DETERMINATION OF THREONINE REQUIREMENTS AND THE METABOLIC AVAILABILITY OF THREONINE FROM FOOD SOURCES IN HEALTHY SCHOOL-AGED CHILDREN

by

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H. B.Sc., The University of Toronto, 2014

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in

THE FACULTY OF GRADUATE AND POSTDOCTORAL STUDIES

(EXPERIMENTAL MEDICINE)

THE UNIVERSITY OF BRITISH COLUMBIA

(Vancouver)

SEPTEMBER 2018

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Determination of threonine requirements and the metabolic availability of threonine from food sources in healthy school-aged children

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the degree of Master of Science in
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Abstract

Threonine, an indispensable amino acid, is required for protein synthesis throughout the body. Due to its quantitative importance in gut mucosal proteins, consuming adequate amounts of threonine is essential for proper digestive and immune function during growth. Currently, the dietary reference intakes (DRIs) recommend an estimated average requirement (EAR) and recommended dietary allowance (RDA) of 19 and 24 mg/kg/d for threonine intake in 6-10y children based on factorial (mathematical) calculations. In addition, it is unknown to what extent dietary threonine is available for protein synthesis from food protein sources in school-aged children.

The primary objective of this thesis was to determine the dietary requirement for threonine in 6-10y healthy children using the minimally invasive Indicator Amino Acid Oxidation (IAAO) method. The secondary objective was to compare threonine metabolic availability from casein (animal protein) and soy (vegetable protein) in the same set of children. Six healthy Canadian children (three boys: three girls) aged 7.5 ± 1.4 y randomly received 6-9 test threonine intakes each, ranging from 1-50 mg/kg/d, with an amino acid mixture patterned after egg protein. Study day diets were complete with protein provided at 1.5 g/kg/d, energy provided at 1.7x the resting energy expenditure. To determine threonine requirements, the oxidation of L-[1-13C]-phenylalanine to 13CO2 (F13CO2) in response to the test intakes was used. To determine threonine metabolic availability, the children were tested at 0 mg/kg/d, and at 11 mg/kg/d from three different sources (threonine as crystalline amino acid, casein and soy), and the oxidation of L-[1-13C]-Phenylalanine to 13CO2 measured.
Threonine requirement was determined to be 21.9 mg/kg/d (95% CI: 10.5 – 33.4 mg/kg/d). The metabolic availability of threonine was determined to be 96.6% and 83.4% from casein and soy, respectively. Threonine requirements in school-aged children were determined for the first time directly, and the results are ~15% higher than current DRI recommendations of EAR 19 mg/kg/d. In addition, the metabolic availability of threonine was found to be higher in casein compared to soy, which needs to be considered when making dietary recommendations.
Lay Summary

Current dietary intake recommendations in school-aged children (6-10y) for the essential amino acid threonine, are set at 19 mg/kg/d, based on mathematical formulas which are derived using adult requirements plus the requirement for growth. It is not known whether the true requirement for threonine is similar. By using the minimally-invasive indicator amino acid oxidation method, we determined a requirement of 21.9 mg/kg/d in healthy 6-10 y old Canadian children. The amount of threonine that is useable for protein synthesis, referred to as metabolic availability, changes depending on the food protein source. Our second experiment determined the relative metabolic availability of threonine when consumed in casein or soy protein. We found a relative availability of 96.6 and 83.4% for threonine from casein and soy, respectively. Thus, threonine requirements in children are only marginally higher (~15%) than recommendations, but diets rich in vegetable protein sources may provide less threonine to the body relative to what can be obtained from animal protein rich sources.
Preface

This study was developed by myself, Peter Radonic, and my supervisor Dr. Rajavel Elango. The study was approved by UBC ethic certificate H15-02691. All research work was carried out primarily by me, with material support from other researchers within the Elango lab.
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<tr>
<td>DAA</td>
<td>Dispensable amino acid</td>
</tr>
<tr>
<td>DAAO</td>
<td>Direct amino acid oxidation</td>
</tr>
<tr>
<td>DIAAS</td>
<td>Digestible indispensable amino acid score</td>
</tr>
<tr>
<td>DRI</td>
<td>Dietary reference intake</td>
</tr>
<tr>
<td>EAR</td>
<td>Estimated average requirement</td>
</tr>
<tr>
<td>IAA</td>
<td>Indispensable amino acid</td>
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<tr>
<td>FAO</td>
<td>Food and agricultural organization</td>
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<tr>
<td>F\textsubscript{13}CO\textsubscript{2}</td>
<td>Rate of label tracer oxidation</td>
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<td>IAAB</td>
<td>Indicator amino acid balance</td>
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<tr>
<td>IAAO</td>
<td>Indicator amino acid oxidation</td>
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<td>kg</td>
<td>Kilogram</td>
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<tr>
<td>MA</td>
<td>Metabolic availability</td>
</tr>
<tr>
<td>mg</td>
<td>Milligram</td>
</tr>
<tr>
<td>mg/kg/d</td>
<td>Milligrams per kilogram body weight per day</td>
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<tr>
<td>PDCAAS</td>
<td>Protein digestibility – corrected amino acid score</td>
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<td>RDA</td>
<td>Recommended dietary allowance</td>
</tr>
<tr>
<td>SPI</td>
<td>Soy protein isolate</td>
</tr>
<tr>
<td>TDG</td>
<td>Threonine dehydrogenase (EC:1.1.1.103)</td>
</tr>
<tr>
<td>TDH</td>
<td>Threonine dehydratase (EC:4.3.1.19)</td>
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<td>µmol</td>
<td>Micromole</td>
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<td>Amino Acid Abbreviations</td>
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Acknowledgements

I would like to acknowledge and thank my supervisor Dr. Rajavel Elango, for all of the help and support that he has given me throughout my program. I know his patience, vision, and guidance have helped me both to complete this research program, but also to develop my knowledge of amino acid requirements and pediatric nutrition. I would also like to thank my committee members Dr. Linda Casey and Dr. James Thompson, for their helpful input and edits to my thesis during the development of the study and writing process. Finally, I cannot thank enough the members of the Elango lab for their unending support, motivation, friendship throughout my time here, and into the future. Madeleine Ennis, Betina Rasmussen, Katia Caballero, Abrar Turki, and Gaia Lepine, my time spent with you has been a pleasure and I look forward to hearing about your success in the future!

It is with great appreciation that I thank the Canadian Institute for Health Research, for their funding of this study. Great science is necessary for all, but it is not free. CIHR made this and many other studies possible, and for that I say thank you. The staff and faculty of the UBC Experimental Medicine Program deserve thanks as well, for their coordinating and organizing the program which I had the privilege to complete.
Dedication

This thesis is dedicated to my parents. Without them, I never would have developed the inquisitive drive to explore and discover that has helped me to where I am today. I would also like to dedicate this to my close friends who kept me grounded and focused on the future.
Chapter 1: Introduction

The quantity and quality of food that people consume has a great impact on long-term health outcomes. In particular, dietary protein and amino acid consumption have been the focus of both nutritional and public health oriented studies due to their impact on growth and development (Arentson-Lantz et al., 2015). Current recommendations for dietary intake of protein and amino acids are based upon previous research using the nitrogen balance method, the results of which are increasingly thought to underestimate true amino acid requirements across the human life-cycle (Campbell et al., 1994; Elango et al., 2010; Gaffney-Stomberg et al., 2009).

An alternative method called the Indicator Amino Acid Oxidation (IAAO) method, using state-of-the-art stable isotope technology has been developed (Pencharz and Ball, 2003). The IAAO method uses an indispensable (essential) amino acid labeled with a stable isotope ($^{13}$C) which is sensitive to small changes in amino acid intake and is a minimally invasive technique safe for use in human populations of all ages. The IAAO method has been applied in school-age children to determine requirements for total protein (Elango et al., 2011), and individual amino acids, including lysine, methionine, and phenylalanine (Elango et al., 2007; Humayun et al., 2006; Pencharz et al., 2012).

The dietary requirements of threonine (Thr), an indispensable amino acid, have not yet been determined using the IAAO method. Current recommended threonine intakes were set using a factorial (mathematical) calculation (Institute of Medicine, 2005). Direct determination of threonine requirements in children is important, as it forms a major part of mucin proteins in the small intestinal mucosa (Faure et al., 2005; Guzman-Aranguex and Argüeso, 2010; Law et al., 2007), and has been shown to be important in growth and development of the small intestinal mucosal layer (Bertolo et al., 1998; Guzman-Aranguex and Argüeso, 2010; Law et al., 2007).
Additionally, threonine is the second limiting amino acid for protein synthesis in cereal and vegetable protein based diets, with lysine being the first limiting amino acid, as shown earlier in animal models (Fernandez et al., 1994; Mavromichalis et al., 1998). While this is not a significant concern in developed countries where there is adequate food available, in developing countries with suboptimal nutrition these inadequate intake of these limiting amino acids can have serious developmental consequences (Pelletier and Frongillo, 2003; Smith and Haddad, 2000). Recent research has shown that lysine requirements in healthy Indian children were virtually identical when compared to normal healthy children living in Canada (Elango et al., 2007). Interestingly, Indian children infected with intestinal parasites demonstrated a 20% increase in lysine requirements (Pillai et al., 2015). Whether threonine requirements are similarly affected in association with parasitic infection is unknown. Therefore, determining basic threonine requirements in healthy children is of great interest because of its basis for further research.

I will now review protein and amino acid metabolism, with a focus on threonine, methods to determine amino acid requirements and metabolic availability of amino acids from foods.
Chapter 2: Literature Review

2.1 Protein and Amino Acids

Of the three macronutrients that are required for life (protein, fat, and carbohydrate), protein has the most complex set of functions. Protein is a general name for a chain of sub-units of amino acids (AA), which are fundamental building blocks comprised of a carbon backbone, an amine group, a carboxyl group, and an active group, the presence of which differentiates the traits and function of each amino acid.

The 20 amino acids which make up human tissue protein, are classified into three subgroups: indispensable, conditionally indispensable, and dispensable amino acids. Nine are indispensable, seven are conditionally indispensable, and four are dispensable (Reeds, 2000). An indispensable amino acid is defined as an amino acid which cannot be synthesized within the body at the rate required for growth and development, and must be consumed from dietary sources (Borman et al., 1946; Reeds, 2000). Conditionally indispensable amino acids are limited in the rate at which they can be synthesized in the body, and become indispensable when demands are increased (Reeds, 2000). Dispensable amino acids are those which can be created in the body in the presence of an adequate source of nitrogen (Reeds, 2000). The nine indispensable amino acids are: histidine (His), methionine (Met), lysine (Lys), phenylalanine (Phe), tryptophan (Tyr), leucine (Leu), isoleucine (Ile), valine (Val), and threonine (Thr). Recommendations for dietary protein and amino acids for healthy people are expressed as one of two guidelines. The first, the Estimated Average Requirement (EAR), is the intake which meets the requirement for ~50% of the population. The second, the Recommended Daily Allowance (RDA) is the intake that meets the requirements for ~97-98% of the population (Institute of Medicine, 2005).
2.2 Threonine

Threonine, as described above, is one of the indispensable amino acids, and possesses a neutral chemical structure, with a methyl group bonded to the active group (Figure 1), with a chemical formula of $\text{C}_4\text{H}_9\text{NO}_3$.

![Zwitterionic structure of threonine](image)

Figure 1: Zwitterionic structure of threonine

The additional methyl group at carbon 3 displaces the OH group and forms a second chiral center, with the centers of chirality being at C2 and C3. As a result of having two chiral centers, during its synthesis there are four racemic conformations of threonine which can result: (2R,3R), (2S,3S), (2R,3S), and (2S,3R) (Chassagnole et al., 2001). Of these, (2S,3R) is the enantiomer which is referred to as L-threonine (Sapoundjieva et al., 2006). (2S,3R) is biologically active in humans.
2.3 Threonine Metabolism

Dietary threonine is either incorporated into body proteins, or broken down and oxidized via two separate but related pathways (Figure 2) (Chapman, 2011). The initial steps of the catabolic process occur with the help of two separate enzymes, threonine dehydratase (TDH) and threonine dehydrogenase (TDG) (Darling et al., 2000). Of the two, TDH is the more active pathway in humans, catalyzing the conversion of threonine into 2-ketobutyric acid (Darling et al., 2000). This occurs in the cytosol of the cell, mostly in the liver (Floc’h et al., 1997), but the subsequent conversion of 2-ketobutyric acid into propionyl-CoA, which results in the release of carbon in the form of CO₂, occurs in the mitochondria of the cell. This conversion is completed by either pyruvate dehydrogenase (PDH) or branched chain α-keto acid dehydrogenase (BCαKD) (Chapman, 2011).

![Threonine catabolism](Figure 2: Threonine catabolism. Adapted from (Bird and Nunn, 1983; Chapman, 2011; Darling et al., 1999))
2.4 Threonine Dietary Sources

Most food protein sources contain threonine, with varied composition. Meat and other animal products are among the highest sources of threonine (Table 1). Although plant sources also provide threonine, it is at a lower concentration relative to total weight. The proportion of an amino acid which is ultimately available from food sources is also different based on the digestibility of the food, which affects the bio-availability in cells (Young and Pellett, 1994).

Table 1: Threonine content of commonly consumed protein sources

<table>
<thead>
<tr>
<th>Threonine</th>
<th>Corn</th>
<th>Sorghum</th>
<th>Sweet potato</th>
<th>Chickpea</th>
<th>Rice</th>
<th>Egg white</th>
<th>Beef</th>
<th>SPI</th>
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<tr>
<td>g/100g food</td>
<td>0.4</td>
<td>0.4</td>
<td>0.1</td>
<td>0.8</td>
<td>0.2</td>
<td>3.7</td>
<td>1.2</td>
<td>3.1</td>
</tr>
<tr>
<td>mg/1g protein</td>
<td>37.6</td>
<td>32.6</td>
<td>53.3</td>
<td>37.4</td>
<td>35.9</td>
<td>45.4</td>
<td>45.3</td>
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</table>

(US Department of Agriculture, Agricultural Research Service, 2015)(Fuller et al., 1989)

2.5 Methods to Determine Amino Acid Requirements

2.5.1 Nitrogen Balance

The traditional method, nitrogen balance, was developed to determine the difference in the amount of nitrogen consumed versus losses in response to different test intakes (Bender, 2012). Since the average content of proteins are about 16% N by weight, the view that nitrogen balance equates to protein balance has been the traditional view. When dietary requirements are met nitrogen balance will be zero, because the amount of nitrogen being consumed is equal to that being excreted as waste. A positive nitrogen balance is determined when the amount of nitrogen consumed is greater than the amount excreted, and is usually observed in conditions of growth. The opposite, negative nitrogen balance, is usually observed when the amount of nitrogen consumed is less than the amount excreted. Figure 3 depicts the movement of nitrogen through the body showing a net zero nitrogen balance. While it is true that the majority of
nitrogen loss is captured in the urinary and fecal excretion, the method does not take into account miscellaneous losses of nitrogen, which includes nitrogen in exhaled breath, sweat and nails.
Figure 3: Nitrogen balance, zero nitrogen balance. Bender et al., 2012.

This diagram depicts the movement of nitrogen through the body during a net zero nitrogen balance, (nitrogen consumed equals nitrogen excreted)
The nitrogen balance method has been used to determine protein requirements in animals (Mitchell et al., 1968) and mostly adult humans (Pencharz et al., 1977; Rand et al., 2003) including a few in vulnerable populations, like children (Gattas et al., 1990; Nakagawa et al., 1961, 1962). While the method has been used to estimate specific amino acid requirements (Nakagawa et al., 1961, 1962), several drawbacks exist. The method is intensive to perform, as the duration of the study has to be over several days for each test intake (Bricker et al., 1949; Garza et al., 1977; Yoshimura, 1972). This is primarily because the nitrogen pool in the body is quite large, and for the nitrogen pool to equilibrate to the change in test protein intake, takes a minimum of 4-7 days (DRI 2005). Nitrogen losses, as described above from all sources like sweat etc are also hard to determine with accuracy (Lopez et al., 1986)(Bender, 2012; Calloway et al., 1971; Howat et al., 1975)(Pencharz et al., 1977; Scrimshaw et al., 1972). While constants are used for the miscellaneous losses, collection of urine and feces over several days becomes a cumbersome task. Furthermore, nitrogen intakes, which has to be over a range of deficient to adequate becomes challenging to perform in vulnerable populations for extended periods of time. Especially in healthy children, the ethical aspects of the conduct of nitrogen balance studies makes it difficult to test very low amino acid test intakes.

### 2.5.2 Plasma Amino Acid Response Method (PAARM)

Measurement of plasma amino acid concentrations was also developed and considered for use in determining the requirements of specific amino acids. This method analyzes changes in the concentration of free plasma amino acids over time in response to changing levels of test intake (Kurpad et al., 2002). The principle behind this method is that when intake of amino acids meets and exceeds the requirement, then the amount of circulating amino acids in plasma will
begin to increase. This method has been used in humans (Kurpad et al., 2002; Tontisirin et al., 1973; Young et al., 1971, 1972) and pigs (Mitchell et al., 1968). The method has not gained wide-spread use due to high variability in the responses (Munro, 1970) (Young et al., 1972). Kurpad 2002 used this method along with the IAAO method, and found a requirement for threonine of 13-15mg/kg/d with a 95% CI of 1-19mg/kg/d. The large range in confidence intervals for the results from this method, coupled with the relative invasiveness of having to collect several blood samples from participants on each study day makes it a less desirable method.

2.5.3 Direct Amino Acid Oxidation (DAAO)

The use of stable isotopes to determine amino acid requirements was pioneered by Young and colleagues (Meguid et al., 1986a, 1986b; Meredith et al., 1986). DAAO refers to the method where the oxidation of a test amino acid directly measures the requirement of the target amino acid, rather than using a secondary marker. Prior to each study day participants are given a two-day adaptation diet which adapts them to the relative amount of amino acid they will receive on the study day. On each study day, the target amino acids are labeled with a stable isotope, $^{13}$Carbon, at the 1-C carbon position (Meguid et al., 1986a, 1986b; Meredith et al., 1986).

$^{13}$Carbon is a stable isotope with an additional neutron, and the additional mass can be detected using mass spectrometers, isotope ratio mass spectrometer (IRMS). The IRMS determines differences in enrichment of $^{12}$carbon and $^{13}$carbon in expired CO$_2$ (Bier, 1997). By maintaining isocaloric and isonitrogenous dietary intakes for each study day, the DAAO allows for directly measuring relative changes in the rate of oxidation of the test amino acid to CO$_2$. To ensure that
the results are measuring only changes in the target amino acid, all other amino acids must be provided in excess.

When a low test amino acid intake is consumed, the majority of the labeled amino acid will be utilized for protein synthesis, with very little being oxidized. As a result, the amount of $^{13}$CO$_2$ which is detected in breath samples is low relative to diets with an excess test amino acid intake (Figure 4) (Elango et al., 2012a). As the test amino acid intake increases, more of the amino acid is utilized for protein synthesis, until the minimum dietary requirement is met. After this threshold, circulating amino acid concentration will increase leading to oxidation, and thus releasing $^{13}$CO$_2$ into the breath. The relative amount of $^{13}$CO$_2$ that is exhaled will increase in proportion to the amount of L-[1-$^{13}$C]-amino acid that is provided above the minimum requirement. This method has been shown to be both reliable and of low invasiveness (Elango et al., 2008). One of the drawbacks of the method is that very low test amino acid intakes cannot be tested, because the isotope doses of the test amino acid has to be at particular doses, in order to be detectable in breath. Furthermore, the method is not suitable for all amino acids, especially for threonine, because threonine has two separate catabolic pathways, as described above; threonine dehydratase (TDH) and threonine dehydrogenase (TDG) pathways (Darling et al., 2000). Thus DAAO cannot be reliably used for determining threonine requirements.
Figure 4: Oxidative response due to increasing amino acid or protein intake, for the indicator amino acid oxidation (IAAO), direct amino acid oxidation (DAAO), indicator amino acid balance methods (IAAB). Adapted from Elango et al., 2012. IAAO determines amino acid requirements by increasing intake until oxidation decreases to a point of leveling out. DAAO determines amino acid requirements by increasing intake until the requirement is met and any additional intake is oxidized. The IAAB method increases intake until the amount of nitrogen that is retained by the body reaches a plateau due to the requirement for protein synthesis being met.

2.5.4 24 h Indicator Amino Acid Balance (24 h IAAB)

The Indicator Amino Acid Balance (24 h IAAB) uses elements of the DAAO method (stable isotope labeled amino acid), as well as the plasma amino acid response method (PAARM) method (blood collection) to determine amino acid requirements. This method is more invasive than the DAAO method, using a 24 h time period in which the labeled amino acid is given intravenously in a continuous dosage. Blood samples are taken to monitor the circulating
plasma levels of L-[1-13C]-amino acid. Additionally, instead of a 2-day adaptation time, a 7-day adaptation time is used to adapt participants to the study day levels of intake (Kurpad et al., 2005).

Due to time requirements and concerns of invasiveness, the 24 h IAAB method is used less frequently than the IAAO and DAAO methods.

2.5.5 Indicator Amino Acid Oxidation (IAAO)

The indicator amino acid oxidation method (IAAO) involves a 2-day adaptation period, and an 8hr study day, similar to the DAAO method. In contrast to the DAAO method though, the IAAO uses an IAA labeled with 13C which is not the amino acid being investigated. Typically, L-[1-13C]-phenylalanine is the amino acid which is used (Buono et al., 2001a; Elango et al., 2007; Mager et al., 2003),

![Diagram of Biphasic linear regression model of IAAO method]

**Figure 5**: Biphasic linear regression model of IAAO method
Demonstrates the decrease in oxidative response, as amino acid intake increases.
although $^{13}$C-leucine and $^{13}$C-lysine are also used in some IAAO studies depending on the target amino acid and how its metabolic pathway interacts with other amino acids (El-Khoury et al., 1998, 2000; Institute of Medicine, 2005). This method determines amino acid requirements by measuring changes in the rate of oxidation of the indicator amino acid in response to varied levels of intake of the test amino acid. When intake of the test amino acid is low, that amino acid becomes the limiting amino acid, and all other amino acids are in excess. Excess amino acids are oxidized, including the oxidation of indicator amino acid in breath. Through the capture and analysis of breath samples both prior to and during the feeding of the indicator amino acid we are able to establish both baseline and plateau levels of oxidation. The difference in measured $^{13}$CO$_2$ content between baseline and plateau samples is the APE (amount % excess) of carbon 13 in breath samples relative to background levels (Elango et al., 2012a). The APE is adjusted based on body weight, and rate of CO$_2$ production as measured by indirect calorimeter ($V$CO$_2$) to produce a value which can be compared between participants and study days: $F$$_{^{13}}$CO$_2$. The rate of label tracer oxidation, $F$$_{^{13}}$CO$_2$ is used to generate a biphasic linear regression model (Figure 5) and the requirement of the test amino acid.

The minimal level of invasiveness has allowed the IAAO method to be used in animals and humans. (Ball and Bayley, 1984; Bertolo et al., 1998; Buono et al., 2001a; Elango et al., 2007, 2011, 2012b; Fuller et al., 1994). Within human populations the method has been applied in multiple vulnerable populations such as neonates and pregnant women to determine the EAR for amino acids (Hogewind-Schoonenboom et al., 2015; Huang et al., 2014; Stephens et al., 2015). Neither of these populations have been studied extensively using either the N-balance method or the plasma amino acid method.
Table 2 outlines the requirements of several indispensable amino acids as determined by the IAAO method, in school-aged children (~6-10y), as well as the current EAR recommended by the DRI (Institute of Medicine, 2005). Elango et al., 2007 found an EAR for lysine of 35 mg/kg/d, which was similar to previous recommendations. Compared to prior determined total sulfur amino acid requirements (Methionine + Cysteine) of 27 mg/kg/d (Nakagawa et al., 1961), Turner et al., 2006 determined an EAR of 12.9 mg/kg/d which was in line with previous findings from young adults (Buono et al., 2001a, 2001b).

Table 2: Requirements of amino acids determined in 6-10y children using IAAO method

<table>
<thead>
<tr>
<th>Reference</th>
<th>Age (Y)</th>
<th>Participants</th>
<th>Nutrient</th>
<th>EAR (mg/kg/d)</th>
<th>DRI (mg/kg/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mager et al. 2003</td>
<td>8.5 ± 1.2</td>
<td>5 M</td>
<td>BCAA’s</td>
<td>147</td>
<td>99</td>
</tr>
<tr>
<td>Humayun et al. 2006</td>
<td>9.4 ± 2.3</td>
<td>5 M, 1 F</td>
<td>MET</td>
<td>5.8</td>
<td>18</td>
</tr>
<tr>
<td>Turner et al. 2006</td>
<td>9.1 ± 2.2</td>
<td>5 M, 1 F</td>
<td>Met and Cys</td>
<td>12.9</td>
<td>18</td>
</tr>
<tr>
<td>Elango et al. 2007</td>
<td>8.4 ± 0.9</td>
<td>4 M, 1 F</td>
<td>LYS</td>
<td>35</td>
<td>37</td>
</tr>
<tr>
<td>Pillai et al. 2010</td>
<td>8.4 ± 0.8</td>
<td>3 M, 3 F</td>
<td>LYS</td>
<td>33.5</td>
<td>37</td>
</tr>
<tr>
<td>Pillai et al. 2015</td>
<td>8.3 ± 0.6</td>
<td>13 M, 8 F</td>
<td>Lys</td>
<td>42.8</td>
<td>37</td>
</tr>
</tbody>
</table>

The EAR data displayed in this table were determined within the respective studies. DRI data was sourced from the Institute of medicine 2005 DRI publication.

Humayun et al., 2006 determined a methionine EAR of 5.8 mg/kg/d when diet contained excess cysteine (21 mg/kg/d cysteine), which was lower than the prior study by (Kurpad et al., 2004) where a requirement of 10 mg/kg/d Met in the presence of only 12 mg/kg/d Cys was found in adult men. Two studies by Pillai et al. (2010; 2015) evaluated lysine requirements in Indian children. Following the determination of the lysine requirement in healthy Canadian children (35 mg/kg/d, Elango et al. 2007), Pillai et al., 2010 determined a similar requirement (33.5 mg/k/d) in a healthy population in India. However, Pillai et al., 2015 in a follow-up study determined that Indian children who had intestinal parasitic infections and were chronically undernourished had a higher lysine requirement (~20%) than the healthy children.
2.6 **Threonine Intake Recommendations and Requirements**

The current recommendations for threonine intake are described by the Dietary Reference Intakes (DRI’s) as the EAR and RDA (Institute of Medicine, 2005). The values are set at 16 and 20 mg/kg/d, as the EAR and RDA, respectively for adults 19 y and older. For young school-aged children (4-8 y) the requirements are set at 19 mg/kg/d for EAR and 24 mg/kg/d for RDA. While the adult values are based on previous experimental data such as the previous IAAO studies for threonine (Table 3), the children estimates are based on a factorial calculation extrapolated from estimated adult requirements.

The requirements for dietary threonine have been previously studied in a few populations, through the use of stable isotope based methods (Borgonha et al., 2002; Campbell et al., 1994; Hogewind-Schoonenboom et al., 2015; Kurpad et al., 2002; Wilson et al., 2000; Zhao et al., 1986) (Table 3). Of these six studies, four studies were carried out in adult populations (Borgonha, Kurpad, Wilson, Zhao), and two in neonatal populations (Chapman, Hogewind-Schoonenboom). While these studies determined threonine requirements using stable isotope methodology, all of them were carried out either in different populations, or using methods which differ from our 8h IAAO method. The importance of our study comes from examining a different population (6-10 y children) which have not yet had a requirement for threonine experimentally determined.

**Table 3**: Threonine requirements determined using the IAAO method in human populations.

<table>
<thead>
<tr>
<th>Reference</th>
<th>Method</th>
<th>Age group (y)</th>
<th>Participants</th>
<th>EAR mg/kg/d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wilson et al. 2000</td>
<td>8h IAAO</td>
<td>26.0 ± 7.2</td>
<td>6 M</td>
<td>19 (upper CI 26.2)$^*$</td>
</tr>
<tr>
<td>Borgonha et al. 2002</td>
<td>24h IAAO</td>
<td>23.4 ± 2.6</td>
<td>9 M, 6 F</td>
<td>15</td>
</tr>
<tr>
<td>Kurpad et al. 2002</td>
<td>24h IAAO/IAAB</td>
<td>19.6 ± 1.2</td>
<td>16 M</td>
<td>15 (CI 11-27)</td>
</tr>
</tbody>
</table>
2.7 Protein Quality and Measurement Techniques

While the sections above addressed the quantitative requirements for amino acids, diet is consumed as foods, with variable composition of amino acids and other nutrients. Thus, the quality of protein in foods is determined by the profile of indispensable amino acids present (Levesque et al., 2011a), and their relative bio-availability for various tissues and cell types in the body. In order for a food protein to be ‘high quality’ it must contain amino acids in sufficient ratios to enable protein synthesis within the body, after accounting for bioavailability. Plant protein sources such as rice, beans, and soy contain relatively lower amounts of indispensable amino acids, such as threonine, compared to animal food sources. Furthermore, plant based protein sources have several anti-nutritional factors such as tannins in legumes and cereals, phytates in cereals, and trypsin (a digestive enzyme) inhibitors in soy beans (Elango et al., 2012c). These factors result in either a decreased ability to breakdown food, or a decreased ability to absorb nutrients once they have been broken down. Thus, not only is it important to establish threonine intake recommendations, it is also crucial to determine the amount of threonine from food sources that is actually available for protein synthesis in-vivo.

2.7.1 Protein Efficiency Ratio (PER)

The Protein Efficiency Ratio (PER), was the earliest method developed for establishing protein bio-availability and its usefulness for growth (Osborne et al., 1919). The protein
efficiency ratio was developed from the observation that rats, given the same caloric intake over the same time period, showed variable growth rate in direct proportion to dietary protein intake. Furthermore, it was observed that the same amount of protein intake from different sources also affected the growth rate. This effect was referred to as the efficiency ratio. The PER method was found to have poor predictability of nutritional value in humans, compared to the original rat studies (Young, 1991, 1992; Young and Pellett, 1994). The use of rat models as a basis of study has been criticized (Elango et al., 2012c; 1991) due to fundamental differences in growth. Rats have a much higher rate of growth compared to humans, which requires them to consume a greater relative quantity of indispensable amino acids to meet requirements. In turn, this reduces the apparent value of plant foods, and undervalues them for human consumption.

2.7.2 Protein Digestibility-Corrected Amino Acid Score (PDCAAS)

The PDCAAS method was developed by the FAO and WHO during a joint committee meeting (FAO, 1991) in an effort to develop a scoring method for humans, as well as determining the relative quality of individual indispensable amino acids when obtained from particular foods. The emphasis was on individual amino acids, because the PER method described above evaluated the overall protein utilization from foods, with no information on limiting amino acid digestibility. Instead of relying on growth rates of laboratory animals, it compares the protein and amino acid content of food sources to a reference amino acid profile, generally casein and the amino acid requirement profile for a growing child. The resulting ratio is known as the PDCAAS. Once a score has been determined, it is corrected by the true digestibility. The true digestibility is determined by comparing the amount of nitrogen that is contained in the consumed food to that which is excreted in feces, and is expressed as a % of
dietary nitrogen intake (Elango et al., 2012c). Nitrogen loss must also be determined independently of food intake, so that the background rate of endogenous nitrogen loss can be quantified (Scrimshaw et al., 1972; Shah et al., 1982). Endogenous nitrogen loss must be accounted for in order for true digestibility to be adjusted and PDCAAS determined. This endogenous nitrogen loss is determined by feeding the test animals foods which contain very little to no nitrogen (Levesque et al., 2011a; Pencharz et al., 1977; Schaafsma, 2005; Scrimshaw et al., 1972; Shah et al., 1982). The urine and feces are measured for nitrogen content, and that is considered the obligatory endogenous excretion, or how much the body will lose due to epithelial cellular sloughing and bacterial loss in the colon and intestines.

Despite the long-time use of PDCAAS, the method has many limitations (Elango et al., 2012c). The major limitation is that the endogenous nitrogen loss values, based on fecal losses, represent mostly nitrogen secreted by the micro-organisms of colonic origin, especially in a rat model. The PDCAAS method does not take into account the large variations in bioavailability values for entire proteins and the individual amino acids (Fuller and Tomé, 2005). For example, in human milk, indispensable amino acid bioavailability ranges from 86% for threonine to 100% for methionine and tyrosine (Fuller and Tomé, 2005). Furthermore, some foods naturally contain antinutritional factors, such as trypsin inhibitors in soya protein, tannins in legumes and cereals, and phytates in cereals. These decrease the bioavailability of amino acids from the food sources (Myrie et al., 2008); the PDCAAS method tends to overestimate the protein quality of products which contain these anti-nutrient compounds (Gilani et al., 2005; Sarwar, 1997). One additional drawback to the PDCAAS method is its truncation of all availability to 100%, regardless of the experimental values. This truncation of PDCAAS values has limited use for most dietary
recommendations, as it assumes that the protein sample in question will be the sole protein source in the diet.

Due to these limitations, the FAO (Ball et al., 2015; FAO, WHO, 2013; Lee et al., 2016) has recommended alternative scoring methods to be developed and used.

2.7.3 Digestible Indispensable Amino Acid Score (DIAAS)

As an alternative to the PDCAAS method, the DIAAS method was proposed.

DIAAS is defined below (FAO, WHO, 2013).

\[
\text{DIAAS} (%) = 100 \times \left( \frac{\text{mg of dietary IAA in 1g of digestible protein}}{\text{mg of dietary IAA in 1g of reference protein}} \right)
\]

The digestibility of amino acids from both reference and test protein is determined by measuring the amount of amino acid which is collected at the end of the ileum, compared to the amount that was administered orally. Thus, compared to the PDCAAS, which uses fecal measures, use of ileal measures were considered a major improvement. However, the process of collecting ileal effluent is highly invasive, involving the insertion of an ileal catheter either alongside a feeding tube, or through surgical insertion. Due to in intensiveness of each of these, the DIAAS method is mainly applicable in animal studies. The DIAAS allows for the measurement of changes in nitrogen excretion, in response to protein intakes from different food sources (Gaudichon et al., 2002). The method involves implanting an ileal catheter for sampling ileal digesta and adapting the animal to the test food. The animal is then fed a mixture of the test food which contains all protein in the diet, and titanium dioxide, or another indigestible but edible marker. As the food passes through the ileum, some of the amino acids present will be absorbed, but at the same time basal endogenous losses will continue to occur as cells and tissue
are sloughed off or lost. This is measured at the distal end through the ileal cannula. The use of an indigestible marker allows for correction of amino acid concentration between samples, so as to account for variances in digestive rates. The endogenous losses are determined based upon the amino acid content of collected ileal samples, compared to the animals that received a protein-free diet (Cervantes-Pahm et al., 2014)

The DIAAS method was developed to use the true ileal digestibility (TID) as a benchmark for nutrients, and is applicable in humans, and a variety of animal models. However, the highly invasive nature of this method makes animal models the preferable choice, compared to human populations. Growing pigs have been used to great effect, as their growth and metabolic requirements are comparable to those of humans, and they are easier subjects to work with, compared to human participants (Stein et al., 2001, 2007a). The DIAAS method was developed with the intent of determining a score for each individual indispensable amino acid, as well as a whole-food approach. The DIAAS method does this without limiting the possible score by truncating it to 100%, the way PDCAAS does, and provides data that is considered to be more reliable and true to human foods (Rutherfurd et al., 2015; Schaafsma, 2000).

2.7.4 Metabolic Availability of Amino Acids

Amino acid metabolism is a complex system of chemical reactions in the body that result in the breakdown (catabolism) or synthesis (anabolism) of proteins and compounds for systemic use. The rate at which amino acids can be used by the body for metabolism is variable depending on many factors such as body temperature, demand for the resulting product, and the availability of all 20 amino acids in sufficient quantities. Other factors include the presence or availability of
co-factors, enzymes, and inorganic nutrients which may be required to facilitate protein and enzyme activity (Ballard and Morrow, 2013; Witard et al., 2014).

The translation from the amount of protein that is consumed to the amount that is available for metabolic purposes is not necessarily 1:1. Throughout the digestive process anti-nutritional factors such as phytic acid (Traylor et al., 2001), enzymatic degradation, physical loss (Stein et al., 2007b) decrease the amount of protein and amino acids which are available for protein synthesis. Plant based foods are composed largely of cellulose. Nutrients in plants have lower availability in humans due to the inability to efficiently break it down cellulose into its constituent glucose monomers, and release other nutrients which are bound to the cellulose cellular walls (Young and Pellett, 1994). If these nutrients are not able to be extracted from the cellulose matrices, they will pass through the digestive system and be excreted (lost) from the body.

Metabolic availability varies in response to differences between consumed foods, as well as due to conditions within the body. These factors, in conjunction with the amino acid profiles (different amounts of amino acids according to food source) (Kar et al., 2016), are what determines how effective and efficient a dietary food source is for individual consumption and health.

While this current FAO recommendation is new, and there are research groups actively conducting studies in pigs to develop a database for ileal digestibility values for various food sources, FAO has also recommended that human based approaches be developed to be able to validate the pig-based estimates being developed. To that end, we have proposed the use of stable isotopes as a non-invasive and reliable method (Elango et al 2012b). The term being used to represent this stable isotope based amino acid availability values is ‘metabolic availability’.
2.7.5 Metabolic Availability Determined using the Indicator Amino Acid Oxidation Method (IAAO)

The IAAO method was first established to determine metabolic availability of lysine from different feed stuffs in pigs (Moehn et al., 2005). The IAAO method is based on the assumption that pure (crystalline) amino acids are 100% available (fully digested and absorbed) and are thus completely available to cells. Thus, a range of test amino acid intakes are used to derive the slope of IAAO (Figure 6). Once an initial slope has been determined for the crystalline amino acids using the IAAO method, a test food protein providing a similar amino acid content is evaluated. All test food proteins are expected to have a shallower response, as they include potential anti-nutritional factors, non-protein components, etc. The difference between the IAAO from the crystalline amino acids vs the test protein is calculated to determine the metabolic availability of the test food. Previously, Humayun et al. (2013) have utilized the IAAO method in adult men to determine metabolic availability of methionine and lysine. They found that casein and soy protein both had lower availabilities of methionine and cysteine when compared to the reference crystalline amino acid mixture. Prolla et al. (2013) examined availability of lysine from cooked and browned white rice, and found that they were respectively 97% and 70% bio-available compared to the reference amino acid mixture.

Thus far, the IAAO method has not been used to determine metabolic availability of amino acid in school-aged children. Different food sources contain varied amounts of threonine. In addition to amino acid content, the metabolic availability also differs based on the quality of protein and other anti-nutritional factors in foods. Whether threonine has the same metabolically
availability from an animal source (casein) vs. a plant source (soy protein isolate) needs to be
determined directly in children and will be of potential significance to dietary threonine
recommendations.

**Figure 6:** Example levels of indicator amino acid oxidation based on level of Threonine intake
and various protein sources. The availabilities of various protein sources are represented by the solid line (crystalline amino
acid), the dashes (Test protein 1, Casein), or the dots (Test protein 1, Casein), or the dots (Test
protein 2, Soy)
Chapter 3: Rationale, Objectives, Hypothesis

3.1 Rationale

Protein and amino acid requirements for school-aged children may be underestimated by current Dietary Intake Recommendations, based on recent research (Elango et al., 2010; Humayun et al., 2007b; Institute of Medicine, 2005). If the recommendations for threonine are low, then intake may be insufficient to meet the requirements for growth and its important role in the gastrointestinal tract. Threonine forms a substantial portion of the core strand of the small intestine protein, mucin (Guzman-Aranguez and Argüeso, 2010). Mucin synthesis is in turn relied upon for the uptake and usage of all other nutrients. Recently the IAAO method has been applied in healthy Indian children (Pillai et al., 2010), and Indian children affected by gut parasites (Pillai et al., 2015). It was shown that the intestinal parasite infestation increased lysine requirements ~20% (33.5 mg/kg/d to 42.8 mg/kg/d). Whether threonine requirements are increased in children with gut parasites is unknown. Prior to examining the impact of gut parasites on threonine requirements, requirements must be established in healthy children. Different food sources contain varied amounts of threonine. In addition to content, the metabolic availability also differs based on the quality of protein and other anti-nutritional factors in foods. Animal based sources such as milk, eggs etc. would have higher availability when compared to plant based sources such as cereals and legumes. Whether threonine is metabolically available to the same amount from an animal source (casein), vs. plant source (soy) needs to be determined directly in children, which will be of potential significance to base dietary threonine intake recommendations.

3.2 Objectives

The objectives of the current proposed work are:
(1) To determine the threonine requirements in healthy school-aged children (6-10 y), by measuring the oxidation of L-[1-13C]-Phenylalanine to 13CO2 (F13CO2) in response to graded intakes of threonine.

(2) To determine the threonine metabolic availability from different food protein sources (casein, soy) in healthy school-aged children (6-10y).

3.3 Hypothesis

We hypothesized that:

(1) The requirements for threonine in school-aged children would not differ from the current DRI (2005) recommendations of 19 mg/kg/d.

(2) The metabolic availability of threonine from casein would be similar to soy in school-aged children.
Chapter 4: Experiment 1: Determination of Threonine Requirements in School-aged Children

4.1 Methods

4.1.1 Participants

6 healthy school-aged children were invited to participate in the study at the Child & Family Research Institute (BCCHR), BC Children’s Hospital, Vancouver, Canada. Previous IAAO studies in children have demonstrated that a robust breakpoint can be obtained by studying 5-6 children in a repeated measures design, with each child participating in 6-9 study days (Elango et al., 2007; Humayun et al., 2006; Mager et al., 2003; Turner et al., 2006). Randomization of children to multiple individual test intakes allows a better definition of the slope and intercept in the two-phased linear regression analysis (Figure 5).

4.1.2 Experimental Design

This requirement study design was based on that used in previous studies which determined IAA requirements in school-aged children (Elango et al., 2011; Mager et al., 2003; Pillai et al., 2010; Turner et al., 2006). The overall design of the study can be seen in Figure 7.
Figure 7: Experimental design flowchart for requirement study

Subject Recruitment
N=6 healthy school-aged children, aged 6-10 y

Pre-study
Informed Consent
Anthropomorphic measurement
Indirect calorimetry
Body composition analysis
3 day diet record

Prescribed 2 day standardization diet

Study day visit
IAAO Protocol using
L-1-$^{13}$C-Phenylalanine

Randomized threonine intakes
1-50 mg/kg/d
(range of deficient to excess)

Breath and urine samples collection

Breakpoint analysis on $^{13}$CO$_2$ in response to test threonine intakes to determine threonine requirements
4.1.3 Recruitment

Participants were recruited using a combination of recruitment posters and word of mouth advertising. Recruitment posters (Appendix 1) were placed in high-traffic areas in and around both British Columbia Children’s Hospital Research Institute (BCCHR), and UBC Vancouver campus. Posters were also placed in coffee shops and community centers throughout Vancouver, and Burnaby, BC. When participants made contact regarding the study, researchers confirmed the age of participants, and then scheduled a pre-study visit to fully confirm eligibility according to inclusion and exclusion criteria. Due to the large time commitment, participants were compensated with an honorarium of $100 per day. This honorarium did not apply to the pre-study visit, but participants were also reimbursed for transit costs or given a parking voucher, to prevent biasing participation based on socioeconomic status.

4.1.4 Pre-Study Assessment

The experimental design was based on the minimally invasive IAAO model which was developed, and has been tested successfully in adults and children (Elango et al., 2011; Humayun et al., 2007b). Prior to collecting any participant data or information, participants were brought in for a pre-study screening visit where informed consent was collected. All pre-studies were conducted at the Clinical Research Evaluation Unit (CREU), at the B.C. Children’s Hospital Research Institute (BCCHR), BC Children’s Hospital, Vancouver, BC. Participants were given the option to either fast for 12 h (overnight) before coming in for the pre-study or to attend in an unfasted state for consent, and then return fasted for baseline data collection. Assent from children and consent from parents was always collected prior to any data collection or study procedures. Participant data was measured and recorded including participant height, weight, and
body fat content measured through bioelectrical impedance (BIA) (Quantum IV RJL systems). BIA was chosen due to the low invasiveness of the method, and the validity of the previous Quantum 2 system (Fakhrawi et al., 2009). The system we used, the Quantum 4 is the more recent update of this validated system. Metabolic rate was measured as resting energy expenditure (REE) using an indirect calorimetry (IC) machine (Vmax Encore; Viasys), which consists of a clear hood placed over the participant’s head and shoulders. Air moves freely in and out of the hood, drawn at a constant rate by a pump in the IC, and changed based on participant rate of respiration. Participants are able to see everything through the hood and breathe normally without any discomfort. This procedure takes about 20 minutes to complete and consists of the participant lying under a one-way gas flow hood and maintaining minimal activity while the rate of breathing and basal activity stabilizes. If the participant wants to stop the test anytime, the hood can be pulled away. This test measures rate of carbon dioxide production by comparing exhaled CO₂ and O₂ values to background air values. The test generates a value for both resting energy expenditure (REE), and respiratory quotient (RQ) values (McClave and Snider, 1992). In addition to anthropometric information collection, a 3-day diet record consisting of 2 week-days and one weekend day was provided to the participants, to be filled out and returned following the pre-study, where it was used to assess normal and routinely consumed foods.

4.1.5 Inclusion Characteristics

- Age between 6 and 10y
- Children with body weight between 3rd and 85th percentiles for their age group for weight (Dietitians of Canada, 2015; World Health Organization, 2006)

4.1.6 Exclusion Characteristics

- Age under 6 years or over 10 years
- Diagnosis of acute or chronic medical condition
- Food allergies
- Current use of prescription medication
- History of weight < 3rd percentile for or above 85th percentile
- Claustrophobia
- Recent illness (Fever, vomiting, intense runny nose within 5 days) or antibiotic consumption

4.2 Study Day Diet

Throughout the course of the study day participants were provided with an experimental diet consisting of 8 hourly shakes. These shakes consisted of a protein-free powder (PFD-1, Mead Johnson) flavored using Koolaid®, and Tang® powder, which also doubled as a carbohydrate energy source. Fat was added in the form of corn oil, and amino acids were added in the form of an L-amino acid mixture formulated to mimic egg protein composition (Table 5), at a rate of 1.5 g/kg/d evenly distributed across all of the meals. Protein free cookies were provided to make up the difference in daily caloric consumption. Daily energy consumption was given at a rate of 1.7 x REE, as determined by the indirect calorimetry readings taken during fasting conditions during the pre-study visit.

The shakes were prepared the day prior to the study day. This was done to ensure cleanliness, and palatability. Shakes were kept in a food-safe refrigerator between preparation and consumption. Preparation of the tracer compounds was completed on the morning of the study day, after the weight of the participant was determined for the day. This was done so that changes in body weight are met with the same dosage of L-[1-13C]-Phe in μmol/kg at each study day.
4.3 Diet Standardization and Test Threonine Intake

Two days prior to the study day, children were provided with dietary recommendations based on the 3-day food record provided at the time of the pre-study visit. The 3-day food records were analyzed using a nutrient analysis database (Food Processor SQL 11, ESHA Research) to determine protein and threonine intake. The reported foods were used to develop a standardized diet to provide energy at a rate of 1.7x resting energy expenditure (REE), and total protein content at 1.5g/kg (Pillai et al., 2010) to be consumed for 2 days prior to each study day. This was also intended to prevent significant dietary changes prior to study day (Moehn et al., 2004) and to standardize all participants to uniform protein intake prior to beginning the study. Prior to the study day, participants were randomly assigned to receive six test intakes between 1 and 50 mg/kg/d to cover a range of intakes from very deficient to surplus. For each study day, an intake amount was randomly selected from the participant’s pool of intakes and removed from further selection. This test intake range was intended to provide intakes both below and above the current recommendations of 10-20 mg/kg/d (W.H.O. FAO, 2007). With this recommendation in mind, the participant intake pools were distributed so that each participant experienced three high (>20 mg/kg/d threonine), and three low (<20 mg/kg/d threonine) study day intakes. While there is no known upper limit (UL) for threonine, the DRI (Institute of Medicine, 2005) shows no adverse effects for the levels of consumption which we used in this study. One previous study (Pencharz et al., 2008) cited the use of quantities of threonine that were up to 4-5 times greater than our largest planned intake, without adverse reactions. The threonine supplementation was given in the form of 8 hourly iso-nitrogenous and iso-caloric meals, each of which were formulated to provide 1/12 of the total daily energy expenditure (TDEE) calculated as the resting energy expenditure (REE)*1.7. Participants consumed 2/3 of their TDEE throughout the course.
of the study day, as it is meant to replace what would be the major meals breakfast and lunch.

Table 5 shows the amino acid profiles of the protein sources that were used.

Table 4: Study Day Protocol

<table>
<thead>
<tr>
<th>Hour</th>
<th>0</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>Food Intake</td>
<td>⚫</td>
<td>⚫</td>
<td>⚫</td>
<td>⚫</td>
<td>⚫</td>
<td>⚫</td>
<td>⚫</td>
<td>⚫</td>
<td>⚫</td>
</tr>
<tr>
<td>Isotope L-[1-13C]-PHE</td>
<td></td>
<td></td>
<td>Ω</td>
<td>Ω</td>
<td>Ω</td>
<td>Ω</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NaH13CO3</td>
<td></td>
<td></td>
<td>Ω</td>
<td>Ω</td>
<td>Ω</td>
<td>Ω</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sampling</td>
<td></td>
<td></td>
<td>Ω</td>
<td>Ω</td>
<td>Ω</td>
<td>Ω</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Breath</td>
<td>BBB</td>
<td>BBB</td>
<td>B</td>
<td>B</td>
<td>BBB</td>
<td>B</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Urine</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
<td>P</td>
</tr>
<tr>
<td>vCO2</td>
<td>Φ</td>
<td>Φ</td>
<td>Φ</td>
<td>Φ</td>
<td>Φ</td>
<td>Φ</td>
<td>Φ</td>
<td>Φ</td>
<td>Φ</td>
</tr>
</tbody>
</table>

Outline of sampling and dose administration during each study day. Adapted from (Elango et al., 2007). ⚫: Meal intake times. Ω: Labeled PHE intake. R: labeled Bi-carbonate intake. B: Breath samples. P: Urine samples. Φ: VCO2
Table 5: Composition of reference egg protein during test day

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>mg/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>87.57</td>
</tr>
<tr>
<td>Asparagine</td>
<td>38.83</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>38.83</td>
</tr>
<tr>
<td>Cysteine</td>
<td>25.77</td>
</tr>
<tr>
<td>Glutamine</td>
<td>66.00</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>66.00</td>
</tr>
<tr>
<td>Glycine</td>
<td>38.83</td>
</tr>
<tr>
<td>Histidine</td>
<td>26.47</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>73.23</td>
</tr>
<tr>
<td>Leucine</td>
<td>97.13</td>
</tr>
<tr>
<td>Lysine</td>
<td>88.27</td>
</tr>
<tr>
<td>Methionine</td>
<td>34.51</td>
</tr>
<tr>
<td>Proline</td>
<td>48.86</td>
</tr>
<tr>
<td>Serine</td>
<td>97.83</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>18.19</td>
</tr>
<tr>
<td>Valine</td>
<td>81.97</td>
</tr>
</tbody>
</table>

Amino acids provided in the same profile as egg protein, except for Phenylalanine, threonine, tyrosine, and alanine which are added to study day meals separately.

Four of the hourly meals were consumed prior to the addition of the tracer compound. The tracer compound is comprised of the stable isotope $^{13}\text{C}$, which is naturally present at a low rate in all carbon containing compounds. The L-$[1-^{13}\text{C}]$-phenylalanine (carbon-13 labeled L-phenylalanine) was administered at a known concentration which is only slightly higher than the background level normally present in food, water, and air. At the time of the fifth hourly meal, a dose of $0.176 \ \mu\text{mol/kg NaH}_{13}\text{CO}_3$ (99 atom% excess; Cambridge Isotope Laboratories, Woburn, MA) was administered orally simultaneously with a priming (3.048 mg/kg/d) and hourly (1.958 mg/kg/h) (Elango et al., 2011) dose of L-$[1-^{13}\text{C}]$-phenylalanine (99 atom% excess; Cambridge
Isotope Laboratories, Woburn, MA) for every hour following (Table 5). This NaH$^{13}$ CO$_3$, or sodium bicarbonate, acts as a priming dose for the body’s bicarbonate buffer system. By priming this buffer system, it ensures that the labeled CO$_2$ is not sequestered into the buffer but is instead expired in breath. The amount of L-phenylalanine that was given in the form of L-[1-$^{13}$C]-phenylalanine was subtracted from the test diet to ensure equal intake throughout the day. Tyrosine is provided at a rate of 61 mg/kg/d in order to ensure an excess amount and proper partitioning of the carboxyl carbon of phenylalanine between protein incorporation in the body vs. oxidation (Shiman and Gray, 1998).

4.4 Study Day Protocol

On the day of the study participants returned to the Clinical Research and Evaluation Unit at the BCCHR. Participants were asked to confirm having fasted for 10-12 hours the previous night. On each study day, participants began by having their height and weight measured, before consuming the first of the hourly test meals. An accurate weight for the study day is required to prepare an accurate and sufficient amount of the labeled L-[1-$^{13}$C]-Phe which acts as a tracer for our IAAO protocol. Details on the collection and processing of breath samples using evacuated tubes and the IRMS, as well as urine sample collection and storage can be found in subsequent sections (4.5-4.5.3). While the study day was estimated to take a minimum of 8 h to complete, the total amount of time that was required of the participant to consume test meals and provide breath and urine samples is substantially less. Therefore, when samples were not being collected or food given, participating children were allowed to watch television, listen to music, read, use a computer, play board games, or color pictures. Participants were also invited to bring their own
activities, homework, or electronic devices for entertainment. Additionally, on each study day between meals 4 and 5 the indirect calorimetry procedure was performed to measure vCO₂.

4.5 Sample Collection and Analysis

Breath and urine samples were collected throughout the study days for the purpose of evaluating the baseline and isotopic steady state measurement of 13C. Methods for collection are outlined in 4.5.1 and 4.5.2 respectively.

4.5.1 Breath Sample Collection

Breath samples were collected at the following nine time points: 3:15, 3:30, 3:45, 6:30, 7, 7:15, 7:30, 7:45, 8 hours (Table 4). These time points encapsulate a background metabolic state (samples 1-3) while ensuring that a proper steady state was reached (samples 4-9) within the 4-hour isotope provision. Samples were collected by trapping expired gas in disposable Labco Exetainer tubes using a breath bag (Single use collection bags, EasySampler System, Quintron, Terumo Medical). Each exetainer collected 10 ml of expired air in triplicate at each time point. Exetainers were prepared by using a vacuum needle pump to bring the interior pressure down to a very low internal pressure (e⁻¹ mbar or less than 1/1000th of normal atmospheric pressure), this ensured that the exetainers draw in a 10 ml breath sample during the sampling process. These samples were labeled with individual subject ID, breath sample number, and set ID, and stored at room temperature following collection, until analyzed (Bross et al., 1998).

While the main concern with breath samples is having them collected prior to upcoming meals instead of following (which can artificially increase labeled CO2 in the breath due to oral
residue), completing all breath samples within a short amount of time, as close to the intended time point is also important. Additionally, ensuring that a proper seal is made by the participant’s mouth around the Quintron bag mouthpiece helps to ensure accuracy of the samples.

4.5.2 Urine Sample Collection

Urine samples were collected periodically during the day in a specimen collection container and transferred into sterile 50 ml specimen jars. A sample of 9.8 ml of urine were added to a 15 ml falcon tube containing 200 µl of 30% HCl for the purpose of being a preservative. After agitation through inversion, 3 x 1ml samples were pipetted out into 1.5 ml Eppendorf tubes. All urine samples were stored at -20 C until analysis (Bross et al., 1998). In the event that participants were unable to produce the small amount of urine per sample time (10 ml for analysis, 30 ml for reserve, 40 ml total), participants were offered a cup of water, and asked to try again in a few minutes. Water was made freely available throughout the course of the study day to ensure proper hydration, and starting 20 min before upcoming urine samples, participants were reminded to drink plenty of fluids. In some instances, participants were still unable to complete the urine sample on time. In the event that a urine sample was collected late, the timing of the subsequent sample was adjusted so that it is at least 20 min after the previous sample collection.

4.5.3 Breath and Urine Analysis

The analytical protocol has been adapted from Turner et al., 2006. Breath enrichment of expired $^{13}$CO$_2$ was measured using the standard operating procedure of our isotope ratio mass spectrometry (IRMS, IsoPrime 100). Urine samples were stored at -20C in case of future analysis (Turner et al., 2006).
4.6 Isotope Kinetics

To determine the rate of production of $^{13}\text{CO}_2$, the equation below was used. The rate of label tracer oxidation to $^{13}\text{CO}_2$ production is denoted by the $F^{13}\text{CO}_2$. $\text{ECO}_2$ is the $^{13}\text{CO}_2$ that is enriched and expired through the breath during a metabolic steady state. The factors 44.6 $\mu$M/mL and 60 min/hour convert $F^{13}\text{CO}_2$ to $\mu$M/h. The body weight is denoted by $W$. The value 0.82 is the rate of carbon fixation due to bicarbonate (Hoerr et al., 1989) and 100 ensures that the resulting number can be evaluated as a fraction (Elango et al., 2011).

$$F^{13}\text{CO}_2 \ (\mu\text{mol}^{-1} * \text{kg}^{-1} * \text{h}) = \frac{(\text{FCO}_2)(\text{ECO}_2)(44.6 \ \mu\text{M}/\text{mL})(60\text{min}/\text{hr})}{(W \ \text{kg})(0.82)(100)}$$

4.7 Statistical Analysis

All participant data was analyzed using statistical software (version 24, SPSS), with significance set at $p \leq 0.05$. Threonine requirements were determined using breakpoint analysis of the $F^{13}\text{CO}_2$ data points, using a bi-phase linear regression crossover model, using SAS (SAS/STAT version 9.4). The point at which the two regression lines meet is the breakpoint and represents the estimated average requirement for threonine. The SAS analysis compares four different models of the data for each cut-point (which are chosen based on visual inspection of the data, followed by separating the dataset into two regression lines: 1 slope unweighted, 1 slope weighted, 2 slopes unweighted, 2 slopes weighted. While the one slope-analysis assumes that there is only a decreasing slope in the intakes below the requirement and a zero-slope after that, the two-slope assumes an initial line with a negative slope, and a second regression line with a slope close to zero (Elango et al., 2007). A weighted analysis is used when the variances are found to be unequal due to heteroscedasticity, or an uneven variance in the variances along
the range of intakes. The weighted and unweighted models were analyzed for several data cut-points. The final model which provided the highest $R^2$, lowest CV, and root mean square error (RMSE) was chosen to represent the breakpoint. Lower and Upper 95% confidence intervals were created using Fiellers theorem (Seber and Lee, 2012)(Elango et al., 2011), which generated the standard error for the breakpoint estimate. The CI was determined from $CI = \text{Breakpoint} \pm t * \text{SE}$.

4.8 Results

Participant Characteristics:

Six children (Table 6) completed a total of 39 study days with randomized threonine study diet intakes ranging from 1 - 50 mg/kg/d. Children completed between 6 and 9 study days each for the requirement study. The children were on average 7.5y old, and were all healthy, based on the pre-study assessments. They were also of normal body composition, as evidenced by their BMI percentiles (10th – 83rd percentiles) and body fat % (~20%) (Williams et al., 2007). On all study days children were ensured to be free of acute/concurrent illness. Also, on each study day children received their energy needs based on their measured resting energy expenditure. Prior to each study day the standardization diets were to ensure children were not deficient in their diets, although participant adherence was poor, and average intake was greater than the 1.5 g/kg/d suggested by our standardization diets, with a mean intake of 2.16 g protein/kg/d (Table 6).
Table 6: Characteristics of children at recruitment, who participated in the requirement study

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value (^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>7.5 ± 1.4</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>25.7 ± 3.8</td>
</tr>
<tr>
<td>Height, cm</td>
<td>129 ± 7.5</td>
</tr>
<tr>
<td>BMI, percentile</td>
<td>38.2 ± 29.2</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>20.7 ± 7.9</td>
</tr>
<tr>
<td>FFM, kg</td>
<td>20.4 ± 3.5</td>
</tr>
<tr>
<td>REE, Kcal</td>
<td>1007 ± 124</td>
</tr>
<tr>
<td>(^2)Protein intake 2 days prior, g/kg/d</td>
<td>2.16 ± 0.4</td>
</tr>
<tr>
<td>(^2)Thr intakes 2 days prior, mg/kg/d</td>
<td>82.8 ± 20</td>
</tr>
</tbody>
</table>

\(^1\)Means ± SD; n=6
\(^2\)FFM calculated based on BIA (Schaefer et al., 1994)
\(^3\)Values derived from 2-day food records collected prior to each study day

L-[1-\(^{13}\)C]-phenylalanine Oxidation:

Oxidation of the tracer (L-[1-\(^{13}\)C]-phenylalanine) was decreased in response to increasing intakes of test threonine intakes until 20 mg/kg/d, after which a plateau in oxidation was observed. The point at which this change occurs, referred to as the ‘Breakpoint’ identifies the minimum requirement for threonine, and represents the EAR. Using bi-phase linear regression analysis we estimated the breakpoint to be 21.9 mg/kg/d (\(R^2=0.237\), 95% CI:10.5 - 33.4 mg/kg/d) (Figure 8a). The final breakpoint was determined based on the model that produced the lowest Akaike information criteria, lowest CV, and highest \(R^2\) using the bi-phase linear regression crossover analysis (Ball and Bayley, 1984; Elango et al., 2007). The Akaike information criteria is used to bolster multi-modal analysis (Akaike, 1987). For visualization boys (Figure 8b) and girls (Figure 8c) are plotted separately with the dotted line representing the breakpoint determined from the entire dataset.
Figure 8: Resulting oxidation graphs for the requirement study.
A: All children, N=39 $^{13}$CO$_2$ values from 1-50 mg/kg/d with breakpoint at 21.9 mg/kg/d; B: Boys n=3, N=21 $^{13}$CO$_2$ values; C: Girls n=3, N=18 $^{13}$CO$_2$ values. Dotted line represents the determined breakpoint from the full dataset. Each color and shape set of data points represents a separate child at unique threonine intakes.
4.9 Discussion

To my knowledge this study was the first to experimentally determine the requirement for threonine in 6-10 y healthy school-aged children using the IAAO method. Due to the unique growth period that this age range experiences, these results should not be applied to other populations. The determined mean threonine requirement of 21.9 mg/kg/d (95% CI: 10.5 – 33.4 mg/kg/d) is 15% higher than the current EAR recommendation of 19 mg/kg/d (with the RDA set at 24 mg/kg/d for children aged 4-8 y (Institute of Medicine, 2005). The DRI EAR recommendations are based on a factorial method, where the amino acid needs for growth (estimated from average rate of protein deposition and average amino acid composition of body protein) are added to the maintenance needs of threonine derived from adult estimates. Previous work in young men by (Wilson et al., 2000) using the IAAO method, and a similar protocol to the current study, found a mean requirement of 19 mg/kg/d (upper 95% CI: 26.2 mg/kg/d), which is ~19% higher than the DRI recommended EAR of 16 mg/kg in 19 y+ adults. Thus, both these studies suggest that the current DRI recommendations could be a slight underestimate of the actual needs. Other studies in young adults living in North America (Borgonha et al., 2002) and India (Kurpad et al., 2002) using L-[1-13C]-Leucine as the indicator amino acid determined a threonine requirement of 15 mg/kg/d. The study design, while modeled using the IAAO method, used a 24 h study day which was preceded by a 6-day adaptation to the test threonine intake (Kurpad et al., 2002) (Table 3). This method referred to as the indicator amino acid balance (IAAB) method takes into consideration both the fed and fasted periods of a study day in response to the test amino acid intake. It has been shown that though both the IAAO and IAAB provide estimates which are greater than the existing requirements, the IAAB method is not practical for some populations which are considered more vulnerable, including young children.
(Elango et al., 2012a; Pencharz and Ball, 2003). The differences which are observed may be due to differences in the location which participants are drawn from (India vs. Canada), as these populations cannot be directly compared due to environmental, biological, and dietary factors, among others.

Using the IAAO method in enterally fed human infants, the estimated mean threonine requirements were determined to be 68 mg/kg/d (95% CI: 32 – 104 mg/kg/d)(Hogewind-Schoonenboom et al., 2015) and in parenterally fed neonates was determined to be 37.6 mg/kg/d (95% CI: 29.9 - 45.2 mg/kg/d) (Chapman et al., 2009). The increased requirement during enteral nutrition is likely due to the increased demand for threonine incorporation in gut mucosal proteins. Using the piglet as a model for human neonates, Bertolo et al., 1998 determined that parenteral feeding reduced the mean requirement for threonine to 190 mg/kg/d, when compared to the orally fed requirement in neonatal piglets of 420 mg/kg/d. Subsequently they also established that this increased requirement during enteral nutrition was mostly for mucin production in the small intestine and colon (Law et al., 2007). When piglets were fed a threonine deficient diet, mucosal mass and total crude mucin content were lower in the colons of the deficient piglets. Histopathological analysis also showed lower numbers of acidic mucin-producing goblet cells in the duodenum and ileum of the deficient pigs when compared to the threonine adequately fed piglets (Law et al., 2007). It has also been shown earlier that during gestation in pigs, the requirement for threonine increases from 6.1 g/d to 13.6 g/d in early and late stage respectively (Levesque et al., 2011b). This data suggests that, during periods of active growth and development the body’s threonine requirements are increased. Previous studies within the Elango lab have recently shown that the protein and lysine requirements are increased by ~40% in late stages of human pregnancy when compared to early stages (Payne, 2014;
Stephen et al., 2015). Whether threonine requirements are increased during human pregnancy remains to be determined, though due to the intestinal development occurring in the fetus this is a likely possibility.

Among the indispensable amino acids, the requirements for school-aged children have been determined for lysine, total branched-chain amino acids (BCAA; isoleucine, leucine, valine), and total sulfur amino acids (TSAA; methionine, cysteine) using the IAAO method (Elango et al., 2007; Mager et al., 2003; Riazi et al., 2003). The mean lysine requirements in healthy children (Elango et al., 2007) of 35 mg/kg/d was found to be similar to the adult mean lysine requirement of 36.9 mg/kg/d, and the mean TSAA requirement in healthy children of 12.9 mg/kg/d was similar to the adult estimates of 12.6 mg/kg/d. For total BCAA however, the school-age children’s requirement was 147 mg/kg/d, or, ~20% higher than the adult mean requirement of 125.7 mg/kg/d, similar to the differences in the current study. The increase in demand for threonine and total BCAA found in these studies can likely be attributed to increased demand in the gut created by a high turnover of proteins. For instance threonine is heavily incorporated into mucosal proteins, and BCAA are oxidized for energy purposes (Chen et al., 2009). Other factors must also be considered when examining these amino acid requirements. The ~20% increase in the requirement for lysine due to parasite infestation can potentially limit the availability of lysine for other body growth and development, as parasite infestation is quite common in low resource settings (Bora et al., 2006; Dambhare et al., 2010; Sethi et al., 2000; Wani et al., 2008). Thus, it will be interesting to examine whether the requirements for threonine, and perhaps the BCAA are increased in children living in malnourished and with active parasite infestation. It is likely that requirements for these amino acids would be higher, and
recommendations may need to be titrated to suit the demands of these vulnerable populations and those who may have undiagnosed infections.

Our experimentally determined mean threonine requirement of 21.9 mg/kg/d in 6-10y children is ~15% higher than the current DRI EAR recommendations of 19 mg/kg/d which leads us to reject our null hypothesis that the requirement for threonine would be similar to the current DRI of 19 mg/kg/d. Identifying threonine requirements is crucial because of threonine’s incorporation in mucin proteins within the GI tract, which we hypothesize may increase threonine demands in certain life-stages and physiological conditions such as Crohn’s disease, colitis or intestinal injury. With the establishment of the minimum requirement for threonine in healthy children living in a developed country, future studies in children living in low socio-economic and less clean environment can now be conducted to determine the impact of parasite infestation. This could lead to a better definition of indispensable amino acid needs in different environmental setting at a global level.
Chapter 5: Experiment 2: Determination of Threonine Metabolic Availability (MA) from Food Sources in School-Aged Children

5.1 Methods

5.1.1 Participants

Six participants (n\textsubscript{boys} = 3, n\textsubscript{girls} = 3) were recruited for this portion of the study. All 6 participants had previously participated in the requirement study. Participants who did not have recent anthropometric data from within a one-month period, were asked to come in for a follow-up pre-study to establish a secondary baseline for diet calculation.

5.1.2 Experimental Design

This metabolic availability study was carried out using a design similar to earlier metabolic availability studies in adults (Elango et al., 2007; Humayun et al., 2007a; Prolla et al., 2013). A representation of the experimental design can be seen in Figure 9.
**Subject Recruitment**
N=6 healthy school-aged children, aged 6-10 y  
(All 6 children from requirement study)

**Pre-study**
*repeated following Requirement study protocol, if child did not have recent data from study visits

Prescribed 2 day standardization diet

Randomized to Threonine intake  
0mg/kg/d, 11mg/kg/d from: pure AA, Casein, or Soy  
(50-60% of the average Threonine requirement)

Randomized to Threonine intake  
0mg/kg/d, 11mg/kg/d from: pure AA, Casein, or Soy  
(50-60% of the average Threonine requirement)

Breath and urine samples collection

Metabolic Availability determined by comparing $^{13}$CO$_2$ in response to crystalline AA vs Casein vs Soy

Figure 9: Study design flowchart for MA study
5.2 Diet Standardization

Diet standardization procedures for the two days prior to each study day were performed similar to the requirement study, with diets adjusted to reflect any changes in body mass (section 4.2). Participant reported diets were analyzed using a nutrient analysis database (Food Processor SQL 11, ESHA Research) and prescribed diets were developed. These standardization diets were designed to provide 1.5 g/kg/d of protein, and to meet the TDEE of 1.7*REE. Participants were asked to keep a 2-day food record for the 2 days prior to each study day, which was collected by the researcher on each study day. These diet records were also analyzed using ESHA, to assess actual intakes.

5.3 Study Day Diet

Similar to the requirement study, study day diets were prepared to contain a specific amount of threonine. Diets were formulated to be isonitrogenous and iso-caloric for each of the eight study day meals. Upon completion of the requirement study, an intake of 11mg/kg/d of threonine was decided as the test level for metabolic availability studies, as it is ~50% of our estimated requirement. The principle of the IAAO method for metabolic availability is that the testing for availability should be on the descending slope of the IAAO response to increasing intakes of the limiting amino acid. Thus, addition of the limiting amino acid as crystalline amino acid, compared to the amino acid addition from a food protein at that chosen level represents the relative availability of the amino acid for whole body protein synthesis (Moehn et al., 2005).

Soy protein isolate and casein protein have different amino acid profiles compared to the egg-protein profile that was used to compose the study diets in the requirement study. The different profiles can be seen in Table 7. To compose a diet that was equal in amino acid
composition across all three study diets (soy, casein, crystalline amino acids), several calculations were made.

First the amount of the protein source (casein or soy) that is needed to make up the 5 mg/kg/d of threonine was determined. An intake of 5 mg/kg/d of threonine from the food protein sources was used, instead of the full 11 mg/kg/d, in order to allow for the remaining profile of amino acids to be uniform between study days. Using the full 11mg/kg/d would have resulted in imbalances in other amino acids, and potentially created a non-isonitrogenous diet. The calculations for casein and soy are presented in appendix D & E respectively. Secondly, we needed to balance phenylalanine, as it is the indicator amino acid used to represent whole body protein synthesis and cannot be variable across intakes. On each study day participants were given phenylalanine at an overall rate of 30.5 mg/kg/d. The total amount to be included at each meal time is determined using:

\[
\text{total phenylalanine (mg)} = (30.5 \times \text{bw})/12
\]

The total phenylalanine calculation is divided by 12, because we formulate each meal as being 1/12th of the total intake for the day. By providing 8 meals, we are providing overall 2/3rds of total daily nutrition (breakfast and lunch) with the remaining 1/3rd coming from the dinner or post-study day meal. Once this was determined, we subtracted the amount of phenylalanine coming from the food source from the total for each meal, as well as the amount of phenylalanine which would be included as the labeled L-[1-\text{^{13}}C]- phenylalanine. The remaining amount was included as crystalline L- phenylalanine, which was added separately to each meal. A similar process was used to equalize the amino acid profile among protein source, with secondary amino acid mixes being included for the casein and soy study days due to low amounts of certain amino acids in each diet.
### Table 7: Amino acid (mg/g protein) of study proteins

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>Reference&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Casein&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Soy&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alanine</td>
<td>61.5</td>
<td>29.81</td>
<td>35.2</td>
</tr>
<tr>
<td>Arginine</td>
<td>75.1</td>
<td>34.62</td>
<td>66.2</td>
</tr>
<tr>
<td>Asparagine</td>
<td>33.3</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>Aspartate</td>
<td>33.3</td>
<td>65.38</td>
<td>103.1</td>
</tr>
<tr>
<td>Cysteine</td>
<td>22.1</td>
<td>7.69</td>
<td>26.4</td>
</tr>
<tr>
<td>Glutamine</td>
<td>56.6</td>
<td>**</td>
<td>**</td>
</tr>
<tr>
<td>Glutamate</td>
<td>56.6</td>
<td>190.38</td>
<td>165.2</td>
</tr>
<tr>
<td>Glycine</td>
<td>33.3</td>
<td>27.88</td>
<td>33.9</td>
</tr>
<tr>
<td>Histidine</td>
<td>22.7</td>
<td>34.62</td>
<td>26.9</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>62.8</td>
<td>55.00</td>
<td>34.6</td>
</tr>
<tr>
<td>Leucine</td>
<td>83.3</td>
<td>86.54</td>
<td>75.2</td>
</tr>
<tr>
<td>Lysine</td>
<td>75.7</td>
<td>70.19</td>
<td>54.3</td>
</tr>
<tr>
<td>Methionine</td>
<td>29.6</td>
<td>36.54</td>
<td>12.6</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>54.7</td>
<td>49.04</td>
<td>38.7</td>
</tr>
<tr>
<td>Proline</td>
<td>41.9</td>
<td>97.12</td>
<td>44.4</td>
</tr>
<tr>
<td>Serine</td>
<td>83.9</td>
<td>57.69</td>
<td>45.5</td>
</tr>
<tr>
<td>Threonine</td>
<td>47.1</td>
<td>44.23</td>
<td>28.8</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>15.6</td>
<td>15.38</td>
<td>15</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>40.7</td>
<td>35.58</td>
<td>31.5</td>
</tr>
<tr>
<td>Valine</td>
<td>70.3</td>
<td>62.31</td>
<td>40.6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reference amino acid profile based on egg protein  
<sup>b</sup> Casein Protein Isolate: Micellar Casein Protein, Protein Co, Quebec, QC, Canada  
<sup>c</sup> Soy Protein Isolate: NOW foods, Bloomingdale, Illinois, USA  
\* not determined separately from Aspartic acid  
** not determined separately from Glutamic acid

### 5.4 Study Day Protocol

The Study day protocol used in the metabolic availability study was identical to that used in the requirement study (section 4.4).
5.5 **Sample Analysis**

Breath and urine samples were collected and analyzed following the same procedures outlined in the first study (section 4.5-4.5.2). All IRMS analysis was carried out by a trained technician, following routine calibration.

5.6 **Urease Assay**

Soy and other plant based protein sources contain anti-nutritional compounds such as: trypsin inhibitor (affects protein digestion); lipoxygenase (oxidation of PUFAs); and phytic acid (binds to minerals and disrupts their absorption) (Yalcin and Basman, 2015). One related compound, urease, is used as a marker for overall anti-nutrient inactivation based on its presence or lack thereof. The absence of active urease indicates that other anti-nutritional compounds are either not present or have also been inactivated in the food source (Wiriyaumpaiwong et al., 2004). If urease is present, it breaks down urea and increases the acidity of the solution, thus decreasing the pH of the sample. A pH difference of 0.3 or lower indicates that adequate inactivation of anti-nutrients was achieved in the product. Anti-nutrient inactivation can be done by cooking food products, or chemical treatment. This technique has been used within the animal nutritional research field, as well as in human food research (Yalcin and Basman, 2015) AACC Method No: 22-90.01([AACC, 2000]) to test the overall safety of food products. The process involves the production of a phosphate-buffer solution with and without urea. The pH of the buffer solution is brought to 7 using HCl, or NaOH; 200mg of the test food product is then added to both of the phosphate-buffer solutions (with and without urea). The solutions are incubated at 30°C, for 20 min., with continuous stirring. A final pH measurement is done after the 30 min of
incubation (Bibby and Hukins, 1992). Residual urease activity analysis was performed using casein, as a control, and soy protein isolate samples. A pH difference of 0.03 and 0.04 respectively, was observed for the casein and soy used in the current study, which indicates a null presence of anti-nutrient components in these food products as it is lower than the threshold pH change of 0.3. This test was performed to rule out the presence of significant anti-nutrients in the test proteins affecting metabolic availability.

5.7 Statistical Analysis and Calculation of Metabolic Availability

Participant characteristics and breath F\textsuperscript{13}CO\textsubscript{2} data are presented as means ± SD. Breath F\textsuperscript{13}CO\textsubscript{2} data was analyzed for normality and outliers (Grubbs, 1950, 1969). The slope of the IAAO response (F\textsuperscript{13}CO\textsubscript{2} data) was analyzed for the range of 0 mg threonine/kg/d to the 11 mg threonine/kg/d. Metabolic availability analysis was based on (Humayun et al., 2007a), where the relative IAAO response at the 11 mg threonine/kg/d from the casein and soy diets were compared to the crystalline amino acid diet, with the crystalline amino acid response being equated to 100% (as the L-crystalline form is assumed to be 100% available). All analysis was performed using GraphPad Prism (GraphPad Software, Inc. CA).

5.8 Results

Participant Characteristics:

Six children (n\textsubscript{boys}=3, n\textsubscript{girls}=3) participated and completed the metabolic availability portion of this study. These participants were the same as those included in the requirement study (Table 6). The children remained healthy and completed all study days with no problems.

Participant characteristics were within normal ranges for the 6-10y age range (Table 8). The
adherence to prescribed diets was low, with an average protein intake of 2.1 ± 0.6 g/kg/d. The intake of threonine for the two days prior was 78 ± 24.4 mg/kg/d, and considerably higher than the requirement determined earlier of 21.9 mg/kg/d. Despite higher than recommended protein intake, we observed a steady state in breath enrichment of $^{13}$C and a plateau on all study days.

Table 8: Characteristics of children who participated in the metabolic availability study

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Value$^1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>8.2 ± 1</td>
</tr>
<tr>
<td>Weight, kg</td>
<td>27.6 ± 4.1</td>
</tr>
<tr>
<td>Height, cm</td>
<td>132.1 ± 6.9</td>
</tr>
<tr>
<td>BMI, percentile</td>
<td>42.2 ± 21.4</td>
</tr>
<tr>
<td>Body fat, %</td>
<td>18.1 ± 7.7</td>
</tr>
<tr>
<td>FFM, kg</td>
<td>21.6 ± 3.5</td>
</tr>
<tr>
<td>REE, Kcal</td>
<td>1001 ± 138</td>
</tr>
<tr>
<td>$^2$Protein intake 2 days prior, g/kg/d</td>
<td>2.1 ± 0.6</td>
</tr>
<tr>
<td>$^2$Thr intake 2 days prior, mg/kg/d</td>
<td>78 ± 24.4</td>
</tr>
</tbody>
</table>

$^1$Means ± SD; n=6
$^2$Values derived from 2-day food records collected prior to each study day

Visual inspection (Figure 10) illustrates the decline in $^{13}$CO$_2$ between 0 mg/kg/d intake and 11 mg/kg/d threonine intake in response to all 3 test protein sources.

Metabolic Availability:

Prior to analyzing the data for metabolic availability, the $^{13}$CO$_2$ data was confirmed as being normally distributed (SPSS ver. 22). Grubbs’ test for outliers (Grubbs, 1950, 1969) displayed outlier values. The Grubbs test is useful when working with smaller sample sizes (for n=6 or similar). Among the different experimental intakes ($n_{\text{crystalline}}=6$, $n_{\text{casein}}=6$, $n_{\text{soy}}=6$), one intake of 11 mg/kg/d crystalline amino acid test intake was found to be an outlier based on a 2-sided Grubbs test (N=6, $\alpha=.2$). Thus, in the final data set the total study days were 17 ($n_{\text{crystalline}}=5$, $n_{\text{casein}}=6$, $n_{\text{soy}}=6$).
L-[1-^{13}C]-phenylalanine oxidation ($F^{13}\text{CO}_2$) values were averaged from participants for each protein source at the 11 mg threonine test intake dose, and the values compared. Crystalline L-threonine is assumed to have 100% metabolic availability (Humayun et al., 2007a), and is therefore the baseline for comparison. Relative to crystalline L-threonine, threonine availability was 96.6% and 83.4% from casein and soy, respectively (Table 9).

![Figure 10: Influence of source of L-Threonine, casein, and soy, on L-[1-^{13}C]-Phenylalanine oxidation. Note: n=5 for 0 mg/kg/d L-Threonine intake; n=5 for 11 mg/kg/d L-Threonine intake; n=6 for casein and soy. Black is crystalline amino acids, green is casein, red is soy protein.]

Table 9: Relative metabolic availability of threonine in healthy school-aged children

<table>
<thead>
<tr>
<th>Threonine Intake</th>
<th>Breath $F^{13}\text{CO}_2$ (µmol/kg/hr)</th>
<th>Metabolic Availability (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-threonine(^1)</td>
<td>0.801 ± 0.187</td>
<td>100</td>
</tr>
<tr>
<td>Casein(^2)</td>
<td>0.828 ± 0.127</td>
<td>96.6</td>
</tr>
<tr>
<td>SPI(^2)</td>
<td>0.934 ± 0.152</td>
<td>83.4</td>
</tr>
</tbody>
</table>

\(^1\) n=5. \(^2\) n=6. $F^{13}\text{CO}_2$ values presented as mean ± SD. Metabolic availability presented as relative amount of each protein source’s threonine which is available relative to crystalline L-threonine.
5.9 Discussion

This is the first study to evaluate any essential amino acid metabolic availability in school-aged children. Current DRI EAR recommendations for protein and amino acids are largely based upon experimental evidence which assume a 100% metabolic availability of amino acids (Elango et al., 2009). Our study in 6-10 y healthy school-aged children using the IAAO method assumes that the purified crystalline amino acid has 100% metabolic availability, while other protein sources are compared are relative to it. We found casein and soy to have metabolic availability of threonine of 96.6% and 83.4% respectively. While a similar threonine availability study has not been previously done in humans, ileal digestibility data from casein and soy in humans (Gaudichon et al., 1999, 2002; Mariotti et al., 1999), and ileal digestibility in pigs (Cervantes-Pahm and Stein, 2010; National Research Council, 1998; Opapeju et al., 2006) are available.

Gaudichon et al., 2002 studied normal weight adult humans using $^{15}$N –labeled proteins to determine the total ileal digestibility (TID) of individual amino acids, with threonine digestibility determined as $93.4 \pm 2.3\%$ for milk and $89.0 \pm 4.9\%$ for soy. $^{15}$N-Milk and $^{15}$N-soy were provided as 30g of protein isolates mixed with water, which is similar to the casein and soy protein isolate in our study. In an earlier study, (Gaudichon et al., 1999) had used the $^{15}$N label to determine overall digestibility and net postprandial protein utilization of milk (NPPU), and determined an ileal digestibility for protein to be 95%. This is concurrent with the value found in the Gaudichon et al 2002 paper, where there was an average amino acid TID of $95.3 \pm 1.8\%$. Similarly, earlier (Mariotti et al., 1999) used a $^{15}$N tracer to determine the NPPU of soy protein isolate, determining an oro-ileal digestibility for protein to be $90.9 \pm 2.2\%$. The use of digestibility as a determinant of availability has been shown to possibly overestimate or in some
cases underestimate true values (Darragh and Hodgkinson, 2000; Elango et al., 2009; National Research Council, 1998). Though ileal digestibility is fundamentally a better measure than fecal digestibility, ileal digestibility only reflects nitrogen/amino acid disappearance from the small intestine and does not reflect actual usage for protein synthesis. The metabolic availability for threonine determined in the current study not only reflects digestion and absorption in these children, it also takes into account the utilization of threonine for cellular protein synthesis (Elango et al., 2012c; Moehn et al., 2005).

Digestibility estimates are routinely conducted in agricultural animals. Pigs in particular are the most well-studied with regards to the digestibility of amino acids from casein and soy. Digestibility is usually determined as apparent ileal digestibility (AID), standard ileal digestibility (SID), which takes into account basal endogenous total nitrogen loss, and true ileal digestibility (TID), which takes into account individual amino acid basal endogenous loss (Stein et al., 2007b). In practice TID is rarely determined due to the several additional experiments needs to be conducted, and accuracy is very low. Cervantes-Pahm and Stein, 2010 looked at weanling pigs weighing 10 kg at the start of the study, and found a SID for casein and soy of 90.9 and 87.8% respectively. (Opapeju et al., 2006) determined a TID of 100 and 87.9 for casein and solvent-extracted soybean meal (SBM) respectively, from 80 kg finishing-barrow pigs. Stein et al., (2007b) state that because of the use of basal endogenous amino acid losses, instead of total amino acid losses, SID should take precedence over TID due to accuracy. SID is still not directly comparable to the values obtained in the current study, but for ileal digestibility values it is the best comparison to IAAO derived metabolic availability values. Furthermore, the soybean meal (SBM) used by Opapeju et al., 2006 is a solvent extracted form of soy meal, and not the same as the soy protein used in our study. The presence of additional compounds and the
difference in processing methods may contribute to different digestibility estimates. Established recommendations from the NRC (National Research Council, 1998) state casein and soy as having apparent ileal digestibility (AID) of 88 and 85% in pigs. These values are similar to the soy threonine metabolic availability estimate determined in this study but differ considerably from our casein metabolic availability results. AID does not take endogenous losses into account when determining digestibility, making it less comparable to metabolic availability determined using the IAAO method, compared to TID and further still SID. When using the IAAO method for determining metabolic availability, all losses through the body, whether occurring during digestion or metabolism are accounted for (Elango et al., 2009). Our metabolic availability data for threonine from casein and soy, while the first of its kind determined in healthy school-aged children, fits with current data from both previous human and animal studies, but more research still remains to be carried out to ensure proper recommendations are being made for this vulnerable population.

The use of the IAAO method to determine metabolic availability has been carried out previously in two human studies. (Humayun et al., 2007a) evaluated the availability of total sulfur containing amino acids (TSAA) from casein and soy protein isolates in healthy adult males (26.5 y, 69.9 kg). It was found that TSAA’s were $87.4 \pm 3.8$ % and $71.8 \pm 3.6$ % metabolically available respectively. Using the same labeled amino acid (L-1\textsuperscript{13}C-Phenylalanine) and a similar protocol, our study determined a difference of 13.2% between threonine availability in casein and soy, compared to the 15.6% difference found for TSAA by Humayun 2007. (Prolla et al., 2013) used the same method in young men, to determine the metabolic availability of lysine in cooked (97%) and oven-browned white rice (70%) finding a difference of 27% between the two food sources. The large difference exhibited in rice is probably due to
the cooking process for the rice impacting metabolic availability. Cooking rice to the point of browning increases the formation of maillard compounds, which limits the availability of amino acids (Sgarbieri et al., 1973). Maillard compounds are formed when carbohydrate and protein molecules meet in high-heat conditions, forming complexes which are not digestible in humans. In both Humayun et al. 2007 and our studies metabolic availability was determined by comparing the relative $F^{13}CO_2$ determined at a set intake of the target amino acids (60% of the requirement for methionine in the TSAA study and 50% of the requirement for threonine in current study), for each protein source. Prolla et al. took a different approach and determined the metabolic availability through comparison of the slope between 10, 15, and 19 mg/kg/d of lysine. The method employed by Humayun 2007 is more appropriate in our case, since we only studied 2 test intake points (0 mg and 11 mg/kg/d threonine intake), which is insufficient to perform a slope analysis. Figure 10 does present a visual representation of what these slopes may look like but is not valid for analytical use. Methods for determining metabolic availability and determining the impact of the values so determined with regard to dietary requirement recommendations will continue to evolve and develop, and issues such as slope analysis vs. relative $F^{13}CO_2$ determination need to be the focus of future studies. Our study is only the third its kind to be carried out in humans, and demonstrates a trend in metabolic availability between plant and animal derived protein sources.

Our study determined a metabolic availability value of threonine of 96.6% and 83.4% for casein and soy protein respectively in healthy school-aged 6-10 y children. This data, while the first study of its kind to be carried out in school-aged children, aligns with the existing body of literature between other human metabolic availability studies, as well as previous animal digestibility studies. It indicates that the available threonine contents of commonly consumed
protein sources may be overestimated. This is of key importance and needs to be more thoroughly researched in various protein sources, especially from vegetable sources. Ensuring proper recommendations regarding protein intake based on differences in metabolic availability between foods may help to provide a more adequate intake for vulnerable populations who may be at risk of deficiencies in threonine and other indispensable amino acids.
Chapter 6: Summary

6.1 Conclusion

This research project was comprised of two objectives. The first was to determine the minimum dietary requirement for threonine in healthy Canadian 6 – 10 y children, using the IAAO method. Prior to this study the requirement for threonine for this population was derived using a factorial calculation based on adult requirements (Institute of Medicine, 2005). Ensuring proper recommendations for intake of threonine is important during this stage of life because of the increased rate of growth which in turn increases the amount of threonine required both for body tissue, and mucosal gut proteins. threonine makes up a significant portion of mucin, a gut mucosal protein, and is required both for regulating gut growth and development, as well as gut health and immune function. The IAAO technique determined a requirement of 21.9 mg/kg/d ($R^2=0.237$, 95% CI:10.5 - 33.4 mg/kg/d) which is ~15% higher than the current EAR of 19 mg/kg/d for this age group.

The increase in threonine requirement determined by our study is likely due to an underestimated requirement for growth present in the factorially derived requirement. By experimentally determining the requirement directly in the target population, we ensured that both the maintenance and growth requirements were adequately measured. However, it is possible that the true values for pediatric requirements could in fact be higher still, due to the slow rate of growth during the 6-10y period, and more studies needs to be conducted to confirm the findings for growth.

The secondary objective was to determine the metabolic availability of threonine from casein protein and soy protein isolate. The IAAO method has been used to determine metabolic availability in previous studies (Humayun et al., 2007a; Prolla et al., 2013) but these were all
done in adult populations. We hypothesized that casein and soy protein would have similar availability. Both test proteins were assumed to have lower availability compared to the crystalline amino acid which is considered to be 100% available based on previous research (Elango et al., 2009, 2012c). The method employed by Humayun et al. 2007, directly compared the $^{13}$CO$_2$ values for the 11mg intakes of crystalline, casein, and soy protein intake study days. This provides a ratio for the resulting amount of oxidation, and therefore availability of the threonine being provided. Prolla et al., 2013 used a different method which compared the slope from an averaged value at 7 mg intake, to an averaged value at 14 mg intake of the different protein sources. Our study used only two data points (0mg, 11mg) which was insufficient to generate a line or a slope, and thus the method used by Prolla is not applicable to our results. However, the method used by Humayun allowed for comparing data points at a single intake threshold, giving us the relative availability of 96.6% (casein) and 83.4% (soy).

Some of the potential limitations of the current studies must be discussed. One limitation which was present in both of our studies is the standardization diets prior to each study day had variable protein intakes. The requirement study had an average intake of 2.16±0.04 g/kg/d, and the metabolic availability study had an average intake of 2.1±0.6 g/kg/d protein. The average intake of both studies were higher than our target of 1.5 g/kg/d, which was determined to be appropriate by previous research (Elango et al., 2011). The standardization diets were simply prescribed based on actual foods that each child liked to habitually consume. We did not provide the actual foods to be consumed which may have standardized the diets better, similar to our adult requirement studies (Humayun et al., 2007a, 2007b). The adult studies are conducted using a milk-shake based standardization diet and were considered invasive for the 6-10y child study. Thus, we prefer to use a food-based prescription approach. In the past it has been shown that
prior protein intake has an impact on phenylalanine kinetics measured using plasma and urine, but not breath (\(F^{13}\text{CO}_2\)) (Thorpe et al., 1999). Although the intakes were variable and it is unknown how this might affect the determined values, we did observe a steady state in breath (\(F^{13}\text{CO}_2\)) on all study days. In addition, our metabolic availability diets were based on a mixture of crystalline amino acids and protein sources with the labeled tracer as a crystalline amino acid as well. While the interactions of amino acids are assumed to be uniform in nature, additional experiments should be carried out using intrinsically labeled tracer amino acids to confirm the mechanistic aspects of the metabolic availability. However these experiments would likely require serial plasma sample collections and would be more invasive than the IAAO model we used. Within the minimally invasive construct, the IAAO model based on breath samples seems like an ideal method, and more studies needs to be conducted to confirm the validity.

In summary, the set of studies presented are the first to both determine threonine requirements in school-age children, as well as to use the IAAO method to determine metabolic availability of an amino acid from school-aged children. We have shown that the requirement for threonine is ~15% higher (21.5 mg/kg/d) than the currently recommended EAR (19 mg/kg/d), and the metabolic availability of threonine is higher from casein (96.6%) than from soy protein isolate (83.4%).

### 6.2 Future Directions

With regards to further research into threonine requirements in children, there are two further stages of research which should be pursued. Current recommendations for threonine intake in 6-10 y healthy Canadian children may not be appropriate for children who are malnourished and have ongoing infections (such as gut parasites), as shown earlier in chronic
malnourished children for lysine needs and may need to be determined to ensure that adequate intake for protein synthesis and growth is being met. As was seen in the series of studies to determine lysine requirements (Elango et al., 2007; Pillai et al., 2010, 2015), establishing the requirement for threonine in a healthy population within a high-risk area would be the next step. This could then be followed by a study in children with intestinal parasites in the same environment, both pre and post-treatment. The total contribution of these studies would be to help better understand the developmental impact of disease and nutrient insufficiency. The nature of disease can be diverse and wide reaching, but Pillai et al., 2015 found children with intestinal parasites to require a greater amount of lysine (20% increase) than similar children who did not have parasites. The current study can be the basis for comparison in other conditions.

The use of the IAAO method for determining metabolic availability of amino acids should also be expanded upon. Following the success of (Humayun et al., 2007a; Prolla et al., 2013) in adults, our study should serve as a pilot study for determining metabolic availability of other amino acids in school-aged children. This research has shown that metabolic availability of amino acids can change greatly depending on the protein source (animal vs vegetable source).

It is important to note that recommendations should not be altered based only on one study. The results of this study should be confirmed in additional participants, both within Canadian and other populations. We also suggest that more studies are carried out into establishing the metabolic availabilities of different food sources, as well as different amino acids. Currently more work is ongoing in our laboratory aiming to determine the metabolic availability of lysine, which is the first limiting amino acids in plant proteins, from multiple food sources in healthy school-aged Canadian children. Similar to the set of for requirement studies, it would also be necessary to carry out metabolic availability studies in other living conditions, to
determine if intestinal parasites or disease alter not only the requirement, but the availability of threonine as well. The impact of the difference in metabolic availability among other indispensable amino acids needs to be investigated, as it is likely to have a great impact on the potential health, growth, and development of children in areas with low food security, or low food diversity.
References


Nutr. 96, 759–767.


Institute of Medicine (2005). DRI.


World Health Organization (2006). WHO child growth standards: length/height for age, weight-
for-age, weight-for-length, weight-for-height and body mass index-for-age, methods and
development. WHO Child Growth Stand. Lengthheight Age Weight--Age Weight--Length
Weight--Height Body Mass Index--Age Methods Dev.
Yalcin, S., and Basman, A. (2015). Effects of infrared treatment on urease, trypsin inhibitor and
Yoshimura, H. (1972). Physiological Effect of Protein Deficiency with Special Reference to
Evaluation of Protein Nutrition and Protein Requirement1. In World Review of Nutrition and
Dietetics, (Karger Publishers), pp. 100–133.
Diet. Assoc. 91, 828–835.
Raton, FL).
59.
Young, V.R., Tontisirin, K., Özalp, I., Lakshmanan, F., and Scrimshaw, N.S. (1972). Plasma
Amino Acid Response Curve and Amino Acid Requirements in Young Men: Valine and Lysine.
J. Nutr. 102, 1159–1169.
AACC International Approved Methods - AACC Method 22-90.01. Measurement of Urease Activity.
Protein Research With Children!

WHAT IS THREONINE?
➢ An amino acid (a building block of protein) that must be part of your diet as you cannot make it yourself

WHY ARE WE INTERESTED IN THREONINE?
➢ Threonine is critical for ensuring proper growth and development in children

We are looking for:
• Healthy Children
• Normal weight
• Aged 6-10 years
• Boys and Girls!

This study involves:
• Screening at BC Children’s Hospital (1 hour)
• 9 visits to BC Children’s Hospital (8 hours each)
• A specialized diet on study days
• Breath and urine samples (minimally invasive)
• Body size, muscle mass evaluation

Compensation for your time, parking and transit passes will be offered
➢ If you would like more information about this study, please contact us today!

Principal Investigator: Dr. Rajavel Elango
Primary Contact: Peter Radonic
Study Email: pradonic@cfri.ca

Contact: 604-875-2000 ext. 4911
Contact: 604-875-2000 ext. 4607
Appendix B  Participant Consent Form

Department of Pediatrics

PARTICIPANT INFORMATION AND CONSENT FORM

Determination of Threonine Requirements and the Metabolic Availability of Threonine from Food Sources in Healthy School-aged Children

Principal Investigator:

Co-Investigator(s):

Sponsors:  Canadian Institutes of Health Research (CIHR)

Site:  Oak Street Campus, UBC  Child & Family Research Institute

Emergency Telephone Number:

Available 24 hours per day and seven days per week

1. INVITATION TO PARTICIPATE IN THIS STUDY

If you are a parent or legal guardian of a child who may take part in this study, permission from you and the assent (agreement) of your child may be required. When we say “you” or “your” in this consent form, we mean you and/or your child; “we” means the researchers and other staff.

You are being invited to take part in this research study because there is currently little information regarding how much threonine children between 6 and 10 years of age need to eat to grow properly. Threonine is an amino acid, which is the building block for protein in our body. We need to eat enough threonine every day from food. Currently it is not known with confidence how much threonine children between 6 and 10 years of age need to eat. Some foods like meat and milk have
more threonine, but vegetables like peas have less. This study is being conducted to find out the minimum amount of threonine that children between 6 and 10 years of age need in a day, and whether some foods will provide more inside the body.

This study is being conducted as part of Peter Radonic’s Master’s Thesis.

2. YOUR PARTICIPATION IS VOLUNTARY IN THIS STUDY

Your participation is voluntary. You have the right to refuse to participate in this study. If you decide to participate, you may still choose to withdraw from the study at any time without any negative consequences to the medical care, education, or other services to which you are entitled or are presently receiving. Before you decide, it is important for you to understand what the research involves. This consent form will describe the study, why the research is being done, what will happen to you during the study and the possible benefits, risks, and discomforts. If you wish to participate, you will be asked to sign this consent form within 7 days. Please take time to read the following information carefully and to discuss it with your child, family and doctor before deciding on participation.

3. WHO IS CONDUCTING THIS STUDY?

The Principal Investigator Dr. Rajavel Elango has received financial compensation from the sponsor Canadian Institutes of Health Research (CIHR) for the work required in doing this clinical research and/or for providing advice on the design of the study/travel expenses/etc. Financial compensation to researchers for conducting the research is associated with obligations defined in a signed contractual agreement between the researchers and the sponsor. Researchers must serve the interests of the participant and also abide by their contractual obligations. For some, the payment of financial compensation to the researchers can raise the possibility of a conflict of interest. You are entitled to request any details concerning this compensation from the Principal Investigator.

4. BACKGROUND INFORMATION ON THE STUDY

Threonine is an indispensable amino acid (nutrient containing nitrogen), which cannot be made in the body and must be consumed from food. Amino acids are the building blocks of protein in your body, and need to be eaten in required amounts to maintain health and growth. The current recommendation for how much threonine children should eat is based on mathematical calculations, which may not be correct. Our study will use a method called the Indicator Amino Acid Oxidation (IAAO) method, which is a new and more accurate method. This method uses a test liquid meal with a specific amount of threonine mixed with a stable isotope tracer. A stable isotope is a labeled amino acid, which is colourless, odourless, and tasteless, as well as being completely safe. They are naturally present in the air we breathe, the food we eat, and the water we drink. Using only air you breathe out and urine measured with specialized equipment we can measure if you are eating enough threonine for protein synthesis to take place, and we can study threonine requirements. This technique has been used to measure the requirements for other amino acids in babies, children, adults, and pregnant women. Establishing these requirements will allow us to improve dietary recommendations for future generations of children, especially those who are at risk for food insecurity.
5. **WHAT IS THE PURPOSE OF THIS STUDY?**  
The purpose of the study is to find out the amount of threonine needed in the diet for school-aged children (between 6 and 10 years of age). The results from this study may allow us to provide better threonine recommendations for children in this age group. Recent animal and human studies have indicated that these mathematical methods are not sufficient for ensuring adequate protein and amino acid intake in children. A secondary purpose is to determine how much of the threonine that is in several common protein sources is digested and used by our bodies to create tissue protein.

6. **WHO CAN PARTICIPATE IN THE STUDY?**  
- Healthy children between 6 and 10 years of age  
- Normal weight and normal eating habits.

7. **WHO SHOULD NOT PARTICIPATE IN THE STUDY?**  
- Children under 6 years old, or over 10 years old.  
- Children who are currently ill, with a fever, cold, vomiting or diarrhea.  
- Children outside of normal weight parameters (3rd-85th percentiles for weight according to Dietitians of Canada).  
- Children with claustrophobia.  
- Children currently or recently taking medication or antibiotics.

8. **WHAT DOES THE STUDY INVOLVE?**  
**Overview of the Study**  
This study will be conducted at the Oak Street Campus of UBC at the Child and Family Research Institute (CFRI) in the Clinical Research Evaluation Unit (CREU). If you agree to participate in this study, then you will be asked to complete the study procedures described below. Your full participation involves 9 separate study days where each study day is 8 hours, and 1 pre-study visit (1 hour). Study days can take place on non-school days, weekends, holidays, during the summer break, or as per your convenience.

If you decide to join this study:  
- **Pre-study day procedures**  
- Participant assent, and parent/guardian consent will be collected at the CREU in the CFRI building located in BC Children’s Hospital. You will be asked to come following an overnight fast (10-12 hr.). The screening visit will take approximately 1 hour to complete.
- The preliminary assessment is done to collect basic information about your child, to make sure you are informed about the study details, and collect the information needed to tailor the study diet to your child’s needs. You will also be asked health related questions to assess your medical history.  
- A research assistant will measure your height, weight, and body fat content (using bioelectric impedance machine and a skin-fold caliper). Metabolic rate will be measured using an indirect calorimetry machine. This consists of a clear hood (like a space-man’s suit) which will be placed on your head. This clear hood has air coming freely in and out. You can see everything through the hood and breathe normally without any discomfort. This will take about 20 minutes to
complete. If you want to stop the test anytime, you can just pull the hood away. This test measures how much carbon dioxide you produce.

☐ You will also be provided a 3-day food record sheet to fill out and return. This consists of recording the food you eat for any 2 days during the week and 1 day on the weekend.

b. Study Day Procedures:
If you agree to participate in this study, then the following procedures will be followed:
For the two days before each study day, we will recommend how much protein your child should eat from foods. This will consist of foods typically consumed by your child, and will be developed based on your reported food intake from the above described 3-day food record. This diet is made to ensure that your child has enough protein in his/her diet before our study day.

☐ The study will be conducted in the CREU at the CFRI, which is where the pre-study screening occurs. For each visit, you will be asked to fast (10-12hrs) before coming to the lab.
☐ Only water may be consumed prior to and during the study day. The study day test diet as described below will provide your daily energy and nutritional needs. At the end of the study day, you are free to resume your normal food intake.
☐ On the study day a research assistant will again measure your weight and height. The research assistant will also measure the rate at which you are breathing out carbon dioxide using the same clear hood.
☐ You will be given the test liquid diet as eight small hourly meals on the study day. Each meal is made up of 1) a mixture of amino acids, 2) an amino acid-free flavoured liquid and amino acid-free cookies that provide energy and other nutrients, and 3) the labeled amino acid which is added to the last four meals. The test meals will be complete for all nutrients, except for threonine.
☐ To measure how your body responds to the test diet we will collect your breath sample 9 times and urine 6 times during the study day. To collect breath, you will have to breathe into a container – just like blowing through a straw into a bag. To collect urine, you will have to pass urine into a urine sample hat in the privacy of the washroom. Urine samples will be kept for 7 years, in a -80C freezer. Samples will only be used for metabolic analysis for this study. Data will be kept in secured cabinets, or password protected computers and deleted after 7 years.

Breath collection bag

☐ When samples are not being collected, your child can watch television, listen to music, read, use a computer, play board games, color pictures, or do homework. Wireless internet access will be available, so personal electronic devices can be brought and used by children.
☐ In total, you can expect to dedicate approximately 8 hours per study day you participate in. You are invited to participate in up to 9 studies. If you choose to participate in all 9 days, your total time commitments will 72 hours.

9. WHAT ARE MY RESPONSIBILITIES?
In order to participate in this study:
☐ Participants must meet all inclusion criteria.
☐ On study days, only consume the test diet during study hours.
☐ Make sure you are fasted before each study day.
Let us know of any negative food or health related events.

10. WHAT ARE THE POSSIBLE HARMS AND DISCOMFORTS?
The test requires you to consume a small dose of isotope. This is a safe form of isotope and is widely used in medical research even in children and pregnant women. During the indirect calorimetry test you are requested to lie down for 20-30 minutes with an open hood/canopy over the face and head. Some children may feel uncomfortable during this period. The hood/canopy has outside air freely moving in and out. If you are uncomfortable, then the test will be stopped immediately. Some children may also find the body fat callipers to be slightly uncomfortable. Our liquid test diet may cause a stomach upset, which usually goes away after one meal. There are no other known risks involved with participating in this research. We recognize that the length of each study day and number of study days may pose an inconvenience to you.
The test amount of threonine in the current study are safe, and within the limits of what is normally eaten by people living in North America.

11. WHAT ARE THE POTENTIAL BENEFITS OF PARTICIPATION?
There are no direct benefits to you or your child for taking part in this study. We hope that the information gained from this study can be used to develop and improve future dietary guidelines for threonine in children.

12. WHAT HAPPENS IF I DECIDE TO WITHDRAW MY CONSENT TO PARTICIPATE?
Your participation in this research is entirely voluntary. You may withdraw from this study at any time and without providing any reasons for your decision. If you decide to enter the study and then withdraw, there will be no penalty or loss of benefits, if any, to which you are otherwise entitled. If you choose to enter the study and then decide to withdraw at a later time, all data collected about you during the enrolment part of the study will be retained for analysis, after which the study information may be shredded.

13. CAN I BE ASKED TO LEAVE THE STUDY?
If you are not able to meet the requirements of the study, the study researcher may withdraw you from the study. If you are asked to leave the study, the reasons for this will be explained to you and you will have the opportunity to ask questions about this decision.

14. HOW WILL MY TAKING PART IN THIS STUDY BE KEPT CONFIDENTIAL?
Your confidentiality will be respected. However, research records and health or other source records identifying you may be inspected in the presence of the Investigator or the University of British Columbia – Children’s & Women’s Health Centre of BC Research Ethics Board for the purpose of monitoring the research. No information or records that disclose your identity will be published without your consent, nor will any information or records that disclose your identity be removed or released without your consent unless required by law.

You will be assigned a unique study number as a participant in this study. This number will not include any personal information that could identify you (e.g., it will not include your Personal
Health Number, SIN, or your initials, etc.). Only this number will be used on any research-related information collected about you during the course of this study, so that your identity will be kept confidential. Information that contains your identity will remain only with the Principal Investigator and/or designate. The list that matches your name to the unique study number that is used on your research-related information will not be removed or released without your consent unless required by law.

Your rights to privacy are legally protected by federal and provincial laws that require safeguards to insure that your privacy is respected. You also have the legal right of access to the information about you that has been provided to the sponsor and, if need be, an opportunity to correct any errors in this information. Further details about these laws are available on request to your study doctor.

15. WHAT HAPPENS IF SOMETHING GOES WRONG?
   By signing this form, you do not give up any of your legal rights and you do not release the study doctor, participating institutions, or anyone else from their legal and professional duties. If you become ill or physically injured as a result of participation in this study, medical treatment will be provided at no additional cost to you. The costs of your medical treatment will be paid by your provincial medical plan.
   In case of a serious medical event, please report to an emergency room and inform them that you are participating in a clinical study and that the following person can then be contacted for further information: Dr. Rajavel Elango at telephone number: 778-986-8655

16. WHAT WILL THE STUDY COST ME?
   Participation in the study will not cost you anything. In appreciation of the time that it takes to complete this study you will receive $100 upon each study day completion to a maximum of $900 for 9 study days. Vehicle parking coupons or transit fare will also be provided depending on method of transportation. Receiving compensation of this amount is taxable and you will therefore be issued a T4A form. In order to issue the T4A for we will collect you SIN number. The SIN number will be used for tax purposes only, and will be stored in a locked secure area separate from your study data. In addition, your SIN number will be given to the UBC financial department in order to compensate you and generate the T4A form.

17. WHO DO I CONTACT IF I HAVE QUESTIONS ABOUT THE STUDY DURING MY PARTICIPATION?
   If you have any questions or desire further information about this study before or during participation, or if you experience any adverse effects, you can contact Dr. Rajavel Elango at 604-875-2000 x4911, or his research assistant Peter Radonic at 604-875-2000 x4607.

18. WHO DO I CONTACT IF I HAVE ANY QUESTIONS OR CONCERNS ABOUT MY RIGHTS AS A PARTICIPANT?
   If you have any concerns or complaints about your rights as a research participant and/or your experiences while participating in this study, contact the Research Participant Complaint Line in the University of British Columbia Office of Research Ethics by e-mail at RSIL@ors.ubc.ca or by phone at 604-822-8598 (Toll Free: 1-877-822-8598).
Determination of Threonine Requirements and the Metabolic Availability of Threonine from Food Sources in Healthy School-aged Children

Participant Consent

My signature on this consent form means:

- I have read and understood the information in this consent form.
- I have had enough time to think about the information provided.
- I have been able to ask for advice if needed.
- I have been able to ask questions and have had satisfactory responses to my questions.
- I understand that all of the information collected will be kept confidential and that the results will only be used for scientific purposes.
- I understand that my participation in this study is voluntary.
- I understand that I am completely free at any time to refuse to participate or to withdraw my child from this study at any time, and that this will not change the quality of care that I receive.
- I understand that I am not waiving any of my legal rights as a result of signing this consent form.
- I understand that there is no guarantee that this study will provide any benefits to my child.

The parent(s)/guardian(s)/substitute decision-maker (legally authorized representative) and the investigator are satisfied that the information contained in this consent form was explained to the child/participant to the extent that he/she is able to understand it, that all questions have been answered, and that the child/participant assents to participating in the research.

I will receive a signed copy of this consent form for my own records.

I consent to participate in this study.

____________________  ______________________  __________
Parent/Guardian Signature  Parent/Guardian Printed Name  Date

____________________  ______________________  __________
Signature of Person Obtaining Consent  Printed Name  Study Role  Date

Investigator Signature

____________________  ______________________  __________
Investigator Signature  Printed name  Date

My signature above signifies that the study has been reviewed with the study participant by me and/or by my delegated staff. My signature may have been added at a later date, as I may not have been present at the time the participant’s signature was obtained.

I consent to be contacted in the future, regarding participation in additional studies.

Yes []  No []
Appendix C  Participant Assent Form

SUBJECT ASSENT FORM
Protein (Threonine) Requirement in Children aged 6 to 10 years

1. INVITATION
I am being invited to be part of a research study. Research studies try to find new information to help boys and girls around the world. Only I can decide if I want to be in this study. No one can make me be part of the study if I do not want to. Even if I agree now, I can change my mind later. I can stop at any time. No one will be mad if I do not want to be a part of the study any more.

2. WHY ARE WE DOING THIS STUDY
I am a healthy boy or girl with no disease. This study is trying to find out how much protein healthy children need to eat. Protein in food and my body is made up of building blocks, and threonine is one of these building blocks. Threonine is needed every day from foods. Still we do not know how much threonine to eat in a day. Using the information from this study, scientists will know how much threonine boys and girls of my age need to eat. This study will not help me directly, but the results may be helpful to other children in the future.

3. WHAT WILL HAPPEN IN THIS STUDY?
- I will have to come to the hospital without eating anything for 12 hours before.
- I will have to come 9 times, plus 1 time for 1 hour before I start the study.
- Each of the 9 study days will take 8 hours.
- Study days can take place on non-school days, weekends, holidays, or during the summer break.
- A researcher will measure my weight and height.
- I will be requested to drink a test liquid (1 small cup eight times during the day). The liquid drink will taste a bit sweet and a bit sour because it has pure protein powders and orange tang added in. Small cookies will be given with each liquid drink, which are regular cookies. The test liquid is very safe to drink.
- I will breathe into a container 9 times, like blowing through a straw into a bag. (Shown at right)
- Urine samples will be collected in a urine hat 6 times during the day.
- During the day I can watch television, listen to music, read, use a computer, play board games (Monopoly, Blockus, Connect4, Clue, Scrabble etc.), card games (Uno, SkipBo, regular playing cards etc.), color pictures (crayons, markers, coloring sheets
will be provided), or do homework. I can also bring my electronic devices to use, as there will be wireless internet available.

- How much muscle I have will be measured. This test will only take 30 seconds to 1 minute. While I lie down on a bed two wires will be put on my right hand and foot, like Band-Aids. I will not feel anything during this test.
- My body fat will also be measured by the researcher using an instrument called calipers. These calipers will gently pinch your skin for 10-15 seconds, and may feel uncomfortable, but will not hurt.
- While I lie down on a bed a clear hood (like a space-man’s suit) will be placed on my head. This is a clear hood with air coming freely in and out. I can watch TV during the testing. This test will take about 20 minutes. If I want to stop the test anytime, I can just pull the hood away. This test measures how much carbon dioxide I produce.
- My parents can stay with me in the Clinical Research Evaluation Unit (CREU) during the study.

4. WHO IS DOING THIS STUDY?
Dr. Rajavel Elango and his research assistant Peter Radonic from B.C. Children’s Hospital will be doing this study. They will be able to answer any questions that I have about the study. I can also call them at 778-986-8655 (Dr. Elango) or at 604-653-1064 (Peter Radonic) if I have any problems or questions, and I cannot call my parents. This study is being carried out as part of Peter Radonic’s master’s thesis.

5. CAN ANYTHING BAD HAPPEN TO ME?
There is nothing in this study, which will make anything bad happen to me. It is possible that I may feel bad on the study day, and if this happens I should tell the researchers or my parents at once. If I feel scared in small spaces (Claustrophobia) I should let the researchers know so that they can take care of me.

6. WHAT SHOULD I DO IF I AM NOT FEELING WELL?
If I do not feel well, I should let the researchers Dr. Elango or Peter Radonic know immediately. I can also call the study supervisor Dr. Elango (778-986-8655) or Peter Radonic at (604-653-1064), anytime.

7. WHO WILL KNOW I AM IN THE STUDY?
Only the researchers involved in the study will know that I am in it. When the study is finished the researchers will write a paper about what was learned. This paper will not say my name or that I was in the study. I do not have to tell anyone that I was in the study if I do not want to.

8. WHEN DO I HAVE TO DECIDE?
I can take up 7 days to decide if I want to be part of the study. I have also been asked to discuss my decision with my parents.

Signatures
By putting my name on the line below, it means that I agree to be a part of the study.

____________________  ________________________  ___________
Printed Name                     Signature                     Date
Appendix D  Casein metabolic availability calculations

<table>
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<th>Study Date</th>
<th>Requirements mg/kg/d</th>
<th>Subject</th>
<th>THR-10</th>
<th>Protein in Casein (g/100g P)</th>
<th>THR in casein (mg/100g P)</th>
<th>Age</th>
<th>Weight</th>
<th>Height</th>
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<td>9</td>
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<td></td>
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<td></td>
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<table>
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<tr>
<th>G P / day</th>
<th>gP/meal</th>
<th>Minus Casein</th>
<th>gP/meal</th>
<th>gP Mix 1/ meal</th>
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<tr>
<td>Protein intake</td>
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<table>
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<th>Threonine</th>
<th>mg/kg/d</th>
<th>CASEIN (mg)</th>
<th>Intake from Casein (mg THR/d)</th>
<th>g THR/ meal</th>
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<th>g of Casein/d</th>
<th>Protein intake (g/d)</th>
<th>g Casein/meal (12)</th>
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<tr>
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<td>3.665536134</td>
<td>3.189016436</td>
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<table>
<thead>
<tr>
<th>Other AA</th>
<th>0.716375069</th>
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</thead>
</table>

<table>
<thead>
<tr>
<th>THR, PHE, TYR, ALA</th>
<th>mg/Day</th>
<th>g/meal (11mg THR)</th>
<th>From Casein (g/meal)</th>
<th>g/meal (6mg THR)</th>
<th>g/meal</th>
<th>g/meal</th>
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<tbody>
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<td>THR</td>
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<td>0.07025</td>
<td>0.012982107</td>
<td>0.050453736</td>
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<tr>
<td>TYR</td>
<td>1.7167695</td>
<td>0.141064125</td>
<td>0.009418391</td>
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| ALA | 0.716375069 | 105.46648  | 8.78887375 | 246.9673524 | 0.246967353 |

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<thead>
<tr>
<th>PHE balance</th>
<th>mg/d</th>
<th>Phe req (mg/d)</th>
<th>Phe from Case</th>
<th>Total PHE in 8 meals</th>
<th>Balance PHE</th>
<th>PHE for meals 1, 2, 3, 4</th>
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<td></td>
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<td>103.8568571</td>
<td>556.38</td>
<td>305.2136569</td>
<td>76.30341421</td>
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<td></td>
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<td>PHE / 12</td>
<td>0.0572767893</td>
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<td>PHE in 8 meals</td>
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<th>Phe 13C</th>
<th>Total PHE in 8 meals</th>
<th>Balance PHE</th>
<th>PHE for meals 1, 2, 3, 4</th>
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<tr>
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<td>85.6488</td>
<td>556.38</td>
<td>305.2136569</td>
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<tr>
<td>Meal 5</td>
<td>55.0198</td>
<td>201.8149429</td>
<td>50.45373572</td>
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<tr>
<td>Meal 6</td>
<td>55.0198</td>
<td>201.8149429</td>
<td>50.45373572</td>
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<td>Meal 7</td>
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<td>50.45373572</td>
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<td>Meal 8</td>
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<td>201.8149429</td>
<td>50.45373572</td>
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<td>Total</td>
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### Appendix E  Soy metabolic availability calculations

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<th>Study Date</th>
<th>Requirements mg/kg/d</th>
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<tr>
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<td>THR in Soy (mg/100g P)</td>
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<td></td>
<td>Protein in Soy (g/100g P)</td>
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<td></td>
<td>83</td>
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<td>REE</td>
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<td>1196</td>
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#### Protein intake

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<tr>
<th>Protein (g/kg/d)</th>
<th>g P/day</th>
<th>gP/meal</th>
<th>Minus Soy</th>
<th>gP/meal ADI for soy</th>
<th>gP Mix 1/ meal</th>
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<td>1.5</td>
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<td>37.29104167</td>
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#### Threonine

<table>
<thead>
<tr>
<th>mg/kg/d</th>
<th>BASE (mg)</th>
<th>Soy (mg)</th>
<th>Intake from Soy (mg THR/d)</th>
<th>mg THR/ meal</th>
<th>g THR/ meal</th>
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<tbody>
<tr>
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**PHE, TYR, ALA**

#### Other AA

<table>
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<th>mg/Day</th>
<th>g/meal (11mg THR)</th>
<th>From Soy (g/meal)</th>
<th>g/meal (6mg THR)</th>
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**THR, PHE, TYR, ALA**

#### PHE balance

<table>
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<th>mg/d</th>
<th>PHE req (mg/d)</th>
<th>PHE to be added</th>
<th>PHE / 12</th>
<th>PHE in 8 meals</th>
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<td>843</td>
<td>0.654203125</td>
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<td>0.436135417</td>
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#### PHE balance in 8 meals

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<th>Balance PHE Diet</th>
<th>PHE for meals 1, 2, 3, 4</th>
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<td>85.6488</td>
<td>556.38</td>
<td>179.8072167</td>
<td>0.044951804</td>
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<thead>
<tr>
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<th>PHE diet</th>
<th>PHE Soy</th>
<th>13C PHE</th>
<th>Total</th>
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<td>55.0198</td>
<td>15.733073</td>
<td>85.648800</td>
<td>101.381873</td>
<td>55.0198</td>
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Appendix F  Subject Code Master List

Subject Code Master List

Application of Stable Isotopes to Determine Threonine Requirements in healthy School-Aged Children

<table>
<thead>
<tr>
<th>Subject Name</th>
<th>Code (Alpha-Numeric)</th>
<th>Comments</th>
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</tbody>
</table>
Appendix G  Pre-Study Assessment

Pre-Study Day Assessment
Determination of Threonine requirements and the Metabolic Availability of Threonine from Food Sources in Healthy School-aged Children

Principal Investigator: Dr. Rajavel Elango  604-875-2000 ext. 4911 (office)
Student Investigator: Peter Radonic  604-875-2000 ext. 4607 (office)
240-938-9886 (cell)

PRELIMINARY ASSESSMENT

Subject ID: __________________________
Date: __________________________
Birthday: ______/_______  Age: ________  Gender: ___
(month) (year)
Height (cm): ___________  Weight (kg): ___________  BMI: ___________

Bioelectrical Impedance Analysis

BIA: R ________________  XC ________________
(resistance)  (reactance)

BIA: Z ________________
(Impedance)

Body Composition Profile

% Body fat (BIA): ___________  Lean body mass (BIA): ___________

Indirect Calorimetry

Measured REE (kcal/day): ___________  Estimated REE (kcal/day): ___________

Daily energy requirement (kcal/day): ___________

VCO₂ (ml/min): ___________  VO₂ (ml/min): ___________

RQ: ___________

Medical History
Details of health condition(s)
________________________________________________________________________

Are you currently taking any prescription medications? Yes______ No_______

List of medications:________________________________________________________________________

Are you currently having vomiting episodes? Yes______ How many/day ___ No______

Are you currently having fever/cold? Yes_____ No_____

Nutritional Supplement Intake

Are you currently taking vitamins? Yes_____ No_____

Are you taking any other nutritional supplements? Yes_____ No_____

If yes, please list all nutritional supplements:
1.__________________________________ 3.__________________________________
2.__________________________________ 4.__________________________________

Activity Level

Daily exercise (minutes)________________________

Sedentary__________ Moderate______________ High__________________

Availability for 9 studies

Yes__________ No____________

Comments:
### Dietary Record

**Subject ID:** ____________________________

**Date:** __/__/____

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Item</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Breakfast</td>
<td></td>
<td>Snack</td>
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</tr>
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<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Dinner</td>
<td></td>
</tr>
<tr>
<td>Snack</td>
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<tr>
<td></td>
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<td></td>
</tr>
<tr>
<td>Lunch</td>
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</tr>
</tbody>
</table>
Appendix I  Study Day Protocol

Determination of Threonine requirements and the Metabolic Availability of Threonine from Food Sources in Healthy School-aged Children

Subject ID: ___________________________  Date: ___________________________

Height (cm): ________________  Weight (kg): ________________

Protein intake (g/kg/d): ________________  Energy intake (kcal/day): ________________

<table>
<thead>
<tr>
<th>Time</th>
<th>Sample Collection/Anthropometry</th>
<th>Meals and isotope tracer</th>
<th>Comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>8:00</td>
<td></td>
<td>Meal #1</td>
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</tr>
<tr>
<td>9:00</td>
<td></td>
<td>Meal #2</td>
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</tr>
<tr>
<td>10:00</td>
<td></td>
<td>Meal #3</td>
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</tr>
<tr>
<td>11:00</td>
<td></td>
<td>Meal #4</td>
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</tr>
<tr>
<td>11:15</td>
<td>1&lt;sup&gt;st&lt;/sup&gt; breath (3x)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1&lt;sup&gt;st&lt;/sup&gt; urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11:30</td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; breath (3x)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>11:45</td>
<td>3&lt;sup&gt;rd&lt;/sup&gt; breath (3x)</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>2&lt;sup&gt;nd&lt;/sup&gt; urine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>12:00</td>
<td>VCO&lt;sub&gt;2&lt;/sub&gt; measurement</td>
<td>Meal #5 – primer dose and 1&lt;sup&gt;st&lt;/sup&gt; oral dose</td>
<td></td>
</tr>
<tr>
<td>13:00</td>
<td></td>
<td>Meal #6 – 2&lt;sup&gt;nd&lt;/sup&gt; oral dose</td>
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</tr>
<tr>
<td>14:00</td>
<td></td>
<td>Meal #7 – 3&lt;sup&gt;rd&lt;/sup&gt; oral dose</td>
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<tr>
<td>14:30</td>
<td>4&lt;sup&gt;th&lt;/sup&gt; breath (3x)</td>
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<td></td>
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<tr>
<td></td>
<td>3&lt;sup&gt;rd&lt;/sup&gt; urine</td>
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<tr>
<td>15:00</td>
<td>5&lt;sup&gt;th&lt;/sup&gt; breath (3x)</td>
<td>Meal #8 – 4&lt;sup&gt;th&lt;/sup&gt; oral dose</td>
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<tr>
<td></td>
<td>4&lt;sup&gt;th&lt;/sup&gt; urine</td>
<td></td>
<td></td>
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<tr>
<td>15:15</td>
<td>6&lt;sup&gt;th&lt;/sup&gt; breath (3x)</td>
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<td>15:30</td>
<td>7&lt;sup&gt;th&lt;/sup&gt; breath (3x)</td>
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<td>5&lt;sup&gt;th&lt;/sup&gt; urine</td>
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<tr>
<td>15:45</td>
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<td>16:00</td>
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<td>6&lt;sup&gt;th&lt;/sup&gt; urine</td>
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