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

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science Graduate Department of Exercise Sciences University of Toronto
Abstract

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2017

We aimed to determine the protein requirements of active adolescents by utilizing the minimally invasive, Indicator Amino Acid Oxidation (IAAO) technique. Seven male participants (n=7; 13.8±0.5 years; 171±2.8 cm; 57.3±4.2 kg; 48.4±5.0 kg FFM; 0.3±0.2 years from peak height velocity (PHV); means±95% CI) each underwent 6 metabolic trials that included an acute bout of a variable intensity exercise, followed by the ingestion of 8 hourly meals providing a variable amount of protein (0.2 – 2.67 g·kg\(^{-1} \cdot d^{-1}\)), 6 g·kg\(^{-1}\) of carbohydrate, and sufficient energy. Protein was provided as crystalline amino acid mixtures, modeled after the contents of egg protein, apart from tyrosine (40 mg·kg\(^{-1} \cdot d^{-1}\)) and phenylalanine (30.5 mg·kg\(^{-1} \cdot d^{-1}\) of which 5.46 mg·kg\(^{-1}\) was L-[\(^{13}\)C] phenylalanine over the final 4 drinks). \(^{13}\)CO\(_2\) enrichment in breath was determined by continuous-flow isotope ratio mass spectrometry and CO\(_2\) production by indirect calorimetry. Bi-phase linear regression indicated a breakpoint of the \(^{13}\)CO\(_2\) excretion (F\(^{13}\)CO\(_2\)) equivalent to an estimated average requirement (EAR) of 1.48 g·kg\(^{-1} \cdot d^{-1}\) (\(r^2 = 0.54\)), with the upper 95% CI, which approximates the recommended dietary allowance (RDA) of 1.78 g·kg\(^{-1} \cdot d^{-1}\). Our estimate exceeds the current protein RDA based on the factorial estimate of nitrogen balance for adolescents (0.9 g·kg\(^{-1} \cdot d^{-1}\)). Therefore, suggesting that variable intensity exercise, as well as, the incorporation of a new determining method, increases daily protein requirements in active adolescents.
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List of Abbreviations

NBAL – Nitrogen Balance
IAAO – Indicator Amino Acid Oxidation
LBM – Lean Body Mass
FM – Fat Mass
PHV – Peak Height Velocity
APHV – Age of Peak Height Velocity
MVPA – Moderate to Vigorous Physical Activity
GH – Growth Hormone
TH – Thyroid Hormone
IGF-1 – Insulin like Growth factor
T – Testosterone
BMC – Bone Mineral Content
EAA – Essential Amino Acids
NEAA – Non-essential Amino Acids
ATP – Adenosine Triphosphate
RDA – Recommended Dietary Allowance
EAR – Estimated Average Requirement
FAO – Food and Agriculture Organization of the United Nations
WHO – World Health Organization
DAAO – Direct Amino Acid Oxidation
PAR-Q – Physical Activity and Readiness Questionnaire
IPAQ – International Physical Activity Questionnaire
LIST – Loughborough Intermittent Shuttle Test
A.P.E. – Atoms Percent Excess
Phe Ox – Phenylalanine Oxidation
Phe Ra – Phenylalanine Flux
CI – Confidence Interval
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Chapter 1. Literature Review

1.1 – Introduction

The growth that occurs during puberty in adolescence includes a linear growth spurt, the accrual of lean body mass (LBM), and other secondary sexual maturations that alter body size, shape, LBM composition, and fat mass (FM) distribution (Tanner et al., 1975). These physiological changes create an increased demand for energy and nutrients. Nutritional needs are higher during adolescence than at any other time in the life cycle (Torun, 2005). Adequate ingestion of nutrients is a requisite for achieving full growth potential and development.

Regular physical activity and sport participation is important for healthy growth and development. When participating in sports, adolescents typically engage in variable intensity team sports (i.e. soccer, basketball, hockey), which leads to greater energy requirements. The consumption of additional nutrients play an important role in counterbalancing the energy expended during sport play (Forbes, 1987). A recent analysis of both pre-adolescent and adolescent Canadian athletes was conducted to determine whether athletes of this population were consuming sufficient (as determined by the general recommendations) macro and micronutrients (Parnell et al., 2016). Results revealed that males between 11-13 years of age (N=26) consume 2.4 ± 0.8 g·kg\(^{-1}\)·d\(^{-1}\) of protein, while males between the 14-18 years of age (N=53) consume 2.0 ± 0.6 g·kg\(^{-1}\)·d\(^{-1}\) of protein (Parnell et al., 2016). These determined values are greater than current recommendations, which is encouraging, seeing that nutrition and physical growth are related. A failure to consume adequate quantities of dietary amino acids can increase the risks of delayed sexual maturation, arrested or attenuated linear growth, and potentially lead to the development of diet-related chronic diseases, such as, cardiovascular and respiratory diseases (Finkelstein et al. 1992, Rogol et al., 2002, Kulin et al., 1982).
A variety of methods have been utilized to estimate protein requirements in the general population, most common being the nitrogen balance technique (NBAL). However, these methods have been primarily used to study requirements in adults, consequently, the protein requirements of adolescents have been generally estimated using the factorial approach. This approach assumes that basal requirements are the same throughout the life cycle. The basal requirements (derived from NBAL data in adults) are added to a growth component which accounts for the body protein changes that occur with age (Pencharz et al., 2006). Recent methodological developments have questioned the adequacy of current protein recommendations for adults that are based on the NBAL technique, which suggests that the recommendations for adolescents are questionable as well, and must be re-evaluated. For example, recent application of the minimally invasive Indicator Amino Acid Oxidation (IAAO) technique to re-evaluate protein requirements in adults and children suggest that the current recommendations determined by the NBAL technique may be underestimations (Humayun et al., 2007; Elango et al., 2011), and therefore the true protein requirements in adults and children remain a point of open debate. Furthermore, the additional demands that physical activity and sport participation elicit deems it essential to determine accurate protein requirements in an active adolescent populations that can be used as guidelines to ensure optimal nutrition, growth and development.

Thus, the aim of this study was to evaluate, for the first time, the impact of variable intensity exercise on protein requirements in active, adolescent males utilizing the IAAO technique. It was hypothesized that our findings would i) yield higher protein requirements than comparative values established using NBAL and the factorial method in adolescents, and ii) confirm that physical activity increases protein requirements in active adolescents compared to what is currently recommended for their sedentary counterparts.
1.2 - Growth and Development

1.2.1 – Normal Growth

Normal growth is a strong testament to the overall good health of a child. Growth can be attributed to many factors, some of which are inherited, while others can be influenced by the environment and lifestyles in which the child developed. These factors can act independently or harmoniously to modify a child’s growth potential. The rate at which a child grows is individualized; however typical growth patterns have been established for comparative purposes (Baxter-Jones et al., 2008).

At birth, rapid growth is observed such that children tend to grow ~25 cm in the first year of life, ~12 cm in the second year, and by ~6 cm each year after that until puberty (Tanner, 1989). Puberty marks the most rapid linear growth since infancy (Tanner et al., 1975) and is characterized by hormonal and physiological changes that alter body size, shape, LBM composition, and FM distribution. These changes are sexually dimorphic given that females generally accumulate more FM whereas males generally develop more LBM. In boys, puberty tends to begin at a chronological age of ~12 years, with the initiation of increased LBM accrual. It is not until ~13.5 years of age when boys hit their peak height velocity (PHV), which is the period in which maximal growth occurs and large physiological changes are observed (Mirwald et al., 2002). Children mature at individualized rates; therefore, chronological age is of limited utility in the assessment of individual growth and maturation. To account for these differences, age of peak height velocity (APHV) is the most commonly used method in determining what stage of development the child is in (Malina, 2000). Maturity offset is the measured value that determines years before or after PHV and can be calculated using specific equations differentiated by sex. (Appendix A). Maturity
offset values range from negative to positive, with a value of 0 indicating the child being at PHV (Mirwald et al., 2002).

The hallmark of puberty is the growth spurt, in which boys on average gain height ~10.3 cm/year, weight at a rate of ~9 kg/year, compared to ~3 kg/year pre-pubertal, and decrease their body fat by ~1.15 kg/year (Tinggaard et al., 2012; Marshall et al., 1970; Malina, 1991). This rapid growth is dependent primarily on hormone level increases in the body. Growth hormone (GH) promotes protein synthesis, inhibition of fat formation, and the proliferation of collagen synthesis responsible for linear growth in the bones (Soliman et al., 2014). Thyroid hormone (TH) promotes cartilage and bone formation. Cortisol increases blood glucose concentration preparing the body for upcoming physiological growth changes. Insulin-like growth factor (IGF-1) promotes cell proliferation, muscle tissue growth through stimulation of glycogen accumulation, and formation of collagen (Soliman et al., 2014). Testosterone promotes sexual maturation, increases in bone density, muscle growth, and decreases in limb fat, which inherently, increases lean body mass (Arslanian et al., 1997; Tanner et al., 1965). Although hormones play unique roles during puberty, the interactions of these hormones are essential for a normal growth spurt and sexual maturation to occur in an adolescent. The rapid physiological changes observed during puberty, specifically the addition of LBM, creates an increased rate of protein turnover (protein synthesis and protein breakdown), requiring an increased supply of endogenous and dietary protein to support the LBM growth (Soliman et al., 2014). While increases in hormone levels play a major role in normal growth and development, exercise and nutrition are vital components for normal growth and development patterns as well.
1.2.2 – Effects of exercise

In adolescents, regular physical activity and sport participation is important for healthy body function, development, and growth (Armstrong et al., 2005). It is recommended for children and adolescents to perform at least 60 minutes of moderate-to-vigorous physical activity (MVPA) daily (Tremblay et al., 2011). Included in this recommendation are aerobic activities such as walking, playing tag with friends, or participating in aerobic team sports (e.g., soccer, basketball) at least 3 times per week. In addition, muscle-strengthening activities such as biking, swimming, hiking, and climbing are also encouraged at least 3 times per week (Tremblay et al., 2011).

Regular physical activity and sport participation has been shown to increase the accrual of LBM (Baxter-Jones et al., 2008), increase bone strength, reduce fat mass, and decrease the risk of developing cardiovascular or respiratory complications (Finkelstein et al., 1992; Rogol et al., 2002; Kulin et al., 1982). A longitudinal study by Baxter et al. (2008) used 109 physically active adolescent males to identify the influence of physical activity on LBM accrual during puberty. Through bi-annual assessment of body mass, physical activity levels, stature, and body composition, results showed that habitual physical activity had a significant independent influence on LBM growth of the total body in adolescent males during puberty (Baxter-Jones et al., 2008). In addition, involvement in sport improves socialization among peers, respect for rules and scholastic learning, as well as, reduce abnormal social behaviors (Ruggeri et al., 2004).

The active load forces, such as the pull on a bone from muscle contraction experienced during stop and go type activities (e.g. running, jumping), causes mechanical strains and deformation of the bones (Vicente-Rodriguez, 2006). These strains cause the body to be in a state of remodeling, and as a response, the body increases bone mass to improve structural support. The continued repetition of these load forces also enhances muscle mass, increases strength and power,
and allows for further LBM growth to occur in addition to that which develops during puberty (Vicente-Rodriguez, 2006). A longitudinal study by Rauch et al. (2004) postulated that the development of LBM precedes the increase in bone strength development (Rauch et al., 2004). This study used 138 adolescents who were examined during pubertal growth, and measured LBM and bone mineral content (BMC) in the arms, lower extremities, and overall total body accrual. Results showed that the development of BMC was secondary to the increase in LBM, and that bone development is driven by the accrued muscle (Rauch et al., 2004). Therefore, the addition of exercise and adequate nutritional consumption helps maximize the LBM growth and development associated with the pubertal growth spurt.

1.2.3 - General Nutrition

Proper nutrition is vital to support normal growth and development. The rapid biological and physiological changes occurring in an adolescent during puberty puts and increased importance on the consumption of adequate amounts of macronutrients to fuel these changes. Consuming a balanced and healthy diet during all stages of growth (infancy, childhood, and most importantly puberty) is necessary for both proper growth and normal pubertal development.

Childhood under nutrition has been shown to delay the onset of puberty, as well as create deficiencies in vitamins, minerals, and micro nutrients that are vital for pubertal development and growth (Tinggaard et al., 2012). This can lead to serious consequences including; impaired growth, osteopenia, and anemia (Finkelstein et al., 1992; Rogol et al., 2002; Kulin et al., 1982). Childhood over nutrition and obesity on the other hand, have been shown to significantly accelerate the onset of puberty, and shorten growth and development duration (Forbes, 1987).
The increased nutritional needs triggered by physiological changes occurring during puberty, along with the possible negative effects that under nutrition and over nutrition can have on adolescent growth and development, makes it imperative that an emphasis be put on providing a well-balanced and healthy diet to every adolescent child. This will provide the nutritional substrates to facilitate the aspects of growth and development that are related to nutrition.

1.3 – Protein Metabolism

1.3.1 – General Protein Metabolism

Protein serves many regulatory and structural purposes to the human body, including being an essential component of enzymes, antibodies, cell receptors, hormones, as well as muscle and bone. Each protein is comprised of a combination of amino acids, all of which contain an amino group (NH₂), a carboxylic acid group (COOH), and an R-group that varies depending on the amino acid. There exist 20 amino acids, of which, 9 are considered essential amino acids (EAA) because they cannot be synthesized in the body and must be obtained from the diet. The 9 EAA are; histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine. The remaining 11 non-essential amino acids (NEAA) can be synthesized within the human body, and thus, do not need to be consumed in large quantities in the diet.

Throughout the body, protein is continuously in a state of metabolic flux, commonly referred to as protein turnover, which is characterized by constant cycles of protein synthesis and protein breakdown (Figure 1; Tarnopolsky, 2004). Protein turnover can be influenced by several stimuli including (but not limited to) exercise, dietary intake, and pubertal growth spurts. The content of whole-body protein is based on the difference between the rate of protein synthesis from
free amino acids, and the rate of protein breakdown into amino acids. Therefore, the pool of free amino acids is constantly being recycled, as it serves not only to provide the necessary amino acids for synthesis, but is also being replenished by the endogenous breakdown of proteins and the exogenous ingestion of dietary proteins. The following sections will outline the major metabolic fates of proteins, and highlight the importance of adequate protein intake as it pertains to adolescent growth, physical activity effects, and the current recommended dietary allowance.

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 free flowing amino acids must be transported to the liver or kidneys to be transaminated and oxidized. – Modified from Tarnopolsky, 2004

1.3.2 – Protein Synthesis

Protein synthesis is a highly regulated process, consisting primarily of transcription and translation. Transcription occurs in the cell’s nucleus, where a signal promotes the expression of DNA encoding for a specific protein by generating a complementary mRNA template. Translation then turns that mRNA template into a functioning protein through the connections of peptide bonds.
The combination of amino acids in the blood, with the extracellular and intracellular fluids make up what is collectively referred to as the free amino acid pool, which can serve as the precursors for protein synthesis (Tarnopolsky, 2004). In a fasted state, protein synthesis occurs, but at a slower rate compared to when the body is in a fed state (Kim et al., 2005). This is because the free amino acids are provided to the amino acid pool by protein breakdown instead of ingested protein. However, some free amino acids may also be used as a source of energy via oxidation (Section 1.3.4). When this occurs, amino acids undergo structural changes and are ultimately removed from the free amino acid pool, and rendered unavailable to participate in protein synthesis (Millward, 1998; Packer et al., 2015).

Upon ingestion, protein is broken down into its amino acid components by the digestive system and absorbed into the blood. This allows the amino acid pool to consist of all 20 different amino acids, the 11 NEAA that can be generated endogenously, along with the 9 EAA that can only be obtained exogenously. Once absorbed, the amino acids enter the free amino acid pool where they can be utilized for protein synthesis. Whole body protein synthesis is augmented in a fed state due to the increased availability of free amino acids for synthesis (Bohe et al., 2003; Svanberg et al., 1998; Tipton et al., 1998). This suggests that being in a fed state maximizes whole body protein synthesis by replenishing the free amino acid pool with both NEAA and EAA through the consumption of protein.

1.3.3 – Protein Breakdown

Within the human body, protein breakdown is accomplished by proteases. Three major systems serve to degrade proteins: the ubiquitin-proteasome pathway, the lysosomal system, and the calpain system (Belcastro et al., 1998; Lecker et al., 1999). Proteases function to hydrolyze
that peptide bond that holds amino acids together, by doing this, it cleaves individual amino acids from the protein molecule. Once cleaved, amino acids then enter the free amino acid pool in the blood or extracellular fluid. Upon entry, these free amino acids can now be re-synthesized into structural or regulatory proteins and/or be oxidized as an energy source. The primary function of protein breakdown is to replenish the free amino acid pool via protein degradation. The rate at which protein breakdown occurs can be altered by factors which include, but are not limited to, exercise, nutritional consumption, and energy demands.

1.3.4 – Amino acid Oxidation

The process of amino acid oxidation uses protein as an energy source to produce adenosine triphosphate (ATP) while also removing the body of any excess amino acids (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 the amino acid to another molecule (usually α-ketoglutarate). This leaves behind a carbon-skeleton of the transaminated amino acid that can be reassembled into a different molecule such as Acetyl CoA, and can enter the Citric Acid Cycle in the mitochondria to be used as a substrate to produce ATP (Belcastro et al., 1998). Deamination is the process that takes place primarily in the liver (Lecker et al., 1999) 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 in the liver via the addition of CO₂ prior to its subsequent urinary or breath 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 via the Citric Acid Cycle (Millward, 1998).
1.3.5 – Effects of nutrition

The consumption of dietary protein and other macromolecules have variable effects on the metabolic fates of protein. The addition of ingested protein puts the body in a fed state, causing the free amino acid pool to expand. The increase in free amino acid availability allows for an accelerated rate of protein synthesis (Tipton et al., 1998). In addition, the replenishment of the free amino acid pool decreases the need for amino acids via protein breakdown, and thus, slows down the rate at which protein breakdown occurs (Bohe et al., 2003). Excessive consumption of dietary protein causes the body to have a surplus of amino acids. Unlike carbohydrate and fat, amino acids cannot be stored to a great extent within the human body. Therefore, any excess amino acids not needed for synthesis or replenishment of the amino acid pool are readily oxidized from the body as a source of energy via deamination and transamination (Millward, 1998; McKenzie et al., 2000; Smith et al., 1996). Conversely, the inadequate consumption of dietary protein results in decreased rates of protein synthesis, and increased rates of protein breakdown. The increase in protein breakdown (especially within skeletal muscle) is to maintain the free amino acid pool in the absence of dietary intake (Rennie et al., 2000). This suggests that the metabolic fates of protein, and the rates at which protein turnover occurs can be altered by the consumption or inadequate consumption of dietary proteins.

1.3.6 – Effects of exercise

The increased energy and protein demands associated with aerobic and muscle strengthening physical activity must be adequately met with optimal consumption of dietary protein in an effort to help build LBM, replenish energy deficits, and recover from repetitive load muscle damage (Baxter-Jones et al., 2008; Vicente-Rodriguez, 2006). The effects of aerobic and
muscle strengthening activity have been well documented in adults (Burd et al., 2009). Exercise elicits an increase in the rate of protein synthesis, primarily within the muscles, while putting a higher demand on the consumption of dietary protein (Burd et al., 2009). Various studies have determined that exercise significantly increases the rate of protein breakdown in the muscles as well, with the intent of replenishing the amino acid pool and providing a source of amino acids to support the increased rates of protein synthesis, and allow for the repair of damaged muscle fibers, and LBM growth (Figure 2; Phillips et al., 1997; Phillips et al., 1999; Biolo et al., 1995; Chesley et al., 1992; Yarasheski et al., 1993). There have been limited studies conducted that pertain to the effects of exercise and physical activity in adolescents (Boisseau et al., 2007; Aerenhouts et al., 2013). It is believed that the effects of physical activity seen in active adults would be similar to those seen in the active adolescent population (Boisseau et al., 2007). This suggests that the increased rates of protein turnover observed with the addition of physical activity is an attempt to support the replenishment of amino acids that were oxidized as a source of energy, recover from exercise induced muscle damage, and help build LBM.

Figure 2. The impact of resistance exercise, and amino acid consumption on protein synthesis and breakdown. – Rasmussen et al., 2003
1.3.7 – Effects of growth

In boys, the pubertal growth spurt begins at ~12 years of age and is characterized by increased rates of linear growth, weight gain, LBM accretion, and overall physiological maturation (Malina, 1991). To support these changes, it was hypothesized that the efficiency of protein utilization is increased during puberty by decreasing protein oxidation and increasing protein retention (Beckett et al., 1997). These efficiency changes are suggested to be related to the myriad of hormonal changes that occur during puberty (Arslanian et al., 1997). Concentrations in plasma free insulin, GH, IGF-1, and IGF binding protein 3 (IGFBP-3) were shown to correlate positively with protein retention, and are assumed to be main candidates for altering the efficiency of protein utilization during puberty (Beckett et al., 1997). Throughout puberty, bone strength increases and LBM growth are promoted by TH and GH respectively, by accelerating the rates of protein synthesis. Muscle tissue growth is promoted by IGF-1, while testosterone promotes increased muscle growth and other physiological maturations through the decrease of postprandial protein breakdown and protein oxidation rates (Tanner et al., 1965; Arslanian et al., 1997). The need for amino acids to support the increased rates of protein synthesis puts a significant importance on the consumption of optimal dietary protein to supplement the physiological growth and changes that naturally occur during the pubertal growth spurt.

1.3.8 – Estimated Average Requirement & Recommended Dietary Allowance

As previously mentioned, the results of NBAL method studies are commonly used to estimate dietary protein recommendations. Unfortunately, however, these studies have been used to investigate the protein requirements of primarily adults. Consequently, the protein requirements of adolescents have generally been estimated using the factorial method (Dewey et al., 1996),
which assumes that basal requirements are the same throughout the life cycle. The basal requirements are then added to a growth component, which estimates the amount of LBM growth accrual that will occur at that stage of life (Pencharz et al., 2006). To be considered in a sufficient nitrogen balance range, enough to support the growth and development associated with the adolescent growth spurt, a positive balance of 11 mg of nitrogen·kg\(^{-1}\)·d\(^{-1}\) is required (Boisseau et al., 2007). As a result, the current recommendations suggest that adolescents should consume a daily estimated average requirement (EAR) of 0.78 g·kg\(^{-1}\)·d\(^{-1}\) (FAO/WHO, 2007). The EAR represents the minimum level of protein required to offset daily losses of nitrogen in 50% of the population (Figure 3). However, the EAR may not sufficiently represent 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 (SD). A single SD is determined by calculating the square root of the variance, variance being the average of the squared differences from the mean. The addition of 2 SD is designed to supply 97.5 % of the population with an adequate amount of dietary protein, and is currently set at 0.90 g·kg\(^{-1}\)·d\(^{-1}\) for the adolescent population (FAO/WHO, 2007). These recommendations in comparison to the adult male (0.66 g·kg\(^{-1}\)·d\(^{-1}\) (EAR) & 0.80 g·kg\(^{-1}\)·d\(^{-1}\) (RDA)), are higher because they were determined using the factorial approach, which assumes that all basal requirements are similar throughout the life cycle and thus can be determined from adult requirements. From there, an addition amount of protein was added to the adolescent requirement in attempt support the LBM growth and development that occurred during the rapid growth spurt associated with puberty (FAO/WHO, 2007).
Figure 3. The EAR and RDA. The EAR corresponds to the average requirement of 50% of the population, while the RDA encompasses the level of protein required to ensure 97.5% of the population is not deficient – Packer et al., 2015

When discussing the protein requirements of active individuals, the addition of aerobic physical activity has been shown to increase protein requirements in the active adult population (Packer et al., 2015; Kato et al., 2016). Subsequently, to help replenish amino acids that were oxidized as energy sources, assist in muscle damage recover, and build greater LBM, it is recommended that active adults consume between 1.64 - 1.83 g·kg\(^{-1}\)·d\(^{-1}\) of dietary protein (Packer et al., 2015; Kato et al., 2016). There has been very limited research conducted in the active adolescent population, but existing studies have concluded that the addition of physical activity, along with the demands of the pubertal growth spurt, increases dietary protein requirements by ~54%, when comparisons are made between the current protein EAR for sedentary adolescents (0.78 g·kg\(^{-1}\)·d\(^{-1}\); FAO/WHO, 2007) and the suggested protein EAR for active adolescents (1.2 g·kg\(^{-1}\)·d\(^{-1}\); Boisseau et al., 2007; Aerenhouts et al., 2013).
1.4 – Methods of Determining Protein Requirements

1.4.1 – Nitrogen Balance (NBAL)

The NBAL method has been the most commonly used method to determine protein requirements for decades (Rand et al., 2003). NBAL involves measuring the total amount of nitrogen from protein taken into the body through dietary nutrient consumption or infusion, and the total amount of nitrogen excreted from the body. The primary sources of nitrogen excretion come from feces, urine, and sweat loss (Rand et al., 2003; Young et al., 1989). The nitrogen difference is obtained when the total amount of nitrogen excreted is subtracted from the total amount of nitrogen taken in. To be considered at “nitrogen balance”, the difference between the two values should be equal to zero. When the total amount of nitrogen taken in is greater than the total amount of nitrogen excreted, the body is said to be in a positive NBAL environment, which would lead to a net gain of protein or to be in an anabolic state (1 g nitrogen is estimated to account for 6.25 g of protein; FAO, 1973). Conversely, when the total amount of nitrogen excreted is larger than the total amount of nitrogen taken in, the body is said to be in a negative NBAL environment, which leads to a loss of protein or to be in a catabolic state (Tarnopolsky, 2004). For individuals aiming to gain LBM, such as growing adolescents, NBAL should be positive.

The NBAL method requires a 7 - 10-day diet adaptation period for each level of protein intake. Upon adaptation, nitrogen losses in feces, and urine are measured while miscellaneous nitrogen losses are estimated for which allows for the calculation of NBAL at each protein intake level (Tarnopolsky, 2004). The values of both nitrogen intake and nitrogen excretion are then plotted and a linear regression analysis is used to determine the x-intercept, which correlates to be the overall zero net balance or EAR. A minimum positive nitrogen balance of 11 mg of
nitrogen·kg\(^{-1}\)·d\(^{-1}\) is suggested to be needed to support the growth and accrued LBM associated with puberty (Boisseau et al., 2007). The single linear regression is traditionally used as the model for analysis, but, it has been shown that using a mixed model bi-phase linear regression, to better model the curvilinear data, delivers more accurate measurements with regards to nitrogen balance and protein requirements (Humayun et al., 2007). From there, a buffer of two standard deviations is added to determine the RDA which should encompass approximately 97.5% of the studied population (Rand et al., 2003; Tarnopolsky, 2004; Young et al., 1989). The current EAR and RDA values of 0.78 g·kg\(^{-1}\)·d\(^{-1}\) and 0.9 g·kg\(^{-1}\)·d\(^{-1}\) respectively, for adolescents provided by the Food and Agriculture Organization of the United Nations (FAO) and the World Health Organization (WHO) are based on the determination of adult protein requirements, using the NBAL method, plus the addition of a growth allowance, which estimates the requirements of growth during adolescence (FAO/WHO, 2007).

### 1.4.2 – Strengths of NBAL

There are multiple advantages associated with the use of the NBAL method for determining protein requirements. First, NBAL presents very minimal risks to participants partaking in studies. NBAL presents a non-invasive method for determining protein requirements. Neither infusion, nor muscle biopsies are required to perform the NBAL method, making it minimal risk to most demographics. Additionally, NBAL presents a relatively simple methodology for determining protein requirements. Dietary consumption can easily be controlled for, and the collection of nitrogen excretion (primarily through urine and feces) can be analyzed with particularly simple methods. Although there are strengths to using the NBAL method, there are some limitations associated with the technique.
1.4.3 – Limitations of NBAL

Although, NBAL is the most commonly used method for determining protein requirements, several limitations exist which may question the accuracy of NBAL in determining estimates of protein requirements. Studies have suggested that NBAL tends to underestimate true values of nitrogen excretion due to the difficult nature of measuring secondary losses of nitrogen (nails, sweat, hair, exhalation) (Forbes, 1973; Humayun et al., 2007). These losses are often estimated at 15 mg of nitrogen·kg⁻¹·d⁻¹ based on data previously studied (Boisseau et al., 2002; Aerenhouts et al., 2013). With an underestimated nitrogen excretion value and an accurate nitrogen intake value, that would result in an abnormally high nitrogen balance, which in turn, would cause for an underestimation of overall protein requirements (Humayun et al., 2007). The underestimated requirements would be even greater when dealing with athletes, who regularly can lose between 21-130 mg of nitrogen per 100 mL of sweat during training (Consolazio et al., 1963). This would lead to an even greater nitrogen loss, and cause the nitrogen ingestion/excretion difference to be even smaller.

The NBAL method requires subjects to consume a controlled adaptation diet for an extensive period (minimum 7 days), in an attempt to adapt to the level of assigned protein intake. In populations such as children and adolescents, potential health risks such as muscle atrophy, bone weakening, loss of immune system strength, and fatigue, arise during intakes of deficient protein for the allotted extensive period. Additionally, adaptation diets of this duration may be prone to compliance issues in a younger population. Deviation from the adaptation diet in any way will cause inaccurate nitrogen intake calculations and result once again in inaccurate protein requirement estimations. In addition, the traditional linear regression statistical method used in NBAL analysis has been shown to produce underestimated values when analyzing data. The
relationship between nitrogen intake and nitrogen balance is curvilinear because the efficiency of protein utilization decreases as a net zero balance is approached (Humayun et al., 2007). Instead, the utilization of a bi-phase linear regression has shown to produce increased estimated protein requirements when applied to data from linear regression nitrogen balance studies (Humayun et al., 2007). Finally, the limited amount of protein intakes tested (between 2-3), leaves room for error when analyzing data and estimating protein requirements. Therefore, the utilization of new methodologies is required to accurately determine protein requirements in the human body.

1.5 - The Indicator Amino Acid Oxidation (IAAO) Technique

1.5.1 – Measuring Whole Body Protein Turnover using Stable Isotope Tracers

As previously mentioned, most studies addressing protein requirements use the NBAL method (Pencharz et al., 2003). However, in the late 1980’s, once the limitations associated with NBAL were acknowledged, different methods for determining protein requirements using stable isotopes were developed (Pencharz et al., 2003). In earlier studies, the L-[1-13C] leucine tracer was given as a continuous infusion for 2 hours until a baseline plateau was reached in arterial leucine enrichment or venous α-ketoisocaproic acid enrichment (Matthews et al., 1980). The enrichment of this amino acid pool was assumed to reflect the same enrichment for all amino acid pools from which whole-body protein exchange occurred (Wagenmaker et al., 1999). The rate of appearance of this free amino acid pool was then calculated from the tracer dilution observed at steady-state according to the formula $Q = \frac{i(E_i/E_p - 1)}{E_i}$, where $Q$ is the turnover, $i$ is the infusion rate of the tracer, $E_i$ is the enrichment of the infused tracer, and $E_p$ is the enrichment of the plasma precursor pool (Wagenmaker et al., 1999). At steady state, $Q$ equals the rate of appearance (synthesis + excretion),
as well as, the rate of disappearance (breakdown + dietary consumption; Figure 4). Therefore, by being able to measure excretion through breath $^{13}$CO$_2$ enrichment, and recording dietary consumption, the protein breakdown and synthesis can then be calculated (Wagenmaker et al., 1999). Overall, the L-[1-$^{13}$C] leucine tracer method is considered a fair estimate of whole-body protein metabolism using stable isotope tracers.

Figure 4. General model of protein metabolism used in the whole-body methods. Q is the whole-body nitrogen turnover. Ra is the rate of appearance in the free amino acid pool. Rd is the rate of disappearance from the free amino acid pool. Phe represents phenylalanine. Tyr represents tyrosine. – Wagenmaker et al., 1999

1.5.2 – History of the IAAO Technique

In the early 1980’s the IAAO technique was first implemented in young pigs in two studies that sought to determine amino acid requirements for feeding (Kim et al., 1983; Ball et al., 1984). In 1986, a group of direct amino acid oxidation (DAAO) studies reported increased amino acid requirements for leucine, valine, and lysine for humans compared to those that were being prescribed by FAO and WHO (Pencharz et al., 2003). The DAAO studies required participants to
ingest crystalline amino acid mixtures in sufficient amounts to maintain nitrogen balance. The \(^{13}\text{C}\) labelled test amino acids (leucine, valine, and lysine) were then infused at various intakes to determine their individual requirements (Meguid et al., 1986; Meredith et al., 1986). The results of the studies warranted future research to develop a stable, non-invasive isotope protocol that could be used to determine protein requirements in humans. In 1993, the IAAO technique was used for the first time in humans (Pencharz et al., 2003). The results of the study were similar to those of the DAAO results, in that the protein requirements previously determined by NBAL were underestimates (Zello et al., 1990; Zello et al., 1993). Since then, the IAAO technique has been modified and utilized in multiple protein requirement studies (Humayun et al., 2007; Elango et al., 2011; Packer et al., 2015; Wooding et al., 2015; Kato et al., 2016).

1.5.2 – *Methodological Application of the IAAO Technique*

The IAAO technique uses a stable isotope (\(^{13}\text{C}\)) to specifically label a single amino acid and use it as an indicator (most commonly Phenylalanine) (Pencharz et al., 2003; Zello et al., 1995). The indicator amino acid will always be given in excess of the protein requirement (30.5 mg · kg\(^{-1}\) · d\(^{-1}\)), while the remaining amino acids will be given in a range from deficient to excess of the protein requirement through the consumption of crystalline amino acids which are based on the amino acid composition of egg protein (FAO/WHO, 2007). During intakes of deficient amino acids, while the indicator amino acid is consumed in excess, all amino acids cannot be used optimally for protein synthesis, therefore, the oxidation of amino acids will increase (Zello et al., 1995). As the limiting test protein intakes are increased, a smaller amount of indicator amino acid oxidation will occur, thus, a larger portion of the amino acids will be utilized for protein synthesis (Figure 5; Zello et al., 1995). However, once the protein requirement for the test amino acid is reached, no further decrease in indicator amino acid oxidation will occur (Zello et al., 1995).
Additionally, no further protein synthesis is expected to be seen once the test protein intake exceeds the protein requirement. The point at which no further decrease in indicator amino acid oxidation (via $^{13}$CO$_2$ production through breath and urine) is seen, regardless of increases in test protein intake, is termed the “breakpoint” (Figure 5; Zello et al., 1995). It is at this point that the EAR of the test protein is determined, and the IAAO technique establishes protein requirements for a given population. From there, two standard deviations can be added to the EAR to determine the RDA.

![Figure 5](image.png)

**Figure 5.** Trends in synthesis and oxidation during IAAO technique. Breakpoint (EAR) has been denoted with the dashed lines. – Packer et al., 2015

The attenuation of F$^{13}$CO$_2$ with greater protein intakes has been used as a proxy for the maximization of whole body protein synthesis and, thus, determination of a physiologically relevant protein requirement. For example, Ball et al., (1986) determined the impact of dietary protein intake on amino acid oxidation in young pigs (Ball et al., 1986). This study measured radioactive phenylalanine through expired carbon dioxide as well as the incorporation into liver protein and observed an inverse relationship (Ball et al., 1986). This suggested that a decrease in expired radioactive carbon dioxide is related to an increase in liver protein synthesis. Additionally,
Moore et al., (2009) measured albumin (a liver export protein) and muscle protein synthesis to determine an ingested protein dose response following resistance exercise (Moore et al., 2009). While determining a dose response, Moore et al., (2009) also displayed a similar pattern between albumin (a liver export protein) and muscle protein synthesis, in that, both increased in a dose dependent manner and plateaued at 20 g of ingested protein (Moore et al., 2009). This would suggest that if F$^{13}$CO$_2$ is minimized then there would be a corresponding maximization of liver (Ball et al., 1986) and potentially muscle protein synthesis (Moore et al., 2009).

1.5.3 – The IAAO Technique & Protein Requirements

Multiple studies have utilized the IAAO technique to determine protein requirements since it was first implemented in 1993. A study by Humayun et al. (2007) utilized the IAAO technique to determine the protein requirements for the first time in healthy, sedentary, young adult males (Humayun et al., 2007). Eight adult males were recruited for the study, in which, as performed in other studies that have used the IAAO technique, L-(1-$^{13}$C)-Phenylalanine was used as the indicator amino acid. Subjects were also given test protein intakes ranging from 0.10 - 1.8 g·kg$^{-1}$·d$^{-1}$ over the 7 trial days. Initially, Humayun et al. (2007) applied a bi-phase linear regression model to the data from 28 previously published NBAL studies that used protein intakes ranging from low to very high (Humayun et al., 2007). It was determined that the protein requirements originally determined using a linear regression model (EAR: 0.66 g·kg$^{-1}$·d$^{-1}$ and RDA: 0.83 g·kg$^{-1}$·d$^{-1}$) were significantly lower than those determined using a bi-phase linear regression model on the same NBAL data (EAR: 0.91 g·kg$^{-1}$·d$^{-1}$ and RDA: 1.0 g·kg$^{-1}$·d$^{-1}$). Humayun et al., (2007) argued that the protein requirement determined by the bi phase linear regression was more reliable because it remained the same for both true and overestimated nitrogen balance values. By applying hypothetical data that represented both a 10% overestimation of nitrogen balance, and
true nitrogen balance values, Humayun et al., (2007) were able to show that protein requirements (i.e. the point at which nitrogen balance crossed the X-axis or neutral balance) changed by 20% when analyzed by a linear regression. In contrast, defining the protein requirement of the same dataset by a bi phase linear regression would have no impact of the EAR (Humayun et al., 2007). Therefore, seeing how true nitrogen balance values are difficult to obtain, Humayun et al., (2007) suggested that it was crucial that a bi phase linear regression be applied to determine protein requirements (Humayun et al., 2007). Humayun et al. (2007) continued to re-evaluate protein requirements using the IAAO technique and thus, the estimated EAR and RDA were determined to be 0.93 g·kg\(^{-1}\)·d\(^{-1}\) and 1.2 g·kg\(^{-1}\)·d\(^{-1}\), respectively (Humayun et al.,2007). Both values are greater than the suggested current EAR and RDA of 0.66 g·kg\(^{-1}\)·d\(^{-1}\) and 0.83 g·kg\(^{-1}\)·d\(^{-1}\), respectively, which were determined using the NBAL method and a linear regression analysis model. The similarity between the bi-phase regression on NBAL determined requirements, and the IAAO determined requirements supported the validity of the IAAO technique as an accurate novel method for determining protein requirements.

A subsequent study by Elango et al. (2011) sought to re-evaluate the recommended protein requirements for healthy, school-age children on a sedentary day using the IAAO technique (Elango et al., 2011). Seven young healthy children (6-11 years old) were randomly given test protein intakes ranging from 0.1 - 2.56 g·kg\(^{-1}\)·d\(^{-1}\) over a minimum of 7 trial days each. Results showed that the EAR and RDA were determined to be 1.3 g·kg\(^{-1}\)·d\(^{-1}\) and 1.55 g·kg\(^{-1}\)·d\(^{-1}\), respectively (Elango et al., 2011). These, are greater values than the current suggested EAR and RDA of 0.76 g·kg\(^{-1}\)·d\(^{-1}\) and 0.95 g·kg\(^{-1}\)·d\(^{-1}\) respectively, for children determined by the NBAL method (FAO/WHO, 2007).
A more recent study by Packer et al. (2015) also utilized the IAAO technique in healthy active adult males, in an attempt to re-evaluate the recommended protein requirements for the healthy, active male population (Packer et al., 2015). Seven active males were instructed to perform a variable intensity aerobic exercise stimulus (Loughborough Intermittent Shuttle Test) on each trial day, followed by the ingestion of test protein intakes ranging from 0.2 - 2.6 g·kg\(^{-1}\)·d\(^{-1}\). Results determined the EAR and RDA to be 1.3 g·kg\(^{-1}\)·d\(^{-1}\) and 1.6 g·kg\(^{-1}\)·d\(^{-1}\), respectively. Following similar trends of previous IAAO studies, the results determined by Packer et al. (2015) are greater than the current EAR and RDA of 0.66 g·kg\(^{-1}\)·d\(^{-1}\) and 0.83 g·kg\(^{-1}\)·d\(^{-1}\), respectively for sedentary adults determined by FAO/WHO using the NBAL method (Packer et al., 2015). This suggests that the current suggested protein recommendations, determined by the NBAL method for active adolescents, may be underestimated as well, and should be re-evaluated using the IAAO technique.

**1.5.4 – Conclusion**

Although, NBAL is the most commonly used method when determining protein requirements, there are multiple flaws that come with the method (Millward, 2001). Even though the non-invasive nature of NBAL is one of its strengths, the uncertain measurements on total nitrogen excretion makes the NBAL method questionable as it may underestimate true protein requirements. As a result, the use of alternative methods has been implemented to determine protein requirements. The results obtained with the IAAO technique suggest that the protein requirements determined by NBAL are underestimating true protein requirement values (Humayun et al., 2007; Elango et al., 2011; Packer et al., 2015). However, protein requirements in adolescents, more specifically, active adolescents, have yet to be determined using the IAAO technique. This makes it essential that the IAAO technique be utilized in an active adolescent
population to re-evaluate the current dietary protein recommendations previously determined by the NBAL method, as well as, evaluate the effects of physical activity on protein requirements.

1.6 – Study Rationale

The combined effects that puberty and physical activity have on protein requirements in adolescents are not well documented. However, the NBAL technique has been used to determine that physical activity may enhance protein requirements in active adolescents to RDA levels of approximately 1.4 – 1.5 g·kg\(^{-1}\)·d\(^{-1}\) (Boisseau et al., 2007; Aerenhouts et al., 2013). More recent studies conducted using the IAAO technique suggests that the protein requirements determined by NBAL may be underestimates (Humayun et al., 2007; Elango et al., 2011; Packer et al., 2015). Therefore, the aim of this study was to utilize the IAAO technique to re-evaluate the protein requirements previously determined by NBAL in the active adolescent population.

The hallmark of puberty is the growth spurt, which is characterized by increases in linear growth, LBM, and secondary maturation. It is suggested that these growths lead to increases in protein requirements, while contradicting studies suggest that these growths lead to an increase in the efficiency of protein utilization (Aerenhouts et al., 2013; Beckett et al., 1997).

The IAAO technique has never been utilized in an adolescent population, thus, it is essential that it be used to re-evaluate protein requirements, in an attempt to determine whether protein requirements or the efficiency of protein utilization are increased during the pubertal growth spurt. As well, our study aimed to evaluate the effects of physical activity on adolescent protein requirements. It was hypothesized that the results from our study would i) yield higher protein requirements than comparative values established using NBAL and the factorial method
in adolescents, and ii) confirm that physical activity increases protein requirements in active adolescents compared to the current recommendation for their sedentary counterparts.
Chapter 2. Methods

2.1 – Introduction

Based on the background knowledge of protein requirements, adolescent nutritional needs, and the effects of physical activity previously described in earlier chapters, the research study was divided into 3 sequential research phases. This section will provide a detailed description of the participants, and the progression from Phase I to Phase III of the study. In addition, the study design, methodology, and materials inherent to each phase will be clarified.

2.2 – General Study Design & Study Participants

The current research study quantified the protein requirements of highly active, adolescent males, proximate to PHV, using the IAAO technique. The protein requirements values obtained were subsequently compared to published values for protein requirements in sedentary and active adolescents, adults, and sedentary children, which were obtained using either the NBAL and IAAO technique. A total of 7 healthy, active, adolescent male participants between -0.5 to +1.0 from PHV were recruited for the study. Participants were recruited from various sports teams, as well as the junior varsity blues recreational camps at The University of Toronto. Prior to study enrollment, prospected participants were given a detailed oral introduction to the study, and were given the opportunity to ask questions pertaining to the study before the participant and parent/guardian were asked to sign consent and assent forms, respectively, in accordance with the university’s ethical committee. The study was approved by the University of Toronto’s Research Ethics Board. The specific participant PHV range distinctly separates the adolescent population from both the child and young adult demographic. This range encompasses the duration of which
maximal growth occurs during the pubertal growth spurt (Mirwald et al., 2002). Any larger of a range (< -0.5 or > +1.0) would have led to finding physiological differences within the group such as large weight and height differences. These differences may have led to varied results, and ultimately, varied protein requirements. Participants were also assessed on their physical fitness level by partaking in an aerobic capacity test (Beep Test). A predetermined level of 8.3 had to be completed to ensure that participants were in the top 20th percentile of their age group (12-15 years of age) in aerobic capacity and physical fitness (Appendix B, unpublished data). The level of 8.3 was determined to be equivalent to a predicted maximal oxygen consumption (VO₂max) of 53.99 ml·min⁻¹·kg⁻¹, which was determined from the average estimated VO₂max of four different equations (Leger et al., 1988; Barnett et al., 1993; Barnett et al., 1993; Matsuzaka et al., 2004). Characteristics of a typical 12 and 15-year old boy (Body weight, kg; body mass index) were used as standard values (FAO/WHO, 2007) to determine VO₂max. The average of the determined VO₂max for both a 12 and 15-year old boy was then used to estimate the equivalent predicted VO₂max of level 8.3 on the Beep Test. Participants then partook in 8 laboratory visits, of which, the final 6 were metabolic trials. The three phases of the study are described as follows.

2.3 – Phase I, Introductory Session

Phase I consisted of a single laboratory visit lasting approximately 1 hour, and had three primary objectives. First, it served to provide the prospective participants and parent/guardian with a comprehensive oral introduction to the study protocol to ensure that they were properly informed before being asked to provide consent and assent. Once participants and parent/guardian were given a detailed overview of the study protocol, they were then given the opportunity to ask questions pertaining to the study, and subsequently signed the required consent and assent.
documents. Second, the first phase of research was also used to obtain background information with respect to the participants’ general health and habitual activity levels using the Physical Activity Readiness Questionnaire (PAR-Q+) and the International Physical Activity Questionnaire (IPAQ) respectively (Packer et al., 2015). Finally, the participants’ standing height, sitting height (cm), and weight (kg) were measured using a Detecto Physician Beam scale. These anthropometric values were used to determine the participants’ biological age, in reference to their PHV (Mirwald et al., 2002; Appendix A). Finally, participants were instructed to record a 3-day dietary log and to wear a Sensewear Body Media Armband Accelerometer for the same 3-days to determine each participant’s habitual dietary behavior and resting energy expenditure (REE), respectively (Appendix C). The REE value recorded by the accelerometer was used to determine total daily energy needs that was given during a 2-day adaptation diet prior to each metabolic trial.

2.4 – Phase II, Body Composition Analysis & Fitness Assessment

Phase II entailed a single laboratory visit that lasted approximately 2 hours, and had three primary objectives. Participants underwent a detailed body composition assessment (Fat mass, FM; kg, Fat-Free mass, FFM; kg, height; cm, and whole body mass; kg) determined by the BodPod (Air Displacement Plethysmography) (Cosmed USA Inc., Chicago, IL). Participants were asked to refrain from consuming any solid or liquid food or drinks 12 hours prior to the body composition analysis to ensure the recording of accurate fasted measurements. Upon completion of the various body composition measurements, participants were required to perform the Beep Test, to characterize their fitness level. Participants were required to complete a minimum predetermined level of 8.3 (predicted VO$_{2\text{max}}$ of 53.99 ml·min$^{-1}$·kg$^{-1}$) to be enrolled in the study. Additionally, participants were given a detailed introduction to the Loughborough Intermittent Shuttle Test
(LIST; Figure 6) exercise stimulus and metabolic trials that they would perform. Finally, participants were informed that they would be required to consume a 2-day study adaptation diet prior to each metabolic trial.

2.5 – Phase III, Metabolic Trials

Once participants were fully enrolled in the study, they were required to visit the lab for six metabolic trials lasting approximately 10 hours each and consisting of two components: 1) the completion of the LIST exercise stimulus, and 2) a subsequent 8-hour metabolic trial. Each metabolic trial followed the same protocol, differing only in the level of protein the participant was randomly assigned to consume. Two days prior to each metabolic trial, participants were required to adhere to a predetermined adaptation diet which provided 1.2 g·kg⁻¹·d⁻¹ of protein and sufficient energy estimated at 1.5 times their REE as previously determined during their 3-day accelerometer recording. Prior to each metabolic trial, participants were required to arrive to the lab following an overnight fast (12h). Participants were also instructed to ingest a liquid-based, protein-free carbohydrate beverage (1 g·kg⁻¹·d⁻¹ of carbohydrate as a 1:1 ratio of maltodextrin (Polycal; Nutricia, Amsterdam, Netherlands) and sports drink powder (Gatorade Endurance Formula; PepsiCo, Purchase, NY)) at least 1 hour before performing the exercise stimulus to: i) prevent the participants from having to exercise in the fasted state; ii) increase ecological validity by adhering to pre-exercise recommended carbohydrate intake (Burke et al., 2011), and; iii) to potentially minimize amino acid oxidation during exercise (Kato et al., 2016).

Participants then performed a modified version of the LIST exercise stimulus. The LIST is a variable intensity exercise test resembling play in organized sports such as soccer (Armstrong et
al., 2006; Castagna et al., 2003; Stroyer et al., 2004). The modified LIST is comprised of 4 segments of 15 minutes of variable intensity exercise including walking, jogging (60% VO$_{2\text{max}}$ speed), running (90% VO$_{2\text{max}}$ speed), and sprinting (Figure 6). 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), which provides participants with the recommended 60 minutes of MVPA daily (Tremblay et al., 2011).

Figure 6. Schematic for the modified Loughborough Intermittent Shuttle Test.

Immediately following the exercise, participants consumed the first of 8 hourly liquid meals along with protein-free cookies. Each metabolic trial provided a randomly assigned amount of protein, 6 g·kg$^{-1}$ of carbohydrate, and a sufficient amount of energy estimated at 1.5 times their REE (Appendix C). The test protein within the liquid meals was provided as crystalline amino acids, which were modeled on the basis of egg protein amino acid composition, with the exception
of tyrosine (40 mg·kg\(^{-1}\)·d\(^{-1}\)) and the indicator amino acid phenylalanine (30.5 mg·kg\(^{-1}\)·d\(^{-1}\); with 5.46 mg·kg\(^{-1}\)·d\(^{-1}\) provided as L-\(^{13}\)C phenylalanine during the 5\(^{th}\) to 8\(^{th}\) hourly meals). On each of the metabolic trials, participants were assigned to consume one of six different test protein intakes in a random order. The test protein intakes covered a range from deficient to excessive (0.2 - 2.67 g·kg\(^{-1}\)·d\(^{-1}\)), according to previous studies that implemented IAAO and NBAL protocols in sedentary and active adults, as well as healthy school aged children (Humayun et al., 2007; Kato et al., 2016; Packer et al., 2015; Elango et al., 2011).

The first four hourly meals consumed following the completion of the LIST exercise stimulus were comprised of the aforementioned test protein intakes without the addition of the oral tracer (L-\(^{13}\)C phenylalanine) to establish baseline enrichment. A priming dose of NaH\(^{13}\)CO\(_2\) (0.176 mg·kg\(^{-1}\)) and L-\(^{13}\)C phenylalanine (1.86 mg·kg\(^{-1}\)) was ingested in the 5\(^{th}\) hourly meal. All subsequent meals during the metabolic trial included 1.2 mg·kg\(^{-1}\) of L-\(^{13}\)C phenylalanine as part of the total intake to maintain isotopic steady state for the remainder of the metabolic trial.

### 2.6 - Sample Collection and Analysis

Three baseline breath samples at 15-minute intervals (15, 30, and 45 min), as well as two baseline urine samples at 30-minute intervals (15 and 45 min), were collected prior to the consumption of the 5\(^{th}\) meal to establish baseline \(^{13}\)CO\(_2\) and L-\(^{13}\)C phenylalanine enrichment values, respectively. During each trial, the rate of CO\(_2\) production (VCO\(_2\)) was measured over 20 minutes following the 5\(^{th}\) or 6\(^{th}\) drink, but before the 7\(^{th}\) drink using indirect calorimetry (MOXUS Metabolic Cart, AEI Technologies) to determine steady state metabolism. It has been shown that baseline \(^{13}\)CO\(_2\) and VCO\(_2\) plateau within ~180 mins after the LIST in male adults (Packer et al.,
2015). Moreover, steady state VCO₂ has been obtained within 6h of constant feeding in adolescents (Beckett et al., 1997), which collectively would suggest our measurements were performed at both isotopic and metabolic steady state. In addition, six plateau breath samples were collected every 15 minutes and three plateau urine samples were collected every 30 minutes beginning 2.5 hours after the 5th meal. Breath samples were collected in disposable Extainer tubes and stored at room temperature prior to measurement of \(^{13}\text{CO}_2\) enrichment by a continuous-flow isotope ratio mass spectrometry. Urine samples were collected in disposable urine collection containers and 3 × 1 mL aliquots were stored at -80°C prior to [1-\(^{13}\text{C}\)] phenylalanine enrichment determined by API 4000 triple quadrupole mass spectrometer.

### 2.7 – Data Organization

42 test protein intakes were tested ranging from 0.20 - 2.67 g·kg\(^{-1}\)·d\(^{-1}\) which were separated into 6 different protein intake ranges (i.e. 0.20 – 0.55 g·kg\(^{-1}\)·d\(^{-1}\); 0.62 – 0.97 g·kg\(^{-1}\)·d\(^{-1}\); 1.04 – 1.39 g·kg\(^{-1}\)·d\(^{-1}\); 1.46 – 1.81 g·kg\(^{-1}\)·d\(^{-1}\); 1.90 – 2.25 g·kg\(^{-1}\)·d\(^{-1}\), and 2.32 – 2.67 g·kg\(^{-1}\)·d\(^{-1}\)). Each protein intake range was comprised of 7 protein intake levels separated by 0.03 – 0.07 g·kg\(^{-1}\)·d\(^{-1}\) increments. For example, protein intakes corresponding to 0.20, 0.24, 0.27, 0.34, 0.41, 0.48, and 0.55 g·kg\(^{-1}\)·d\(^{-1}\) represented the 7 protein intake levels within the range of 0.20 – 0.55 g·kg\(^{-1}\)·d\(^{-1}\).

Each participant was randomly assigned one protein intake level within each of the 6 protein intake ranges over the 6 metabolic trials.
2.8 - Tracer Kinetics

Phenylalanine flux (Phe Ra, μmol·kg\(^{-1}\)·h\(^{-1}\)), the rate of appearance of \(^{13}\)CO\(_2\) in breath (F\(^{13}\)CO\(_2\), μmol·kg\(^{-1}\)·h\(^{-1}\)), and phenylalanine oxidation (Phe Ox, μmol·kg\(^{-1}\)·h\(^{-1}\)) were calculated by using the following equations:

\[
Phe Ra = i \cdot \frac{E_i}{E_u} - I
\]

Where \(i\) is the rate of L-[\(^{1-13}\)C] phenylalanine ingested (μmol·kg\(^{-1}\)·h\(^{-1}\)), \(I\) is the rate of L-phenylalanine ingested (μmol·kg\(^{-1}\)·h\(^{-1}\)), \(E_i\) and \(E_u\) are the isotopic enrichments as mole fractions (A.P.E) of the test meal and urinary phenylalanine, respectively, at isotopic plateau.

\[
F^{13}CO_2 = (VCO_2) \cdot (ECO_2) \cdot (44.6) \cdot (60) \cdot BW^{-1} \cdot (0.82) \cdot (100)
\]

Where \(VCO_2\) is the CO\(_2\) production rate (mL·min\(^{-1}\)), \(ECO_2\) is the \(^{13}\)CO\(_2\) enrichment in expired breath at isotopic steady state (APE), BW is the body weight of the participant (kg). The constants 44.6 (μmol·mL\(^{-1}\)) and 60 (min·hour\(^{-1}\)) were used to convert \(VCO_2\) into μmol·h\(^{-1}\). The factor 0.82 is the correction for CO\(_2\) retained in the bicarbonate pool of the body in the fed state (Elango et al., 2011). The factor 100 changes the APE to a fraction.

\[
Phe Ox = F^{13}CO_2 \cdot \left(\frac{1}{E_u} - \frac{1}{E_i}\right) \cdot 100
\]

2.9 - Statistical Analysis

Unless indicated otherwise, all results are expressed as means ± SD. All statistical analysis was performed with SAS (SAS/STAT version 9.3; SAS Institute) for Windows. A mixed model bi-phase linear regression with participants as a random variable was used to
analyze the effects of protein intake on F^{13}CO_2 and Phe Ox, while a linear regression was used to analyze the effects of protein intake on Phe Ra. Significance was established at P < 0.05. To determine mean protein requirements and recommended protein intakes, a bi-phase linear regression crossover analysis was performed on F^{13}CO_2 (as the primary outcome) and Phe Ox (as a secondary outcome) in agreement with previous studies (Humayun et al., 2007; Elango et al., 2011; Packer et al., 2015; Kato et al., 2016).
Chapter 3. Results

3.1 - Participants

Participant characteristics are provided in Table 1. The participant PHV range distinctly separates the adolescent population from both the child and young adult demographics. This range encompasses the duration of which maximal growth occurs during the pubertal growth spurt (Mirwald et al., 2002).

**Table 1.** Participant anthropometric measures and characteristics

<table>
<thead>
<tr>
<th>Participant characteristics</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>13.8 ± 0.5</td>
</tr>
<tr>
<td>Height, cm</td>
<td>171 ± 2.8</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>57.3 ± 4.2</td>
</tr>
<tr>
<td>FFM, kg</td>
<td>48.4 ± 5.0</td>
</tr>
<tr>
<td>Percent Body Fat, %</td>
<td>15.4 ± 7.2</td>
</tr>
<tr>
<td>Years from PHV, y</td>
<td>0.3 ± 0.2</td>
</tr>
<tr>
<td>Predicted VO\textsubscript{2max}, ml O\textsubscript{2}kg\textsuperscript{-1}· min\textsuperscript{-1}</td>
<td>54.2 ± 1.9</td>
</tr>
<tr>
<td>Habitual MVPA, min·d\textsuperscript{-1}</td>
<td>178 ± 57.5</td>
</tr>
<tr>
<td>Habitual Energy Expenditure, kcal·d\textsuperscript{-1}</td>
<td>2886 ± 306.9</td>
</tr>
<tr>
<td>Resting Energy Expenditure, kcal·d\textsuperscript{-1}</td>
<td>1463.7 ± 102.7</td>
</tr>
<tr>
<td>Habitual Energy Intake, kcal·d\textsuperscript{-1}</td>
<td>2131.4 ± 106.5</td>
</tr>
<tr>
<td>Habitual Protein Intake, g·kg\textsuperscript{-1}·d\textsuperscript{-1}</td>
<td>1.8 ± 0.4</td>
</tr>
<tr>
<td>Habitual Carbohydrate Intake, g·kg\textsuperscript{-1}·d\textsuperscript{-1}</td>
<td>4.7 ± 0.2</td>
</tr>
<tr>
<td>Habitual Fat Intake, g·kg\textsuperscript{-1}·d\textsuperscript{-1}</td>
<td>1.3 ± 0.3</td>
</tr>
<tr>
<td>Trial Day Energy Intake, kcal·d\textsuperscript{-1}</td>
<td>2868.8 ± 202.5</td>
</tr>
</tbody>
</table>

Values are means ± 95% CI, n=7
3.2 – Breakpoint (F\textsuperscript{13}CO\textsubscript{2} Excretion)

F\textsuperscript{13}CO\textsubscript{2} decreased in all participants with the increase of protein intakes with bi-phase linear regression crossover analysis revealing a breakpoint (EAR) at 1.48 g·kg\textsuperscript{-1}·d\textsuperscript{-1} (Figure 7A, R\textsuperscript{2} = 0.54). The population safe intake estimated by the upper 95% confidence interval (CI) was determined to be 1.78 g·kg\textsuperscript{-1}·d\textsuperscript{-1}, while the lower 95% CI was determined to be 1.18 g·kg\textsuperscript{-1}·d\textsuperscript{-1}. Additionally, when F\textsuperscript{13}CO\textsubscript{2} was normalized to FFM the breakpoint was 1.73 g·kg FFM\textsuperscript{-1}·d\textsuperscript{-1} (Figure 7B, R\textsuperscript{2} = 0.62) with the upper and lower 95% CI determined to be 2.03 and 1.43 g·kg FFM\textsuperscript{-1}·d\textsuperscript{-1}, respectively. A comparison between our results and other recommended protein requirements determined using the IAAO technique show that our EAR and upper 95% CI are physiologically greater than all other determined values for various studied populations, except for the endurance trained adult male population (Table 2). Our determined EAR is also statistically greater than the EAR determined for non-exercising adults (Table 2).
Figure 7A: Relationship between F\textsuperscript{13}CO\textsubscript{2} and protein intake when normalized to body weight in active adolescent males. The breakpoint represented the EAR and was determined to be 1.48 g·kg\textsuperscript{-1}·d\textsuperscript{-1}, with an upper 95% CI (RDA) of 1.78 g·kg\textsuperscript{-1}·d\textsuperscript{-1}, and a lower 95% CI of 1.18 g·kg\textsuperscript{-1}·d\textsuperscript{-1} (R\textsuperscript{2}=0.54). B: Relationship between F\textsuperscript{13}CO\textsubscript{2} and protein intake when normalized to FFM in active adolescent males. The breakpoint represented the EAR and was determined to be 1.73 g·kg FFM\textsuperscript{-1}·d\textsuperscript{-1}, with an upper 95% CI (RDA) of 2.03 g·kg FFM\textsuperscript{-1}·d\textsuperscript{-1}, and a lower 95% CI of 1.43 g·kg FFM\textsuperscript{-1}·d\textsuperscript{-1} (R\textsuperscript{2}=0.62).
**Table 2.** Comparison between the EAR and upper 95% CI of multiple studies that have utilized the IAAO technique in various populations.

<table>
<thead>
<tr>
<th>Study</th>
<th>Sex</th>
<th>Age</th>
<th>Exercise Stimulus</th>
<th>EAR (g·kg⁻¹·d⁻¹)</th>
<th>Upper 95% CI (g·kg⁻¹·d⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sedentary Adults (Humayun et al., 2007)</td>
<td>M</td>
<td>26.8 ± 2.0</td>
<td>N/A</td>
<td>0.93*</td>
<td>1.2</td>
</tr>
<tr>
<td>Sedentary Children (Elango et al., 2011)</td>
<td>M/F</td>
<td>8.4 ± 1.4</td>
<td>N/A</td>
<td>1.3</td>
<td>1.55</td>
</tr>
<tr>
<td>Endurance Adult Males (Kato et al., 2016)</td>
<td>M</td>
<td>28.0 ± 4.0</td>
<td>20 km treadmill run</td>
<td>1.65</td>
<td>1.83</td>
</tr>
<tr>
<td>Active Adult Males (Packer et al., 2015)</td>
<td>M</td>
<td>22.9 ± 0.8</td>
<td>LIST</td>
<td>1.35</td>
<td>1.64</td>
</tr>
<tr>
<td>Active Adult Females (Wooding et al., 2015)</td>
<td>F</td>
<td>21.4 ± 0.8</td>
<td>LIST</td>
<td>1.36</td>
<td>1.68</td>
</tr>
<tr>
<td>Active Adolescent Females (Brooks et al., 2017)</td>
<td>F</td>
<td>12.2 ± 0.3</td>
<td>LIST</td>
<td>1.28</td>
<td>1.46</td>
</tr>
<tr>
<td>Active Adolescent Males (Brooks et al., 2017)</td>
<td>M</td>
<td>13.8 ± 0.5</td>
<td>LIST</td>
<td>1.48</td>
<td>1.78</td>
</tr>
</tbody>
</table>

* Represents statistical difference from active adolescent male EAR. Statistical difference was determined by the equation \( \text{Mean}_1 - \text{Mean}_2 \pm 1.96 \times (\text{SE}_1^2 + \text{SE}_2^2)^{^0.5} \). Intervals that did not contain zero were considered statistically different.

### 3.3 – Phenylalanine Oxidation

Phenylalanine oxidation (Phe Ox) decreased in all participants with the increase in protein intakes with bi-phase linear regression crossover analysis revealing a breakpoint (EAR) at 1.48 g·kg⁻¹·d⁻¹ (Figure 8A, \( R^2 = 0.62 \)). The upper 95% CI (RDA) was determined to be 1.73 g·kg⁻¹·d⁻¹, while the lower 95% CI was determined to be 1.23 g·kg⁻¹·d⁻¹. When Phe Ox was normalized to
FFM the breakpoint was 1.72 g·kg FFM⁻¹·d⁻¹ (Figure 8B, R² = 0.69), with an upper and lower 95% CI determined to be 1.98 g·kg FFM⁻¹·d⁻¹ and 1.46 g·kg FFM⁻¹·d⁻¹, respectively.
Figure 8A: Relationship between Phe Ox and protein intake when normalized to body weight in active adolescent males. The breakpoint and upper 95% CI were revealed to be at 1.48 g·kg⁻¹·d⁻¹ and 1.73 g·kg⁻¹·d⁻¹, respectively. B: Relationship between Phe Ox and protein intake when normalized to FFM in active adolescent males. The breakpoint and upper 95% CI were revealed to be at 1.72 g·kg⁻¹·d⁻¹ and 1.98 g·kg FFMM⁻¹·d⁻¹, respectively.

3.4 – Phenylalanine Flux

A requirement for studies that utilize the IAAO technique is that Phe Ra is not influenced by protein intake. This would provide evidence that the precursor pool for indicator oxidation does not change in size in response to test protein intakes (Humayun et al., 2007). Our determined phenylalanine flux (Phe Ra) decreased with increasing protein intake when normalized to body weight (Figure 9A, \( P < 0.001 \), mean Phe Ra = 58.3 ± 8.5 μmol·kg⁻¹·h⁻¹) and FFM (Figure 9B, \( P < 0.01 \), mean Phe Ra = 68.9 ± 11.6 μmol·kg FFMM⁻¹·h⁻¹).
Figure 9A: Relationship between phenylalanine rate of appearance (Phe Ra) and protein intake when normalized to body weight in active adolescent males following the LIST exercise stimulus. Mean Phe Ra was measured to be $58.3 \pm 8.5 \, \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ($R^2 = 0.26$, slope = $-5.24 \pm 1.4$, y-intercept = $64.6 \pm 2.1 \, \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$, $P = 0.0008$).

Figure 9B: Relationship between Phe Ra and protein intake when normalized to FFM in active adolescent males following the LIST exercise stimulus. Mean Phe Ra was measured to be $68.9 \pm 11.6 \, \mu\text{mol} \cdot \text{kg} \cdot \text{FFM}^{-1} \cdot \text{h}^{-1}$ ($R^2 = 0.19$, slope = $-5.74 \pm 1.9$, y-intercept = $78.4 \pm 3.5 \, \mu\text{mol} \cdot \text{kg} \cdot \text{FFM}^{-1} \cdot \text{h}^{-1}$, $P = 0.0009$).
Chapter 4. Discussion

4.1 – Introduction

The aim of the present study was to utilize the minimally invasive IAAO technique for the first time in active adolescent males to evaluate the impact of variable intensity exercise on protein requirements. Our data shows that on a day in which an acute bout of variable intensity exercise (modified LIST exercise) is performed that provides the recommended 60 minutes/day of MVPA, the EAR and RDA (as determined by the upper 95% CI) for protein were 1.48 g·kg⁻¹·d⁻¹ and 1.78 g·kg⁻¹·d⁻¹, respectively, in active adolescent males. When normalized to FFM, our data revealed the EAR and RDA for protein to be 1.73 g·kg FFM⁻¹·d⁻¹ and 2.03 g·kg FFM⁻¹·d⁻¹, respectively. Thus, our estimates of protein requirements (normalized to body weight) are ~90% and 98% greater than the current EAR and population safe intake for non-exercising adolescents (0.78 and 0.9 g·kg⁻¹·d⁻¹, FAO/WHO, 2007), respectively. When comparing our estimated to suggested EAR and RDA estimated by Boisseau et al. (2007) for active adolescent males (1.2 and 1.4 g·kg⁻¹·d⁻¹, Boisseau et al., 2007), our determined results are ~23 and 27% greater, respectively. Additionally, when comparing our estimates to suggested mean protein intake for active adolescents to stay in a positive nitrogen balance (1.5 g·kg⁻¹·d⁻¹, Aerenhouts et al., 2013), our determined results are ~19% greater in protein RDA as well. All recommendations for non-exercising and active adolescents have been previously determined using the factorial method and/or NBAL technique. The following sections will contextualize our results considering previous studies that have determined protein requirements using the NBAL and IAAO techniques. Suggestions for avenues of future research will also be provided in light of a discussion of the strengths, limitations, and implications of the present study.
4.2 – Phenylalanine Flux

In comparison to the study conducted by Humayun et al. (2007), the average Phe Ra for our study of ~69 μmol·kg FFM⁻¹·h⁻¹ when normalized to FFM was generally in agreement with non-exercising adults (~70 μmol·kg FFM⁻¹·h⁻¹, Humayun et al., 2007). The similarities seen between our results and those determined by Humayun et al. (2007) may be attributed to the increase in protein utilization that has been seen in adolescents during puberty (Beckett et al., 1997), as well as, the addition of physical activity. Increasing protein utilization would inherently decrease Phe Ra across all protein intakes, while, research has suggested that increased levels of whole body protein turnover are seen in response to exercise (Rennie et al., 1981).

In support of the ability of exercise to increase Phe Ra, a study conducted by Packer et al. (2015) incorporated the LIST exercise stimulus into the IAAO protocol in active adult males and reported a mean Phe Ra when normalized to FFM of 89.1 μmol·kg FFM⁻¹·h⁻¹ (Packer et al., 2015). The Phe Ra determined by Packer et al. (2015) was ~27% greater than what was previously reported in non-exercising adults (~70 μmol·kg FFM⁻¹·h⁻¹, Humayun et al., 2007). Having similar participant characteristics and methodologies, the main difference between these two studies was the incorporation of exercise. This would suggest that exercise was the main contributor to the increase seen in mean Phe Ra. Additionally, a study conducted by Elango et al. (2011) incorporated the IAAO technique in non-exercising school-age children, and saw a mean Phe Ra when normalized to FFM of 49.4 μmol·kg FFM⁻¹·h⁻¹ (Elango et al., 2011). The Phe Ra determined by Elango et al. (2011) is ~41% less than what was determined in our study (~69 μmol·kg FFM⁻¹·h⁻¹). Both study’s participants differed most in active level, with our participants performing the LIST exercise stimulus on trial days. This would further support the explanation of exercise being the main contributor to increasing Phe Ra.
Interestingly, when comparing the mean Phe Ra of the Elango et al. (2011) and Humayun et al. (2007) studies, it is evident that the mean Phe Ra determined by Humayun et al. (2007) is ~43% greater than what was determined by Elango et al. (2011). Participants in both studies were inactive, and both utilized the IAAO technique, hence, the only difference between the two is the populations that were investigated. The difference in mean Phe Ra can potentially be explained by the increased rates of growth that are occurring during childhood. It has been suggested that during periods of rapid growth and development, protein synthesis rates have been shown to excessively increase (Pencharz et al., 1981). An increase in protein synthesis would require greater amino acid incorporation from protein breakdown to be utilized in creating new proteins. This increased incorporation would result in a reduction of amino acids being released into circulation in the free amino acid pool, and inherently reflect a decrease in whole body Phe Ra.

Therefore, because our adolescent males were going through puberty, their utilization of protein may have been increased, which would cause Phe Ra to decrease. However, because our participants were also performing the LIST exercise stimulus on trial days, which was previously suggested to increase Phe Ra when comparing non-exercising to exercising adults (Humayun et al., 2007; Packer et al., 2015), our observed mean Phe Ra is greater than the mean Phe Ra determined by Elango et al. (2011), similar to the mean Phe Ra determined by Humayun et al. (2007), and less than the mean Phe Ra determined by Packer et al. (2015).

A requirement for studies that utilize the IAAO technique is that Phe Ra is not influenced by protein intake. This would provide evidence that the precursor pool for indicator oxidation does not change in size in response to test protein intakes (Humayun et al., 2007). Results from our study display a slight but significant decrease in Phe Ra in response to increasing test protein intakes (Figure 9). While there was a visual decrease in Phe Ra in response to protein intake in the
study conducted by Humayun et al. (2007), this apparent difference was not statistically significant. Nevertheless, a significant difference in Phe Ra has also been seen in response to increasing protein intakes in adult males after the LIST (Packer et al., 2015). This would suggest that our precursor pool was affected by increased protein intakes. This inverse relationship between Phe Ra and test protein intake seen in our results may be caused by a dietary protein-induced suppression of protein breakdown, which has been previously reported at the whole-body protein level (Kim et al., 2016). If this were true, an induced suppression of protein breakdown by dietary protein consumption would attenuate the appearance of unlabeled amino acids in the plasma (and hence urine), at steady state, which would consequently translate into a decrease in Phe Ra.

To estimate the potential impact of the observed decrease in Phe Ra with increased protein intakes in adult males after the LIST, a reanalysis of Phe Ox data was conducted using an average participant Phe Ra value to determine if the breakpoint would change under the circumstance where Phe Ra was constant throughout all test protein intakes (Packer et al., 2015). Upon reanalysis, a breakpoint that was ~8% greater (1.56 g·kg⁻¹·d⁻¹) than the originally established breakpoint (1.44 g·kg⁻¹·d⁻¹) was revealed. This increase suggested that the results originally established may have represented a minor underestimation of true protein requirements (Packer et al., 2015). However, in the present study the EAR was identical (1.48 g·kg⁻¹·d⁻¹) when estimated from F¹³CO₂ and Phe Ox breakpoints. Since calculation of Phe Ox requires Phe Ra whereas F¹³CO₂ does not, the identical EAR by Phe Ox and F¹³CO₂ would suggest that the protein-induced decrease in Phe Ra (and subsequently the potential impact on precursor pool size) did not influence our determined protein requirements for active adolescent males.
4.3 – Requirement relative to non-exercising adolescents

To date, limited research has directly investigated the protein requirements of adolescents. Additionally, this is the first study to-date that has employed the IAAO technique in the active adolescent male population in the presence of an exercise stimulus. The current EAR for protein in non-exercising adolescents, which was determined using the factorial method (0.78 g·kg\(^{-1}\)·d\(^{-1}\), FAO/WHO, 2007), is ~90% less than the EAR that was determined by our study (1.48 g·kg\(^{-1}\)·d\(^{-1}\)). The increase in EAR may be attributed in part to the introduction of aerobic physical activity (i.e., the LIST exercise stimulus in our study), which has been shown to increase protein requirements in adults (Wooding et al., 2015; Packer et al., 2015; Kato et al., 2016). Alternatively, the greater estimate may also be related to methodological differences given that IAAO technique has been shown to display increased protein requirements in non-exercising adults and children compared to the NBAL technique (Humayun et al., 2007; Elango et al., 2011).

Physical activity has been shown to influence the growth of LBM, independent from the pubertal growth spurt, in adolescents (Baxter-Jones et al., 2008). Provided energy needs are met, this activity-induced augmentation of LBM growth would ultimately need to be supported by adequate protein intake. The introduction of physical activity has been suggested to contribute ~0.8% of additional LBM growth over the span of a year when an adolescent is at PHV (Baxter-Jones et al., 2008). In reference to our participants (~48 kg FFM), physical activity can be responsible for ~380g of FFM accrual over the course of a year, which would translate into ~1.06 g LBM·d\(^{-1}\). Assuming LBM consists of ~75% water and only ~25% lean dry mass, it can be suggested that the addition of physical activity in an adolescent can be responsible for increasing protein requirements by ~0.27 g·kg\(^{-1}\)·d\(^{-1}\) to support this growth. Therefore, the activity-induced increase in LBM growth may contribute to the greater protein requirements in active adolescents.
The high muscle forces that are associated with resistance exercise or weight-bearing (stop-and-go) endurance exercise have been shown to induce muscle damage. It has been suggested that exercise induced muscle damage is caused by mechanical and metabolic factors (Tee et al., 2007). The mechanic stress has been suggested to stem from physical stress on the muscle fibre, while the metabolic effects of exercise induced muscle damage are suggested to be prolonged glycogen depletion, and increased metabolic rates (Tee et al., 2007). Thus, the consumption of dietary protein is required to assist in repairing and remodeling the resulting damaged muscle fibre (Tee et al., 2007; Ascensao et al., 2008). Given that our exercise stimulus was modelled after the play of soccer, lasted ~75 minutes, and consisted of varying intervals of walking, jogging, and sprinting, our LIST exercise had a highly aerobic nature, and required participants to expend considerable amounts of energy. Although, glucose and glycogen stores are the primary fuel source for aerobic glycolysis, amino acid oxidation may contribute ~5% of total energy expenditure with total oxidation scaling with oxygen consumption (Lamont et al., 2001). During prolonged endurance exercises that primarily rely on endogenous carbohydrates, the subsequent decrease in muscle glycogen may result in amino acids providing up to ~10% of energy needs in adults (Lemon et al., 1980). When comparing fat oxidation rates to those of adults, it has been suggested that pre-pubertal males (~11 years of age) generally oxidize fat at a higher rate, similar to that of children (Riddell et al., 2008). As males develop through puberty (~15 years of age), their fat oxidation rates slow down and begin to mimic the oxidation rates of adults (Riddell et al., 2008). With our participants having a similar age to those already developing through puberty, it is possible that fat oxidation rates, similar to adults, would occur. With similar fat oxidation rates, it can be assumed that pubertal male amino acid oxidation rates would be similar to those of adults as well. This would suggest that these pubertal males have the potential to also supply up to 10.4% of total
energy needs during our exercise stimulus. In reference to our study, using the mean body weight of our participants (57.3 kg), the average energy expenditure during the LIST exercise was 612.4 kcal. Assuming 5% of our energy was contributed by protein, and that each gram of protein is equivalent to 4 kcal, ~0.13 g·kg\(^{-1}\) of protein would have been oxidized during our exercise stimulus. The potential increased utilization of amino acids as a fuel source during exercise, would ultimately lead to an increased protein requirement, and the need for additional consumption of dietary protein for these exercising individuals.

When comparing non-exercising to exercising adults using similar methodologies, exercising adults had an EAR that was ~105% greater than what was determined for non-exercising adults. This demonstrates that active adults require additional protein over non-exercising adults when put under similar test protocols (Humayun et al., 2007; Packer et al., 2015). It is believed that the increase in protein requirements caused by physical activity seen in exercising adults would be similar to those seen in the active adolescent population, due to amino acid oxidation rates being suggested to be similar in adolescents and adults (Boisseau et al., 2007; Baxter-Jones et al., 2008).

Protein requirements may also be increased in an attempt to support recovery from exercise-induced muscle damage (Lemon et al., 1992). When investigating the impact of a soccer match, which our LIST exercise stimulus was modelled after, it was determined that performing a soccer match increases oxidative stress and induces muscle damage for up to 72 hours in adults (Ascensao et al., 2008). With amino acids being the building block for skeletal muscle, an increase in muscle damage could potentially increase the need for additional exogenous amino acids to help repair and rebuild the damaged skeletal muscle. Therefore, not only can amino acids be utilized as an energy source during prolonged endurance exercise, they can also be used to support recovery
from exercise induced muscle damage, which could translate into increased amino acid requirements.

4.4 – Requirement relative to exercising adolescents

The present study also aimed to compare our results, which utilized the IAAO technique, to the current suggested protein recommendations for exercising adolescents, which were determined by the NBAL technique (Boisseau et al., 2007; Aerenhouts et al., 2013). A study by Boisseau et al. (2007) aimed to determine general protein requirements in adolescent male soccer players (N = 11, Age = 13.8 ± 0.1 years; Body weight = 54.2 kg; FFM = 47.7 kg). In this study, participants were provided diets at protein intakes of 1.0, 1.2, and 1.4 g·kg⁻¹·d⁻¹. Comparatively, our determined EAR is ~23% greater than that which was determined by Boisseau et al. (2007). Similarly, a longitudinal study by Aerenhouts et al. (2013) also aimed to estimate general protein requirements for active adolescents using 29 sprint female (Age = 14.7 ± 1.6; Body weight = 53.7 kg; FFM = 44.6 kg) and 31 sprint male athletes (Age = 14.8 ± 1.7 years; Body weight = 59.4 kg; FFM = 53.9 kg, Aerenhouts et al., 2013). Seven-day diet and physical activity diaries were completed to estimate energy balance and protein intake, while, NBAL was calculated by 24-hour urine sample analysis. An EAR of 1.2 g·kg⁻¹·d⁻¹ and 1.13 g·kg⁻¹·d⁻¹ was deemed sufficient to yield a positive NBAL for active male and female adolescents respectively. When comparing our results to the EAR for protein in active male adolescents derived by Aerenhouts et al. (2013), our determined EAR is ~23% greater than what was determined by Aerenhouts et al. (2013).

Previous studies have suggested that NBAL can underestimate total nitrogen excretion, due to miscellaneous losses of nitrogen (i.e. hair, sweat, exhalation) being estimated at 15 mg of
nitrogen·kg\(^{-1}\)·d\(^{-1}\) (Boisseau et al., 2002) since they are difficult to measure accurately (Forbes, 1973; Humayun et al., 2007). This may be a significant source of variability that could contribute to the differences seen in determined protein requirements by NBAL and IAAO studies given that an underestimation of nitrogen excretion will result in a higher than normal NBAL and, consequently, an underestimation of true protein requirements. For example, the estimation of 15 mg of nitrogen·kg\(^{-1}\)·d\(^{-1}\) losses through miscellaneous avenues may be an even greater underestimation for active individuals. Athletes may excrete 21 – 130 mg of nitrogen/100 ml of sweat during physical activity (Consolazio et al., 1963). With athletes excreting up to almost ten times more nitrogen through sweat than what is currently estimated for total miscellaneous losses, the underestimation of nitrogen excretion may be a major source of error for NBAL studies when determining protein requirements, especially when utilized in active populations.

When comparing our results to the EAR determined by Boisseau et al. (2007) (1.2 g·kg\(^{-1}\)·d\(^{-1}\), Boisseau et al., 2007), it is important to note that both our study and the study by Boisseau et al. (2007) investigated active male adolescents of similar age (13.8 ± 0.5 and 13.8 ± 0.1 years of age, respectively). Having similar participants, with similar fitness levels, the primary potential explanation for the observed discrepancy in protein EAR is the difference in methodology between the two studies. As previously mentioned, the NBAL technique has been suggested to underestimate total nitrogen excretion which leads to underestimations of true protein requirements. The current EAR for non-exercising adults, determined by the NBAL technique (0.66 g·kg\(^{-1}\)·d\(^{-1}\), FAO/WHO, 2007), is ~41% less than the EAR revealed by Humayun et al. (2007) for non-exercising adults, determined by the IAAO technique (0.93 g·kg\(^{-1}\)·d\(^{-1}\), Humayun et al., 2007). Synonymous to the difference seen between the study conducted by Boisseau et al. (2007) and ours, the major difference between the current EAR for non-exercising adults, and the study
by Humayun et al. (2007) is the utilization of the IAAO technique instead of NBAL. Humayun et al. (2007) explains the visible difference in EAR by mentioning that the NBAL technique may underestimate true protein requirements by underestimating total nitrogen excretion (Humayun et al., 2007). In addition, Humayun et al. (2007) further explain that another source of error for the NBAL technique is the utilization of a linear regression when analyzing the data, as compared to the bi-phase linear regression, which is used by the IAAO technique (Humayun et al., 2007). During their study, Humayun et al. (2007) were able to re-analyze (using a bi-phase linear regression) the data collected from previous NBAL studies and determined a re-evaluated EAR for sedentary adults to be 0.91 g·kg\(^{-1}\)·d\(^{-1}\) (Humayun et al., 2007). The re-evaluated EAR was similar to the EAR determined using the IAAO technique (0.93 g·kg\(^{-1}\)·d\(^{-1}\), Humayun et al., 2007), which identified a source of error for the NBAL technique, as well as, validated the IAAO technique as an accurate method for determining protein requirements. Consequently, our determined EAR being ~23% greater than the EAR determined by Boisseau et al. (2007) can potentially be explained by the multiple sources of error found with the NBAL technique.

Aerenhouts et al. (2013) estimated total protein intake through free-living participant dietary recall diaries, as opposed to providing subjects with diets consisting of set protein quantities. It has been shown that dietary recalls can be difficult and can lead to misrepresentation of true dietary intake (Shim et al., 2014). Participants can report up to a 14% difference between actual and reported total energy and protein intake (Baker et al., 2014). To put into context, if a participant was to report ingesting 140 g of protein, while actually ingesting 150 g of protein (~7% difference), they would be reporting 1,600 mg of nitrogen less than what was being ingested (1 g of protein = 160 mg of nitrogen). Having underreported total nitrogen intake, NBAL would subsequently seem more positive at a lower reported intake, when in fact more protein was
ingested. This underreporting would then lead to an overestimation of NBAL, which would ultimately result in an underrepresentation of true protein requirements. By trusting in participant dietary recall diaries, Aerenhouts et al. (2013) may have misrepresented true nitrogen intake, which, along with the sources of errors found in the NBAL technique (i.e. estimating miscellaneous nitrogen loss), may explain the potential difference in protein EAR seen when compared to our determined results.

4.5 – Requirements relative to IAAO Studies

While other studies have utilized the IAAO technique with a similar exercise stimulus in other demographics, our study was the first to employ the IAAO technique in an active adolescent male population. The results from our study displayed the greatest protein EAR (1.48 g·kg⁻¹·d⁻¹) when compared to previous IAAO studies.
Figure 10: A schematic displaying comparisons between IAAO studies that investigated protein requirements in various populations. Each study is represented by their lead author followed by their determined protein EAR in parentheses as g·kg\(^{-1}·d^{-1}\). Approximate differences were calculated between studies EAR. Potential explanations as to why there are differences are suggested in parentheses beside approximate values. G represents growth as the main contributor to protein EAR difference. Ex represents exercise as the main contributor to protein EAR difference. G + Ex represents a combination of both growth and exercise as main contributors to protein EAR difference.

4.5.1 – Requirements relative to non-exercising adults

When comparing our results to those determined by Humayun et al. (2007), who investigated protein requirements in non-exercising adults, it is apparent that our determined EAR of 1.48 g·kg\(^{-1}·d^{-1}\) is ~60% greater than the EAR value established for non-exercising adults (0.93 g·kg\(^{-1}·d^{-1}\), Figure 10; Humayun et al., 2007). The increase in our EAR compared to the EAR determined for non-exercising adults may be explained in part by the addition of physical activity, which has been demonstrated to increase protein requirements in adults after a similar bout of exercise (Packer et al., 2015), potentially as a result of, increased amino acid oxidation, development of exercise-induced muscle damage, and/or supporting increased LBM growth.
Alternatively, the greater EAR in the present study compared to the previous study in non-exercising adults (Humayun et al., 2007) may also be explained by the nutritional demands of the physiological changes that occur during the pubertal growth spurt (i.e. LBM accrual). When comparing the current EAR for non-exercising adults (0.66 g·kg⁻¹·d⁻¹, FAO/WHO, 2007) to non-exercising adolescents (0.78 g·kg⁻¹·d⁻¹, FAO/WHO, 2007), which were determined by NBAL and the factorial method, respectively, an ~18% increase is observed. This increase in protein requirements is suggested to allow the growing adolescent body to be supplied with the adequate amount of protein it needs to optimally grow during puberty.

4.5.2 – Requirements relative to exercising adults

When comparing our results to the previously mentioned study by Packer et al. (2015), which utilized the IAAO technique and LIST exercise stimulus in the active adult male population, it is evident that our determined EAR (1.48 g·kg⁻¹·d⁻¹) is ~10% greater than the EAR established for active adult males (1.35 g·kg⁻¹·d⁻¹, Figure 10; Packer et al., 2015). When comparing our determined EAR, after normalizing to FFM (1.73 g·kg FFM⁻¹·d⁻¹), to the EAR determined by Packer et al. (2015), normalized to FFM (1.60 g·kg FFM⁻¹·d⁻¹, Packer et al., 2015), a slightly smaller ~8% difference is observed. Active adult males generally have larger amounts of LBM compared to active adolescent males. The need to maintain LBM can potentially explain the reason for the evident smaller difference seen between protein EAR normalized to FFM (~8%) compared to protein EAR normalized to body weight (~10%). However, the effects of the pubertal growth spurt, and the physiological changes that occur, may justify the larger protein requirements in our active adolescent population, in comparison to the active adult population. As supported by the
discrepancy seen between current protein recommendations for adults (0.66 g·kg\(^{-1}\)·d\(^{-1}\), FAO/WHO, 2007) and adolescents (0.78 g·kg\(^{-1}\)·d\(^{-1}\), FAO/WHO, 2007), the requirement to consume additional protein is necessary to primarily support LBM accrual during puberty.

4.5.3 – Requirements relative to non-exercising children

Finally, when comparing our results to those determined for healthy school aged children on a non-exercising day using the IAAO technique, our determined EAR is \(~14\%\) greater than what was determined for children (1.3 g·kg\(^{-1}\)·d\(^{-1}\), Figure 10; Elango et al., 2011). As previously mentioned, outside of the first year of life, adolescence is the period of life in which the greatest relative change in LBM accrual occurs (Mirwald et al., 2002). The additional dietary protein is required to supply the body with sufficient amounts of protein to allow for optimal growth to occur during puberty. However, adolescents have been suggested to increase dietary protein utilization during the pubertal growth spurt (Beckett et al., 1997), which could explain the minimal difference observed between the current protein EAR for non-exercising adolescents (0.78 g·kg\(^{-1}\)·d\(^{-1}\), FAO/WHO, 2007) and children (0.76 g·kg\(^{-1}\)·d\(^{-1}\), FAO/WHO, 2007).

Our participants were active adolescents and performed the LIST exercise stimulus on trial days, while the participants in Elango et al. (2011) study were healthy children that were not exercising on trial days. As previously stated, endurance exercise lasting 60 minutes or greater can require protein to be oxidized as a source of energy, as well as, utilized in repairing exercise induced muscle damage (Lemon et al., 1980; Ascensao et al., 2008). In addition, the incorporation of physical activity has been shown to increase LBM growth, beyond that of which accrues during the pubertal growth spurt (Baxter-Jones et al., 2008). Therefore, the increase in protein EAR
observed between our results and those determined by Elango et al. (2011) can be attributed to the addition of physical activity, which increases protein oxidation as a fuel source, uses protein to help repair exercise induced muscle damage, and requires protein to support addition LBM accrual. However, the increase in protein requirements is minimized by the increased protein utilization that has been suggested to occur during the pubertal growth spurt.

4.6 – Current Protein Consumption

Participants from our study were able to receive the recommended 60 minutes/day of MVPA (Tremblay et al., 2011) through the incorporation of the LIST exercise stimulus. Our determined protein requirement (1.48 g·kg\(^{-1}\)·d\(^{-1}\)), which was determined using the IAAO technique, is greater than current protein recommendations for adolescents (0.78 g·kg\(^{-1}\)·d\(^{-1}\)), which was determined by the factorial method (FAO/WHO, 2007). Interestingly, a macronutrient analysis of both pre-adolescent and adolescent Canadian athletes was conducted to determine whether these athletes were consuming sufficient (as determined by the general RDA) macro and micronutrients (Parnell et al., 2016). Results revealed that males between 11-13 years of age (N=26) consume 2.4 ± 0.8 g·kg\(^{-1}\)·d\(^{-1}\), while males between the 14-18 years of age (N=53) consume 2.0 ± 0.6 g·kg\(^{-1}\)·d\(^{-1}\) (Parnell et al., 2016). When compared to the habitual protein intakes of our participants (1.8 ± 0.4 g·kg\(^{-1}\)·d\(^{-1}\)), similar values are observed. Although, these consumption values are greater than our determined RDA (1.78 g·kg\(^{-1}\)·d\(^{-1}\)), which represents a potential excess of protein consumption, it is encouraging to see that male adolescent athletes are consuming adequate/more than adequate amounts of protein daily. It has been suggested that protein intakes are generally adequate or excessive because protein is overvalued by coaches and athletes.
Additionally, a Canadian Community Health Survey (2004) also confirmed that children and adolescents between the ages of 4-18 are within the acceptable consumption range for all macronutrients, including protein (Canadian Community Health Survey, 2004). For those active children and adolescents who may not be consuming adequate protein, nutritional adjustments must be made to ensure optimal physiological growth and development.

When compared to the current protein consumption trends of individuals in developing countries, it has been shown that individuals from these countries are not only consuming a lesser quantity of protein in their daily diet, but also, a lower quality of protein (Schonfeldt, 2012; Lee et al., 2014). A recent statistical analysis by FAO determined that individuals from developing countries consume 0.89 g·kg\(^{-1}\)·d\(^{-1}\) while individuals from Africa consume only 0.69 g·kg\(^{-1}\)·d\(^{-1}\) (FAO, 2011). It is interesting to note that these values were determined by calculating protein that disappears from food supply, rather than protein consumed (Schonfeldt, 2012), suggesting that these values may overestimate true protein consumption. A more in-depth health survey determined that based on 24-hour dietary recall, true protein consumption was more than 20% less than what was determined from the food supply disappearance calculations (Schonfeldt, 2012). Therefore, this could suggest that individuals from developing countries including those in Africa may only consume 0.55 - 0.71 g·kg\(^{-1}\)·d\(^{-1}\) (Schonfeldt, 2012). A recent study determined that 37% of African adolescents (age 12 – 18) engaged in the recommended 60 mins of MVPA/day (Oyeyemi et al., 2016), which is much greater than the determined 7% of Canadian adolescents that perform 60 mins of MVPA/day (La rose, 2014). With the physical activity level of these adolescents being so great, the daily protein that they are consuming may be even more substandard than what they require. When comparing these protein consumption values to our determined RDA (1.78 g·kg\(^{-1}\)·d\(^{-1}\)), it is evident that individuals from developing countries may
not be consuming adequate dietary protein for their lifestyles and could be at risk for malnourishment. Thus, our protein requirement may be especially important for active growing adolescents worldwide to ensure all youth consume a diet to support their optimal growth and development.

In addition, a study conducted by Lee et al., (2014), determined that children in Southeast Asia are consuming a poorer quality of protein, which is resulting in their malnourishment (Lee et al., 2014). The predominant food in the Southeast Asian diet, rice and other cereals, contain lower utilizable protein, compared to animal and dairy protein, and are specifically low in the essential amino acid lysine (Lee et al., 2014). Even though these individuals may be consuming adequate quantity of protein, the utilizable nature of these proteins puts these children at risk for malnourishment and possibly optimal growth and development. Therefore, although Canadian children and adolescents may consume protein at or above the level determined herein, the same may not be said about comparable populations in developing countries. As previously mentioned, there are more than five times more adolescents from developing countries who are active (based on current recommendations) compared to Canadian youth (Oyeyemi et al., 2016), thus, nutritional adjustments should be made to allow for optimal growth and development of these children and adolescents.

4.7 – Strengths, Limitations, and Future Avenues of Research

Several strengths exist when discussing the design and application of the present study. First, the IAAO technique is a minimally invasive means of determining protein requirements, deeming it practical for both healthy and at-risk populations. Second, the utilization of the IAAO
technique to determine protein requirements only requires a two-day dietary adaptation period prior to each metabolic trial (Humayun et al., 2007). This not only allows for a practical application of the technique in younger or at risk participants, but, allows for a single participant to be tested several times over a range of deficient to excess protein intakes. Comparatively, the NBAL technique requires participants to adhere to a 7-14-day dietary adaptation period, which often prevents participants from consuming more than three protein intakes, and can become impractical to younger participants during the deficient protein intake trials (Rand et al., 2003; FAO/WHO, 2007). As a result, our study was able to provide protein intakes at a wider range, which potentially led to a more accurate determination of protein requirements.

Our study was the first to utilize the IAAO technique in an active adolescent population to determine protein requirements. As previously mentioned, limited research has been conducted pertaining to the protein requirements of adolescents, more specifically, active adolescents. Thus, our study gives further insight as to what the nutritional demands are for this active population, not only allowing for optimal performance in sport, but maximizing full growth potential as well.

Additionally, our study utilized an exercise stimulus (LIST exercise) that is modelled after the play of a soccer match and satisfies the recommendation of at least 60 minutes of MVPA per day for children and adolescents (Tremblay et al., 2011). The results from our study would presumably reflect the needs of generally active adolescent males, based on current physical activity recommendations, and highlights the importance of consuming adequate dietary protein to support LBM development and optimal growth. It should be noted that recent reports have determined that in 2005, 56% of males between the ages of 5 and 14 participated in team oriented sports (Clark, 2008). During our study, we modelled our exercise stimulus after a team oriented sport (i.e. soccer), which allowed us to investigate the effects of typical physical activity for this
population. Our study was able to display the benefits of physical activity in the adolescent population, and supports the recommendation of encouraging children and adolescents to perform at least 60 minutes of MVPA daily to ensure healthy growth and development.

Although, the IAAO technique was first utilized to determine protein requirements in the active adolescent population in our study, there may be some limitations inherent to its ecological validity. For example, participants were required to consume 8 hourly liquid meals on trial days with each representing one-twelfth of the participant’s daily energy requirements; this was done to ensure isotopic and metabolic steady state was achieved in a non-invasive manner (Bross et al., 1998). Even though these meals provided a sufficient amount of daily total energy, they may not mimic a typical dietary regimen, which generally consist of three large meals with unbalanced protein intakes (de Castro et al., 1997). It has been demonstrated that the pattern of protein intake can influence protein metabolism after exercise in adults as the repeated consumption of moderate amounts of protein (~20 g) spread out over regular intervals (~3h) has been shown to maximize muscle protein synthesis and whole body net balance (Moore et al., 2012; Areta et al., 2013). In addition, previous studies have measured the effects of a three-large equal meal feeding pattern on 24-hour leucine oxidation and determined that leucine is oxidized 16% less during this feeding pattern compared to an hourly meal feeding pattern (Raguso et al., 1999). This would suggest that protein requirements are decreased during this feeding pattern. Therefore, the hourly feeding pattern in the present study may overestimate true protein requirements which would warrant future studies to further investigate the effects of various feeding patterns on protein requirements.

Future studies could utilize the IAAO technique in several other demographics, with and without an exercise stimulus. Incorporating the IAAO technique in a sedentary adolescent population without an exercise stimulus would provide insight on whether the increase seen in
protein requirements in the present study may be due to growth demands, the addition of physical activity, or both. It can be speculated that protein requirements would be greater in this population than other sedentary populations based on current protein recommendations (FAO/WHO, 2007), which suggests adolescents require the greatest amount of protein compared to other sedentary populations to support the large physiological changes that occur during adolescence (Mirwald et al., 2001). However, even though LBM accrual will be greatest during this time of life, protein requirements are not speculated to increase greatly due to the increased utilization of protein found in adolescents during puberty (Beckett et al., 1997). It can also be speculated that protein requirements would be less than active populations, due to the increasing effects physical activity has on protein requirements. Whether due to increased amino acid oxidation during endurance exercise (Lamont et al., 2001), or the demands of increased LBM accrual during exercise (Baxter-Jones et al., 2008), protein requirements can be speculated to be greater in an active population compared to any sedentary population.

Additionally, a study utilizing the IAAO technique with an exercise stimulus, similar to the LIST exercise, in the active children population will determine if physical activity affects a child’s protein requirements in a similar manner as it does other populations. Children are recommended to perform at least 60 minutes of physical activity daily, which would primarily consist of MVPA. Therefore, it is important to determine how exercise affects their nutritional requirements to ensure optimal growth and development. Adult women have been shown to oxidize more fat, and subsequently, less amino acids than adult males (Knechtle et al., 2004). Similarly, children have been suggested to oxidize more fat, and less amino acids, when compared to adolescents (Riddell et al., 2008). Therefore, it can be speculated that based on their preference to oxidize fat more than amino acids, and the addition of a more endurance based exercise stimulus,
the protein requirements of this population would be greater than active adults but less than active adolescents.

Furthermore, a study utilizing the IAAO technique with a feeding protocol similar to the westernized feeding pattern (three meals per day), as opposed to the eight meals per trial in our study, can help determine if protein requirements are different based on various feeding patterns. As previously mentioned, Raguso et al. (1999) determined that leucine oxidation is 16% less during a three-meal feeding pattern compared to an hourly meal feeding pattern (Raguso et al., 1999). This suggests that the three-meal feeding pattern apparently spares leucine from oxidative losses and thus, would decrease overall protein requirements. By utilizing a participant in a non-steady state, with repeated breath sample collections, one would be able to capture the area under the \( F^{13}\text{CO}_2 \) curve to determine protein requirements. Additionally, the oxidation rates of a single meal have been shown to be reflective of three similar sized meals (el-Khoury et al., 1995). Thus, trial days can be shortened by measuring the oxidation of a single meal and predicting what would happen with three meals. It can be speculated that the suggested study would determine decreased protein requirements compared to our current, hourly meal feeding pattern, due to the decrease in amino acid oxidation.

Moreover, a study utilizing the IAAO technique with a similar protocol in adolescents from different countries (i.e. Africa and Southeast Asia), as opposed to Canadian and US adolescents, can help determine if protein requirements of adolescents are similar regardless of individualized habitual dietary intake. As previously mentioned, the modern-day trend of individuals in developing countries is the consumption of a lesser quantity of protein in their habitual diet (Schonfeldt, 2012). Consuming a poor diet with inadequate amounts of protein has been shown to delay the onset of puberty and create deficiencies in vitamins and minerals, which are crucial for
normal pubertal growth and development (Tinggaard et al., 2012). In addition, adolescents from developing countries have been shown to be more active than North American adolescent. Therefore, understanding whether adolescents from different countries have lower daily protein requirements, or highlighting that they may be malnourished, is important in assuring that all adolescents, regardless of country of residence, are not hindering their growth and development because of nutritional deficiency.

Lastly, a study that incorporates the IAAO technique but with different protein sources (i.e. soy, plant based, casein), as opposed to the egg based protein used in our study, can help determine if protein requirements are affected by the type, and quality of protein being consumed. Not all individuals have a daily regime that include ideal protein sources, as highlighted by Lee et al., (2014). Therefore, it would be important to determine whether protein requirements are increased based on the quality of dietary protein. It can be speculated that consuming low utilizing proteins, such as rice (low in lysine) or soy (low in Sulphur containing amino acids) would result in a greater daily protein requirement. Individuals, specifically children and adolescents, that habitually consume these types of proteins must intake larger amounts to ensure they are providing the body with sufficient amounts of all essential amino acids to support normal growth and development (Kalman, 2014).

Results from these, and other similar suggested future studies, can provide additional protein requirements for various populations as well as help explain the discrepancies seen between protein requirements in other populations. Our study will serve as a benchmark for which all other subsequent IAAO studies and previous NBAL studies can be compared to.
4.8 – Conclusion

In conclusion, we demonstrate using the novel IAAO technique for the first time in active adolescent males that recommended protein intakes are greater than those previously established for adolescents, which was determined using NBAL, as well as, sedentary adults, active adults, and school aged children which were determined using the IAAO technique. Our EAR (1.48 g·kg\(^{-1}\)·d\(^{-1}\)) and RDA (1.78 g·kg\(^{-1}\)·d\(^{-1}\)) demonstrate the need for increased consumption of protein for active adolescents, compared to what is currently recommended for general adolescents. This increased protein consumption supports the replenishment of the amino acid pool from oxidative aerobic losses, provides a source of amino acids to support recovery from exercise induced muscle damage, and/or helps LBM growth of this active population. Adequate consumption of protein will also allow for optimal physiological growth and development during a time in which rapid changes are occurring. Our results can provide a framework for future studies to elucidate protein requirements in comparative populations, both active and sedentary.
Appendices

Appendix A - Maturity offset Calculations

Boys:

\[ Maturity \ Offset = -9.236 + (0.0002708 \times \text{Leg Length and Sitting Height interaction}) - (0.001663 \times \text{Age and Leg Length interaction}) + (0.007216 \times \text{Age and Sitting Height interaction}) + (0.02292 \times \text{Weight by Height ratio}). \]

Girls:

\[ Maturity \ Offset = -9.376 + (0.0001882 \times \text{Leg Length and Sitting Height interaction}) + (0.0022 \times \text{Age and Leg Length interaction}) + (0.005841 \times \text{Age and Sitting Height interaction}) - (0.002658 \times \text{Age and Weight interaction}) + (0.07693 \times \text{Weight by Height ratio}). \]
Appendix B – Adolescent male Beep Test Results

Grades 7-10 (Ages 12-15): Adolescents

80th Percentile: L8-3

<table>
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<th>Percentiles</th>
<th>Lengths</th>
<th>Levels</th>
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<td>95</td>
<td>81.0</td>
<td>L9-9</td>
</tr>
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</table>
Appendix C – *Study Diet Energy Intake Equation*

\[
\text{Study Diet Energy Intake} = (\text{REE} \times 1.5) + [(0.1425 \times \text{Weight(kg)} \times 75(\text{min})) \times 1.1]
\]

\textbf{REE} = Resting Energy Expenditure During Sleep (kcal) (Recorded Using SenseWear BodyMedia Armband Accelerometer; Calabro, 2009)

1.5 = Activity Factor

0.1425 = Average Energy Expenditure During LIST Exercise Stimulus (kcal/kg/min)

75 Minutes = Duration of the LIST Exercise Stimulus

1.1 = 10\% Buffer for Energy Expended During the LIST Exercise Stimulus
Appendix D – Adolescent Female Data

Participants

A total of 7 active, female participants between -0.5 to +1.0 years from PHV were recruited for the present study. Participant characteristics are provided in Table 1. The participant PHV range distinctly separates the adolescent population from both the child and young adult demographics. This range encompasses the duration of which maximal growth occurs during the pubertal growth spurt (Mirwald, 2002).

Participant anthropometric measures and characteristics

<table>
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<th>Participant characteristics</th>
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</thead>
<tbody>
<tr>
<td>Age, y</td>
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<tr>
<td>Height, cm</td>
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<tr>
<td>Body weight, kg</td>
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<td>FFM, kg</td>
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<tr>
<td>Percent Body Fat, %</td>
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<tr>
<td>Years from PHV, y</td>
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<tr>
<td>Predicted VO₂max, ml/kg/O₂/min</td>
<td>47.5 ± 2.0</td>
</tr>
</tbody>
</table>

Values are means ± SD, n=7

Phenylalanine Flux

Phe Ra was not affected by protein intake (Figure 3, P = 0.14, average Phe Ra = 57.7 ± 2.4 μmol·kg⁻¹·h⁻¹). This indicates that the phenylalanine pool for the IAAO did not change in response to increasing test protein intakes. This would suggest that any change observed in Phe Ox will be reflective of whole-body protein synthesis.
Relationship between phenylalanine rate of appearance (Phe Ra) and protein intake in female adolescents following the LIST exercise stimulus.

**Average Protein Requirement and Recommended Protein Intake**

The use of a bi-phase linear regression crossover analysis revealed a breakpoint (estimated average requirement (EAR)) at 1.28 g·kg\(^{-1}\)·d\(^{-1}\) (Figure 4A, \(R^2 = 0.70\)). The upper 95% CI (recommended dietary allowance (RDA)) was determined to be 1.46 g·kg\(^{-1}\)·d\(^{-1}\). Additionally, when normalized for FFM, a breakpoint was revealed at 1.47 g·kg FFM\(^{-1}\)·d\(^{-1}\) (Figure 4B, \(R^2 = 0.64\)). The upper 95% CI was determined to be 1.78 g·kg FFM\(^{-1}\)·d\(^{-1}\). Subsequently, a bi-phase linear regression crossover analysis of Phe Ox revealed breakpoints at 1.32 g·kg\(^{-1}\)·d\(^{-1}\) (Figure 5A, \(R^2 = 0.75\)) and 1.49 g·kg FFM\(^{-1}\)·d\(^{-1}\) (Figure 5B, \(R^2 = 0.69\)), when normalized for body weight and FFM, respectively. The upper 95% CI was determined to be 1.49 g·kg\(^{-1}\)·d\(^{-1}\) and 1.74 g·kg FFM\(^{-1}\)·d\(^{-1}\), when normalized for body weight and FFM, respectively. Although the estimated EAR and RDA are similar between the breakpoint analysis of F\(^{13}\)CO\(_2\) and Phe Ox, F\(^{13}\)CO\(_2\) data is generally
considered more accurate as they most closely resemble phenylalanine hydroxylation and, thus, reflect true intercellular whole-body protein synthesis.

**Figure A:** Relationship between $F^{13}CO_2$ and protein intake when normalized to body weight. **B:** Relationship between $F^{13}CO_2$ and protein intake when normalized to FFM

**Figure A:** Relationship between Phe Ox and protein intake when normalized to body weight. **B:** Relationship between Phe Ox and protein intake when normalized to FFM
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resistance training in youth randomized clinical trial. *JAMA Pediatrics, 168*(11), 1006-1014.


