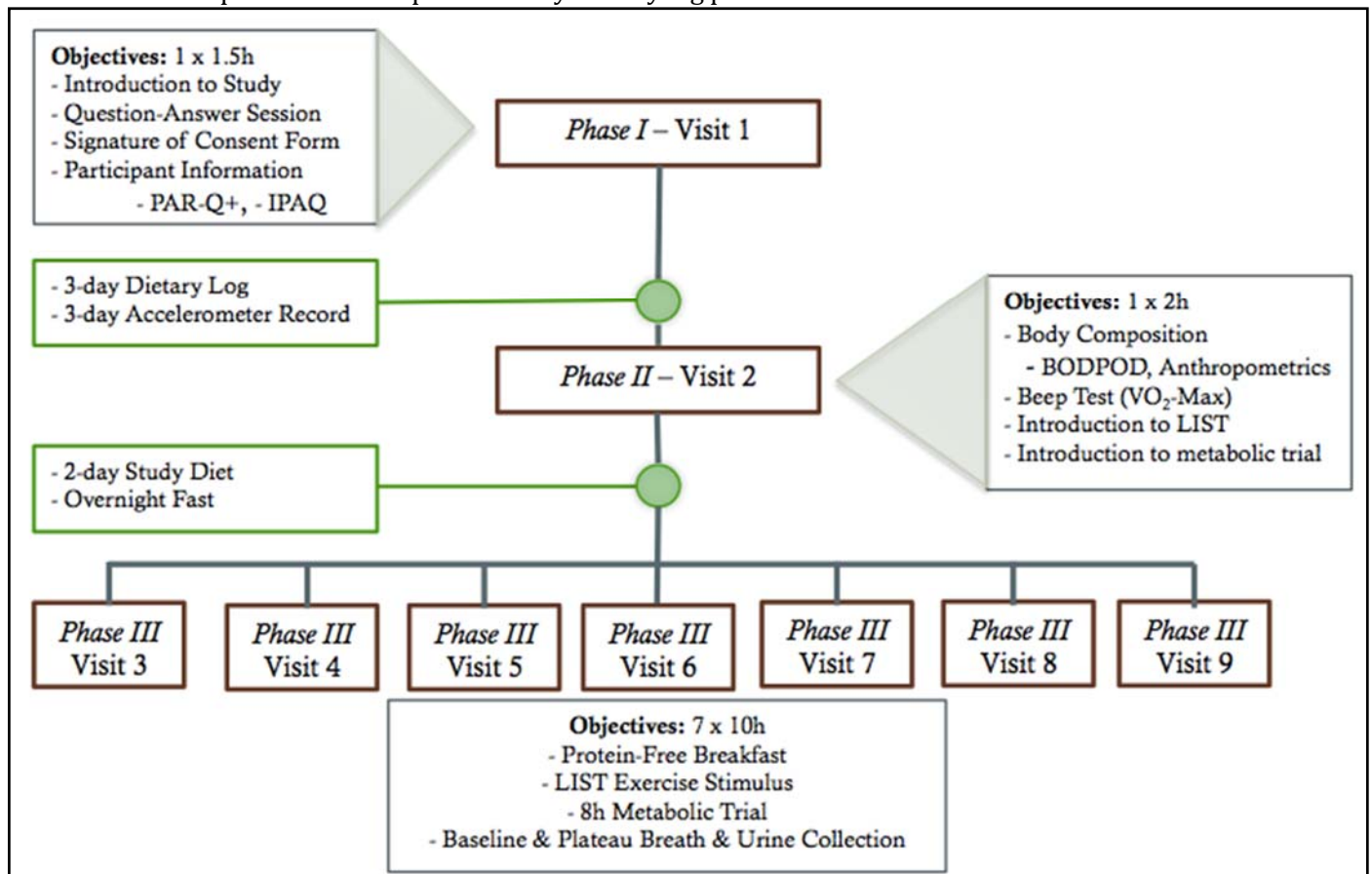
The purpose of the present study is to, for the first time, utilize the minimally invasive IAAO technique to evaluate of the impact of a variable intensity exercise on protein requirements in active populations. It is hypothesized that the study's results will yield higher protein requirements than those analogous values established using NBAL.

## Section 2. Phase I Protocol, Introductory Session:

A total of 7 participants will be recruited from each demographic group (i.e. for young, adult males, 7 participants aged 18-35 will be recruited). There are three phases of the present study. Each participant will take in two preliminary trials, each lasting one-to-two hours (*Phase I and Phase II*), followed by 7 metabolic trials (*Phase III*).

**2.1 Trial** - The purpose of Phase I will be to provide an introduction to the study to the participants.

- 1) Subjects arrive at the Goldring Metabolism and Sport Science Lab
- 2) Provide introduction to the study & provide an opportunity for participants to ask questions
- 3) Participants (and their parents if applicable) are provided with consent + assent forms to sign (See Appendix)
- 4) Participants complete the PAR-Q+ survey to assess their health (See Appendix)
- 5) Participants complete the IPAQ survey to assess their habitual activity levels (See Appendix)
- 6) Screen the surveys to ensure that the participants meet the requirements for the study
  - *Criteria:* No health risks (PAR-Q+), & perform moderate to vigorous exercise  $\geq 5$  times per week (IPAQ)
- 7) Once participants have been screened, and meet the inclusion criteria, they are officially enrolled in the study
- 8) Set-Up the Sensewear Accelerometer with participant's information to be worn prior to Phase II
  - Participants must wear the accelerometer for 3 days prior to Phase II
- 9) Explain the Dietary Log form to be filled out prior to Phase II (See Appendix)
  - Participants must complete a 3-day dietary log prior to Phase I



## 2.2 Phase I Data Table

Participant Number:

Date:

Height (cm):

Weight (kg):

<b>Event</b>	<b>Date (Time)</b>	<b>Checklist</b>
1) Subjects arrive at Goldring		
2) Introduction to study & opportunity to ask questions		
3) Participants sign consent & assent forms (if applicable)		
4) Participants complete the PAR-Q+ and IPAQ surveys		
5) Screen surveys to ensure participants meet inclusion criteria - <i>Inclusion Criteria:</i> No health risks & moderate-vigorous $\geq 5$ /week		
6) Set Up Sensewear accelerometer with participant information - Instruct participants to wear accelerometer for 3d prior to Phase II		
7) Provide participants with dietary log - Instruct participants to complete a 3-day dietary log prior to Phase II		

## **Section 3. Phase II Protocol, Body Composition & Fitness Assessment:**

### **3.1 Pre-Trial**

*\* Subjects will have completed the 3-day dietary log and having worn the Sensewear Body Media Armband Accelerometer for 3 days prior to reporting to the Goldring Lab for Phase II.*

- 1) Input the accelerometer data to the lab computer, and save
- 2) Keep file of dietary log for future reference
- 3) Ensure that BodPod is available for use
- 4) Ensure that Field House at the Athletic Centre, or the gym at Goldring is available for use

**3.2 Trial** – *Phase II will serve to collect body composition measurements, and to provide a fitness test to participants*

- 1) Subjects arrive at the Goldring Metabolism and Sport Science Lab
- 2) Record subject's height (cm) and weight (kg)
- 3) Provide subjects with a verbal introduction to the BodPod, beep test, and LIST exercise stimulus
- 4) Perform BODPOD analysis
- 5) Place Sensewear Body Media Armband accelerometer on the participants left arm
- 6) Participants perform the Beep Test
  - Record max-HR (using carotid palpation) and beep test level obtained
  - Timestamp both before and after the Beep Test
- 7) Provide 15-20 minutes of rest before partaking in the LIST exercise stimulus introduction (See Appendix)
  - Participants complete the LIST exercise stimulus
- 8) Participants return the Goldring Metabolism and Sport Science Lab
- 9) Provide brief explanation of the metabolic trial days in Phase III
- 10) Provide 2-day study diet to be consumed the two-days prior to the first metabolic trial

### **3.3 Post-Trial**

- 1) Ensure that participants report to lab for Phase III following an over-night fast
- 2) Ensure that participants refrain from consuming alcohol, or caffeine prior to each metabolic trial
- 3) A time-period of at least 72h must occur between Phase II and Phase III
- 4) Save all accelerometer data
- 5) Ensure that participants meet beep test inclusion criteria

### 3.4 Phase II Data Tables

Participant Number:

Date:

Height (cm):

Weight (kg):

Beep Test Level Obtained:

Max-HR (bpm):

Event	Date (Time)	Checklist
1) Subjects arrive at the Goldring Metabolism and Sport Science Lab - Save accelerometer data & collect 3-day dietary log		
2) Record subject's height (cm) and weight (kg) above		
3) Provide an introduction to the BODPOD, beep test, and LIST exercise		
4) Perform BODPOD analysis		
5) Outfit Sensewear Accelerometer on participants		
6) Participants perform the beep test (timestamp before + after beep test) - Record Beep Test Level Obtained & Max-HR on Beep Test		
7) Participants rest for 15-20 minutes prior to LIST introduction test		
8) Participants perform one block of the LIST for familiarization		
9) Participants return to the Goldring Metabolism and Sport Science Lab		
10) Provide a brief explanation to the trial days in Phase III		
11) Provide participants with adaptation diet to be consumed 2-d prior to each trial in Phase III		
<i>POST-TRIAL</i> Save all accelerometer data from Phase II - Ensure that participants meet the beep test inclusion criteria ( $\geq$ level 10)		

## **Section 4. Phase III Protocol. Metabolic Trials:**

### **4.1 Pre-Trial**

- 1) Subjects must report to lab having consumed the 2-day study diet, and having fasted overnight
- 2) Ensure that all study day meals have been portioned (See Appendix)
- 3) Ensure that MOXUS is set-up, calibrated, and ready for use
- 4) Ensure that the Field House or (preferably) the Goldring Gym can be used for the LIST exercise stimulus
- 5) Have protein-free breakfast ready for consumption (See Appendix)
- 6) Prepare all exertainers for the trial (~40), and urine eppendorfs (~25)

### **4.2 Trial**

- 0:00 - Participants arrive to the Goldring Lab in the early morning  
- Subject must have consumed the 2-d adaptation diet, and fasted overnight
- 0:00 - Participants are outfitted with the accelerometer, consume the PRO-free breakfast, and the trial is explained
- 1:00 to 2:30 - Participants complete the LIST Exercise Stimulus (See Appendix)  
- Remember to timestamp the accelerometer both before and after the LIST
- 2:30 - Participants consume the first of 8 hourly meals
- 3:30 - Participants consume the second of 8 hourly meals
- 4:30 - Participants consume the third of 8 hourly meals
- 5:30 - Participants consume the fourth of 8 hourly meals (collect baseline breath and urine samples)  
- *Breath*: 3 Exertainers @ 5:30, 5:45, 6:00, 6:15 (3 x 4 = 12 Baseline Breath Samples from 4 time-points)  
- *Urine*: 3 Eppendorfs @ 5:30, 6:00, 6:30 (3 x 3 = 9 Baseline Urine Samples from 2 time-points)
- 6:30 - Participants consume the fifth of 8 hourly meals  
- Participants consume the Isotope Primer, the Bicarbonate Primer, and the first Isotope CI
- 7:30 - Participants consume the sixth of 8 hourly meals  
- Participants consume the second Isotope CI
- 8:00 - Breath (CO<sub>2</sub>) analysis for 20-30 minutes on MOXUS metabolic cart  
- Ensure to save all data
- 8:30 - Participants consume the seventh of 8 hourly meals (collect plateau breath and urine samples)  
- Participants consume the third Isotope CI  
- *Breath*: 3 Exertainers @ 8:30, 8:45, 9:00, 9:15, 9:30, 9:45, 10:00, 10:15 (3x8 = 24 Plateau Breath Samples)  
- *Urine*: 3 Eppendorfs @ 8:30, 9:00, 9:30, 10:00, 10:30 (3 x 5 = 15 Plateau Urine Samples)
- 9:30 - Participants consume the eighth of 8 hourly meals (continue to collect breath and urine as above)
- 10:30 - Metabolic Trial is complete  
- Provide 2-day study diet to participants for the next metabolic trial if needed

### **4.3 Post-Trial**

- 1) Input all accelerometer data into the lab computer & save metabolic cart data
- 2) Place necessary samples in the appropriate fridge or freezer
- 3) Return breath and urine samples to SickKids for analysis

A minimum of a 3-day washout period must occur between Phase III trials

















## **Section 5. Appendices**

5.1 Consent, Assent Forms

5.2 PAR-Q+ Questionnaire

5.3 IPAQ Questionnaires (Children + Adults)

5.4 Description & Use of the Sensewear Body Media Accelerometer

5.5 Dietary Log Sheet (x3) & Instructions

5.6 The LIST Exercise Stimulus

5.7 Description of the 2-Day Study Diet

5.8 Description of the Protein-Free Breakfast

5.9 How to Make the 1kg Test-Protein Mix, 1kg Amino Acid Mix (Composition)

5.10 How to Make Study Day Meals

5.11 Cookie Recipes



## Appendix L – Metabolism Study Consent Form for Adults

### Title of Research Project:

- Amino acid metabolism and basic requirements in active, young adults

### Investigators

### Contact number

Dan Moore                      Professor, Faculty of Kinesiology, University of Toronto  
416-946-4088

Glenda Courtney-Martin              PhD, Sick Kids Hospital, 416-813-5744

Jeff Packer                      MSc Student, Faculty of Kinesiology, University of Toronto  
647-825-7032

### Purpose of the Research:

Protein is an essential nutrient we need to eat enough of in our diet to maintain important bodily functions and to support normal growth in children and adolescents and the recovery from exercise. Currently how much protein is needed in healthy, active school-age children and adolescents is not known exactly. Additionally, active children tend to have greater amounts of muscle and stronger bones than children who are inactive. Since protein provides the building blocks for the growth of muscle and bone, the effect that exercise has on how much protein children and adolescents need to eat is not well understood with current recommendations being based mostly on estimates from adults. Better understanding of the specific needs for protein during the important growth periods of childhood and adolescence up to and including young adulthood is needed to ensure young individuals are meeting the body's protein needs, including that for optimal growth and for the recovery from exercise.

### Description of the Research:

If you decide to enter this study we will measure the total amount of protein you need and use that information to determine what the requirement is for young adults. The results from this study will be compared to that of children and adolescents to determine if protein requirements change with growth and maturation. This study will involve a total of 9 separate trial days including today's information session. The study days will be separate but will be completed over a period spanning a couple of months.

In the event that you agree to participate in the study, the remainder of this session will serve to assess your physical activity levels and general health through the completion of two distinct surveys. Following the completion of these surveys, we will provide you with an accelerometer (a device used to measure normal activity patterns and energy expenditure), and 3 days of food record documentation along with the appropriate instructions. For the 3 days prior to returning the Cardiovascular Regulation Lab here at the Athletic Centre at the University of Toronto for Session 2, you will be required to wear the accelerometer, as well as fill out a 3-day food record of what you normally eat according to the instructions we have provided you with.

On the second session, you will return to the Cardiovascular Regulation Lab. We will then assess your body composition (amount of fat and fat-free mass) using one non-invasive measure called the BODPOD. The BODPOD is a non-invasive method used to estimate your body composition, and does not cause any pain. The BODPOD requires you to sit in a chamber for less than a minute to establish the amount of air you displace within the chamber. This allows us to estimate your body composition. Upon completion of the body composition measures, you will engage in an aerobic fitness test called the Beep Test. The beep test requires you to run back and forth between cones spaced 20-metres apart until volitional fatigue, and has been validated in all age groups. Once the beep test assessment has been completed, you will be given an opportunity to rest prior to performing additional fitness assessments including a handgrip strength exercise and a vertical jump to assess lower limb muscle function. You will then be introduced to the exercise test that will be conducted on all subsequent trial days, which will involve running at different speeds that will be similar to what you would encounter during a soccer game.

Prior to sessions three to nine, you will wear the accelerometer for 2 days and will consume a controlled liquid diet designed to provide adequate energy and protein. You will then return to the Cardiovascular Regulation Lab in the morning having refrained from consuming any breakfast. You will then be equipped with the accelerometer and will consume a small liquid breakfast. You will then be permitted to rest for 30 minutes prior to engaging in the exercise protocol. The exercise protocol will be conducted in open environment in the Field House at the Athletic Centre at the University of Toronto. It will consist of running at different speeds spanning from a sprint, to a run, to a jog, to a walk, which will be similar to the type of activity patterns that would occur during a game of soccer. The exercise protocol will take approximately 1 hour to complete with a total of 15min of rest throughout. Following the completion of the exercise protocol, you will consume a meal every hour for 8 consecutive hours, which will provide you with your energy needs and a variable amount of protein. You will be required to occasionally breathe into a gas collection chamber and to provide urine samples for analysis. Once all 8 meals have been consumed and all data has been collected, the trial day will be complete.

**Potential Harms:**

- We know of no harm that taking part in this study could cause you.

**Potential Discomforts or Inconvenience:**

- While this study does not cause harm we recognize that the length of the study day, the number of days required to complete the study and travel to the University of Toronto might pose an inconvenience to you.

**Potential Benefits:**

- You will not benefit directly from participating in this study.
- However the results of this study will be used to determine the requirement for protein in healthy active young adults. A separate group of children and adolescents will also be tested in a parallel study to determine their protein requirements. This will help us understand how much protein different age groups (i.e. children, adolescents, and young adults) should consume in their diets to support growth during an active lifestyle.

**Confidentiality:**

- We will respect your privacy. No information about who you will be given to anyone or be published without your permission, unless required by law. For example, the law could make us give information about you have been abused, if you have an illness that could spread to others, if you or someone else talks about suicide (killing themselves), or if court orders us to give them the study papers.

The data produced from this study will be stored in a secure, locked location. Only members of the research team (and maybe those individuals described above) will have access to the data. This could include external research team members. Following completion of the research study the data will be kept as long as required then destroyed as required by University of Toronto policy. Published study results will not reveal your identity.

**Reimbursement:**

- The proposed compensation is meant to adequately reimburse you for any costs incurred (e.g. parking and a small post-study meal) and to provide a token gift of appreciation for your effort. Compensation will be \$100 per metabolic trial

**Participation:**

- It is your choice to take part in this study. You can stop at any time. You will be compensated for all trials that you partakes in.
- New information that we get while we are doing this study may affect your decision to take part in this study. If this happens, we will tell you about this new information. And we will ask you again if you still want to be in the study.

**Conflict of Interest:**

- I, and the other research team members have no conflict of interest to declare.

**Consent :**

By signing this form, I agree that:

- 1) You have explained this study to me. You have answered all my questions.
- 2) You have explained the possible harms and benefits (if any) of this study.
- 3) I know what I could do instead of having myself take part in this study. I understand that I have the right to refuse to let myself take part in the study. I also have the right to take myself out of the study at any time
- 4) I am free now, and in the future, to ask questions about the study.
- 5) I understand that no information about myself will be given to anyone or be published without first asking my permission.
- 6) . I have also been provided the study timeline and been given demonstrations of all the measures to be used.
- 7) I agree, or consent, that I \_\_\_\_\_ may take part in this study.

\_\_\_\_\_  
Printed Name of Adult

\_\_\_\_\_  
Adult signature & date

\_\_\_\_\_  
Printed Name of person who explained consent

\_\_\_\_\_  
Signature of Person who explained consent & date

\_\_\_\_\_  
Printed Witness' name (if the parent/legal guardian Witness' signature & date does not read English)

If you have any questions about this study, please call \_\_\_\_\_ at \_\_\_\_\_

**If you have questions about your rights as a subject in a study or injuries during a study, please contact either of the investigators or the ethics review board at [ethics.review@utoronto.ca](mailto:ethics.review@utoronto.ca) or 416 946 3273.**

# PAR-Q+

## The Physical Activity Readiness Questionnaire for Everyone

Regular physical activity is fun and healthy, and more people should become more physically active every day of the week. Being more physically active is very safe for MOST people. This questionnaire will tell you whether it is necessary for you to seek further advice from your doctor OR a qualified exercise professional before becoming more physically active.

### SECTION 1 - GENERAL HEALTH

Please read the 7 questions below carefully and answer each one honestly: check YES or NO.		YES	NO
1.	Has your doctor ever said that you have a heart condition OR high blood pressure?	<input type="checkbox"/>	<input type="checkbox"/>
2.	Do you feel pain in your chest at rest, during your daily activities of living, OR when you do physical activity?	<input type="checkbox"/>	<input type="checkbox"/>
3.	Do you lose balance because of dizziness OR have you lost consciousness in the last 12 months? Please answer NO if your dizziness was associated with over-breathing (including during vigorous exercise).	<input type="checkbox"/>	<input type="checkbox"/>
4.	Have you ever been diagnosed with another chronic medical condition (other than heart disease or high blood pressure)?	<input type="checkbox"/>	<input type="checkbox"/>
5.	Are you currently taking prescribed medications for a chronic medical condition?	<input type="checkbox"/>	<input type="checkbox"/>
6.	Do you have a bone or joint problem that could be made worse by becoming more physically active? Please answer NO if you had a joint problem in the past, but it does not limit your current ability to be physically active. For example, knee, ankle, shoulder or other.	<input type="checkbox"/>	<input type="checkbox"/>
7.	Has your doctor ever said that you should only do medically supervised physical activity?	<input type="checkbox"/>	<input type="checkbox"/>

If you answered NO to all of the questions above, you are cleared for physical activity.



Go to Section 3 to sign the form. You do not need to complete Section 2.

- › Start becoming much more physically active – start slowly and build up gradually.
- › Follow the Canadian Physical Activity Guidelines for your age ([www.csep.ca/guidelines](http://www.csep.ca/guidelines)).
- › You may take part in a health and fitness appraisal.
- › If you have any further questions, contact a qualified exercise professional such as a CSEP Certified Exercise Physiologist® (CSEP-CEP) or CSEP Certified Personal Trainer® (CSEP-CPT).
- › If you are over the age of 45 yrs. and NOT accustomed to regular vigorous physical activity, please consult a qualified exercise professional (CSEP-CEP) before engaging in maximal effort exercise.



If you answered YES to one or more of the questions above, please GO TO SECTION 2.



Delay becoming more active if:

- › You are not feeling well because of a temporary illness such as a cold or fever – wait until you feel better
- › You are pregnant – talk to your health care practitioner, your physician, a qualified exercise professional, and/or complete the PARmed-X for Pregnancy before becoming more physically active OR
- › Your health changes – please answer the questions on Section 2 of this document and/or talk to your doctor or qualified exercise professional (CSEP-CEP or CSEP-CPT) before continuing with any physical activity programme.

## SECTION 2 - CHRONIC MEDICAL CONDITIONS

Please read the questions below carefully and answer each one honestly: check YES or NO.		YES	NO
1.	Do you have Arthritis, Osteoporosis, or Back Problems?	<input type="checkbox"/> If yes, answer questions 1a-1c	<input type="checkbox"/> If no, go to question 2
1a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking medications or other treatments)	<input type="checkbox"/>	<input type="checkbox"/>
1b.	Do you have joint problems causing pain, a recent fracture or fracture caused by osteoporosis or cancer, displaced vertebra (e.g., spondylolisthesis), and/or spondylolysis/pars defect (a crack in the bony ring on the back of the spinal column)?	<input type="checkbox"/>	<input type="checkbox"/>
1c.	Have you had steroid injections or taken steroid tablets regularly for more than 3 months?	<input type="checkbox"/>	<input type="checkbox"/>
2.	Do you have Cancer of any kind?	<input type="checkbox"/> If yes, answer questions 2a-2b	<input type="checkbox"/> If no, go to question 3
2a.	Does your cancer diagnosis include any of the following types: lung/bronchogenic, multiple myeloma (cancer of plasma cells), head, and neck?	<input type="checkbox"/>	<input type="checkbox"/>
2b.	Are you currently receiving cancer therapy (such as chemotherapy or radiotherapy)?	<input type="checkbox"/>	<input type="checkbox"/>
3.	Do you have Heart Disease or Cardiovascular Disease? This includes Coronary Artery Disease, High Blood Pressure, Heart Failure, Diagnosed Abnormality of Heart Rhythm	<input type="checkbox"/> If yes, answer questions 3a-3e	<input type="checkbox"/> If no, go to question 4
3a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking medications or other treatments)	<input type="checkbox"/>	<input type="checkbox"/>
3b.	Do you have an irregular heart beat that requires medical management? (e.g. atrial brillation, premature ventricular contraction)	<input type="checkbox"/>	<input type="checkbox"/>
3c.	Do you have chronic heart failure?	<input type="checkbox"/>	<input type="checkbox"/>
3d.	Do you have a resting blood pressure equal to or greater than 160/90 mmHg with or without medication? (Answer YES if you do not know your resting blood pressure)	<input type="checkbox"/>	<input type="checkbox"/>
3e.	Do you have diagnosed coronary artery (cardiovascular) disease and have not participated in regular physical activity in the last 2 months?	<input type="checkbox"/>	<input type="checkbox"/>
4.	Do you have any Metabolic Conditions? This includes Type 1 Diabetes, Type 2 Diabetes, Pre-Diabetes	<input type="checkbox"/> If yes, answer questions 4a-4c	<input type="checkbox"/> If no, go to question 5
4a.	Is your blood sugar often above 13.0 mmol/L? (Answer YES if you are not sure)	<input type="checkbox"/>	<input type="checkbox"/>
4b.	Do you have any signs or symptoms of diabetes complications such as heart or vascular disease and/or complications affecting your eyes, kidneys, and the sensation in your toes and feet?	<input type="checkbox"/>	<input type="checkbox"/>
4c.	Do you have other metabolic conditions (such as thyroid disorders, pregnancy-related diabetes, chronic kidney disease, liver problems)?	<input type="checkbox"/>	<input type="checkbox"/>
5.	Do you have any Mental Health Problems or Learning Difficulties? This includes Alzheimer's, Dementia, Depression, Anxiety Disorder, Eating Disorder, Psychotic Disorder, Intellectual Disability, Down Syndrome)	<input type="checkbox"/> If yes, answer questions 5a-5b	<input type="checkbox"/> If no, go to question 6
5a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking medications or other treatments)	<input type="checkbox"/>	<input type="checkbox"/>
5b.	Do you also have back problems affecting nerves or muscles?	<input type="checkbox"/>	<input type="checkbox"/>

Please read the questions below carefully and answer each one honestly: check YES or NO.		YES	NO
6.	Do you have a Respiratory Disease? This includes Chronic Obstructive Pulmonary Disease, Asthma, Pulmonary High Blood Pressure	<input type="checkbox"/> If yes, answer questions 6a-6d	<input type="checkbox"/> If no, go to question 7
6a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking medications or other treatments)	<input type="checkbox"/>	<input type="checkbox"/>
6b.	Has your doctor ever said your blood oxygen level is low at rest or during exercise and/or that you require supplemental oxygen therapy?	<input type="checkbox"/>	<input type="checkbox"/>
6c.	If asthmatic, do you currently have symptoms of chest tightness, wheezing, laboured breathing, consistent cough (more than 2 days/week), or have you used your rescue medication more than twice in the last week?	<input type="checkbox"/>	<input type="checkbox"/>
6d.	Has your doctor ever said you have high blood pressure in the blood vessels of your lungs?	<input type="checkbox"/>	<input type="checkbox"/>
7.	Do you have a Spinal Cord Injury? This includes Tetraplegia and Paraplegia	<input type="checkbox"/> If yes, answer questions 7a-7c	<input type="checkbox"/> If no, go to question 8
7a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking medications or other treatments)	<input type="checkbox"/>	<input type="checkbox"/>
7b.	Do you commonly exhibit low resting blood pressure significant enough to cause dizziness, light-headedness, and/or fainting?	<input type="checkbox"/>	<input type="checkbox"/>
7c.	Has your physician indicated that you exhibit sudden bouts of high blood pressure (known as Autonomic Dysreflexia)?	<input type="checkbox"/>	<input type="checkbox"/>
8.	Have you had a Stroke? This includes Transient Ischemic Attack (TIA) or Cerebrovascular Event	<input type="checkbox"/> If yes, answer questions 8a-c	<input type="checkbox"/> If no, go to question 9
8a.	Do you have difficulty controlling your condition with medications or other physician-prescribed therapies? (Answer NO if you are not currently taking medications or other treatments)	<input type="checkbox"/>	<input type="checkbox"/>
8b.	Do you have any impairment in walking or mobility?	<input type="checkbox"/>	<input type="checkbox"/>
8c.	Have you experienced a stroke or impairment in nerves or muscles in the past 6 months?	<input type="checkbox"/>	<input type="checkbox"/>
9.	Do you have any other medical condition not listed above or do you live with two chronic conditions?	<input type="checkbox"/> If yes, answer questions 9a-c	<input type="checkbox"/> If no, read the advice on page 4
9a.	Have you experienced a blackout, fainted, or lost consciousness as a result of a head injury within the last 12 months OR have you had a diagnosed concussion within the last 12 months?	<input type="checkbox"/>	<input type="checkbox"/>
9b.	Do you have a medical condition that is not listed (such as epilepsy, neurological conditions, kidney problems)?	<input type="checkbox"/>	<input type="checkbox"/>
9c.	Do you currently live with two chronic conditions?	<input type="checkbox"/>	<input type="checkbox"/>

Please proceed to Page 4 for recommendations for your current medical condition and sign this document.

# PAR-Q+



If you answered NO to all of the follow-up questions about your medical condition, you are ready to become more physically active:

- › It is advised that you consult a qualified exercise professional (e.g., a CSEP-CEP or CSEP-CPT) to help you develop a safe and effective physical activity plan to meet your health needs.
- › You are encouraged to start slowly and build up gradually – 20-60 min. of low- to moderate-intensity exercise, 3-5 days per week including aerobic and muscle strengthening exercises.
- › As you progress, you should aim to accumulate 150 minutes or more of moderate-intensity physical activity per week.
- › If you are over the age of 45 yrs. and NOT accustomed to regular vigorous physical activity, please consult a qualified exercise professional (CSEP-CEP) before engaging in maximal effort exercise.



If you answered YES to one or more of the follow-up questions about your medical condition:

- › You should seek further information from a licensed health care professional before becoming more physically active or engaging in a fitness appraisal and/or visit a or qualified exercise professional (CSEP-CEP) for further information.



Delay becoming more active if:

- › You are not feeling well because of a temporary illness such as a cold or fever – wait until you feel better
- › You are pregnant - talk to your health care practitioner, your physician, a qualified exercise professional, and/or complete the PARmed-X for Pregnancy before becoming more physically active OR
- › Your health changes - please talk to your doctor or qualified exercise professional (CSEP-CEP) before continuing with any physical activity programme.

## SECTION 3 - DECLARATION

- › You are encouraged to photocopy the PAR-Q+. You must use the entire questionnaire and NO changes are permitted.
- › The Canadian Society for Exercise Physiology, the PAR-Q+ Collaboration, and their agents assume no liability for persons who undertake physical activity. If in doubt after completing the questionnaire, consult your doctor prior to physical activity.
- › If you are less than the legal age required for consent or require the assent of a care provider, your parent, guardian or care provider must also sign this form.
- › Please read and sign the declaration below:

I, the undersigned, have read, understood to my full satisfaction and completed this questionnaire. I acknowledge that this physical activity clearance is valid for a maximum of 12 months from the date it is completed and becomes invalid if my condition changes. I also acknowledge that a Trustee (such as my employer, community/fitness centre, health care provider, or other designate) may retain a copy of this form for their records. In these instances, the Trustee will be required to adhere to local, national, and international guidelines regarding the storage of personal health information ensuring that they maintain the privacy of the information and do not misuse or wrongfully disclose such information.

NAME \_\_\_\_\_ DATE \_\_\_\_\_

SIGNATURE \_\_\_\_\_ WITNESS \_\_\_\_\_

SIGNATURE OF PARENT/GUARDIAN/CARE PROVIDER \_\_\_\_\_

For more information, please contact:  
Canadian Society for Exercise Physiology  
[www.csep.ca](http://www.csep.ca)

### KEY REFERENCES

1. Jamnik VJ, Warburton DER, Makarski J, McKenzie DC, Shephard RJ, Stone J, and Gledhill N. Enhancing the effectiveness of clearance for physical activity participation; background and overall process. APNM 36(S1):S3-S13, 2011.
2. Warburton DER, Gledhill N, Jamnik VK, Bredin SSD, McKenzie DC, Stone J, Charlesworth S, and Shephard RJ. Evidence-based risk assessment and recommendations for physical activity clearance; Consensus Document. APNM 36(S1):S266-s298, 2011.

The PAR-Q+ was created using the evidence-based AGREE process (1) by the PAR-Q+Collaboration chaired by Dr. Darren E. R. Warburton with Dr. Norman Gledhill, Dr. Veronica Jamnik, and Dr. Donald C. McKenzie

(2). Production of this document has been made possible through financial contributions from the Public Health Agency of Canada and the BC Ministry of Health Services. The views expressed herein do not necessarily represent the views of the Public Health Agency of Canada or BC Ministry of Health Services.

# INTERNATIONAL PHYSICAL ACTIVITY QUESTIONNAIRE

(August 2002 version)

## SHORT LAST 7 DAYS TELEPHONE FORMAT

For use with Young and Middle-aged Adults (15-69 years)

The International Physical Activity Questionnaires (IPAQ) comprises a set of 4 questionnaires. Long (5 activity domains asked independently) and short (4 generic items) versions for use by either telephone or self-administered methods are available. The purpose of the questionnaires is to provide common instruments that can be used to obtain internationally comparable data on health-related physical activity.

### *Using IPAQ*

Use of the IPAQ instruments for monitoring and research purposes is encouraged. It is recommended that no changes be made to the order or wording of the questions as this will affect the psychometric properties of the instruments.

### *Translation from English and Cultural Adaptation*

Translation from English is supported to facilitate worldwide use of IPAQ. Information on the availability of IPAQ in different languages can be obtained at [www.ipaq.ki.se](http://www.ipaq.ki.se). If a new translation is undertaken we highly recommend using the prescribed back translation methods available on the IPAQ website. If possible please consider making your translated version of IPAQ available to others by contributing it to the IPAQ website. Further details on translation and cultural adaptation can be downloaded from the website.

### *Data Entry and Coding*

Attached to the response categories for each question are suggested variable names and valid ranges to assist in data management and interviewer training. We recommend that the actual response provided by each respondent is recorded. For example, "120 minutes" is recorded in the minutes response space. "Two hours" should be recorded as "2" in the hours column. A response of "one and a half hours" should be recorded as either "1" in hour column and "30" in minutes column.

### *Further Developments of IPAQ*

International collaboration on IPAQ is on-going and an *International Physical Activity Prevalence Study* is in progress. For further information see the IPAQ website.

*More Information*

More detailed information on the IPAQ process and the research methods used in the development of IPAQ instruments is available at [www.ipaq.ki.se](http://www.ipaq.ki.se) and Booth, M.L. (2000). Assessment of Physical Activity: An International Perspective. Research Quarterly for Exercise and Sport, 71 (2): s114-20. Other scientific publications and presentations on the use of IPAQ are summarized on the website.

## Short Last 7 Days Telephone IPAQ

READ: I am going to ask you about the time you spent being physically active in the last 7 days. Please answer each question even if you do not consider yourself to be an active person. Think about the activities you do at work, as part of your house and yard work, to get from place to place, and in your spare time for recreation, exercise or sport.

READ: Now, think about all the *vigorous* activities which take *hard physical effort* that you did in the last 7 days. Vigorous activities make you breathe much harder than normal and may include heavy lifting, digging, aerobics, or fast bicycling. Think only about those physical activities that you did for at least 10 minutes at a time.

1. During the last 7 days, on how many days did you do vigorous physical activities?  
\_\_\_\_\_ Days per week [VDAY; Range 0-7, 8,9]  
8. Don't Know/Not Sure  
9. Refused

[Interviewer clarification: Think only about those physical activities that you do for at least 10 minutes at a time.]

[Interviewer note: If respondent answers zero, refuses or does not know, skip to Question 3]

2. How much time did you usually spend doing vigorous physical activities on one of those days?  
\_\_\_ \_\_\_ Hours per day [VDHRS; Range: 0-16]  
\_\_\_\_\_ Minutes per day [VDMIN; Range: 0-960, 998, 999]  
998. Don't Know/Not Sure  
999. Refused

[Interviewer clarification: Think only about those physical activities you do for at least 10 minutes at a time.]

**[Interviewer probe:** An average time for one of the days on which you do vigorous activity is being sought. If the respondent can't answer because the pattern of time spent varies widely from day to day, ask: "How much time in total would you spend **over the last 7 days** doing vigorous physical activities?"

\_\_\_\_\_ Hours per week [VWHR; Range: 0-1121]  
\_\_\_\_\_ Minutes per week [VWMIN; Range: 0-6720, 9998, 9999]  
9998. Don't Know/Not Sure  
9999. Refused

**READ: Now think about activities which take *moderate physical effort* that you did in the last 7 days. Moderate physical activities make you breathe somewhat harder than normal and may include carrying light loads, bicycling at a regular pace, or doubles tennis. Do not include walking. Again, think about only those physical activities that you did for at least 10 minutes at a time.**

3. During the **last 7 days**, on how many days did you do **moderate** physical activities?

\_\_\_\_\_ Days per week [MDAY; Range: 0-7, 8, 9]  
8. Don't Know/Not Sure  
9. Refused

**[Interviewer clarification:** Think only about those physical activities that you do for at least 10 minutes at a time]

**[Interviewer Note:** *If respondent answers zero*, refuses or does not know, skip to Question 5]

4. How much time did you usually spend doing **moderate** physical activities on one of those days?

\_\_\_\_\_ Hours per day [MDHRS; Range: 0-16]  
\_\_\_\_\_ Minutes per day [MDMIN; Range: 0-960, 998, 999]  
998. Don't Know/Not Sure  
999. Refused

**[Interviewer clarification:** Think only about those physical activities that you do for at least 10 minutes at a time.]

**[Interviewer probe:** An average time for one of the days on which you do moderate activity is being sought. If the respondent can't answer because the pattern of time spent varies widely from day to day, or includes time

spent in multiple jobs, ask: "What is the total amount of time you spent over the **last 7 days** doing moderate physical activities?"

- \_\_\_\_\_ Hours per week [MWHRS; Range: 0-1121]  
\_\_\_\_\_ Minutes per week [MWMIN; Range: 0-6720, 9998, 99991]  
9998. Don't Know/Not Sure  
9999. Refused

**READ: Now think about the time you spent walking in the last 7 days. This includes at work and at home, walking to travel from place to place, and any other walking that you might do solely for recreation, sport, exercise, or leisure.**

5. During the **last 7 days**, on how many days did you **walk** for at least 10 minutes at a time?  
Days per week [WDAY; Range: 0-7, 8, 91]  
8. Don't Know/Not Sure  
9. Refused

**[Interviewer clarification: Think only about the walking that you do for at least 10 minutes at a time.]**

**[Interviewer Note: *If respondent answers zero*, refuses or does not know, skip to Question 7]**

6. How much time did you usually spend **walking** on one of those days?  
\_\_\_\_\_ Hours per day [WDHRS; Range: 0-161]  
\_\_\_\_\_ Minutes per day [WDMIN; Range: 0-960, 998, 999]  
998. Don't Know/Not Sure  
999. Refused

**[Interviewer probe: An average time for one of the days on which you walk is being sought. If the respondent can't answer because the pattern of time spent varies widely from day to day, ask: "What is the total amount of time you spent walking over the **last 7 days**?"**

- \_\_\_ \_\_\_ \_\_\_ Hours per week [WWHRS; Range: 0-1121]  
\_\_\_\_\_ Minutes per week [WWMIN; Range: 0-6720, 9998, 9999]  
9998. Don't Know/Not Sure  
9999. Refused

**READ: Now think about the time you spent sitting on week days during the last 7 days. Include time spent at work, at home, while doing course work, and during leisure time. This may include time spent sitting at a desk, visiting friends, reading or sitting or lying down to watch television.**

7. During the last 7 days, how much time did you usually spend *sitting* on a week day?

\_\_ \_\_ Hours per weekday [SDHRS; 0-1s1

\_\_\_\_\_ Minutes per weekday [SDMIN; Range: 0-960, 998, 9991

998. Don't Know/Not Sure

999. Refused

[Interviewer clarification: Include time spent lying down (awake) as well as sitting]

[Interviewer probe: An average time per day spent sitting is being sought. If the respondent can't answer because the pattern of time spent varies widely from day to day, ask: "What is the total amount of time you spent *sitting* last Wednesday?"

\_\_\_\_\_ Hours on Wednesday [SWHRS; Range 0-161

\_\_ \_\_ \_\_ Minutes on Wednesday [SWMIN; Range: 0-960, 998, 9991

998. Don't Know/Not Sure

999. Refused

## Food Records

Name: \_\_\_\_\_

Height: \_\_\_\_\_ Weight: \_\_\_\_\_

Notes: \_\_\_\_\_

Age: \_\_\_\_\_

Date: \_\_\_\_\_

Phone #: \_\_\_\_\_

First Meal		Second Meal		Third Meal	
Description of Food or Drink	Amount Eaten	Description of Food or Drink	Amount Eaten	Description of Food or Drink	Amount Eaten
Morning Snack		Afternoon Snack		Evening Snack	

Supplements and/or Medications: \_\_\_\_\_

## INSTRUCTIONS FOR KEEPING A FOOD INTAKE RECORD

1. Please record all food and drink consumed, except water, for 3 consecutive days.  
It is most accurate and easiest to record directly after a meal or snack. Record any vomit or “spit up”.
2. Include the time of day when the food is eaten and the place where the food is eaten.  
*For example:* Home, School, Restaurant, Watching TV, etc.
3. Describe the foods accurately and give brand names if possible.  
*For example:* Margarine (Becel) – 1 teaspoon.
4. State whether fruits and vegetables are fresh, canned (water packed, heavy or light syrup), cooked or frozen.
5. Record the amount of food consumed by using household measures such as: cups, teaspoons, tablespoons, or simply weigh the food.  
*For example:* Homo milk – ½ cup or 4 ounces (oz) or 125 grams (gm)  
2 % Cottage cheese – 4 level tablespoons or 50 grams (gm)
6. If weighing meat on a food scale, give detailed description. If there is a bone in the meat or if there is leftover food, weigh after eating and subtract from original weight.  
*For example:* Broiled pork chop with bone – 75 gm, bone weighs – 21 gm (after eating), total weight of pork chop eaten is 54 gm. If weighing is unnecessary, approximate the number of ounces or record the measured size of meat:  
2” x 2” x 1”
7. Describe sandwiches in detail.  
*For example:* Bologna sandwich

Whole wheat bread	2 slices
Bologna	1 slice (50 gm)
Light mayonnaise (Kraft)	1 teaspoon
Lettuce (Iceberg)	1 leaf
Processed cheese (Kraft)	1 slice (30 gm)
8. Be sure to record amounts of additional foods served with cereals, desserts, etc.  
*For example:* Cereal

Rice Krispies (Kellogg’s)	½ cup (15 gm)
Milk 2%	¼ cup
Brown Sugar	2 level teaspoons
9. Include how the food is prepared especially for meats, fish, poultry, eggs, and vegetables. Methods of preparation include Boiling, Roasting, Baking, Broiling, Frying or Steaming. When frying, be sure to mention the type of fat or oil used and quantity (be sure to measure any fat or oil left over).  
*For example:* Scrambled eggs

Eggs	1 large
Milk 2 %	1 tablespoon
Margarine – fried in (Becel)	1 teaspoon
Left over margarine	¾ teaspoon
10. For mixed dishes such as casseroles, stews and baked goods (homemade cookies, cakes, pies, and other desserts), provide recipes on a separate sheet. State the amount of ingredients in the recipe, the number of servings made and the portion eaten by the child.
11. If using commercial (store bought) baked goods, state brand name and amount eaten.
12. If eating out, name the restaurant/chain and record foods eaten with portion sizes.
13. Give the name and amount of vitamin/mineral supplements and of medications/formulas if taken. Please inform us if you are using a special supplement, food replacement or medication. Further information and breakdown of these products may be requested.

## Sensewear Armband Instructions

### Overview:

The Sensewear Armband (SWA) analyzes physiological parameters and uses algorithms to report daily movement, steps taken, degree of physical activity and energy expenditure. The armband detects triaxial acceleration, as well as galvanic skin response, skin temperature and heat flux. This information is analyzed using algorithms performed by the SenseWear Professional 7.0 software. An excel file can be exported from the Sensewear software, containing all of the important results including energy expenditure.

### Instructions for Use:

The armband must be initialized and charged prior to use for each participant on each day. Remove the white square face from the armband strap by pushing down on the front face with two thumbs. Insert the Sensewear software program USB chip to the computer and connect the armband to the computer using the USB wire connection. Open the SenseWear Software and click the "Configure Armband & Display" tab at the top. Type in all subject info and ensure "Data Channels" are set to "Research" (ensures the armband will collect at the default rate of 1 sample/min). Click "OK" and allow the armband to initialize before unplugging.

Once initialized, remove the computer connection and reinsert the armband face into the strap. Wear on the upper left triceps with the logo facing in the readable direction and the sensors on the backside in direct contact with the skin. Adjust the strap so it fits comfortably. Within 10 minutes of wearing (usually less), the armband audio tones will sound indicating the beginning of data collection. There is no power button. After removal from the body, the armband tone will sound again, indicating the end of data collection.

The armband must be removed at least one hour per day (23 hours maximum of continuous wear). There is sufficient memory for approximately 28 days of steady use, and enough battery power for 5-7 days of use if fully charged. Memory and battery lights on the front face indicate the following:

#### ***Battery:***

Green (solid) = more than 24 hours of battery life remain

Amber (flashing) = less than 24 hours of battery life remain

Red (flashing) = battery needs to be charged immediately and the armband cannot collect data

#### ***Memory:***

Green (solid) = more than 24 hours of memory life remain

Amber (flashing) = less than 24 hours of memory life remain

Ref (flashing) = memory is full and the armband cannot collect additional data

The 'timestamp' function (main button on the front face) allows the user to flag time points in the data during the collection process. For example, the researcher might have the user put on the armband upon arrival at the lab, then 'timestamp' at the beginning of an exercise protocol, and 'timestamp' the end of the protocol. Then the data of interest can be easily and accurately located within the minute-by-minute excel summary.

### Data Review:

Plug the armband into the computer and open the software. Click "Retrieve" to save all existing data from the armband to the computer, and keep the "clear armband for next use, after data has been saved" option selected, so the armband memory is cleared for next use. This will save data as a BodyMedia file. To save as an excel file, proceed to the "View & Annotate Armband Data" tab, and click "Export". This will save the data in excel format, which can then be viewed and analyzed on other computers without the SenseWear software.

Column A in the excel file shows the time of each minute-by-minute sample. Column R shows EE in kcal/min and Column W shows METs. Column X will display timestamps as a "1".



### The 2-Day Adaptation Study Diet:

Resting energy expenditure, and habitual energy expenditure will be measured through the use of the Sensewear Body Media Armband Accelerometer. This will provide us the necessary information that will be used to design the 2-day adaptation study diet. The adaptation study diet will be consumed for two days prior to each metabolic trial day. Participants will also complete a 3-day food record, which will provide important information regarding dietary intake, and the typical foods that each participant consumes.

For 2-days before each metabolic trial, participants will consume a diet providing 1.2 g/kg/d of complete protein, with enough energy to cover the REE and habitual energy expenditure as measured by the accelerometer. The adaptation diet will be comprised of normal solid foods. The background <sup>13</sup>C content will be kept relatively stable through the feeding of foods with similar <sup>13</sup>C compositions.

### The PRO-Free Breakfast:

The protein-free breakfast will be comprised half of polyose, and half of Gatorade powder dissolved in water. Polyose (a glucose polymer) provides a carbohydrate source that is rapidly absorbed within the body. The dosage of both polyose and the Gatorade powder in the PRO-free breakfast will be 0.5g of polyose per kg of body mass of the participant (i.e. an 80kg participant will consume 40g of polyose dissolved in water, 40g Gatorade powder). This will provide enough energy for the participant to complete the exercise stimulus after fasting overnight (i.e. 80g CHO = ~320kcal). Non-sugar sweeteners may be used to enhance the taste of the taste of the solution.

How to Make 1kg Amino Acid Mix:

- The study-day amino acid mix will be comprised of all amino acids **EXCEPT L-PHE and L-TYR**
- It is modeled from the amino acid composition of egg protein
- The quantities of each amino acid required for the mix in mg are listed below under '**AA MIX #1**'
- Ensure to use a precise scale (at least 3 decimal places) when adding each amino acid to the mix
- **IMPORTANT:** Wash all implements after adding each amino acid to avoid contamination
- **IMPORTANT:** Ensure to clean the scale (with a brush) from amino acid residue frequently
- **IMPORTANT:** Before partitioning the amino acid mix into meals for study day, shake the mix thoroughly
- **FOR PILOT:** The corresponding amounts of L-PHE AND L-TYR will be added in to the mix separately

Amino Acid Composition for Female Elderly Protein Requirement				
Amino Acid	Egg Protein from GSH Study (g/kg)	Egg Protein from Raja's Children Prot Req Study (g/kg)	Amino Acid	AA MIX #1 (mg/kg/d)
L-Alanine	61.00	61.50	<b>L-Alanine</b>	61.50
L-Arginine-HCL	74.54	75.10	L-Arginine-HCL	75.10
L-Asparagine	33.00	33.30	L-Asparagine	33.30
L-Aspartic Acid	33.00	33.30	L-Aspartic Acid	33.30
L-Cysteine	21.94	22.10	L-Cysteine	22.10
L-Glutamine	56.20	56.60	L-Glutamine	56.60
L-Glutamic Acid	56.20	56.60	L-Glutamic Acid	56.60
L-Glycine	33.00	33.30	L-Glycine	33.30
L-Histidine	22.53	22.70	L-Histidine	22.70
L-Isoleucine	62.35	62.80	L-Isoleucine	62.80
L-Leucine	82.64	83.30	L-Leucine	83.30
L-Lysine-HCL	75.12	75.70	L-Lysine-HCL	75.70
L-Methionine	29.45	29.60	L-Methionine	29.60
L-Phenylalanine	54.24	54.70	<b>L-Phenylalanine</b>	
L-Proline	41.62	41.90	L-Proline	41.90
L-Serine	83.24	83.90	L-Serine	83.90
L-Threonine	46.73	47.10	L-Threonine	47.10
L-Tryptophan	15.48	15.60	L-Tryptophan	15.60
L-Tyrosine	40.42	40.70	<b>L-Tyrosine</b>	
L-Valine	69.72	70.30	L-Valine	70.30
<b>Total</b>	<b>992.42</b>	<b>1000.10</b>		<b>904.70</b>
	<b>100%</b>	<b>100%</b>		<b>90.46%</b>

## Making the Study Day Meals:

### **The Study Day Meals are Comprised of 9 Ingredients:**

- 1) Water
- 2) Tang & Polycose
- 3) Protein Free Powder
- 4) Grapeseed Oil
- 5) Amino Acid Mix
- 6) Tyrosine
- 7) Unlabeled Phenylalanine
- 8) <sup>13</sup>C-PHE (Labeled Phenylalanine)
- 9) Bicarbonate

### **Directions for Making Study Day Meals:**

- \* The breakdown of all meal components is provided in the excel document, and will be printed prior to each trial
- \* The components in each meal will change depending on the participant's weight (kg), energy expenditure (kcal), and test protein intake (g/kg) that they will be consuming on that particular trial day
- \* Ensure to weigh cookies for each trial day to be consumed in addition to the formula

- 1) Weigh and partition the amino acid mix powder into each of the 8 study day containers
- 2) Weigh the tyrosine, phenylalanine, tracer PHE, and bicarbonate required for each of the 8 study day containers  
- Tracer PHE only in meals 5-8, Bicarbonate only in meal 5 (**BE VERY PRECISE**)
- 3) Weigh and add the required amount of water to the blender first (to prevent sticking)
- 4) Weigh and add Tang & Polycose to the blender, blend until homogeneous
- 5) Weigh the protein free powder, *but do not add it to the blender*
- 6) Before adding the protein-free powder to the blender, dissolve the oil in the protein-free powder
- 7) Scrape the protein-free powder and oil into the blender
- 8) Blend until all ingredients are uniformly dissolved into a homogenous solution
- 9) Pour the formula equally into each of the 8 study day containers using the weight on the food balance  
- Blend the mix periodically between pouring it into each of the 8 study day containers
- 10) Shake well, and store in freezer, or fridge (if using drinks within 24 hours)

## ***CORNFLAKE CHERRY COOKIES***

<b>WEIGHT:</b>	<b>INGREDIENTS:</b>
306 GM	WHEAT STARCH
178 GM	MILK FREE MARGARINE
100 GM	WHITE SUGAR
10.5 GM	CORN SYRUP
10 GM	WHIPPED TOPPING
0.5 ML	SALT
5 ML	ALMOND EXTRACT
34 GM	CORNFLAKES-crumbled
100 GM	MARASCHINO CHERRIES-finely chopped

### PREP METHOD:

Cream together sugar and margarine, add corn syrup, almond extract & whipped topping.

Stir in wheat starch, salt and crumbled cornflakes in creamed mixture.

Add chopped cherries.

Roll into 1 tbsp balls and place onto cookie sheet. Using a fork, lightly press down each cookie to flatten dough.

Bake at 350 F for approximately 10-12 minutes or until fluffy and firm to the touch.

\*\*\*when weighing cookies, weigh frozen. These cookies crumble easily\*\*\*

## ***BUTTERSCOTCH COOKIES***

<b>WEIGHT:</b>	<b>INGREDIENTS:</b>
240 GM	WHEAT STARCH
113 GM	BUTTERSCOTCH PUDDING MIX
95 ML	COLD WATER
89 GM	VEGETABLE SHORTENING
59 GM	MILK FREE MARGARINE
41 GM	BROWN SUGAR
25 GM	WHITE SUGAR
7.5 ML	PURE VANILLA EXTRACT
7.5 ML	BAKING POWDER
6 GM	EGG REPLACER
2 ML	SALT

### PREP METHOD:

Cream together shortening and margarine. Add white, brown sugar, pudding mix and egg replacer.

Add water and vanilla extract. Mix well.

Mix in wheat starch, salt and baking powder to creamed mixture. Mix until fluffy.

Roll into 1 tbsp balls and place onto cookie sheet. Using a fork, lightly press down each cookie to flatten dough.

Bake at 375 F for approximately 10-12 minutes or until fluffy and golden brown.