## PROTEIN METABOLISM FOLLOWING EXERCISE IN CHILDREN

## THE REGULATION OF WHOLE BODY PROTEIN METABOLISM FOLLOWING EXERCISE IN CHILDREN

By

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### ABSTRACT

General nutritional strategies to promote whole body protein retention, particularly with relation to exercise, have been largely based on adult research that does not consider the dynamic process of growth and often ignores scenarios commonly experienced by children (e.g., exercise in the heat). Therefore, the aim of the experiments outlined in this thesis was to investigate the importance of post-exercise protein quantity and timing in active children, specifically with respect to whole body protein turnover.

**Chapter 4** demonstrated that a whole-food protein-containing beverage can impact whole body protein balance (WBPB) in healthy children following exercise. Specifically, consuming ~ $0.40 \text{ g} \cdot \text{kg}^{-1}$  of protein in the form of skim milk enhanced whole body nitrogen turnover (Q) and protein synthesis, resulting in a less negative WBPB compared with a carbohydrate electrolyte solution (CES) and water (W) over 16 h. It was also found that puberty and sex significantly affected WBPB.

In order to elucidate the specific effects of protein, participants in **Chapter 5** were provided with isoenergetic mixed macronutrient beverages containing graded levels of protein (0 g·kg<sup>-1</sup>, ~0.18 g·kg<sup>-1</sup>, and ~0.32 g·kg<sup>-1</sup>) following an acute bout of exercise. Net protein balance was increased in a dose-dependent manner early in recovery, but in order to sustain a net anabolic environment over an entire 24 h period it appears that larger protein intakes (~0.32 g·kg<sup>-1</sup>) are required.

Finally, in **Chapter 6**  $[1^{-13}C]$ leucine infusion was used to confirm previous conclusions. Following an acute bout of exercise, graded levels of protein (0 g·kg<sup>-1</sup>, ~0.12 g·kg<sup>-1</sup>, ~0.22 g·kg<sup>-1</sup> and ~0.33 g·kg<sup>-1</sup>) acutely increased whole body leucine balance

in a dose-dependent manner. However, in order to attain a positive WBPB over 24 h, multiple protein feedings following exercise may be more important than the absolute quantity of protein ingested in the post-exercise period.

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## LIST OF ABBREVIATIONS

AMDR	Acceptable macronutrient distribution range
APHV	Age of peak height velocity
AUC	Area under the curve
В	Whole body protein breakdown
BCAA	Branched-chain amino acid
BCAT	Branched-chain aminotransferase
BCKAD	Branched-chain ketoacid dehydrogenase
BMI	Body mass index
<b>C</b> ( <i>t</i> )	Mean plasma leucine concentration
CES	Carbohydrate-electrolyte solution
СНО	Carbohydrate
CO <sub>2</sub>	Carbon dioxide
dC/dt	Time difference in plasma leucine concentration
dEi <sub>IV</sub> /d <i>t</i>	Time difference in plasma leucine enrichment for the
	intravenous tracer
dEi <sub>PO</sub> /d <i>t</i>	Time difference in plasma leucine enrichment for the oral
	tracer
Diet Leu Ei	Leucine enrichment of the total dietary leucine
DRI	Dietary reference intakes
EAR	Estimated average requirement
$\mathrm{Ei}_{\mathrm{IV}}(t)$	Mean plasma leucine enrichment for the intravenous tracer
$Ei_{PO}(t)$	Mean plasma leucine enrichment for the oral tracer
eIF	Eukaryotic initiation factor
Endo R <sub>a</sub>	Endogenous rate of leucine appearance
Exo R <sub>a</sub>	Exogenous rate of leucine appearance
GCMS	Gas chromatographer-mass spectrometer
IV	Intravenous
IRMS	Isotope ratio mass spectrometry
KIC	α-ketoisocaproate
KIV	α-ketoisovalerate
KMV	α-keto-β-methylvalerate
Leu <sub>Ra</sub>	Leucine rate of appearance
Leu <sub>Rd</sub>	Leucine rate of disappearance
MLP	Mid- to late-pubertal
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis

MVPA	Moderate to vigorous physical activity				
Ν	Nitrogen				
N <sub>BAL</sub>	Nitrogen balance				
N <sub>EX</sub>	Total nitrogen excretion				
NH <sub>3</sub>	Ammonia				
N <sub>IN</sub>	Total nitrogen intake				
NOLD	Non-oxidative leucine disposal				
PDCAAS	Protein digestibility corrected amino acid score				
PEP	Pre- to early-pubertal				
pV	Volume of leucine effectively mixing in the body				
RDA	Recommended dietary allowance				
RPE	Rate of perceived exertion				
Q	Whole body nitrogen flux				
Q <sub>A</sub>	Estimate of whole body nitrogen flux based on ammonia				
$\mathbf{Q}_{\mathrm{U}}$	Estimate of whole body nitrogen flux based on urea				
Q <sub>Leu</sub>	Whole body leucine flux				
S	Whole body protein synthesis				
SM	Skim milk				
ТСА	Tricarboxylic acid				
VO <sub>2</sub>	Oxygen consumption				
VO <sub>2max</sub>	Maximal oxygen consumption				
WBPB	Whole body protein balance				

### **CHAPTER 1 : General Introduction**

Proper nutrition has an important role in optimizing health during childhood and throughout life. Dietary protein, in particular, plays a central role in somatic growth as it provides the substrates necessary to build body proteins (Jacob and Nair 2012). For active children and youth, nutrition is particularly important as a result of the increased nutritional requirements to meet the combined physiological demands for the normal processes of growth, as well as exercise (Steen 1996a, 1996b). As a result, establishing the optimal relationship between physical activity (hereafter referred to as 'exercise') and protein is of utmost importance for the pediatric age group (Jacob and Nair 2012).

Understanding the benefits of a nutritionally adequate diet to support exercise and growth is of particular importance now, as there has been an attempt in recent years to promote exercise among youth. Given the overwhelming concern of an inactivity crisis (ParticipATION 2014) that undoubtedly has contributed to the tripling rate of childhood obesity over the last 30 years (Shields 2005), it is important to equip young people with the skills and mindsets needed to lead healthy, active lifestyles, which can carry over into adulthood (Kelder et al. 1994, Hallal et al. 2006). Important benefits of exercise include (but are not limited to): reduced anxiety and depression (Nieman 2002), increased selfesteem (Nieman 2002), development of positive family interactions (Nieman 2002), increased musculoskeletal health (Tobias et al. 2007, Baxter-Jones et al. 2008, Goulding et al. 2009), and decreased risk of obesity and related co-morbidities, cardiovascular disease, various cancers, and type 2 diabetes (Sigal et al. 2006).

With the aim of stimulating children and youth to lead more active lifestyles, it is important to understand how to optimize benefits from specific episodes of exercise. Specifically, it is critical to gain an understanding of how this exercise can be complemented by appropriate protein intake in order to support developing bodies. Thus, the combined effects of protein and exercise on protein metabolism and the promotion of protein accretion for growth deserve further study.

Unfortunately, due to the paucity of scientific information available to formulate pediatric-specific nutritional recommendations, active children and youth must look to adult guidelines that do not consider the dynamic process of growth. In adults, the majority of the literature elucidating the synergies between protein and exercise has focused primarily on optimizing adaptations to exercise in order to increase skeletal muscle size and strength, or to enhance specific sport performance measures (Schoenfeld et al. 2013, Pasiakos et al. 2015). Although this may have relevance for a subset of elite athletes aiming to optimize performance, these specific adaptations may be less important for the average child. For children experiencing concurrent growth in the majority of all bone and lean tissues, it is arguably more important to study the combined effects of protein and exercise on protein metabolism at the whole body level, because this will lead to an understanding of how to best support proper whole-body growth and development. Therefore, pediatric-specific studies are required to clarify issues specific to active youngsters that cannot be otherwise resolved by relying on adult research. For this reason, the fundamental basis for this thesis was to advance our knowledge regarding the impact of protein ingestion on whole body protein turnover, specifically whole body protein balance (WBPB) acutely, following aerobic exercise in healthy, active children.

#### **1.1. NUTRITION AND GROWTH**

### **1.1.1.** The Importance of Protein for Growth

Children, unlike adults, experience periods of accelerated growth which would ostensibly require extensive tissue remodeling and would presumably be reflected by higher rates of whole body protein turnover (i.e., both protein synthesis and protein breakdown) (National Research Council 2005). As a result, a child's basal need for dietary protein is increased. Children must also attain a positive WBPB (a whole body state where the anabolic pathways are activated to a greater extent than that catabolic pathways), rather than the net zero balance experienced by adults, in order to sustain the accrual of protein-containing tissue that is necessary for growth. This is particularly important during periods of development where growth occurs very rapidly (i.e. puberty). Consequently, it is critical to ensure sufficient protein intake during these times.

The timing of the pubertal growth spurt can vary considerably between individuals (Rogol et al. 2000), and typically lasts for 2 to 3 years. The period during this growth spurt at which the rate of skeletal and muscle growth is the greatest is defined as the age of peak height velocity (APHV) (Rauch et al. 2004). In a healthy normally developing child, this often occurs between the ages of 11 and 13 (11.8 years for females, 13.4 years for males) (Rauch et al. 2004). The change in growth velocity during the

pubertal growth spurt is quite evident; normal growth velocity up to  $\sim 10$  years of age is  $\sim 5$  cm and  $\sim 3$  kg per year, after which it may increase up to 3-fold during the early pubertal growth spurt (Tanner and Whitehouse 1976). As a result, a child achieves 15-25% of their adult height and acquires 45% of their skeletal growth during the pubertal growth spurt (Rees and Christine 1989).

The majority of body mass increase during this time is a result of increases in lean mass. During childhood, nonathletic females and males have a protein accrual of  $1.0 \text{ g} \cdot \text{d}^{-1}$ <sup>1</sup> and 2.0 g·d<sup>-1</sup>, respectively; however, these values can reach up to 2.3 g·d<sup>-1</sup> and 3.8 g·d<sup>-1</sup>, respectively, during the peak of the pubertal growth spurt (Forbes 1981). During puberty, the peak in lean body mass accrual often coincides with peak rates of height gains, but occurs before peak gains in bone mineral content (Rauch et al. 2004). There are also various factors that may influence protein accretion throughout the pubertal growth spurt. For example, it has been suggested that during puberty ingested protein is metabolized more efficiently, as demonstrated by an enhanced net leucine retention in pubertal compared with pre-pubertal children, which may be attributed to the influence of hormones (Beckett et al. 1997). Therefore, given the growth-related changes that occur during puberty, the studies in this thesis categorized children according to 3 distinct pubertal stages. The first study examined the protein requirements of pre- to earlypubertal (PEP; 8-10 years of age) and mid- to late-pubertal (MLP; 14-16 years of age) children in an attempt to assess children pre- and post-pubertal growth spurt, respectively. Given the rapid growth that occurs during childhood and the importance of understanding how to maximize the accretion of lean body mass while ensuring the attainment of adequate protein needs in children who are at or approaching APHV, the second and third studies in this thesis examined children within ~2 years of APHV (~10-13 years of age).

There are also sex differences that occur during growth with respect to the distribution of synthesized tissues. More specifically, boys have a characteristic rapid acquisition of lean mass and a modest increase in fat mass during early puberty which declines throughout adolescence (National Research Council 2005); whereas girls experience a more modest increase in lean mass with a continual accumulation of fat mass during adolescence (National Research Council 2005). The result is a greater lean mass to height ratio in boys, and a larger deposition of fat mass in girls (Haschke 1989). Given the sex differences in body composition that occur throughout growth, the aim of the first study in this thesis was to investigate potential sex-based differences in the quantity of protein required to synthesize the respective tissues in PEP and MLP children and youth.

Difference between muscle and whole body protein metabolism. Protein turnover within the body can vary substantially between specific tissues (McNurlan and Garlick 1980, Reeds and Garlick 1984, Waterlow 1984). Despite skeletal muscle's importance to the regulation of proper growth and overall health, as well as its significant contribution to whole body protein turnover (Wagenmakers 1999), muscle protein synthesis (MPS) and muscle protein breakdown (MPB), which are commonly used endpoints for studying protein metabolism in adults, are only reflective of one organ. WBPB, on the other hand, is an overall marker of protein status and represents the sum of all protein anabolic and catabolic processes across the body, rather than in one single organ or tissue. This is an especially important consideration within the context of a growing child as a child experiences growth throughout the whole body and is not aiming to maximize growth only at the level of the skeletal muscle. In spite of this, however, it has been proposed that there is a preferential utilization for amino acids to enhance rates of MPS immediately post-exercise, whereas whole-body proteins (including those in the gut) are remodeled as a secondary process (Moore et al. 2014). Thus, it is possible that if amino acids are preferentially utilized for MPS, changes in WBPB could be a reflection of what is happening within the muscle or lean body mass. Moreover, adults do not experience concurrent growth in all tissues. Instead, adults are only "growing" following resistance training, which has proven to increase MPS (Wilkinson et al. 2008) and markers of WBPB (Hartman et al. 2006). While it is unclear whether the results from adult studies would also occur in a normally developing child, this may be further evidence that whole body metabolism during periods of anabolism could reflect changes in "growing tissues" (e.g., muscle).

### 1.1.2 The Energy Cost of Growth

The process of growth is energetically very costly (Butte 2000) and consists of the energy expended to synthesize new tissues, as well as the energy being deposited into these newly synthesized tissues (National Research Council 2005). The energy deposited into newly accrued tissues varies throughout childhood (averaging ~ 20 kcal·d<sup>-1</sup>), and increases markedly around the pubertal growth spurt (up to ~30 kcal·d<sup>-1</sup> at peak velocity) (Butte 2000, National Research Council 2005). Therefore, in children (particularly those

who are at or approaching APHV) the demands for energy are also increased (Torun 2005). Ensuring adequate energy intake throughout growth is extremely important, as chronic energy deficits can result in a reduction in total body mass, particularly lean body mass (i.e., muscle) (Moore et al. 2014), and lead to physiological alterations including short stature, stunted growth, delayed bone age, increased risk of fractures and anemia, and increased susceptibility to infections (National Research Council 2005). Since exercise also increases energy requirements, active youth have the unique demand of ensuring adequate energy for both activity and growth.

In summary, children (particularly those who are at or approaching APHV) have distinct nutritional needs of dietary protein compared with adults (Rodriguez 2005) in order to meet the protein and energy requirements for the optimal deposition of lean body mass during growth and to support a physically active lifestyle. To date, however, the literature is sparse regarding the interactive effects of exercise and nutrition (particularly post-exercise protein consumption) in the pediatric population and, as such, the experiments in this thesis fill gaps in the current literature by investigating the acute protein needs (quantity and timing) of active children following a single bout of exercise.

#### **1.2. DIETARY REQUIREMENTS FOR PROTEIN AND AMINO ACIDS**

## 1.2.1. Protein Requirements in Growing Children

As noted in **Table 1.1**, children and youth require more protein than do adults on an average per body weight basis (Meyer et al. 2007). One limitation to the current protein recommendations for children and youth is that they are based on estimates that are assumed to be adequate for ensuring a positive N-balance (N<sub>BAL</sub>) in all children. For example, the Estimated Average Requirement (EAR) for children ages 1 to 13 years was estimated using the factorial method, which sums the maintenance protein requirements according to body weight (derived from individual estimates for children) to the estimated needs for protein deposition. For children older than 14 years, there was insufficient data available to determine the maintenance protein requirements. As it was argued that the N requirements for maintenance did not vary considerably between youth and adults, the EAR for protein in this age group was based instead on estimates from adults. In adults, protein requirements were established from a review of the existing scientific evidence (specifically the recent meta-analysis (Rand et al. 2003) of N<sub>BAL</sub> studies) with the criteria for adequate protein intake defined as the level of protein intake predicted to achieve a zero N<sub>BAL</sub> (National Research Council 2005). One limitation of the current protein requirements for children older than 14 years of age is the fact that in order to account for the additional amount of protein required for growth, the factorial model was used rather than direct measures (National Research Council 2005).

There are several other limitations to the current protein requirements for children and youth, particularly for those children at or approaching APHV. Estimates of the efficiency of N utilization are based on the adult literature (Rand et al. 2003) and, therefore, may not be accurate given the differences in protein metabolism that occur during growth (Beckett et al. 1997). Another limitation is that very few girls were included in the studies used to estimate requirements, resulting in protein requirements for boys and girls that are estimated to be the same despite the known sex differences in growth (Tanner 1955, Ellis et al. 1997, Ellis 1997). Caution must also be taken when considering the use of DRIs in youth since the recommendations represent the minimal required intake, rather than the level of intake necessary to optimize growth. Finally, as exercise increases the oxidation of amino acids, chiefly the BCAAs, it is unclear whether these recommendations can accurately be applied to active children.

Ages	Sex	EAR	RDA	RDA	AMDR
<b>(y)</b>		$(g \cdot kg^{-1} \cdot d^{-1})$	$(g \cdot d^{-1})$	$(g \cdot kg^{-1} \cdot d^{-1})$	(%)
1-3	Boys and Girls	0.87	13	1.05	5-20
4-8	Boys and Girls	0.76	19	0.95	10-30
9-13	Boys and Girls	0.76	34	0.95	10-30
14-18	Males	0.73	52	0.85	10-30
14-18	Females	0.71	46	0.85	10-30
19-70+	Males	0.66	56	0.8	10-35
19-70+	Females	0.66	46	0.8	10-35

Table 1.1. Dietary Reference Intakes (DRIs) for protein across various age groups and sexes.

Estimated Average Requirement, EAR; Recommended Dietary Allowance, RDA; Acceptable Macronutrient Distribution Range, AMDR. Although no upper limit for protein intake has been established due to limited or conflicting data, individuals should use caution when consuming protein quantities significantly above a typical food-based diet (National Research Council 2005).

## 1.2.2. Amino Acid Requirements

The EARs and Recommended Dietary Allowances (RDAs) for the individual essential amino acids in boys and girls aged 4-18 years are provided in **Table 1.2**. Unfortunately, there was limited empirical data available to determine the requirements for children and youth, with the bulk of available data coming from  $N_{BAL}$  studies published by Nakagawa and coworkers in the 1960s (Nakagawa et al. 1961a, 1961b, 1962, 1963, 1964). Due to the considerable uncertainty regarding the accuracy of these estimates (National Research Council 2005), the EAR for each of the essential amino acids in children and youth was instead determined using the factorial model. The factorial model accounts for estimates of growth at different ages, the amino acid composition of whole body protein, the efficiency of protein utilization (as derived from adult studies) and the estimated maintenance requirement (National Research Council 2005). The RDA for each essential amino acid was then determined using the variance for maintenance and for protein deposition derived from adults (National Research Council 2005).

Unfortunately, the current amino acid recommendations for children may be inadequate (Mager et al. 2003). In one of the few studies that has attempted to directly quantify the amino acid needs of children, Mager *et al.* (2003) used the indicator amino acid oxidation approach and reported that pre-pubertal children require 147 mg·kg<sup>-1</sup>·d<sup>-1</sup> of BCAA, a value 48% higher than the current recommendations (see **Table 1.2**). Thus, despite the establishment of the DRIs for essential amino acids, some uncertainty still remains with respect to their accuracy (Millward 2012).

 Table 1.2. The Estimated Average Requirement (EAR) and Recommended Dietary

 Allowance (RDA) for the individual essential amino acids in boys and girls ages 4-18

 years.

	4-8 y	9 -13 y		14-1	8 y
	<b>Boys and Girls</b>	Boys	Girls	Boys	Girls
Histidine	13 [16]	13 [17]	12 [15]	12 [15]	12 [14]
Isoleucine <sup>1</sup>	18 [22]	18 [22]	17 [21]	17 [21]	16 [19]
Leucine <sup>1</sup>	40 [49]	40 [49]	38 [47]	38 [47]	35 [44]
Lysine	37 [46]	37 [46]	35 [43]	35 [43]	32 [40]
Methionine + Cystein	18 [22]	18 [22]	17 [21]	17 [21]	16 [19]
Phenylalanine + Tyrosine	33 [41]	33 [41]	31 [38]	31 [38]	28 [35]
Threonine	19 [24]	19 [24]	18 [22]	18 [22]	17 [21]
Tryptophan	5 [6]	5 [6]	5 [6]	5 [6]	4 [5]
Valine <sup>1</sup>	23 [28]	23 [28]	22 [27]	22 [27]	20 [24]
<sup>1</sup> BCAAs	81 [99]	81 [99]	77 [95]	77 [95]	71 [87]

Values presented as EAR [RDA] in mg·kg<sup>-1</sup>·d<sup>-1</sup> according to the current Dietary Reference Intakes (National Research Council 2005). <sup>1</sup>Branched-chain amino acids (BCAAs).

### **1.3. REGULATION OF PROTEIN METABOLISM**

#### **1.3.1.** Protein Turnover

Protein within the body is in a constant state of turnover and is constantly being synthesized and degraded. One factor that can affect protein turnover is age; on a body-weight basis, the amount of protein turnover each day decreases from infancy to old age (National Research Council 2005). Protein turnover can also be affected by the complex interactions of various stimuli from a number of metabolic and physiologic conditions including substrate availability, hormonal signaling, and exercise (Millward et al. 2008). For example, in a resting adult, ~25-30% of body protein turnover occurs in skeletal muscle tissue (Reeds and Garlick 1984, Waterlow 1984); this value can increase to upwards of 45% after nutrient intake and exercise (Rennie and Tipton 2000). Unfortunately, however, limited information is available regarding the effects of these stimuli on protein turnover in the pediatric population. Thus, this thesis will investigate the combined effects of metabolic (protein) and physiologic (acute exercise) stimuli on protein turnover in healthy children and youth.

#### 1.3.2. Effects of Amino Acids on Protein Metabolism

The ingestion of dietary protein and/or amino acids can have a significant impact on the biochemical responses of protein metabolism. When a mixed protein-containing meal is consumed, protein synthesis is increased while protein breakdown is simultaneously reduced (Wagenmakers 1999). It appears that the change in protein synthesis is a result of the increased availability of plasma amino acids and an increase in plasma insulin. The change in protein breakdown, on the other hand, is more likely mediated by the increase in insulin alone (Rooyackers and Nair 1997, Wagenmakers 1999). Thus, the fed state results in net protein deposition (where the rate of protein synthesis > protein breakdown). On the other hand, when the body does not receive a source of dietary amino acids (i.e., after an overnight fast or between meals) the rate of protein breakdown exceeds that of protein synthesis. This catabolic environment is maintained until an adequate supply of dietary protein is consumed, and the cycle repeats resulting in a diurnal fluctuation in protein turnover.

Interestingly, increasing the level of dietary protein intake during the fed state may not have influences on protein balance over the course of a day. For instance, Pacy *et al.* (1994) found that when the level of protein intake increased, the amplitude of both protein deposition and protein breakdown increased. Despite these increases, the mean daily rate of protein turnover was not affected. Therefore, to manipulate daily rates of WBPB and obtain the anabolic environment required for growth in children, it is possible that additional stimuli (e.g. exercise) are needed in combination with protein feeding.

The composition of the dietary protein ingested can also influence the magnitude of the biochemical response, given that certain amino acids have the ability to regulate key metabolic pathways (Wu 2009). For example, decreased or insufficient intakes of essential amino acids can reduce the rate of protein synthesis and impair the use of other amino acids for protein synthesis. Therefore, if the diet contains an unbalanced proportion of essential amino acids, even if only one essential amino acid is in limited supply, or the overall protein intake is insufficient, a negative protein balance can occur (Pike and Brown 1984).

On the other hand, an increased concentration of essential amino acids in the blood can be particularly effective at stimulating MPS (Biolo et al. 1997, Volpi et al. 1998, 2000, Bohé et al. 2003, Paddon-Jones 2006). Specific amino acids, which have been referred to as functional or signal amino acids (see **APPENDIX I**), are also beneficial for stimulating the protein anabolic pathways and optimizing metabolism (Wu 2009). Leucine in particular is a potent regulator of metabolic activity (Layman 2003, Garlick 2005). High levels of leucine intake may have direct effects on muscle protein turnover (particularly, enhancing MPS) and tissue sensitivity, which is thought to occur through synergistic effects in signal transduction resulting from an increase in insulin release (Elia and Livesey 1983).

### **1.4. EXERCISE AND PROTEIN METABOLISM**

Regular exercise has a multitude of health benefits that stem from adaptations to both the cardiovascular system [e.g., an increase in vascular compliance, cardiac output, and capillary number as well as an expansion of plasma volume (Rivera-Brown and Frontera 2012) resulting in an enhanced transport of oxygen to the active muscle] and skeletal muscle tissue [e.g. increased expression and deposition of the myofibrillar proteins leading to muscle hypertrophy (Wagenmakers 1999, Tipton and Wolfe 2004)] in response to the exercise stimulus. These chronic changes likely arise from an accumulation of the acute responses to each exercise bout; however, these adaptive responses are not permanent and can be reversed in the absence of the stimulus (Wagenmakers 1999). For the purpose of this thesis, 'acute' exercise effects will refer to short-term effects occurring within minutes, hours or even up to a day (within 24 h) following the exercise period. 'Chronic' exercise effects (i.e., training) will refer to longer-term effects occurring over a period of days, weeks or even months.

The type of exercise performed (e.g. resistance- vs. endurance-based) has major implications for the alterations in protein metabolism that occur in response to an exercise stimulus. In general, it is less likely that young children will partake in high intensity resistance training programs, and the majority of active youth are less interested in obtaining the muscle hypertrophy characteristic of resistance exercise (Wagenmakers 1999, Tipton and Wolfe 2004). Instead, children tend to engage in free-play, or structured exercise programs that are more aerobic in nature [i.e., jump rope and dancing for girls, and team sports for boys (Faucette et al. 1995)]. Therefore, the findings from aerobic-based studies may be more applicable to the active child. For this reason, the following sections focus primarily on the effects of aerobic-based exercise with respect to protein metabolism. Given the infancy in our understanding of the effects of dietary protein ingestion following aerobic-based exercise, evidence from the resistance training literature will also be incorporated, where appropriate, to supplement the gaps in our knowledge.

## 1.4.1. Effects of Exercise on Protein Metabolism in Children

Only a handful of studies have directly examined protein metabolism and exercise in children (using the [<sup>15</sup>N]glycine method), and a number of the existing studies have focused on overweight or obese children (Ebbeling and Rodriguez 1999, Gillis et al. 2012). Since protein metabolism in response to exercise training may differ between normal weight and overweight children (Bolster et al. 2001, Pikosky et al. 2002), this further limits the literature available to understand the physiological response in healthy, non-obese children. Indeed, there are currently only 3 studies that have used the <sup>15</sup>N]glycine method to examine the effects of exercise on protein metabolism in nonobese children and, unfortunately, these studies present conflicting results. The first study was conducted by Bolster et al. (2001) and examined the effects of a 6-wk walking program in nonathletic 8- to 10-year-old children (both boys and girls). Participants were asked to walk 5 d·wk<sup>-1</sup> (~45-60 min) and encouraged to cover a distance ranging from 3.2 to 6.4 km during each session. Another study, conducted by Pikosky et al. (2002), investigated the effects of a 6-wk resistance exercise training program in 7- to 10-year-old boys and girls. Exercise sessions occurred semiweekly and consisted of both body weight supported and machine exercises. In both of these studies the authors found that protein synthesis and protein breakdown were significantly decreased, while WBPB was not affected. Both of these studies, however, are limited by the lack of a control group that did not exercise.

A study by Boisseau *et al.* (2005) examined the effects of gymnastic training (defined as "routines of short and intensive exercise involving mainly upper and lower

limb strains") on protein metabolism in 10 young (7-12 years) female gymnasts and included an age-matched control group (n = 10). The young gymnasts had trained an average of 8-10 h·wk<sup>-1</sup> over the previous year, while the controls engaged in less than 3  $h \cdot wk^{-1}$  of mild levels of exercise. No differences in protein flux, protein synthesis or protein breakdown were found between the two groups. Unlike the previously described studies (Bolster et al. 2001, Pikosky et al. 2002), the authors reported that the gymnast group demonstrated decreased net protein balance (in  $g \cdot d^{-1}$ ,  $g \cdot kg^{-1} \cdot d^{-1}$  and  $g \cdot kg^{-1}$  of  $FFM \cdot d^{-1}$ ) compared with the controls. There are, however, a number limitations and possible explanations for these discrepancies. For one, as Boisseau et al. (2005) reported that the protein intake  $(g \cdot d^{-1} \text{ and } g \cdot kg^{-1} \cdot d^{-1})$  in the gymnast group was less than that of the control group, the decreased net balance may have been a result of the lower protein intake rather than differences in protein oxidation or other mechanisms (i.e., insufficient energy intake in exercising gymnasts) related to regular exercise. Moreover, the time at which the protein measures were assessed differed between studies. The previously cited interventions (Bolster et al. 2001, Pikosky et al. 2002) assessed children during the course of a 10-h overnight fast, whereas Boisseau et al. (2005) examined the girls during both the post-absorptive and post-prandial states over the course of a day. This led Boisseau *et* al. (2005) to conclude that protein metabolism may be more related to dietary aspects (e.g. energy and protein consumption) than to the level of exercise and training.

Another limitation that cannot be overlooked is the fact that during the training programs (Bolster et al. 2001, Pikosky et al. 2002), energy intake was not increased despite increases in activity-related energy expenditure. This may have significant
implications given the relationship between energy and protein metabolism. In an attempt to meet an individual's energy requirements, the body can interchange energy sources from macronutrients; thus, the availability of protein to serve as a substrate for protein synthesis is affected by its need to provide energy to the body. When an individual consumes adequate energy to be in a state of energy balance, the dietary proteins that are ingested can be used for protein-specific anabolic processes (e.g., repairing muscles damaged by exercise or increasing lean mass). On the other hand, when insufficient dietary energy is consumed, there are alterations in both protein and N metabolism and, subsequently, increased requirements (Calloway 1975). For example, when the body is faced with a reduction in available energy, the process of protein breakdown exceeds that of protein synthesis. Thus, in addition to ensuring an adequate consumption of protein, enough non-protein energy (i.e., CHOs and fat) must also be consumed. Given that exercise is known to further alter the complex interactions between energy requirements and protein metabolism, it is unknown whether the protein-energy balance affected the modulations in protein metabolism during the training programs.

Due to the paucity of information in this area of pediatric research and the number of limitations in the available studies, there remains a large gap in our knowledge regarding the effects of exercise on protein metabolism for non-obese children and youth. Given the importance for children lead an active lifestyle while attaining a positive WBPB for proper development, there is a need to better understand this relationship in order to formulate appropriate recommendations for protein for active youth. As each of the aforementioned pediatric studies have examined the effects of chronic exercise training/intervention, there is currently no evidence to inform the effects of a specific bout of exercise on subsequent protein metabolism. Moreover, as the existing literature has focused on children prior to their pubertal growth spurt (7-10 years of age) or non-athletic children undergoing an exercise intervention, the experiments outlined in this thesis are aimed to fill the gaps in our knowledge by examining the relationship between a specific bout of exercise and subsequent protein metabolism in healthy, active youth at different stages of growth.

#### **1.5. NUTRITION, EXERICSE AND PROTEIN METABOLISM**

The human body can incur significant metabolic demands as a result of an acute bout of exercise. As a result, athletes have commonly used nutrition to focus on 3 interrelated approaches to recovery: refueling, rehydration, and repair (Moore et al. 2014). Many athletes already appreciate the need to replenish fuel stores and rehydrate after training (Burke et al. 2004, American College of Sports Medicine et al. 2007); however, repairing and regenerating damaged proteins and reconditioning skeletal muscle are also important determinants of post-exercise recovery, and are aspects that are often neglected by athletes involved in aerobic- or endurance-based sports (Moore et al. 2014).

When an individual is resting and fasted, the rate of MPB exceeds that of MPS and results in a negative net muscle protein balance (Biolo et al. 1995b, Phillips et al. 1997, Pitkanen et al. 2003). In adults, exercise alters muscle protein metabolism by stimulating both MPS and MPB during acute post-exercise recovery (Biolo et al. 1995b,

1999, Phillips et al. 1997, Pitkanen et al. 2003, Kumar et al. 2009). However, in order to achieve a net gain in muscle mass with exercise, MPS must be chronically elevated above MPB breakdown. Despite exercise increasing MPS more than MPB, which creates a less negative net balance, post-exercise protein provision is necessary in order to obtain a positive protein balance following exercise (**Figure 1.1**) (Biolo et al. 1995b, Phillips et al. 1997, Pitkanen et al. 2003). Given the need to attain a net accrual of lean body mass, this fact is particularly important to a growing child.

The synergies between exercise and nutrition in enhancing protein remodeling and protein accretion has not been as well studied in children as it has in adults (Betts and Williams 2010, Hawley et al. 2011). As such, data from adults is extrapolated in order to make recommendations for post-exercise nutrition for active children. Such extrapolations are inappropriate as children have distinct nutritional needs from adults (Meyer et al. 2007), have different physiological adaptations to exercise (Timmons et al. 2003), and the interaction between exercise and nutrition may also be different in the pediatric population. Therefore, the experiments in this thesis have been designed to examine the effects of post-exercise nutrient intake on subsequent measures of whole body protein metabolism in healthy, active children.

## 1.5.1. Dietary Carbohydrates

During exercise the predominant sources of fuel for the body are CHO-based (i.e., muscle and liver glycogen, blood glucose, and blood, muscle, and liver lactate) (Romijn et al. 1993, van Loon et al. 2001). As a result, much research to date has focused on

nutritional strategies to optimize early post-exercise muscle glycogen repletion, and the importance of ingesting CHOs in the early post-exercise recovery period is well established (van Loon et al. 2000b, Jentjens and Jeukendrup 2003, Burke et al. 2004, 2006). In addition to replenishing muscle glycogen, a number of studies have found that the post-exercise consumption of CHOs also affects skeletal muscle protein balance. The positive effects of CHO consumption on net protein balance are likely related to the insulin-induced decrease in protein breakdown, and not changes in protein synthesis. For example, post-exercise CHO consumption inhibits the increase in MPB normally seen following exercise (Miller et al. 2003, Børsheim et al. 2004), which creates a less negative net muscle protein balance (Figure 1.1) (Børsheim et al. 2004). This attenuation in MPB results from the associated rise in plasma insulin concentrations characteristic of CHO administration (Denne et al. 1991, Fryburg et al. 1995, Biolo et al. 1999). Although studies have demonstrated a stimulation of MPS from elevated plasma insulin (Biolo et al. 1995a, Hillier et al. 1998, Fujita et al. 2006), this only occurs with hyperaminoacidemia (Fujita et al. 2006). Moreover, other studies found that a modest increase in insulin did not affect MPS, but rather had permissive qualities (Gelfand and Barrett 1987, Denne et al. 1991, Koopman et al. 2007b, Greenhaff et al. 2008),



**Figure 1.1. Muscle protein metabolism in response to nutrition and exercise.** Responses of muscle protein synthesis (S), muscle protein breakdown (B), and net muscle protein balance (N) at rest in the fasted state, following exercise in the fasted state, following exercise with carbohydrate (CHO) consumption, and following exercise with protein (PRO) supplementation. This figure has been recreated with permission from Beelen, M., Burke, L. M., Gibala, M. J., and van Loon L. J. C. (2010). Nutritional strategies to promote postexercise recovery. *Int J Sport Nutr Exerc Metab* 20(6), 515-532. (License # 3699400694989).

# **1.5.2. Dietary Protein**

Despite other nutrient sources (e.g., CHO) having positive effects on WBPB following exercise, net protein balance will remain negative unless a dietary source of amino acids is consumed (**Figure 1.1**) (Biolo et al. 1995b, Phillips et al. 1997). There is an abundance of adult research that supports the importance of consuming adequate dietary amino acids and/or protein during recovery from resistance-based exercise (Churchward-Venne et al. 2012). Specifically, there exists a dose-dependent relationship between protein intake and rates of post-exercise MPS (Moore et al. 2009); as little as 5-10 g of protein is sufficient to increase MPS and WBPB, with the response becoming saturated at doses of ~20-25 g of intact protein (or ~9 g of essential amino acids) (Moore et al. 2009, Witard et al. 2014).

Amino acid and/or protein ingestion also increases the rates of MPS after endurance-type exercise in adults (Levenhagen et al. 2002, Gibala 2007, Howarth et al. 2009, Breen et al. 2011, Lunn et al. 2012) (see **Appendix II**). Similarly, the enhanced rates of MPS after endurance exercise occur with as little as 10 g of protein (Levenhagen et al. 2002). To the author's knowledge, however, there have been no studies that have investigated the dose-response characteristics of protein ingestion following endurance exercise. Nonetheless, observations from acute exercise studies have led to the generally accepted notion that incorporating protein into a post-exercise recovery regime following endurance exercise is a practical approach to enhance recovery (Berardi et al. 2006, 2008, Betts et al. 2007, Rowlands et al. 2007, Thomson et al. 2011, Ferguson-Stegall et al. 2011b, Breen et al. 2011, Lunn et al. 2012). When considering post-exercise protein intake, the consumption of nonessential amino acids might not be required to elicit the protein-anabolic response in the immediate post-exercise period (Tipton et al. 1999a). Similar to the response seen with complete protein ingestion, the ingestion of essential amino acids exhibit a positive dose-dependent effect on MPS (Børsheim et al. 2002). Moreover, the adult literature implicates the role of BCAAs (leucine, in particular) in enhancing rates of MPS (Blomstrand et al. 2006) and, when an increase in blood insulin concentration is present, attenuating rates of MPB (Biolo et al. 1997, 1999, Tipton et al. 2003, Børsheim et al. 2004).

One factor that influences the utilization of amino acids in response to a bout of exercise is a low availability of muscle glycogen (Lemon and Mullin 1980, Howarth et al. 2010); when exercise is performed in a glycogen-depleted state, the degradation and utilization of specific amino acids as metabolic fuel increases (Wagenmakers 1998). Since the amino acids that become oxidized are unable to contribute to the protein synthetic response during recovery (Lemon 1996), additional dietary protein is required to replace those amino acids lost from the body. Thus, a sufficient intake of both CHO, to restore muscle glycogen (Burke et al. 2004, 2006), and dietary protein or amino acids (Rennie and Tipton 2000, Tipton and Wolfe 2004, Hawley et al. 2006, Koopman et al. 2007b), to produce a positive protein balance, are required in the post-exercise period.

#### 1.5.3. Combining Carbohydrates and Protein

It is now recognized that combined CHO-protein ingestion in the post-exercise period can have significant benefits for athletes, as the co-ingestion of CHO and protein stimulates the rate of amino acid uptake following exercise (Levenhagen et al. 2001, Miller et al. 2003, Goforth et al. 2003, Howarth et al. 2009). Moreover, when protein, protein hydrolysates and/or free amino acids are ingested together with CHOs, postprandial insulin secretions are increased above the levels that are observed when CHO is consumed alone (Rabinowitz et al. 1966, Pallotta and Kennedy 1968, Nuttall et al. 1984, van Loon et al. 2000c, 2000a, Kaastra et al. 2006). Moreover, adding additional free leucine has shown to further increase endogenous insulin release above levels seen with CHO alone (Kaastra et al. 2006).

The strong insulinotropic response elicited by the combined ingestion of CHO and protein/amino acids following exercise (van Loon et al. 2000c, 2000a) can have a stimulating effect on net protein balance (Beelen et al. 2010). However, CHO-protein co-ingestion during the early post-exercise period may not have further stimulating effects on MPS when sufficient protein is consumed, despite further increases in plasma insulin (Koopman et al. 2007a, Greenhaff et al. 2008). Rather, the resultant increases in plasma insulin concentrations might be effective at attenuating MPB (Gelfand and Barrett 1987, Biolo et al. 1999, Greenhaff et al. 2008), which would ultimately improve post-exercise net muscle protein balance and stimulate muscle protein accretion. Therefore, although the consumption of CHOs and protein were originally considered mutually exclusive post-exercise nutritional strategies, it is now generally accepted that optimal post-exercise nutrition should contain a combination of the two. Nevertheless, considerable debate still remains regarding the optimal quantity, type, and timing of ingestion of these nutrients and their subsequent impact on post-exercise protein metabolism (Burd et al. 2009,

Beelen et al. 2010, Moore et al. 2014). Both type and timing of protein ingestion are discussed in Chapter 1.8.

Despite the important role of exercise in the remodeling of lean tissue and enhancement of lean body mass, as well as the known importance of dietary proteins to enhance the protein anabolic response to exercise, there is currently little knowledge pertaining to the relationship between exercise and nutrient intake (e.g., protein or protein-CHO co-ingestion) in children, notwithstanding the abundance of data in the adult populations. Given the significance of dietary protein and protein-CHO ingestion after exercise in adults, it is important to investigate how protein metabolism is similarly affected by nutrition after exercise in children. As a result, this thesis will aim to generate a preliminary understanding of the effects of consuming whole-foods containing protein, and CHO beverages with varying levels of protein on changes in protein metabolism in the post-exercise period. It is the hope that this information will help in the development of pediatric-specific nutritional recommendations that aim to maximize potential positive effects of exercise for this population.

# 1.6. RECOMMENDED PROTEIN INTAKE FOR ACTIVE CHILDREN AND YOUTH

Despite the importance of protein for active children and youth, the current nutritional recommendations for this population are derived from data used to establish the recommendations for adults. Adult athletes who engage in aerobic endurance exercise were estimated to require 1.67 times more protein than sedentary controls (Tarnopolsky et al. 1988), while intakes in elite endurance athletes can reach values nearly twice as high (1.6-1.7 g·kg<sup>-1</sup>·day<sup>-1</sup> (Burke et al. 2007)) as their sedentary counterparts. Even individuals who are only modestly trained in endurance sports appear to require protein intakes that are higher (1.2-1.4 g·kg<sup>-1</sup>·d<sup>-1</sup>) than the general population (Gaine et al. 2006). Due to the paucity of pediatric-specific data, there remains much controversy as to whether (or to what extent) these adult recommendations should be used for active younger persons (Meyer et al. 2007). Additionally, there are no specific guidelines for children or youth that engage in high levels of exercise (at least 50% higher than the general population) on a consistent basis. To further complicate the issue, there are a number of factors that can affect nutritional needs, including: age, sex, body mass and composition, habitual exercise, health status, sport modality, level of athletics, volume of training, training intensity, duration and timing of the exercise session, overall energy intake and CHO availability.

Currently, the limited data examining the relationship between protein intakes and exercise in youth largely consists of investigations that determined whether or not active youth consume enough protein on a theoretical basis by comparing their actual protein intake to the current recommendations. In a review of the literature, Petrie *et al.* (2004) found that young athletes reported daily protein intakes that ranged between 0.96 g·kg<sup>-1</sup>·day<sup>-1</sup> in wrestlers (Horswill et al. 1990) and 2.32 g·kg<sup>-1</sup>·day<sup>-1</sup> in swimmers (Spodaryk 2002), with protein intake in the majority of studies ranging between 1.0-2.0 g·kg<sup>-1</sup>·day<sup>-1</sup> (Spodaryk 2002, Papadopoulou et al. 2002, Petrie et al. 2004, Boisseau et al. 2007, Aerenhouts et al. 2008, Kabasakalis et al. 2009, Aerenhouts et al. 2013). The highly heterogeneous results of these studies is likely due to the inclusion of a variety of sport modalities, ages, and sexes. Nonetheless, it appears as though active children and youth across a variety of sport modalities are ingesting adequate, or even excess, protein compared to what is recommended in the current DRIs (Delistraty et al. 1992, Ziegler et al. 1998).

Caution, however, must be taken when interpreting these results. One important consideration is the difference between meeting recommendations, and having an adequate nutritional status. A good example of this comes from a study of adolescent wrestlers. Although these youths (14-17 years) reported having sufficient protein intakes based on the RDA, their protein status (as measured by the ratio of total essential amino acids to total amino acids and dietary energy-containing nutrient intakes) decreased as their season progressed (Horswill et al. 1990). This may have been a consequence of the chronic effects of weight loss in order to "make weight" (Horswill et al. 1990). As a result, these youths may be at risk for a reduction of fat-free mass despite their apparently adequate protein intake.

Emerging evidence supports the thesis that the current DRIs for protein for youth may underestimate the true needs for this population. For example, Boisseau *et al.* (2007) proposed that for 14-year-old, highly trained male soccer players, an RDA of 1.4 g·kg<sup>-1</sup>·d<sup>-1</sup> (taking into account the protein needed for accretion with growth) would be more appropriate than the current recommendations. Similarly, one of the only studies to investigate protein requirements (as opposed to protein intake) in active youth –

conducted in Flemish adolescent sprint athletes - reported that a protein intake of 1.46  $g \cdot kg^{-1} \cdot day^{-1}$  in girls and 1.35  $g \cdot kg^{-1} \cdot day^{-1}$  in boys was required to achieve positive N<sub>BAL</sub>, even during periods of peak growth (Aerenhouts et al. 2013). These values are considerably higher than the guidelines for their non-active peers, which suggests that the traditional RDA for protein may be insufficient to meet the protein requirements of these active individuals (Boisseau et al. 2007). These findings also shed light on possible differences in daily protein needs between active boys and girls. It has, however, been suggested that the majority of active children and youth likely already consume protein intakes above the minimal recommended levels based on diet alone (without additional supplementation) (Tarnopolsky 2004) when a regular energy-balanced diet with 10-35% of total calories coming from protein is consumed (American Dietetic Association et al. 2009). Therefore, it is possible that additional factors including the type of protein consumed and the timing of intake are also critical to consider when aiming to maximizing recovery from, and adaptation to, exercise (see Section 1.8) (Tarnopolsky 2004). For this reason, in addition to determining the quantity of protein required in the post-exercise period, the experiments in this thesis aimed to investigate additional factors including the timing of protein intake around exercise in order to gain a more thorough understanding of protein requirements following exercise in healthy, active youth.

# 1.7. ADDITIONAL CONSIDERATIONS FOR PROTEIN RECOMMENDATIONS RELEVANT TO THIS THESIS

There are a number of other key factors to consider when targeting optimal postexercise recovery. Some of these factors include an individual's habitual protein intake, the mode of protein administration, the type of protein consumed (accounting for variations in protein quality and digestibility), and the timing of protein intake. The following section addresses some additional factors affecting post-exercise protein metabolism that are pertinent to this thesis.

## 1.7.1. Habitual Dietary Protein Intake

Habitual dietary protein intake can influence the protein synthesis response following exercise. For example, individuals with chronically high dietary protein intakes (i.e.,  $> 1.8 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ) have a decreased rate of mixed MPS in the fasted state following exercise compared with those who consume more modest protein diets (Bolster et al. 2005). It is possible that the post-exercise changes in protein synthesis demonstrated in the fasted state may be the result of an up-regulation of amino acid oxidative capacity in response to feeding which, in turn, could result in a decreased availability of intracellular amino acids that are required to repair and remodel muscle tissue without an exogenous dietary source (Bolster et al. 2005, Gaine et al. 2007). Therefore, habitual dietary protein intake was taken into account when investigating the response of post-exercise protein intake on protein metabolism in the current thesis.

# 1.7.2. Mode of Protein Administration

After consuming a meal, the levels of amino acids in circulation differ depending on the pattern of administration of a dietary protein and the type of protein administered. Independent of the amino acid profile, consuming equivalent amounts of protein as a single meal vs. multiple small repeated boluses can exert different effects on protein metabolism (West et al. 2011). When a single protein meal is consumed, the appearance of amino acids in the plasma is fast and short-lived. This often results in an increase in whole body protein synthesis and amino acid oxidation, and no change in whole body protein breakdown (Boirie et al. 1997). In contrast, when a pulse feeding is used (where an equivalent amount of the same protein is consumed in a continuous manner), the effects mimic a slower absorption: a slower, lower and prolonged appearance of amino acids. This, in turn, results in a slight increase in whole body protein synthesis and oxidation, and a marked inhibition of whole body protein breakdown (Boirie et al. 1997).

Other considerations that may affect optimal protein feeding are the form in which the protein is administered, as well as other characteristics of the meal (e.g., whether one consumes whole foods or isolates). As it is more common for a child to consume whole foods rather than protein supplements following a bout of exercise, it is relevant to the context of this thesis to consider how the protein from whole foods affect the protein responses of the body. Regardless of nutritional composition, when a food protein in whole foods is administered in liquid form (e.g., soy beverage, skim milk) it is digested more rapidly than those in solid food matrices (e.g., egg, steak, and protein bar) (Burke et al. 2012). This rapid digestion can help to achieve peak concentrations of amino acids twice as quickly after ingestion, which is likely due to the greater stimulatory effect of the earlier and sustained aminoacidemia (Burke et al. 2012). Though it is difficult to assess the individual and interactive contributions of different amino acid compositions in protein-rich foods, as well as the digestibility of proteins or protein-rich meals with the pattern of delivery of these amino acids (Burke et al. 2012), it is likely that athletes who wish to enhance protein metabolism during the early recovery process could meet these goals with the ingestion of rapidly digested proteins sources (Breen et al. 2011). As studies directly comparing different protein sources during the recovery from endurance exercise are currently lacking, the studies in this thesis supplied the post-exercise whole-food protein in a liquid form.

#### 1.7.3. Dietary Protein Quality

Another important factor to consider is the quality of the dietary protein consumed. Protein quality is a measure of the absorption and utilization of a protein, and takes into consideration the digestibility and amino acid concentration of the protein source in question (Darragh and Hodgkinson 2000, Schaafsma 2005, Boye et al. 2012, Millward 2012). A "Protein Digestibility Corrected Amino Acid Score" (PDCAAS) was developed to evaluate the quality of a source of dietary protein (National Research Council 2005). A "high quality" protein (i.e., PDCAAS > 1) is considered to be a source of protein that is well balanced in its amino acid content in relation to human needs (Schaafsma 2000). When considering protein quality in nutritional studies such as those included in this thesis, it is often recommended that high-quality proteins (e.g., the milk

proteins casein and whey) be used, so that total N is considered the limiting dietary component rather than a negative  $N_{BAL}$  occurring due to an insufficient intake of a specific essential amino acid (National Research Council 2005).

Protein quality can vary considerably across a range of dietary proteins (Boirie et al. 1997, Pennings et al. 2011) and can result in variations in plasma amino acid responses similar to those observed following different modes of protein administration (Burke et al. 2012). Even subtle difference in protein quality can have measureable effects on the enhancement of post-exercise MPS (Burd et al. 2009). As such, protein quality has a major influence on the type of protein that is recommended following exercise; however, much debate remains regarding which protein or amino acid source is most effective in promoting the protein anabolic response in the post-exercise period.

Milk and associated dairy-based proteins (whey, in particular) are generally associated with greater muscle remodeling and recovery after exercise in adults (Phillips et al. 2009), by supporting greater rates of MPS and net protein balance after exercise compared with plant-based proteins (i.e., soy) (Wilkinson et al. 2007, Tang et al. 2009). What is interesting is that the greater anabolic effect seen following dairy consumption (compared with soy) in adults occurs notwithstanding the fact that these protein sources both have high [albeit artificially truncated (Schaafsma 2000)] protein quality ratings according to the PDCAAS (Phillips et al. 2009). The beneficial effects of milk compared with soy may be a reflection of differences in the make-up of amino acids that are delivered to peripheral tissues (Phillips et al. 2005). It remains unknown, however, whether these differences are a consequence of different amino acid profiles of the two protein sources, different rates of digestion, or differences in amino acid oxidation and deamination (greater for soy than milk) resulting from variations in amino acid flow through the splanchnic bed following consumption (Millward et al. 2008).

The individual protein constituents of milk also have distinct effects on the protein anabolic response. Bovine milk contains the protein sub-fractions casein and whey in a ~4:1 ratio. Differences in anabolic properties between these two proteins is largely attributed to variations in digestion and absorption kinetics (Boirie et al. 1997, Dangin et al. 2001a). Whey protein, the liquid portion of coagulated milk, has independently received much attention given its high biological value and solubility (in addition to its influence on the immune system and its antioxidant properties). Intact casein, on the other hand, is located within the semisolid portion of the milk and clots in the stomach, resulting in a delayed rate of gastric emptying of the solution (Koopman et al. 2009). Due to differences in solubility between whey and casein, the pattern of the amino acid response in the bloodstream also differs; amino acids from whey enter the bloodstream more quickly, whereas the amino acid response of casein is more sustained (Koopman et al. 2009).

Whey protein (compared with casein and soy protein) results in a more effective stimulation of MPS in the postprandial period (Pennings et al. 2011). This response has been found both at rest and after resistance exercise (Tang et al. 2009). Given that a protein's content of essential amino acids is the primary regulator of MPS (Tipton et al. 1999b, 1999a) and the fact that leucine has the ability to provide substrates for protein synthesis and activate the cellular signaling pathways for protein synthesis (Drummond and Rasmussen 2008, Phillips and Van Loon 2011), it is possible that the higher leucine content in whey protein is responsible for the observed increase in MPS (Tang and Phillips 2009). There may, however, be additional factors that are responsible for the observed response as Koopman *et al.* (2005) found that when leucine was added to a protein hydrolysate, post-exercise MPS rates did not increase; rather, protein oxidation decreased and WBPB was enhanced. Regardless of the mechanism(s) responsible, milk proteins (and their isolated forms) have the ability to provide an anabolic advantage compared with other protein sources (Beelen et al. 2010). Moreover, the combination of proteins in milk can have beneficial effects; whey protein can induce a rapid aminoacidemia to allow for the acute stimulation of MPS (Pennings et al. 2011), while casein has a more transient aminoacidemia providing a continual supply of amino acids over a prolonged period of time.

In short, milk and milk-based proteins possess many characteristics that, in theory, make them suitable to enhance the protein anabolic response following exercise. In addition to the well-known health benefits (Huth et al. 2006), as well as the positive effects on rehydration (Volterman et al. 2014), there are a number of reasons milk was chosen as the whole-food post-exercise beverage for this thesis: 1) due to its nutritional value, milk can provide the body with CHOs to replenish muscle glycogen, and amino acids to promote protein synthesis, following exercise (Lunn et al. 2012); 2) milk is considered a "high-quality" protein (i.e., PDCAAS > 1) and, therefore, provides all of the essential amino acids in relation to human needs; 3) milk exists in a liquid form, which

allows for more rapid digestion compared with solid foods (Burke et al. 2012); and 4) it is easily accessible and can be commonly found in the home (Shirreffs et al. 2007).

#### 1.7.4. Timing of Protein Intake

The timing of protein intake in relation to the time of exercise or competition is another important consideration in sports nutrition (Rasmussen et al. 2000, Levenhagen et al. 2001, Hartman et al. 2007). The timing of dietary intake and nutrient delivery not only regulates post-exercise protein hemostasis, but can also affect the capacity for peak performance, training efficiency, the magnitude of adaptations to the same training stimulus, and the optimal recovery from exercise (Cotugna, et al. 2005, van Loon 2014). Despite the growing interest in the timing of nutrient delivery, considerable debate still remains regarding the optimal timing of protein ingestion needed to maximize the anabolic effects in response to exercise. For the purpose of this thesis, a primary focus will be placed on protein intake in the post-exercise period.

Dietary protein ingestion following endurance exercise has similar results as those from the resistance exercise literature (Burd et al. 2009) – an increase in post-exercise MPS. Following a bout of resistance exercise, the sensitivity of skeletal muscle to an exogenous source of amino acids is relatively prolonged (i.e., > 24 h) (Burd et al. 2011). In contrast, the period immediately following exercise cessation may be more important in enhancing protein metabolism when a bout of endurance exercise is performed. For example, Levenhagen *et al.* (2001) reported that following 60 min of moderate-intensity exercise (60% VO<sub>2max</sub>), the anabolic effect of dietary protein on both net whole body and muscle protein homeostasis were enhanced when a nutritional supplement (10 g protein, 8 g CHO, 3 g fat) was consumed immediately after cessation of exercise as opposed to delaying the ingestion by 3 h. As a result, protein intake immediately following exercise may become increasingly important for athletes who have minimal rest time between training and/or competition sessions – a common scenario experienced by children due to the nature of youth sports (i.e., tournament play) (Beelen et al. 2010).

While there is a need for further information regarding the optimal timing of postexercise protein ingestion, the current evidence that protein synthesis is attenuated when feeding is delayed by several hours following a 60 min bout of moderate intensity cycling (Levenhagen et al. 2001) suggests that dietary protein should be consumed in the first 30-60 min following aerobic-based exercise in order to maximize the effects on protein accrual (Moore et al. 2014). Although it is possible that a similar response may be seen in children, there is currently no data available on the importance of post-exercise protein ingestion or the optimal timing (i.e. immediately post-exercise) of ingestion in children (Meyer et al. 2007). Given the distinct nutritional needs of children, and the added complexity of the relationship between growth and protein metabolism, it is unclear whether these recommendations can be appropriately extrapolated to the pediatric population. Therefore, this thesis will aim to obtain a more thorough understanding of the interaction between exercise and the timing of post-exercise protein consumption on WBPB in active children.

# **CHAPTER 2 : Objectives and Hypotheses**

The paucity of research on the protein needs of children has made it impossible to derive evidence-based nutritional recommendations for physically active youth. The aim of this thesis was to advance our knowledge of the acute protein needs of active children by examining protein intake and changes in whole body protein turnover following a single bout of aerobic exercise, while highlighting the importance of studying postexercise protein quantity and timing relative to exercise. This information is critical for providing nutritional advice to active children and allowing for the development of childspecific recommendations in order to ensure proper recovery from exercise is obtained and the growth of muscle and lean tissue is optimized. Combined, the studies included in this thesis addressed some of the gaps in the literature by answering the following questions:

#### **Objective #1:**

- A) Can a whole-food protein-containing beverage (i.e., milk) impact WBPB in healthy children following exercise?
- B) Are there effects of puberty or sex on milk's ability to maintain WPBPB?

Hypothesis #1: It was hypothesized that:

A) When compared with a typical protein-free post-exercise beverage, milk would more effectively maintain a positive WBPB following exercise in healthy children. B) Both puberty and sex would have an impact on the regulation of whole body protein metabolism.

**Objective #2:** Does the quantity of post-exercise protein intake acutely affect WBPB following exercise in healthy children?

**Hypothesis #2:** It was hypothesized that when compared with a protein-free beverage, protein intake would be more effective at enhancing WBPB, with a high-protein intake being the most effective.

**Objective #3:** To what extent does protein intake alter whole body amino acid (particularly leucine) oxidation and utilization in response to exercise in healthy children?

**Hypothesis #3:** It was hypothesized that a graded dosing of protein intake in children would result in altered leucine kinetics reflecting greater whole body leucine balance in a dose-dependent manner.

**Objective #4:** How does the timing of protein intake (immediate vs. delayed) after exercise affect WBPB?

**Hypothesis #4:** It was hypothesized that protein consumption during the acute recovery phase (within 3 h of exercise) would increase WBPB compared with a delayed ingestion.

# **CHAPTER 3 : Methodological Considerations**

#### **3.1. TECHNIQUES TO MEASURE PROTEIN METABOLISM IN CHILDREN**

A number of minimally invasive techniques exist that can be safely and ethically applied to estimate protein metabolism in children. Although these methodologies provide valuable information on whole body protein metabolism, a reflection of what is happening to thousands of protein molecules across a variety of different tissues (Darmaun and Mauras 2005), they provide little information regarding the contribution of specific tissues and proteins to the whole-body changes observed (e.g., whether the increase in protein synthesis was a result of the muscle or splanchnic tissues) which might otherwise be determined in more invasive procedures such as those with muscle biopsies (Wagenmakers 1999). Considering ethical constraints in pediatric research, it is not possible to perform stable isotope methods with concurrent muscle biopsies. It could be argued that since the entire body of children is growing, it may be more important to know what is happening in lean tissue mass throughout the body (which would dominate amino acid metabolism) rather than what is happening in isolated skeletal muscles. Regardless of the method used, there still remains a large gap in our knowledge regarding the metabolic adaptions to nutrition and exercise in children, as well as the mechanisms involved, at both the whole body level and that of individual tissues and proteins.

The studies in this thesis were aimed at studying protein metabolism by two primary methods: (1)  $N_{BAL}$ , which provides a static measure of protein balance, and (2) stable isotopes techniques (i.e., oral [<sup>15</sup>N]glycine and intravenous (IV) [1-<sup>13</sup>C]leucine

infusion), which provide kinetic measures of protein metabolism. Each of these techniques has its own benefits and shortfalls (discussed in further detail below).

#### **3.1.1.** Nitrogen Balance

Classically, this method has been viewed as theoretically the most suitable tool to determine protein requirements (National Research Council 2005). As previously mentioned, the current DRIs for protein intake in healthy individuals are based on a careful review of the recent meta-analysis (Rand et al. 2003) of  $N_{BAL}$  studies, as this is the only assessment technique to date that has produced enough data to make reasonable estimates of total protein (N) requirements (National Research Council 2005). One major benefit of the  $N_{BAL}$  technique is its non-invasive nature and its ability to be administered under free-living conditions.

 $N_{BAL}$  is calculated as the difference between N intake ( $N_{IN}$ ) and N losses ( $N_{EX}$ ). As the only N entering the body comes from dietary protein,  $N_{IN}$  can be easily estimated by assessing the total intake of dietary protein, assuming 16% of which is made up of N (Imura and Okada 1998).  $N_{EX}$ , on the other hand, is calculated as the amount of N excreted in urine, feces, skin and other miscellaneous sources (i.e., sweat, hair, nails, saliva and breath) (National Research Council 2005).

Urea serves as the major end-product for the degradation and disposal of amino acid N and is the dominant form (> 80%) of urinary  $N_{EX}$ . Thus, the amount of urinary urea excreted from the body is an indicator of the amount of N being excreted from the body and, as such, an indicator of protein metabolism. For example, if deamination were increased (which would indicate an overconsumption of dietary protein and/or an increase in protein breakdown), more NH<sub>3</sub> would be produced as part of the urea cycle, which would then increase the amount of urea in the urine. Other N-containing constituents of urine include NH<sub>3</sub>, creatinine, amino acids and, to a much lesser extent, uric acid (National Research Council 2005).

As  $N_{EX}$  is primarily (> 84%) a result of urinary metabolites (i.e., urea, creatinine, and ammonia) (Tomé and Bos 2000), the concentration of urinary metabolites in this thesis were measured directly using specific assay methods (e.g., spectrophotometry). A small amount of N-containing metabolites can also be excreted in the feces and through the integumentary system and, therefore, to acquire a comprehensive indication of N excretion these routes of egress must also be considered (National Research Council 2005). Since fecal and miscellaneous sources of  $N_{EX}$  are often small compared with urinary metabolites, and pose technical and logistical challenges in their determination, these measures were estimated according to previously published values (Gattas et al. 1990), rather than measured directly.

Another means by which N can be lost is through the sweat. Although sweat  $N_{EX}$  contributes minimally to total  $N_{EX}$  at resting conditions, it can contribute up to ~10% of N loss in active adults (Tarnopolsky et al. 1992). As the studies in **Chapter 4** and **Chapter 5** of this thesis had the exercise performed in a warm environment (~34°C, 47% relative humidity), it was decided that sweat  $N_{EX}$  should be considered. Therefore, estimates of sweat  $N_{EX}$  using the change in body mass as a result of exercise in combination with the average sweat nitrogen concentration (Alexiou et al. 1979) were used in the calculation.

Despite the widespread use of the N<sub>BAL</sub> methodology, there are various practical limitations and problems to the determination of N<sub>BAL</sub>. The first limitation is that in order to attain a proper steady state for N<sub>EX</sub> and to allow for sufficient turnover of urea, individuals should be adapted for several days to each new level of dietary protein intake being investigated (Rand et al. 1976, Meakins and Jackson 1996). Second, N<sub>BAL</sub> often overestimates  $N_{\text{IN}}$  and underestimates  $N_{\text{EX}}$  (Tomé and Bos 2000) resulting in a consistent overestimation of N<sub>BAL</sub> reported in the literature (Rand et al. 2003). A third limitation of this technique is that an individual should be studied at various levels of protein consumption (i.e., spanning deficiency to excess) in order to interpolate estimates of individual requirements, as the exact amount of protein required to produce zero N<sub>BAL</sub> varies between individual (Rand et al. 1976). Finally, the N<sub>BAL</sub> technique has been criticized with regards to the linear modeling used to estimate protein requirements, due to the decreased efficiency of protein utilization approaching zero balance (Humayun et al. 2007). Notwithstanding these limitations,  $N_{BAL}$  is still used today as an indicator of an individual's nutritional state and to establish requirements for protein. Although this technique provides useful information regarding differences in net N utilization in response to variations in N<sub>IN</sub>, it is not possible to infer the metabolic role of the N within the body (i.e., protein synthesis, breakdown, and oxidation) (Jeejeebhoy 1986, Bolster et al. 2001). As such, in this thesis the measurement of  $N_{BAL}$  was chosen as an adjunct to stable isotope methodologies.

## 3.1.2. Whole Body Protein Turnover: The Use of Stable Isotopes

Stable isotopes are heavier forms of a given element containing one or several extra neutrons in their nucleus. Most elements, both in nature (e.g., foodstuffs) and in the human body (e.g., tissues), already contain a mixture of various stable isotopes at low, yet significant, natural levels (Darmaun and Mauras 2005). For application in the study of physiology and metabolism stable isotopes are commonly used in the form of a tracer. Tracers are produced by chemically attaching the isotope to a compound of interest, and then administering the tracer by either ingestion or infusion (Mahon and Timmons 2014). As the mass difference between the stable isotope and predominant natural form is often small for common isotopes (e.g. <sup>15</sup>N vs. <sup>14</sup>N and <sup>13</sup>C vs. <sup>12</sup>C), the physicochemical properties of the tracer are nearly identical to the unlabeled tracee under investigation (Koletzko et al. 1998, Mahon and Timmons 2014). The assumption, therefore, is that both the tracer and the tracee will act in a similar manner with respect to metabolism (Timmons et al. 2003). By measuring the amount of tracer and tracee, as well as their subsequent metabolites, estimates of the utilization of the compound of interest can be determined (Bodamer and Halliday 2001).

As proteins and their associated amino acids are constantly undergoing turnover (**Figure 3.1**), static measures provide little information on the dynamic processes taking place (Darmaun and Mauras 2005). Tracers, on the other hand, provide more detailed information regarding the metabolic fate of the particular nutrient of interest (e.g., N or specific amino acids) and, therefore, were chosen for use in this thesis to provide estimates of kinetic measures (i.e., synthesis, breakdown, and turnover) (Darmaun and

Mauras 2005). Moreover, in contrast to radioisotopes, these isotopes are not radioactive when they disintegrate (Koletzko et al. 1998, Darmaun and Mauras 2005) which allows them to be safely used in infants and children as well as other special populations (i.e., pregnant women) (Koletzko et al. 1998).

Though there are a number of stable isotopes that can be applied in human studies, the methods used in this thesis were limited to the use of [<sup>15</sup>N]glycine and L-[1-<sup>13</sup>C]leucine as these are the most commonly used stable isotopes tracers to assess protein metabolism (Wagenmakers 1999), especially in pediatric research.

Figure 3.1. Simplified scheme of the exchange between body proteins and the free amino acid pools within the human body as a whole. Protein turnover is important both for remodeling proteins within the body and for maintaining the reserve of free amino acids (Imura and Okada 1998, Duggleby and Waterlow 2005). In this scheme, all body proteins and proteins within the circulation are grouped as tissue proteins, while all amino acids dissolved in body fluids are grouped within the free amino acid pool. These free amino pools have important roles in controlling the nutritional and metabolic responses of the body's proteins. Proteins are continually degraded and resynthesized (i.e., protein turnover) as shown by the arrows connecting the free amino acids to the tissue proteins; thus, amino acids within the body get recycled. The recycling process, however, is not completely efficient. Amino acids can be lost from the free amino acid pool by excretion, oxidation, or the conversion of amino acids to other non-protein metabolites. Amino acids can also be lost by tissue proteins through the skin, hair, and feces. In order to replenish these losses, new amino acids can enter the free amino acid pool through the *de novo* synthesis in cells (e.g., the production of non-essential amino acids from the gut). However, in order to re-supplement the essential amino acids lost from the body, the consumption and breakdown of dietary proteins is required. Reprinted with permission from Trumbo, P., Schlicker, S., Yates, A. A., Poos, M., Food and Nutrition Board of the Institute of Medicine, The National Academies. (2002). Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids. J Am Diet Assoc 102(11), 1621-1630. (License # 3687840570755).



# 3.1.3. Oral [<sup>15</sup>N]Glycine Tracer

The 'end-product' approach with [<sup>15</sup>N]glycine is a commonly used method to measure components of whole body protein turnover including whole body N flux (Q), protein synthesis, protein breakdown and subsequently WBPB. The end-product method is based on the assumption that the proportions of the tracer dose that go to synthesis and excretion are identical to the proportions of flux that go to synthesis and excretion (Duggleby and Waterlow 2005).

Briefly, an initial spot urine sample is taken for the measurement of background <sup>15</sup>N-enrichment. Then, the isotope is commonly mixed with a beverage (in this thesis we chose water) and ingested orally at a dose of 2 mg of isotope per kg body weight (Ebbeling and Rodriguez 1999, Bolster et al. 2001, Pikosky et al. 2002, Boisseau et al. 2005). All subsequent urine produced over the defined assessment period (following the consumption of the isotope) is then collected in order to assess  $N_{EX}$  (i.e., urea and creatinine) and <sup>15</sup>N-enrichment (Wagenmakers 1999). As with the calculations for  $N_{BAL}$ , the concentration of urinary metabolites were measured directly with spectrophotometry, while fecal and miscellaneous sources of  $N_{EX}$  were estimated according to previously published values (Gattas et al. 1990). Finally, to calculate components of whole body protein turnover, measurements of  $N_{IN}$  (i.e., diet) must also be made.

One important thing to note when using the end-product method is the time period of the collection, as the time course of the excretion of <sup>15</sup>N in urinary ammonia and urea is not uniform (**Figure 3.2**) (Duggleby and Waterlow 2005). Ammonia undergoes a rapid turnover, and by 9-12 h the excretion of [<sup>15</sup>N]ammonia is essentially complete (Duggleby

and Waterlow 2005). When measurements exceed this point, a small but considerable error can be introduced due to the breakdown of labeled protein and, thus, recycling of the tracer into the ammonia pool (Duggleby and Waterlow 2005). Urea, on the other hand, undergoes a slower turnover whereby about 25% of [<sup>15</sup>N]urea from the administration of the tracer is still retained in the body after 9-12 h (Duggleby and Waterlow 2005). Therefore, for periods of collection between 9-24 h, the risk of tracer recycling in the urea pool is negligible (Duggleby and Waterlow 2005). The difference in time course of excretion between N-ammonia and N-urea is based on the notion that rather than a single homogeneous 'metabolic' pool, there are instead two pools that are (to some extent) spatially and metabolically separate (Fern et al. 1985a, Duggleby and Waterlow 2005). It has been further proposed that  $Q_A$  represents the metabolism of the peripheral tissues (i.e., muscle) (Pitts and Pilkington 1966), whereas  $Q_U$  better represents the splanchnic metabolism (Duggleby and Waterlow 2005).

As the estimates of Q based on ammonia  $(Q_A)$  and urea  $(Q_U)$  vary (**Figure 3.3**), there are two different approaches that can be used. The decision on which approach to use often depends on the time frame of interest when assessing whole body protein turnover (i.e., whether measuring the response to exercise and nutrition during the acute recovery period, or whether assessing the effects over the course of a full 24-h period).

The first is the <sup>15</sup>N-ammonia end-product method (Grove and Jackson 1995), which only uses the value for ammonia-N in the calculation of  $N_{EX}$ . This method was chosen in **Chapters 5 and 6** when assessing WBPB over the early acute exercise and recovery period. Based on the constraints of the oral [<sup>15</sup>N]glycine methodology and the

specified time course of ammonia excretion (Grove and Jackson 1995), **Chapter 5** utilized a 9-h collection period (encompassing both the exercise and early ~7-h recovery period). In **Chapter 6**, however, we only used a 6-h monitoring period (encompassing both the exercise and early ~4-h recovery period) to measure WBPB. The decision to measure protein metabolism over this 6-h period was made on an exploratory basis, while acknowledging the constraints of the oral [<sup>15</sup>N]glycine methodology and the fact that the ammonia excretion likely underestimated protein flux (particularly at higher protein intakes), which would result in an overestimation in WBPB.

The other approach that is often used measures both urea-N and ammonia-N, and estimates total N-excretion using an average (either arithmetic or harmonic) of the two to best represent a 'true' Q (Fern et al. 1981, 1985b). This approach was chosen to assess whole body protein metabolism over longer (i.e., 16 h in **Chapters 4**, and 24 h in **Chapters 5** and **6**) measurement periods.



Figure 3.2. Urinary excretion of [<sup>15</sup>N]ammonia and [<sup>15</sup>N]urea over 48 h following a single oral dose of [<sup>15</sup>N]glycine in healthy adults. [<sup>15</sup>N]ammonia is represented by circles, while [<sup>15</sup>N]urea is represented by squares. Reprinted with permission from Grove, G., Jackson, A. A. (1995). Measurement of protein turnover in normal man using the end-product method with oral [<sup>15</sup>N]glycine: comparison of single-dose and intermittent-dose regimens. *Br J Nutr* 74(4), 491-507. (License # 3674530354882).



Figure 3.3. Measures of whole body nitrogen flux (Q) across time as derived from either the concentration of  $[^{15}N]$ ammonia or  $[^{15}N]$ urea in urine following a single oral dose of  $[^{15}N]$ glycine in healthy adults.  $[^{15}N]$ ammonia is represented by open circles, while  $[^{15}N]$ urea is represented by closed circles. Reprinted with permission from Grove, G., Jackson, A. A. (1995). Measurement of protein turnover in normal man using the end-product method with oral  $[^{15}N]$ glycine: comparison of single-dose and intermittent-dose regimens. *Br J Nutr* 74(4), 491-507. (License # 3674530354882).

One of the major benefits of the [<sup>15</sup>N]glycine end-product approach, particularly for use in children, is that it is non-invasive in nature, whereby all metabolic end-points can be collected and measured using urine samples (Duggleby and Waterlow 2005). Moreover, participants are unrestricted in their movements throughout the procedure (aside from when urine samples are collected) allowing them to perform collections outside of the laboratory (Duggleby and Waterlow 2005). Finally, one of the major reasons for choosing to use this technique in this thesis is the ability for protein kinetics to be measured over relatively long time frames (e.g., up to 24 h) (Grove and Jackson 1995), that would not be ethically feasible using IV techniques.

Indeed, there are also a number of limitations that must be considered when using the [<sup>15</sup>N]glycine methodology (Wagenmakers 1999). One setback of the end-product approach is the practical difficulties in collecting samples outside of the laboratory and the assumption that the sample has been collected in its entirety. For this reason, it is often easier for the assessment to occur over night which, for children, would eliminate the need for the collection and storage of urine samples during the day while they are at school (Mahon and Timmons 2014). Another limitation to this technique is with respect to the sensitivity of the oral [<sup>15</sup>N]glycine tracer in its ability to detect small differences in protein turnover that might otherwise be detected by methodologies such as IV infusions. Finally, the [<sup>15</sup>N]glycine technique generally has a low time resolution (due to the necessity to adequately collect the metabolic end-products) over which changes in protein turnover can be measured. Despite its limitations, it has been argued that if the
[<sup>15</sup>N]glycine technique is applied in a strict and standardized way, reasonable estimates of whole-body protein turnover can be determined (Duggleby and Waterlow 2005).

# 3.1.4. Intravenous L-[1-<sup>13</sup>C]Leucine Infusion

Currently, the 'gold standard' for assessing whole-body protein kinetics and quantifying rates of protein turnover in humans is the 'precursor' method. Leucine is typically used as the labeled amino acid as it *1*) is an essential amino acid and, therefore, cannot be produced *de novo* by the human body; *2*) has a relatively greater rate of oxidation; and *3*) is preferentially (**Figure 3.4**) and completely oxidized within the skeletal muscle (Brosnan and Brosnan 2006, Monirujjaman and Ferdouse 2014). This method can provide reliable estimates of leucine oxidation (Leu<sub>OX</sub>), as well as estimates of leucine balance (Leu<sub>BAL</sub>) and flux at the whole-body level, by using only short-term measurements during either the fed or post-absorptive periods (Duggleby and Waterlow 2005).

The most common use of this method includes a short (4-6 h) constant IV infusion of labeled leucine ([1-<sup>13</sup>C]leucine) (Darmaun and Mauras 2005), which is given until the precursor pool for protein synthesis reaches isotopic steady-state (~1-2 h) (Wagenmakers 1999, Duggleby and Waterlow 2005). An initial priming dose of [1-<sup>13</sup>C]leucine is often administered to help reach an early plateau (Wagenmakers 1999, Duggleby and Waterlow 2005). Throughout the procedure, participants also have a second IV line for blood sampling (Duggleby and Waterlow 2005). An initial blood sample is taken once the participant has reached isotopic steady state to obtain baseline values.

In order to achieve metabolic steady-state, participants are often monitored in the post-absorptive period or during a near-steady-state condition using constant nasogastric or nasojejunal infusions, or repeated small 'bolus' meal feedings (Boirie et al. 1996). However, the physiological relevance of this approach is arguably limited as feeding in normal humans is generally in more of a single meal bolus, where the difference in plasma amino acid and insulin responses following a single meal would need to be taken into account (Boirie et al. 1996). Therefore, to gain an understanding of the physiological changes to protein synthesis, breakdown and amino acid oxidation in response to a single meal, the use of oral ([<sup>2</sup>H<sub>3</sub>]leucine) and IV ([1-<sup>13</sup>C]leucine) labeled leucine tracers, in combination with non-steady-state equations was chosen for use in this thesis.

Following the administration of the test meal, a number of metabolic measures are sampled over 20-30 minute intervals throughout the remainder of the monitoring period. In order to assess the amino acids entering the free amino acid pool (either from exogenous sources or as a result of protein breakdown), the plasma concentration of unlabeled leucine, as well as the plasma enrichment of  $[1-^{13}C]$ leucine and  $[^{2}H_{3}]$ leucine, are determined. Though free amino acid pools are located both within cells (i.e., intracellular pool) as well as the body fluids (i.e., plasma pool), the plasma pool is often used as an indicator of the concentration of free amino acids in the body as it is more easily sampled (National Research Council 2005). To determine the amino acids leaving the amino acid pool (either for protein synthesis or oxidation) the plasma leucine concentration is again determined, as well as the plasma enrichment of  $\alpha$ - $[^{13}C]$ ketoisocaproate acid. Plasma  $\alpha$ - $[^{13}C]$ ketoisocaproate acid. Plasma  $\alpha$ - $[^{13}C]$ ketoisocaproate acid enrichment was chosen as

the precursor as it is a good reflection of the immediate intracellular precursor for the irreversible decarboxylation of leucine (Matthews et al. 1982). In addition to plasma samples, breath samples are also required to determine both the <sup>13</sup>CO<sub>2</sub> enrichment in expired CO<sub>2</sub> and average CO<sub>2</sub> production. To estimate Leu<sub>BAL</sub>, the total dietary leucine intake (accounting for the  $[1-^{13}C]$ leucine IV infusion, dietary beverage leucine content, and oral [<sup>2</sup>H<sub>3</sub>]leucine tracer) as well as the leucine enrichment of the total dietary leucine, should be known.

The use of IV infusion of  $[1^{-13}C]$ leucine to assess whole-body protein kinetics relies on several assumptions (**Appendix III**) (Darmaun and Mauras 2005). The majority of these assumption are generally accepted and considered valid in most physiological conditions even after years of intense scrutiny (Wagenmakers 1999, Darmaun and Mauras 2005). Due to the collection of blood samples, one advantage of this technique is the ability to measure changes in circulating amino acid and insulin concentrations that are not available when using the end-product method. Another advantage of this technique is that is has a better time resolution than the [<sup>15</sup>N]glycine methodology over which changes in protein turnover can be measured. In conjunction with non-steady-state kinetics, this technique can assess changes in Leu<sub>OX</sub>, endogenous rate of appearance (Endo  $R_a$ ; marker of protein breakdown) and non-oxidative leucine disposal (NOLD; marker of protein synthesis) over 20-30 min intervals (Boirie et al. 1997), which makes it ideally suited to determine the acute effects of a single nutrition intervention on protein metabolism. Figure 3.4. Representation of the distribution of branched-chain amino acid (BCAA) catabolic capacity by (A) branched-chain aminotransferase (BCAT) and (B) branched-chain ketoacid dehydrogenase (BCKD) across different human tissues. Although the majority of amino acid degradation occurs in the liver (Berg et al. 2002), amino acid degradation also occurs in other tissues. For example, a special subset of essential amino acids known as BCAAs (i.e., leucine, isoleucine and valine) are unique in that only a small fraction of their catabolic capacity exists in the liver, while the majority occurs in extra-hepatic tissues (Brosnan and Brosnan 2006, Monirujjaman and Ferdouse 2014). Skeletal muscle is a major site of BCAA catabolism as approximately half of BCAA catabolic capacity resides in this tissue. The BCAT enzyme catalyzes the reversible transamination of BCAAs, whereas BCKD is the rate-limiting enzyme for BCAA oxidation located in the mitochondrial membrane that catalyzes a decarboxylation of each of the three  $\alpha$ -ketoacids and commits them to oxidation (Danner et al. 1979). Reprinted with permission from Brosnan, J. T., Brosnan, M. E. (2006). Branched-chain amino acids: enzyme and substrate regulation. J Nutr 136(1 Suppl), 207S-211S. (License # 3674501134387).



Despite the academic advantages, however, there still remain some practical disadvantages of the technique. Compared with the end-product method, the precursor method is more invasive in nature; participants are subjected to two IV lines (one for the infusion of the tracer and a second for blood sampling) (Duggleby and Waterlow 2005). Though this technique is still considered to be minimally invasive, it does pose the risk of physiological discomfort and potential emotional distress to the child (Mahon and Timmons 2014). Furthermore, a collection of breath samples is also needed for the [1-<sup>13</sup>C]leucine method. Although this may be easy to perform and convenient for healthy subjects, it becomes more complex in patients who are ill, weak, ventilated or anaesthetized (Wagenmakers 1999). Finally, complex and relatively expensive equipment is needed to perform the technique and analyze the collected samples, with participants being required to remain in the laboratory setting for continual access to the IV and a metabolic cart (for sampling of CO<sub>2</sub> output) (Duggleby and Waterlow 2005). As participants have restricted movement during the time in the laboratory, it also places limitations on the exercise procedures that can be used (Duggleby and Waterlow 2005). The  $[1-^{13}C]$  leucine method requires the use of three analytical techniques: 1) indirect calorimetry to determine total CO<sub>2</sub> production; 2) an isotope-ratio mass spectrometer (IRMS) to assess the isotope enrichment in expired  $CO_2$ ; and 3) a gas chromatographmass spectrometer (GCMS) to measure the isotope enrichment of the precursor pool (plasma KIC) (Wagenmakers 1999, Darmaun and Mauras 2005). Fortunately, major methodological advancements of these analytical methods (particularly IRMS and GCMS) allows for the detection of labeled isotope tracers and their metabolites with highly accurate measurements using very small amounts of sample material (Koletzko et al. 1998). This makes their use in pediatrics a particular benefit, as very little biological sample is required to be collected. Despite the increased popularity of using these methods and increased overall accessibility of the technology (Darmaun and Mauras 2005), the data in this thesis are the first with the use of the [1-<sup>13</sup>C]leucine infusion technique to measure the effects of exercise and nutrition in a population of healthy, active children.

# CHAPTER 4 : Effects of Postexercise Milk Consumption on Whole Body Protein Balance in Youth

#### 4.1. FOREWORD

This study addressed **Objective #1** of this thesis and investigated whether or not a protein-containing beverage such as skim milk (SM) enhances WBPB following exercise in healthy, active children to a greater degree than other beverages commonly consumed following exercise: a carbohydrate-electrolyte solution (CES) or water. This study also investigated whether there were effects of puberty or sex on milk's ability to enhance WBPB. The findings of the study demonstrate that both Q and protein synthesis are increased following consumption of SM, whereas protein breakdown is less affected. This resulted in an enhanced WBPB when SM was consumed postexercise. However, additional protein intake may be required to sustain a net anabolic environment over 16 h postexercise. Moreover, both puberty (pre- to early puberty (PEP) vs. mid- to late puberty (MLP)) and sex (girls vs. boys) may influence protein requirements.

This work was published in the Journal of Applied Physiology (Volume 117, Issue 10, pages 1165-1169, September 2014). The authors are the following: Kimberly A. Volterman, Joyce Obeid, Boguslaw Wilk, and Brian W. Timmons. The corresponding author is Dr. Brian W. Timmons. Boguslaw Wilk and Brian W. Timmons conceptualized and designed the research. Kimberly A. Volterman performed the experiments with assistance from Joyce Obeid. Kimberly A. Volterman analyzed the data in collaboration with Brian W. Timmons. Kimberly A. Volterman, Boguslaw Wilk, and Brian W. Timmons interpreted the results of the experiment. Kimberly A. Volterman prepared tables and drafted the manuscript, with manuscript edits and revisions from Joyce Obeid, Boguslaw Wilk, and Brian W. Timmons. The complete reference is as follows: Volterman, K.A., Obeid, J., Wilk, B., and Timmons, B.W. (2014). Effects of postexercise milk consumption on whole body protein balance in youth. *J Appl Physiol*, 117(10): 1165-1169. Permission is not required for reprint in thesis (see **Appendix IV**).

#### 4.2. ABSTRACT

In adults, adding protein to a postexercise beverage increases muscle protein turnover and replenishes amino acid stores. Recent focus has shifted towards the use of bovine-based milk and milk products as potential postexercise beverages; however, little is known about how this research translates to the pediatric population. Twenty-eight (15 girls) pre- to early-pubertal (PEP, 7-11 yr) and mid- to late-pubertal (MLP, 14-17 yr) children consumed an oral dose of  $[^{15}N]$ glycine prior to performing 2 × 20-min cycling bouts at 60% VO<sub>2peak</sub> in a warm environment (34.5°C, 47.3% relative humidity). Following exercise, participants consumed either water (W), a carbohydrate-electrolyte solution (CES), or skim milk (SM) in randomized, cross-over fashion in a volume equal to 100% of their body mass loss during exercise. Whole body nitrogen turnover (Q), protein synthesis (S), protein breakdown (B), and whole body protein balance (WBPB) were measured over 16 h. Protein intake from SM was  $0.40 \pm 0.10$  g/kg. Over 16 h, O and S were significantly greater (P < 0.01) with SM than W and CES. B demonstrated a trend for a main effect for beverage (P = 0.063). WBPB was more negative (P < 0.01) with W and CES than with SM. In the SM trial, WBPB was positive in PEP while it remained negative in MLP. Boys exhibited significantly more negative WBPB than girls (P < 0.05). Postexercise milk consumption enhances WBPB compared to W and CES; however, additional protein intake may be required to sustain a net anabolic environment over 16h.

## 4.3. INTRODUCTION

One of the main goals of a postexercise beverage, in addition to rehydration (replacing fluid and electrolytes), is to restore muscle glycogen stores that have been utilized during the preceding exercise. The addition of protein to a postexercise beverage also increases muscle protein turnover and replenishes amino acid stores (14). Therefore, aside from the beneficial effects on rehydration and fluid balance (12), a postexercise beverage rich in proteins could also contribute to improved recovery from exercise and exercise performance, while providing the nutrients necessary to enhance lean tissue remodeling and increase lean body mass (27).

In adults, much of the focus in recent years has shifted towards the use of bovinebased milk and milk products as potential postexercise beverages (12; 17; 18; 25); however, very little is known about how this research translates to the pediatric population. While the combined effects of milk (more specifically, calcium) and exercise have been recognized in the promotion of optimal bone development in children (6; 20), the protein needs of this population are not well understood as they remain relatively understudied. This is an important topic when one considers the potential for milk-based products to enhance the anabolic effects of exercise, while facilitating the remodeling and rebuilding process in active, growing children.

Milk has distinct compositional differences compared with beverages typically consumed following exercise, for example water and sports drinks (23). One important characteristic of bovine-milk is the presence of protein and amino acids, which contribute to the maintenance of muscle protein synthesis (MPS) and enhancement of protein balance following exercise (17). Milk protein contains ~20% whey protein and 80% casein protein. Whey and casein protein have distinct structural differences that affect their speed of absorption and catabolic properties; they are referred to as "fast" and "slow" proteins, respectively (3). Upon digestion of whey protein, there is a rapid and transient increase in the appearance of amino acids in the plasma, leading to an acute stimulation of protein synthesis (3; 27). Casein protein, on the other hand, results in a delayed and prolonged rise in plasma amino acids, allowing for the release of insulin and down regulation of muscle protein breakdown (3; 8). The composition of milk protein seemingly produces a beneficial response with respect to MPS and muscle accretion (8). Indeed, adult studies demonstrate that milk enhances MPS to a greater extent than a carbohydrate-electrolyte solution (CES) (27).

The extent to which the beneficial effects of postexercise milk consumption apply to the pediatric population remains unknown. Given its protein content, milk has the potential to enhance protein balance following exercise. Understanding the role of milk in protein balance is especially important in the pediatric years so as to allow for the promotion of an active lifestyle, while maintaining optimal growth and development. Therefore, the aim of this study was to examine whether milk, a protein-containing beverage, could favourably impact whole body protein balance (WBPB) following exercise in healthy children. Our hypothesis was that due to its protein content, milk would maintain a more positive WBPB following exercise when compared with water and a CES. Additionally, the secondary objective of this study was to assess the effects of puberty and sex, as well as their interaction, on milk's ability to maintain WBPB.

## 4.4. METHODS

*Participants*. Twenty-eight pre- to early-pubertal (PEP, 7-11 yr) and mid- to latepubertal (MLP, 14-17 yr) children participated in this study, approved by the Hamilton Health Sciences/Faculty of Health Sciences Research Ethics Board and conducted in compliance with the standards set by the Declaration of Helsinki. All participants and their parents were informed of the study protocol and provided written informed assent and consent, respectively, prior to study enrollment. Participants were recruited from the local community through schools and sporting clubs. General medical and activity questionnaires were used to ensure all participants were healthy and habitually physically active. Participant characteristics are summarized in **Table 4.1**.

	PEP		MLP		
	Boys Girls		Boys	Girls	
п	6	8	7	7	
Age, y	$9.4 \pm 1.0$	$9.5 \pm 0.8$	$15.6 \pm 0.5*$	$14.8 \pm 0.4*$ †	
Stature, cm	$137 \pm 8$	$136 \pm 9$	171 ± 8*	$169 \pm 4*$	
Body mass, kg	$34.2\pm7.7$	$29.6 \pm 5.7$	$59.4 \pm 9.0*$	$60.5 \pm 8.4*$	
Body fat, % <sup>a</sup>	$14.5 \pm 8.4$	$14.3 \pm 6.1$	$15.7 \pm 8.4$	21.3 ± 5.5	
Tanner stage	2 (1)	1 (0)	4 (1)	4 (1)	

## Table 4.1. Participant characteristics.

PEP, pre- to early-pubertal; MLP, mid- to late-pubertal. \*Significant difference from prepubertal, P < 0.001, †Significant difference between sexes, P < 0.05. <sup>a</sup>Determined using bioelectrical impedance analysis as described in (20). Data are presented as mean  $\pm$  SD or median (interquartile range).

General Overview. The present data are secondary outcomes of a study evaluating the effect of milk on rehydration after exercise-induced fluid loss in the heat. Using a randomized, repeated measures cross-over design, participants reported to the laboratory on four separate occasions, separated by 4-10 days. The first session was a preliminary screening visit where we obtained basic anthropometrics and aerobic fitness measurements. For an estimate of habitual dietary intake, participants were asked to complete a 3-day dietary record, which was analyzed with The Food Processor SQL (ESHA, Salem, Oregon) software for energy and macronutrient intakes. The following three sessions, which took place two weeks after the initial visit, were performed in a counterbalanced manner and consisted of an identical experimental protocol with the exception of the postexercise beverage consumed. During each of the three experimental sessions, participants consumed one of three experimental beverages following exercise: 1) plain water (W); 2) a commercially available CES, designed for the postexercise period (Powerade, Coca Cola Ltd, Toronto, Canada); 3) skim milk (SM) (0.1% Skim Milk; Beatrice, Parmalat, Toronto, Canada). The volume consumed was equal to 100% of the body fluid lost during the previous exercise, as previously described (24). The noninvasive oral  $[^{15}N]$ glycine technique, with samples collected over a 16-h period (the time in the laboratory for each experimental session plus the subsequent overnight period), was used to determine the effect of beverage consumption on whole body nitrogen turnover (O), whole body protein synthesis (S), whole body protein breakdown (B) and WBPB.

Preliminary visit. Children attended an initial screening visit, during which we obtained basic anthropometric and aerobic fitness measurements including stature

(Harpenden wall-mounted Stadiometer), body mass (Tanita BWB-800S digital scale, Tanita Corp., Japan), and body composition (InBody520 bioelectrical impedance analyzer; Biospace Co., California, USA). Maturational status was self-assessed according to Tanner criteria (21) using pubic hair development for boys and breast development for girls. To measure aerobic fitness, we determined peak oxygen uptake (VO<sub>2peak</sub>) using the McMaster All-Out Progressive Continuous Cycling Test. The VO<sub>2peak</sub> test was performed in a thermoneutral environment (22°C, 54% relative humidity). The highest 30-s VO<sub>2</sub> was considered the VO<sub>2peak</sub>. The test was terminated when the child could no longer maintain the pre-set cadence of 60 revolutions per minute despite strong verbal encouragement by the investigator. Participants performed each of their sessions on the same mechanically or electromagnetically braked cycle ergometer (Fleisch-Metabo, Geneva, Switzerland or Lode Corival, The Netherlands, respectively). Expired gases were examined throughout the exercise over 30-s intervals in the mixing chamber setting on a calibrated metabolic cart (Vmax 29, SensorMedics, Yorba Linda, CA, U.S.A) with appropriately sized pediatric mouthpieces.

*Experimental protocol.* Children reported to the laboratory at ~3:30 P.M. for each of their experimental sessions. On the day of the first experimental session, parents were given a log book to record everything the child ate and drank throughout the day, before arrival to the laboratory. Participants were then asked to replicate this diet as closely as possible prior to each of the subsequent experimental sessions. Participants were also asked to avoid eating at least 1 h before arriving to the laboratory, to avoid any strenuous physical activity on the days of experimental testing, and to avoid caffeine for 12 h prior

to each visit. Upon arrival, each child was asked to empty his or her bladder and provide a spot urine to measure background [<sup>15</sup>N] enrichment of urinary ammonia. Participants then consumed 2 mg/kg body mass of [<sup>15</sup>N]glycine dissolved in 5 ml/kg body mass of tap water, along with a preexercise standardized meal. This was followed by 1 h of rest before entering a climate chamber set to 35°C and 48% relative humidity to perform  $2 \times$ 20-min bouts of cycling at 60% of their previously determined VO<sub>2peak</sub>.

Upon completion of the exercise, participants exited the climate chamber and rested in a thermoneutral room. At 0, 15 and 30 min following the completion of exercise, participants consumed three equal aliquots of the experimental beverage in a volume equal to 100% of the body fluid lost during exercise, as previously described (24). Participants were then asked to rest in the laboratory for 2 h before ingesting their postexercise standardized meal.

*Urine collection.* All urine produced while in the laboratory, following ingestion of the [<sup>15</sup>N]glycine, was collected at scheduled time points, pooled, and stored at 4°C until the following day. Upon leaving the laboratory, participants were provided with a urine collection container and were instructed to collect all urine produced during the evening until the first urination the following morning (inclusive). Participants were instructed to store the container at 4°C. All urine from the laboratory and home were then pooled and the total volume measured to the nearest milliliter. Two 3-ml aliquots representing the 16-h measurement period were stored at -20°C until subsequent analysis.

*Diet.* Each participant was provided with a pre- and postexercise meal so as to standardize nutrition throughout the 16-h urine collection. These meals, consumed in the

laboratory, consisted of a piece of toast with raspberry jam, an apple, a Nutrigrain bar and a Boost meal replacement drink. All food was weighed so that each participant received the same amount of food relative to his or her body mass (i.e., gram of food or fluid per kilogram of body mass). The total nutrition over the 16 h measurement period also included the experimental beverages; thus, due to the nature of the trial, protein intake during the SM trial was higher than during the W and CES trials.

Analysis of samples. To estimate urinary nitrogen excretion, the sum of the major nitrogen-containing metabolites urea and creatinine were determined by colorimetric analysis using commercially available kits (Quantichrom, Bioassay Systems, USA). The enrichments (i.e. ratio of tracer:trace, t:Tr) of urinary [<sup>15</sup>N]ammonia (in baseline and 16h samples) were determined in duplicate by isotope ratio mass spectrometry by Metabolic Solutions Incorporated (Nashua, NH, USA). O. determined by the [<sup>15</sup>N]ammonia endproduct method, was then calculated as Q (g N/kg) = d/corrected t:Tr/BM, where d is the dose of oral [<sup>15</sup>N]glycine, corrected t:Tr is the baseline corrected [<sup>15</sup>N] enrichment of urinary ammonia, and BM is the participant's body mass. S was calculated as S (g protein/kg) =  $[Q-(E/BM)] \times 6.25$  g protein/g N, where E is nitrogen excretion expressed as the sum of both measured and estimated nitrogen excretion. Measured nitrogen excretion was calculated as the sum of urinary urea and creatinine nitrogen excretion over the 16-h period. Estimated nitrogen excretion was calculated using estimated average sweat nitrogen and amino acid concentrations (1) with an average  $\sim 15\%$  nitrogen content of amino acids (13), multiplied by fluid loss estimated by change in body mass for each participant. In agreement with previously published values in children consuming a 1.2 g protein/kg/d diet, fecal nitrogen excretion was estimated to be 0.9 mg/kg/h (10). B was calculated as B (g protein/kg) =  $[Q-(I)/BM)] \times 6.25$  g protein/g N, where I is nitrogen intake determined by analysis of the standardized meals provided along with the experimental beverage. Finally, WBPB was determined as WBPB (g protein /kg) = S – B.

Statistical analysis. All data were analyzed using Statistica version 5.0. To determine differences in protein intake in the SM trial, a two-way (puberty × sex) ANOVA was performed. To assess the effects of beverage, a separate one-way repeated measures ANOVA was used for Q, S, B and WBPB (total of 4 ANOVAs). To assess the effects of puberty and sex on milk's ability to maintain protein balance, Q, S, B and WBPB from the SM trial were analyzed using separate two-way (puberty × sex) ANOVAs (a total of four ANOVAs). When main effects or interactions were significant, the source of statistically significant differences was determined using Tukey's post hoc test. The significance level for all tests was set at P < 0.05. All data are presented as mean  $\pm$  SD, as well as 95% confidence intervals where appropriate. Effect sizes for primary outcome variables were calculated using eta squared ( $\eta^2$ ) and interpreted according to Cohen's guidelines (5).

#### 4.5. RESULTS

Thirty-eight participants were initially recruited to participate in the study. Six participants were excluded due to failure to provide an overnight urine sample, two participants were excluded due to missing data, and two participants excluded due to values that were greater than 2 SD from the mean value for their puberty and gender for

each of the following variables: Q, S, B and WBPB. As such, our sample size was reduced to 28 participants.

*Experimental diet.* The experimental beverages in both the W and CES trials provided an absolute and relative protein intake of  $0 \pm 0$  g and  $0 \pm 0$  g/kg, respectively. The absolute protein intake from the SM beverage was  $18.1 \pm 7.0$  g, with PEP children consuming a smaller absolute amount of protein than MLP children (12.2  $\pm$  3.8 g and 24.0  $\pm$  3.7 g, respectively; P < 0.001), by virtue of lower sweating rates during the previous exercise. However, when expressed relative to body mass, protein intake from the SM beverage (0.40  $\pm$  0.10 g/kg) did not differ between pubertal groups or between sexes. Macronutrient intake is summarized in **Table 4.2**. As a result of the differences in beverage composition (24), macronutrient intake over the 16-h observation period (which included the pre- and postexercise standardized meals, and the experimental beverages) differed between experimental trials for energy (P < 0.05), carbohydrate (P < 0.001), fat (P < 0.05) and protein (P < 0.001) intakes.

Whole body protein metabolism. Rates of Q, S, B and WBPB over 16 h are summarized in **Table 4.3**. A main effect for beverage was observed for Q (P < 0.001), S (P < 0.01), and WBPB (P = 0.01), whereas B demonstrated a trend for a main effect for beverage (P = 0.063). Rates of Q, S, B, and WBPB according to puberty and sex in the SM trial are summarized in **Table 4.4**. There were no main effects for puberty or sex, nor were statistically significant puberty × sex interactions observed for Q, S or B. WBPB demonstrated a main effect for both puberty (P < 0.001) and sex (P < 0.05), however, no puberty × sex interaction was found.

#### Table 4.2. Dietary intakes.

	Intake, 16 h				Intake, 24 h			
-	W	CES	SM	W	CES	SM	Habitual Intake, 24 h	
Energy, kcal/kg	$29.14\pm5.33^a$	$33.49\pm6.82^b$	$31.95\pm5.37^a$	$50.25 \pm 6.31^{a}$	$51.22 \pm 6.41^{a}$	$50.26\pm5.86^a$	$44.0 \pm 11.2$	
Carbohydrate, g/kg	$5.54\pm1.00^{a}$	$6.14 \pm 1.24^{b}$	$5.89\pm0.98^{c}$	$8.63 \pm 1.15^{a,b}$	$8.75 \pm 1.15^{a}$	$8.42 \pm 1.04^{\text{b}}$	$6.27\pm2.09$	
Fat, g/kg	$0.44\pm0.08^{\text{a}}$	$0.44\pm0.09^a$	$0.42\pm0.07^{b}$	$1.16\pm0.10^a$	$1.17\pm0.10^{\text{a}}$	$1.15\pm0.10^{a}$	$1.40 \pm 0.39$	
Protein, g/kg	$0.83\pm0.16^a$	$0.82\pm0.16^a$	$1.24\pm0.23^{b}$	$1.58\pm0.23^a$	$1.62\pm0.23^{a}$	$2.05\pm0.29^{b}$	$1.60 \pm 0.45$	

Intake over 16 h consisted of controlled diet consumed within the laboratory; Intake over 24 h consisted of the 16-h inlaboratory diet and an extrapolated analysis consumption in the 8 h prior to arrival at the laboratory analyzed by dietary logs; habitual intake over 24 h was the average of the 3-day diet log prior to study commencement. W, water; CES, carbohydrateelectrolyte solution; SM, skim milk. Data reported as means  $\pm$  SD. Conditions with different letters are significantly different from each other within the respective measurement time period, P < 0.05.

	W	CES	SM	Р	$\eta^2$
Q, g N/kg	$0.62 \pm 0.11^{a} (0.58, 0.67)$	$0.61 \pm 0.12^{a} (0.57, 0.67)$	$0.69 \pm 0.12^{b} (0.65, 0.74)$	< 0.001	0.080
S, g/kg	$2.94 \pm 0.59^{a}$ (2.77, 3.22)	$2.90 \pm 0.72^{a} (2.67, 3.22)$	$3.33 \pm 0.64^{\mathrm{b}}(3.10, 3.64)$	< 0.01	0.081
B, g/kg	$3.30 \pm 1.12^{a,b} (3.06, 3.54)$	$3.26 \pm 0.14^{a} (2.98, 3.54)$	$3.56 \pm 0.15^{\mathrm{b}}(3.26, 3.85)$	0.06	0.034
WBPB, g/kg	$-0.32 \pm 0.28^{a}(-0.41, -0.20)$	$-0.33 \pm 0.25^{a}(-0.42, -0.22)$	$-0.19 \pm 0.36^{b} (-0.32, -0.05)$	0.01	0.043

Table 4.3. Whole body protein metabolism over 16 h.

Q, whole body nitrogen turnover; S, protein synthesis; B, protein breakdown; WBPB, whole body net protein balance determined using the [<sup>15</sup>N]ammonia end-product method. Data reported as means  $\pm$  SD and (95% confidence interval). Conditions with different letters are significantly different from each other within the respective variable group, P < 0.05.

	PEP girls	PEP boys	MLP girls	MLP boys	Puberty $P(\eta^2)$	$\operatorname{Sex}_{P(\eta^2)}$	Interaction $P(\eta^2)$
Q, g N/kg	$\begin{array}{c} 0.73 \pm 0.13 \\ (0.61, 0.84) \end{array}$	$0.71 \pm 0.15$ (0.55, 0.86)	$0.62 \pm 0.11$ (0.52, 0.72)	$0.72 \pm 0.11$ (0.62, 0.82)	0.336 (0.036)	0.405 (0.026)	0.234 (0.055)
S, g/kg	$3.59 \pm 0.84$	$3.15 \pm 0.65$	$3.08 \pm 0.52$	$3.62 \pm 0.68$	0.938	0.852	0.076
	(2.89, 4.29)	(2.47, 3.83)	(2.60, 3.56)	(2.99, 4.24)	(0.002)	(0.001)	(0.125)
B, g/kg	$3.42 \pm 0.79$	$3.26 \pm 0.91$	$3.43 \pm 0.63$	$4.09 \pm 0.61$	0.144	0.381	0.161
	(2.76, 4.07)	(2.30, 4.22)	(2.85, 4.02)	(3.52, 4.66)	(0.079)	(0.027)	(0.072)
WBPB, g/kg	$0.17 \pm 0.20^{a}$	$-0.11 \pm 0.42^{a,b}$	$-0.35 \pm 0.14^{b}$	$-0.47 \pm 0.14^{b}$	<0.001	0.040	0.402
	(0.00, 0.34)	(-0.55, 0.33)	(-0.49, -0.22)	(-0.60, -0.35)	(0.420)	(0.086)	(0.013)

Table 4.4. Whole body protein metabolism across pubertal groups and sex over 16 h.

WBPB, whole body net protein balance determined using the [ $^{15}$ N]ammonia end-product method during the skim milk trial. Data reported as means ± SD and (95% confidence interval). Groups with different letters are significantly different from each other within the respective variable group, *P* < 0.05.

#### 4.6. **DISCUSSION**

The potential benefits of adding protein to a postexercise beverage to enhance lean tissue remodeling and increase lean body mass in growing children cannot be overlooked. In this study, we demonstrate that SM, a protein-rich beverage, stimulates protein synthesis to a greater extent than W and a CES, and as a result creates a less catabolic environment over 16 h following exercise in a warm environment. Despite the improvement in WBPB, it is apparent that children require a higher protein intake than that of the current study to achieve a net anabolic state during an overnight period. Furthermore, it is important to consider age- and sex-specific recommendations; we demonstrated that PEP and MLP children – boys and girls both – showed differences in postexercise WBPB following the consumption of SM. For example, although all children received the same relative dose of protein following exercise, MLP children experienced a more negative WBPB than PEP children. Furthermore, only the PEP girls were able to attain a positive WBPB over the 16-h postexercise recovery period.

Over a 16-h recovery period, the postexercise consumption of SM significantly increased the rate of S, and had a tendency to increase B. While exercise training has known effects on protein metabolism in children, including a decrease in protein turnover and increase in nitrogen balance (4; 16), we are not aware of studies examining the acute protein response to specific episodes of exercise. Our results suggest that the postexercise ingestion of SM had a greater effect on the stimulation of protein synthesis than of protein breakdown. Although we lack the ability to determine the extent to which the metabolism within the skeletal muscle of the children influenced changes in WBPB, the

observation that postexercise protein synthesis was stimulated by postexercise protein ingestion is consistent with previous adult studies (14; 28). Because changes in protein synthesis are a large contributing factor to changes in protein balance (15), it is not surprising that children had a significantly more negative WBPB following the ingestion of protein-free beverages, such as W and CES.

An important consideration with regards to growth in active children is the attainment of a positive net protein balance, whereby the anabolic pathways are activated to a greater extent than the catabolic pathways. However, a large proportion of children in the present study, regardless of experimental condition (25 of 28 in W, 24 of 28 in CES, 19 of 28 in SM), experienced a negative net WBPB over the 16-h recovery period. This observation was made despite all children in the SM trial consuming a significantly greater amount of protein than the relative dose of dietary protein shown to maximally stimulate postexercise muscle protein synthesis in older versus young adults (~0.40 g/kg vs. ~0.25 g/kg, respectively) (14). Although it is possible that children require a larger relative protein dose due to higher rates of tissue remodeling, the negative WBPB observed is more likely a result of the observation period used. In our study, children spent a large portion of the recovery period in the post-prandial and overnight fasted states. Despite the elevated rate of protein turnover as a result of the SM beverage, it is possible that the lack of additional feeding periods resulted in an insufficient stimulation of protein synthesis to offset the fasted losses that were experienced. It is unclear whether the children in the present study would have reached a positive WBPB over a 24h observation period that takes into account additional feedings. These findings emphasize

the need for future studies to investigate the impact of postexercise milk consumption over an entire 24-h period to further our understanding of optimal energy and protein intake in active children. In addition, it is possible that the oral [<sup>15</sup>N]glycine methodology used was not sensitive enough to detect relatively small, albeit potentially physiologically relevant, differences in protein turnover between conditions that may have been seen with other methodology (e.g. intravenous infusion). Moreover, a potential limitation of oral tracers, including  $[^{15}N]$ glycine, is they represent the net sum of all nitrogen metabolism in the body (e.g. within muscle, splanchnic bed, etc.), whereas other stable isotope methodologies, such as [<sup>13</sup>C]leucine infusion, are preferentially metabolized within the skeletal muscle. The decision to utilize the  $[^{15}N]$ glycine methodology in the present study was based on the following: 1) the relatively low within-subject variability (9); 2) the ease of measuring protein kinetics over relatively long time frames (i.e. 16 h) (11); and 3) its feasible application in healthy children (7). Future studies are needed to gain a better understanding of postexercise protein requirements using alternative tracer methodologies in healthy, active children.

In healthy children, puberty is characterized by a number of metabolic and hormonal changes (19), including an increase in insulin resistance that is highest during midpuberty (2). Although we did not assess insulin resistance in the present study, it is possible that the MLP group may have been in a state of relative insulin resistance. As a result, the MLP children may have experienced a reduction in sensitivity to both the insulin-induced stimulation of protein synthesis and to amino acid feeding, which would explain the resultant negative WBPB over the 16-h recovery period that was not experienced by the PEP group. Although the precise mechanisms for the relatively more negative WBPB in MLP is unknown, our findings suggest that higher protein doses (>0.40 g/kg) or the frequency and timing of protein intake may be more important in this group compared with pre- and early pubertal youth. Future studies are needed to examine the relationship between protein dose and timing of protein intake in pubertal children in order to maximize WBPB.

PEP girls were able to attain a positive WBPB over the 16-h recovery period, whereas the PEP boys remained in a net negative WBPB, suggesting that sex-specific differences should also be considered. However, it is important to note that in the present study, we did not control for menstrual cycle nor did we assess hormonal markers, thus, we cannot decipher the mechanism by which these differences might exist. Indeed, the effect of testosterone and growth hormone on protein metabolism remains controversial (22; 26); however, it is possible that hormonal differences between the girls and the boys contributed to the differences in WBPB between groups. Regardless of the mechanisms, it is apparent that further studies involving a greater sample size are needed to appropriately compare boys and girls by maturity status. Another limitation of this study is that we examined only one type of protein, because both protein source and protein quality are important factors to consider in dietary recommendations for growing children (16). SM, the protein source of the present study, is considered to be a high-quality, nutrient dense protein source (16) with a number of additional essential micronutrients. Adult studies have shown that in general, proteins of higher quality are better able to support muscle protein accretion and enhance WBPB after exercise (15; 27). To date, there are no studies examining the effects of protein source or protein quality on protein metabolism following exercise in children. Therefore, whether different protein sources (e.g. plant-based) would have similar effects of postexercise protein metabolism is unknown and should be investigated in future studies.

In conclusion, this is the first study to investigate the effects of postexercise milk ingestion on protein metabolism in active youth. SM consumption resulted in elevated Q, S and WBPB, and a trend toward elevated rates of B compared with W and a CES. Despite the relatively large dose of protein ingested in SM, children were unable to attain a positive WBPB over the 16-h recovery period, probably as a result of the timing of our meals and nitrogen assessments. Regardless, our findings suggest that SM is more effective than W or a commercially available sport drink at stimulating protein synthesis and promoting a more favorable environment for the remodeling of lean tissues following exercise in a hot environment. This study highlights the fact that youth can benefit from consuming a high-quality protein source postexercise for enhancements of WBPB. Future studies should seek to assess graded levels of protein intake to gain a better understanding of the doses required for healthy, active youth.

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# 4.9. DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

# 4.10. REFERENCES

- 1. Alexiou D, Anagnostopoulos A and Papadatos C. Total free amino acids, ammonia, and protein in the sweat of children. *Am J Clin Nutr* 32: 750-752, 1979.
- Ball GD, Huang TT, Gower BA, Cruz ML, Shaibi GQ, Weigensberg MJ and Goran MI. Longitudinal changes in insulin sensitivity, insulin secretion, and betacell function during puberty. *J Pediatr* 148: 16-22, 2006.
- Boirie Y, Dangin M, Gachon P, Vasson MP, Maubois JL and Beaufrere B. Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proc Natl Acad Sci U S A* 94: 14930-14935, 1997.
- 4. **Bolster DR, Pikosky MA, McCarthy LM and Rodriguez NR**. Exercise affects protein utilization in healthy children. *J Nutr* 131: 2659-2663, 2001.
- 5. **Cohen J.** *Statistical power analysis for the behavioral sciences*. Hillsdale, NJ: Erlbaum, 1988.
- De SS, Michels N, Polfliet C, D'Haese S, Roggen I, de HS and Sioen I. The influence of dairy consumption and physical activity on ultrasound bone measurements in Flemish children. *J Bone Miner Metab* 2014.
- Duggleby SL and Waterlow JC. The end-product method of measuring wholebody protein turnover: a review of published results and a comparison with those obtained by leucine infusion. *Br J Nutr* 94: 141-153, 2005.
- Elliot TA, Cree MG, Sanford AP, Wolfe RR and Tipton KD. Milk ingestion stimulates net muscle protein synthesis following resistance exercise. *Med Sci Sports Exerc* 38: 667-674, 2006.

- Fern EB, Garlick PJ, Sheppard HG and Fern M. The precision of measuring the rate of whole-body nitrogen flux and protein synthesis in man with a single dose of [15N]-glycine. *Hum Nutr Clin Nutr* 38: 63-73, 1984.
- Gattas V, Barrera GA, Riumallo JS and Uauy R. Protein-energy requirements of prepubertal school-age boys determined by using the nitrogen-balance response to a mixed-protein diet. *Am J Clin Nutr* 52: 1037-1042, 1990.
- Grove G and Jackson AA. Measurement of protein turnover in normal man using the end-product method with oral [15N]glycine: comparison of single-dose and intermittent-dose regimens. *Br J Nutr* 74: 491-507, 1995.
- James LJ, Clayton D and Evans GH. Effect of milk protein addition to a carbohydrate-electrolyte rehydration solution ingested after exercise in the heat. *Br J Nutr* 105: 393-399, 2011.
- 13. Miller L and Houghton JA. The micro-Kjeldahl determination of the nitrogen content of amino acids and proteins. *J Biol Chem* 159: 373-383, 1945.
- Moore DR, Robinson MJ, Fry JL, Tang JE, Glover EI, Wilkinson SB, Prior T, Tarnopolsky MA and Phillips SM. Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. *Am J Clin Nutr* 89: 161-168, 2009.
- Phillips SM, Tang JE and Moore DR. The role of milk- and soy-based protein in support of muscle protein synthesis and muscle protein accretion in young and elderly persons. *J Am Coll Nutr* 28: 343-354, 2009.
- Rodriguez NR. Optimal quantity and composition of protein for growing children. J Am Coll Nutr 24: 1508-1548, 2005.
- 17. Roy BD. Milk: the new sports drink? A Review. J Int Soc Sports Nutr 5: 15, 2008.

- Shirreffs SM, Watson P and Maughan RJ. Milk as an effective postexercise rehydration drink. *Br J Nutr* 98: 173-180, 2007.
- Smith CP, Archibald HR, Thomas JM, Tarn AC, Williams AJ, Gale EA and Savage MO. Basal and stimulated insulin levels rise with advancing puberty. *Clin Endocrinol (Oxf)* 28: 7-14, 1988.
- 20. Specker B and Vukovich M. Evidence for an interaction between exercise and nutrition for improved bone health during growth. *Med Sport Sci* 51: 50-63, 2007.
- 21. Tanner JM. Growth at Adolescence. Oxford, UK: Blackwell Scientific, 1962.
- Tipton KD and Ferrando AA. Improving muscle mass: response of muscle metabolism to exercise, nutrition and anabolic agents. *Essays Biochem* 44: 85-98, 2008.
- 23. Volterman KA and Moore DR. Hydration for optimal health and performance in athletes. *Agro FOOD Industry Hi-Tech Sport Nutrition* 25: 4-7, 2014.
- 24. Volterman KA, Obeid J, Wilk B and Timmons BW. Effect of milk consumption on rehydration in youth following exercise in the heat. *Appl Physiol Nutr Metab* in press: 2014.
- Watson P, Love TD, Maughan RJ and Shirreffs SM. A comparison of the effects of milk and a carbohydrate-electrolyte drink on the restoration of fluid balance and exercise capacity in a hot, humid environment. *Eur J Appl Physiol* 104: 633-642, 2008.
- West DW and Phillips SM. Anabolic processes in human skeletal muscle: restoring the identities of growth hormone and testosterone. *Phys Sportsmed* 38: 97-104, 2010.

- 27. Wilkinson SB, Tarnopolsky MA, MacDonald MJ, MacDonald JR, Armstrong D and Phillips SM. Consumption of fluid skim milk promotes greater muscle protein accretion after resistance exercise than does consumption of an isonitrogenous and isoenergetic soy-protein beverage. *Am J Clin Nutr* 85: 1031-1040, 2007.
- 28. Yang Y, Breen L, Burd NA, Hector AJ, Churchward-Venne TA, Josse AR, Tarnopolsky MA and Phillips SM. Resistance exercise enhances myofibrillar protein synthesis with graded intakes of whey protein in older men. *Br J Nutr* 108: 1780-1788, 2012.

# CHAPTER 5 : Postexercise Protein Ingestion Increases Whole Body Net Protein Balance in Healthy Children

## 5.1. FOREWORD

It was previously demonstrated that, following exercise, a protein-containing beverage such as SM enhances WBPB to a greater degree than protein-free beverages. In the following study, **Objective #2** of this thesis was addressed whereby the effects of the level of acute protein intake on WBPB following exercise in healthy children were investigated. This study demonstrated that WBPB in healthy children was acutely increased by the postexercise ingestion of protein in a dose-dependent manner. Furthermore, larger protein intakes (~ $0.32 \text{ g} \cdot \text{kg}^{-1}$  body mass) appear necessary to sustain a net anabolic environment over an entire 24 h period.

This work was published in the Journal of Applied Physiology (Volume 117, Issue 2, pages 1493-1501, December 2014). The authors are the following: Kimberly A. Volterman\*, Daniel R. Moore\*, Joyce Obeid, Elizabeth A. Offord, and Brian W. Timmons. \*These authors are co-first authors for this paper, and have contributed equally to this work. The corresponding author is Dr. Daniel R. Moore. Daniel R. Moore, Elizabeth A. Offord, and Brian W. Timmons conceptualized and designed the research. Kimberly A. Volterman carried out the study with the assistance of Joyce Obeid and Brian W. Timmons. Kimberly A. Volterman conducted laboratory analyses with support from Daniel R. Moore and Joyce Obeid. Daniel R. Moore, Kimberly A. Volterman and Brian W. Timmons analyzed and interpreted data, and wrote the manuscript. The

complete reference is as follows: Moore, D.R.\*, Volterman, K.A.\*, Obeid, J., Offord, E.A., and Timmons B.W. (2014). Postexercise protein ingestion increases whole body net protein balance in healthy children. *J Appl Physiol*, 117(2): 1493-1501. Permission is not required for reprint in thesis (see **Appendix V**).

#### 5.2. ABSTRACT

Postexercise protein ingestion maximizes whole body and muscle protein anabolism in adults. No study has specifically investigated the combined effects of exercise and protein ingestion on protein metabolism in healthy, physically active children. Under 24-h dietary control, thirteen (seven males, six females) active children (~11 yr old;  $39.3 \pm 5.9$  kg) consumed an oral dose of [<sup>15</sup>N]glycine prior to performing a bout of exercise. Immediately after exercise, participants consumed isoenergetic mixed macronutrient beverages containing a variable amount of protein [0, 0.75, and 1.5 g/100 ml for control (CON), low protein (LP), and high protein (HP), respectively] according to fluid losses. Whole-body nitrogen turnover (O), protein synthesis (S), protein breakdown (B), and protein balance (WBPB) were measured throughout exercise and the early acute recovery period (9 h combined) as well as over 24 h. Postexercise protein intake from the beverage was ~0.18 and ~0.32 g/kg body mass for LP and HP, respectively. O, S, and B were significantly greater (main effect time, all P < 0.001) over 9 h as compared to 24 h with no differences between conditions. WBPB was also greater over 9 h as compared to 24 h in all conditions (main effect time, P < 0.001). Over 9 h, WBPB was greater in HP (P < 0.05) than LP and CON with a trend (P = 0.075) toward LP being greater than CON. WBPB was positive over 9 h for all conditions but only over 24 h for HP. Postexercise protein ingestion acutely increases net protein balance in healthy children early in recovery in a dose-dependent manner with larger protein intakes (~0.32 g/kg) required to sustain a net anabolic environment over an entire 24 h period.
# 5.3. INTRODUCTION

Physical activity is an essential component for the optimal growth and development of the musculoskeletal system in children. Greater levels of physical activity, especially those which are weight bearing and of higher intensity, have been associated with higher bone mineral density, lean mass, and strength in children (5, 18, 43). The net gain in lean mass with physical activity would require alterations in whole body protein turnover that would favour the net synthesis of body proteins, or, in other words, protein synthesis would be chronically elevated above protein breakdown.

Despite the important role physical activity plays in remodeling lean tissues and enhancing lean body mass, few studies have determined the impact of specific episodes of exercise on protein metabolism in children. Whole body protein metabolism is characterized by the continuous turnover and renewal of body proteins through the simultaneous processes of protein synthesis and protein breakdown. Interestingly, previous research by the Rodriguez laboratory (8, 33) demonstrated that 6 wk of structured exercise (either resistance or aerobic) resulted in a downregulation of overnight fasted whole body protein metabolism in healthy children. Despite this potentially counterintuitive suppression of protein metabolism, 24 h net nitrogen balance, a period that would include both fasted and fed states, was elevated with a concomitant increase in lean body mass and height over the 6-wk exercise intervention that would be consistent with the normal growth velocity of the children (8, 33). The discrepancy between overnight fasted and daily net protein balance in these studies (8, 33) could be the result of a potentially increased exercise-induced anabolic sensitivity during the daily fed state, an effect that has been observed previously in exercising adults (12). Therefore, the measurement of protein metabolism in the fasted state may underestimate the anabolic effects of exercise in children.

Provided energy and micronutrient needs are met, dietary protein plays a central role in somatic growth as it provides the substrates necessary to build muscle and other body proteins (21). It is well-established in adults that the postexercise ingestion of protein is essential to maximize muscle protein synthesis (24, 25, 30) and increase whole body protein balance during the acute recovery period (23, 25). The increase in muscle and whole body protein anabolism during this early (e.g. over 3 h) postexercise period facilitates the recovery process and can be sustained over 24 h (36, 42). This synergy between exercise and nutrition for protein anabolism ultimately provides the basis for training adaptations such as lean mass growth (11). Despite the synergies between exercise and protein ingestion in facilitating muscle and whole body protein remodeling in adults, there are a lack of studies that have specifically addressed the combined effects of exercise and protein ingestion on protein metabolism in children. Therefore, the present study evaluated, for the first time in children, the effect of postexercise protein ingestion on the ability to modify whole body protein metabolism during the exercise and early (i.e. over 9 h) and late (i.e. over 24 h) recovery periods after physical activity. Given that postexercise protein ingestion improves muscle protein balance in a dose-dependent manner (9, 30) and that this enhances 24h net protein balance in adults (36, 42), we hypothesized that whole body protein balance in the present study would demonstrate a similar ingested protein dose-response after activity in children. We present here novel data as secondary outcomes from a larger study that yield important, hypothesisgenerating information on the importance of postexercise protein ingestion to increase whole body net protein balance in healthy children.

## 5.4. METHODS

*Participants*. Thirteen (six females, seven males) volunteers participated in this study, which conformed to the standards set by the Declaration of Helsinki and carried with it approval from the Faculty of Health Sciences/Hamilton Health Sciences Research Ethics Board. All participants and their parents were informed of the purpose, procedures and potential risks of the study both verbally and with a written copy of the information sheet. Each participant provided written informed assent and written informed consent was obtained from each parent prior to enrolment in the study. All participants were healthy and physically active as determined by medical and activity questionnaire.

*General overview.* As indicated previously, participants were a subset of a larger study evaluating the effect of protein in a beverage on rehydration after exercise-induced fluid loss, and, therefore, the present data are secondary outcomes from that study. Participants reported to the laboratory on five separate occasions: a preliminary visit, a familiarization visit, and three intervention visits, the latter of which were separated by a 4- to 10-d washout period (for further details, see below). The intervention visits were conducted in a randomized double-blind cross-over fashion (**Figure 5.1**). Following exercise on the intervention visits, participants ingested a mixed-macronutrient beverage containing a variable amount of protein at a volume equivalent to 150% of their exercise-

induced fluid loss. For each intervention visit, participants were provided with energy and macronutrient-matched controlled diets (see below for details). Whole body protein turnover was measured during the exercise and early (i.e. 9 h) acute recovery period as well as the complete 24-h period by oral [<sup>15</sup>N]glycine to determine the effect of postexercise protein ingestion on whole body protein synthesis, breakdown, and net protein balance.

*Preliminary visit.* Participants completed a preliminary session during which height (Harpenden wall-mounted Stadiometer), body mass (BWB-800, Tanita Corp., Japan), percent body fat (InBody520 bioelectrical impedance analyzer; Biospace Co., California, USA), body mass index (BMI), chronological age, and maturity offset were determined. Maturity offset was determined as the estimated years from age at peak height velocity (28). Maximal aerobic power ( $VO_{2max}$ ) was then assessed on a cycle ergometer (Fleisch-Metabo, Geneva, Switzerland), as previously described (41). Physical characteristics of the participants are presented in **Table 5.1**.

*Familiarization visit.* The purpose of this visit was to 1) familiarize the participants with the exercise equipment, 2) confirm appropriate exercise intensities, 3) ensure participants could successfully complete the exercise, and 4) determine individual estimates of fluid loss during exercise to be expected during subsequent trials. During this visit, the fastest running speed on a treadmill was determined based on a modified version of Bundle et al. (10). Briefly, prior to beginning the test, participants ran at a low-moderate speed (between 3.0 to 4.0 mph) for 30 s to allow them to become comfortable with running on a treadmill. Participants then rested for 2 min. The test then began

between 4.0 to 4.5 mph and increased at 0.1 mph every 3 seconds until the participant 1) strongly stated verbally that they could not continue, 2) grabbed onto the handrails to stop, 3) drifted significantly backwards, or 4) pressed the stop button. The test lasted between 48 and 141 s with the fastest running speed of the participants averaging  $7.4 \pm 1.1$  mph. Following the determination of fastest running speed, participants rested for 7 min prior to beginning the exercise protocol [using the same protocol as the intervention visit (see below) with a shortened postexercise recovery period]; this familiarization session permitted the estimation of the exercise-induced fluid loss in order to design the controlled diet for each subsequent trial (see below for details).

# Figure 5.1. Schematic representation of the trial day and exercise protocol.

*A*: schematic representation of the trial day with a solid time line representing time spent in the laboratory and a hashed time line representing time spent in a free-living setting with a controlled diet. The total postexercise macronutrient intake was consumed in three equal volumes every 15 min after exercise. The meals represent food that was consumed within the laboratory. *B*: schematic representation of the exercise protocol. PMP, peak mechanical power.



	Females $(n = 6)$	Males $(n = 7)$
Age, yr	11.7 (0.5)	11.7 (0.5)
Height, cm	149 (6)	148 (7)
Body mass, kg	40.2 (6.3)	38.3 (5.5)
Body mass, percentile	51.2 (31.5)	43.3 (22.4)
BMI, kg/m <sup>2</sup>	17.5 (1.9)	17.2 (1.4)
BMI, percentile	47.5 (27.8)	41.3 (23.7)
Lean body mass, kg	32.4 (3.9)	33.5 (4.1)
Maturity offset <sup>*</sup>	-0.4 (0.3)	-2.0 (0.5)†
Tanner stage 1/2/3 (n)	1/3/2	2/5/1
Maximal aerobic capacity, ml/kg/min	40.0 (3.7)	50.3 (4.6)†

#### Table 5.1. Participant characteristics.

Tanner staging was self-assessed based on breast development for girls and pubic hair development for boys. Percentile rankings relative to 2000 CDC growth charts for the United States (22). \*Maturity offset defined as the difference between chronological age and estimated age of peak height velocity, the latter of which was calculated as previously described (28). †Different between sexes, P < 0.05. With the exception of Tanner stage (presented as *n* per stage), data are presented as mean (SD).

Intervention visit. Participants recorded their habitual dietary intake for the 24 h prior to reporting to the laboratory in an overnight fasted condition and were asked to match as closely as possible this diet prior to all subsequent trials. After providing a morning spot urine for baseline [ $^{15}$ N]ammonia and urea enrichment (see below), participants consumed 2 mg/kg of [ $^{15}$ N]glycine dissolved in water and a light breakfast providing ~12 and ~15% of each participant's total daily energy and protein intake (details for controlled diet provided below), respectively, from food (i.e., not including the experimental beverage intake). After resting quietly for 1 h, participants then performed three blocks of 15 min of exercise for a total of 45 min (**Figure 5.1***B*). Each exercise block comprised 5 min of running at 50% of their maximal cycling power interspersed with 10-s sprints at 100% of their maximal cycling power.

At 15, 30, and 45 min after exercise, participants consumed equal aliquots of a beverage that contained 0 g [control (CON)], 0.76 g [low protein (LP)], or 1.5 g [high protein (HP)] of bovine skim milk protein/100 ml, which subsequently contained both whey and casein protein fractions in a ratio ~1:4. Beverages were also isoenergetic (28 kcal/100 ml) and provided a variable amount of carbohydrate (7.0, 5.3, and 5.3 g/100 ml) and fat (0, 0.46, and 0.1 g/100 ml) for CON, LP, and HP, respectively. The beverages were provided in a volume equal to 150% of the participant's measured fluid loss (based on the change in body mass with 100 g = 100 ml of fluid) during the previous bout of exercise, which, because of interindividual differences in sweat rates, resulted in a variable amount of protein being ingested between each participant. However, because

fluid loss and subsequent beverage intake were similar between crossover trials, this resulted in a graded amount of protein ingested within each participant. Participants then rested comfortably in the lab for 4 h before consuming lunch, which provided ~46 and ~45% of each participant's total daily energy and protein intake, respectively, from food (i.e., not including the experimental beverage intake).

All urine produced within the lab and after the initial morning spot urine was collected, pooled, and stored at 4°C until the following day (see below). Participants were then provided with the remainder of the controlled diet and allowed to leave the laboratory. Participants were provided with 2 urine containers and were instructed to collect all urine produced up to their dinner meal in one container and all urine produced in the evening and overnight fasted period, including the first urination the following morning, in the second container and store at 4°C before returning them to the laboratory the following day. All urine produced prior to the dinner meal (i.e. first urine container) was pooled with the urine collected within the laboratory, and its volume was measured to the nearest ml, after which an aliquot was taken and stored at -80°C prior to analysis; this sample represented the 9-h exercise and early recovery period. Following this, the evening and overnight fasted urine was then pooled with the remaining 9-h sample to generate an aggregate 24-h urine sample, which was measured to the nearest ml prior to taking a second aliquot for storage at -80°C and subsequent analysis; this sample represented the complete 24-h period.

*Controlled diet.* Participants were provided with a controlled diet for the 24-h period during which protein metabolism measures were performed. Resting energy

requirements were estimated using standard equations (21a) and were corrected with an activity factor of 1.5. Based on the exercise-induced fluid loss measured during the familiarization session and assuming a similar fluid loss during all experimental trials, it was estimated that the rehydration beverages (which were all isoenergetic) would provide  $\sim$ 14% of each participant's daily energy. The energy content of the beverages were subsequently added to the food portion of each participant's controlled diet in order to calculate 24-h energy intake. In addition, the protein intake during the 24-h controlled diet was targeted to be  $\sim 1.4$ g/kg/d when participants were ingesting the estimated fluid intake (i.e. 1.5x fluid loss) of the LP beverage (0.76 g/100 ml); hence, due to the doubleblind nature of the trial, protein intakes during the controlled diet were subsequently lower and higher than 1.4  $g \cdot kg^{-1} \cdot d^{-1}$  during the CON and HP trials, respectively. However, all trials were targeted to provide adequate protein (i.e.,  $\geq 0.95$  g·kg<sup>-1</sup>·d<sup>-1</sup>) according to current recommendations (21b). Aside from the energy and macronutrient profiles of the test beverages, the 24-h controlled diets were supplied as isoenergetic breakfast and lunch meals (consumed within the laboratory, providing  $\sim 11$  and 40% of 24-h energy intake, respectively) and dinner meals (consumed outside the laboratory, providing  $\sim$ 35% of 24-h energy intake: Figure 5.1B) with the remaining  $\sim$ 14% of energy coming from the test beverages. The breakfast, lunch, and dinner meals were also isoprotein and provided ~15, 45, and 40% of the 24h food protein intake, respectively, with the test beverages providing a variable amount of protein in addition to the meal protein intake. Participants were instructed not to ingest the dinner meal away from the laboratory until the final 9h urine sample was collected. To estimate habitual dietary intakes, participants completed a 3-d dietary record that was analyzed with The Food Processor SQL (ESHA, Salem, Oregon) software for energy and macronutrient intakes.

Analysis. The concentration of the major nitrogen-containing metabolites urea and creatinine were determined colormetrically by commercially available kits (Quantichrom, Bioassay Systems, USA) as an estimate of urinary nitrogen excretion. The  $[^{15}N]$ enrichments (i.e. ratio of tracer-trace, t/Tr) of urinary ammonia (at baseline, 9 h, and 24 h) and urea (at baseline and 24 h) were determined in duplicate by isotope ratio mass spectrometry by Metabolic Solutions Incorporated (Nashua, NH) to determine whole body nitrogen turnover and protein metabolism (19). Whole body nitrogen turnover (Q) by the  $[^{15}N]$  ammonia end-product was calculated as Q (g N·kg<sup>-1</sup>·h<sup>-1</sup>) = d/corrected t:Tr·t<sup>-</sup> <sup>1</sup>·BM<sup>-1</sup>, where d is the dose of oral  $[^{15}N]$ glycine, corrected t/Tr is the baseline corrected [<sup>15</sup>N] enrichment of urinary ammonia, t is the time (i.e., 9 or 24 h), and BM is the participant's body mass. O using  $[^{15}N]$  urea as the end product was calculated in an identical manner except no correction of time was used (i.e., rates were expressed in units of g N·kg<sup>-1</sup>·d<sup>-1</sup>). Whole body protein synthesis (S) was calculated as S (g protein·kg<sup>-1</sup>·h<sup>-1</sup>) =  $(Q - E)/(t \times BM) \times 6.25$ , where E is measured and estimated nitrogen excretion. Measured nitrogen excretion was the sum of urinary urea nitrogen and creatinine nitrogen excretion over the 9- and 24-h periods, as required. Sweat nitrogen excretion was estimated from estimated fluid loss (i.e., preexercise body mass – postexercise body mass = body mass loss in 100 g; 100 g = 100 ml fluid loss) multiplied by estimated sweat nitrogen and amino concentrations (1) with an average ~15% nitrogen content of amino acids (26). Fecal nitrogen excretion was estimated at 22 mg·kg<sup>-1</sup>·d<sup>-1</sup> (or 0.9 mg·kg<sup>-1</sup>·h<sup>-1</sup>,

as required) according to previously published values in children consuming a 1.2 g protein  $kg^{-1} d^{-1}$  diet (17). Whole body protein breakdown (B) was calculated as B (g protein  $kg^{-1} d^{-1}$ ) = (Q – I)/(t × BM) × 6.25, where I is nitrogen intake provided by the controlled diet and the experimental beverages. S and B using the harmonic mean of Q was calculated using 24-h nitrogen excretion with no correction of time (i.e., rates were expressed in units of g protein  $kg^{-1} d^{-1}$ ). Whole body protein balance (WBPB) was determined as WBPB = S – B.

Statistics. Whole body Q, S, B, and WBPB measured by [<sup>15</sup>N]ammonia end product method were analyzed using a two-way (time × condition) repeated measures ANOVA. Whole body Q, S, B, and WBPB measured by [<sup>15</sup>N]urea end product and the harmonic mean approach were analyzed using a one-way (condition) repeated measures ANOVA. Differences between means for significant main effects or interactions were determined using a Holm-Sidak post-hoc test. To determine if WBPB was significantly different from zero, a paired *t*-test was performed for each condition. Pearson productmoment correlation coefficients were determined for WBPB (determined by [<sup>15</sup>N]ammonia end product method) and energy and protein intake over both 9 and 24 h. Statistical significance was established at P < 0.05, and all data are expressed as mean  $\pm$  SD.

### 5.5. RESULTS

*Beverage macronutrient intake.* Absolute protein intake from the beverages was  $0 \pm 0$ , 7.1  $\pm$  1.5, and 12.8  $\pm$  3.6 g for CON, LP, and HP, respectively. This resulted in a

relative protein intake of  $0 \pm 0$ ,  $0.18 \pm 0.03$ , and  $0.32 \pm 0.07$  g/kg for CON, LP, and HP, respectively. The drinks provided similar amounts of energy ( $268 \pm 70$ ,  $275 \pm 65$ , and  $242 \pm 62$  kcal; CON = LP = HP, respectively; P = 0.38) but differing amounts of carbohydrate ( $67 \pm 17$ ,  $51 \pm 12$ , and  $46 \pm 12$  g; CON > LP = HP, respectively; P < 0.01) and fat ( $0 \pm 0$ ,  $0.9 \pm 0.2$ , and  $4.4 \pm 1.1$  g; CON < HP < LP; P < 0.01).

Dietary macronutrient intake. When the drinks were included in the diets, energy intake over 9 and 24 h was identical for all conditions (Table 5.2). However, there were expected differences in protein intake between conditions (HP > LP > CON, P < 0.001) and subtle differences in carbohydrate and fat intakes for the 9 and 24 h periods (Table Twenty-four-hour controlled diets were similar in energy to the participants' **5.2**). habitual intakes. Moreover, protein intake during the 24-h period for HP was similar to habitual intakes, whereas CON and LP were ~26 and 14% lower, respectively (P < 0.05). Because of a low-protein intake during dinner on the LP trial that was replicated on all other trials, one participant consumed a daily protein intake that was less than the current recommended daily allowance during the CON (~ $0.81 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ ) but not LP or HP trials (1.04 and 1.18  $g \cdot kg^{-1} \cdot d^{-1}$ ). However, this participant was included in all analysis as their exclusion did not alter the results given that, because of the within-subject design, they had the expected graded protein intake over 9 and 24 h. Relative to the habitual dietary intake, carbohydrate intake was 17-25% higher, whereas fat intake was 13-19% lower for all controlled diets (all P < 0.05).

Whole body protein metabolism by  $[^{15}N]$ ammonia end product enrichment. Whole body Q was not different (main effect for condition, P = 0.19; interaction, P = 0.26) between conditions over 9 h (CON =  $59 \pm 21 \text{ mg N} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ; LP =  $53 \pm 14 \text{ mg N} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ; HP =  $64 \pm 23 \text{ mg N} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) and 24h (CON =  $28 \pm 8 \text{ mg N} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ; LP =  $26 \pm 7 \text{ mg}$ N $\cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ; HP =  $31 \pm 9 \text{ mg N} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ). However, there was a significant effect of time (main effect, P < 0.001) with rates of Q being ~50% lower after 24 h as compared to the early 9-h period. Similarly, there were no differences between conditions for S (main effect for condition, P = 0.15; interaction, P = 0.22) or B (main effect for condition, P =0.32; interaction, P = 0.36) during the 9- or 24-h periods (**Figure 5.2**). S and B were ~58 and 55% lower, respectively, during the 24-h measurement period (main effect for time, P< 0.001). Rates of Q, S and B were greater when expressed relative to LBM but revealed similar differences between conditions and across time as when expressed relative to body mass (data not presented).

There was a main effect of condition (P < 0.05) for WBPB with HP being greater than LP and CON (**Figure 5.3***A*); this was due primarily to a significant difference at 9 h (P < 0.01) but not 24 h ( $P \ge 0.13$ ). There was also a trend (P = 0.075) for a greater WBPB at 9 h for LP compared to CON. Regardless of condition, WBPB was greater during the 9-h period compared with the 24-h period (main effect for time, P < 0.001). WBPB was significantly different ( $P \le 0.013$ ) from zero for all conditions at 9 h but was only different (P < 0.05) from zero at 24 h in HP.

When collapsed across conditions, linear trend analysis revealed no relation between the WBPB over 9 h (r = 0.26, P = 0.11) or 24 h (r = 0.05, P = 0.74) and energy intake (data not shown). However, there were expected linear relationships between WBPB and protein intake over 9 h (r = 0.51, P < 0.01) and 24 h (r = 0.33, P < 0.05; Figure 5.3, *B* and *C*, respectively).

Whole body protein metabolism by harmonic mean of  $[^{15}N]$ ammonia and  $[^{15}N]$ urea end product enrichment. Similar to rates calculated by the  $[^{15}N]$ ammonia end product method, there were no differences between conditions for Q (P = 0.40), S (P = 0.27), B (P = 0.63) or WBPB (P = 0.13) calculated by the harmonic mean of urinary  $[^{15}N]$ ammonia and  $[^{15}N]$ urea (**Table 5.3**). WBPB was, however, significantly different from zero for HP (P < 0.05) but not LP (P = 0.83) or CON (P = 0.74).

## Table 5.2. Dietary intakes.

	9-h Intake			24-h Intake			
	CON	LP	HP	CON	LP	HP	24-h Habitual Intake
Energy, kcal/kg	31.5 (4.0)	31.4 (3.2)	31.0 (3.5)	48.7 (5.9)	48.6 (5.1)	48.2 (5.6)	47.1 (11.7)
Carbohydrate, g/kg	5.34 (0.81)a	4.91 (0.71)b	4.83 (0.74)b	8.11 (1.35)a*	7.69 (1.18)b*	7.61 (1.22)b*	6.5 (2.1)
Fat, g/kg	0.82 (0.22)a	0.92 (0.24)b	0.84 (0.22)a	1.29 (0.21)a*	1.39 (0.21)b*	1.31 (0.20)a*	1.6 (0.5)
Protein, g/kg	0.69 (0.11)a	0.87 (0.09)b	1.02 (0.09)c	1.16 (0.15)a*	1.35 (0.12)b*	1.49 (0.12)c	1.56 (0.44)

Dietary intakes over the 9- and 24-h controlled diets include the contribution from the experimental beverages. Twenty-fourhour habitual intake was determined by a 3-d diet log and was only available for nine subjects. Comparisons to the 24-h habitual intake were only performed for the 24-h controlled diet. Means with different letter are significantly different from each other within the respective time period, P < 0.05. CON, control; LP, low protein, HP, high protein. \*Different from 24-h habitual intake, P < 0.05.



Figure 5.2. Whole body protein synthesis (S) (A) and protein breakdown (B) (B) over 9 and 24 h calculated using urinary [ $^{15}N$ ]ammonia end product enrichment. \*\*\*Main effect for time, P < 0.01. CON, control; HP, high protein; LP, low-protein.

### Figure 5.3. Whole body protein balance (WBPB) over 9 and 24 h.

*A*: whole body protein balance (WBPB) over 9 and 24 h calculated using urinary [<sup>15</sup>N]ammonia end product enrichment. *B*: WBPB relative to protein intake over 9 h expressed relative to protein intake. Linear correlation (collapsed across all conditions): r = 0.51, P < 0.01; line of best fit: y = 73.7x - 32.2, *x*-intercept = 0.44 g·kg<sup>-1</sup>·9 h<sup>-1</sup>. *C*: WBPB relative to protein intake over 24 h expressed relative to protein intake. Linear correlation (collapsed across all conditions): r = 0.33, P < 0.05; line of best fit: y = 20.0x - 24.5, *x*-intercept = 1.23 g·kg<sup>-1</sup>·24 h<sup>-1</sup>. \*Significantly different from zero, P < 0.01. \*\*\*Main effect from time, P < 0.001. #Significantly different from CON and LP at 9 h, P < 0.01. Main effect for condition (HP > LP = CON), P < 0.005.



	CON	LP	НР
Q, g N·kg <sup>-1</sup> ·d <sup>-1</sup>	$0.84\pm0.22$	$0.80\pm0.12$	$0.90 \pm 0.22$
S, $g \cdot kg^{-1} \cdot d^{-1}$	$4.16 \pm 1.46$	$3.78\pm0.62$	$4.45 \pm 1.28$
B, $g \cdot kg^{-1} \cdot d^{-1}$	$4.13 \pm 1.42$	$3.71\pm0.82$	$4.18 \pm 1.37$
WBPB, $g \cdot kg^{-1} \cdot d^{-1}$	$-0.03 \pm 0.31$	$0.02 \pm 0.25$	$0.21 \pm 0.28*$

Table 5.3. Twenty-four-hour whole body protein metabolism.

Whole body nitrogen turnover (Q), protein synthesis (S), protein breakdown (B), and net protein balance (WBPB) calculated using the harmonic mean of [<sup>15</sup>N]ammonia and [<sup>15</sup>N]urea as the end-product. \*Significantly different from zero, P < 0.05.

### 5.6. **DISCUSSION**

Adherence to an active lifestyle is associated with greater strength and lean body mass in children (5), which ultimately would necessitate alterations in protein turnover and tissue remodeling that would favor net anabolism. While previous studies have investigated the impact of physical activity on fasted (8, 33) or daily rates (7) of whole body protein metabolism in healthy active children, to the best of our knowledge, we present here the first data to address the cumulative effect of exercise and nutrition. Specifically, we show that postexercise protein ingestion has little effect on whole body protein synthesis and protein breakdown yet induces a dose-dependent increase in WBPB during the early 9-h exercise and recovery period. However, while net protein balance was positive for all conditions during the early 9-h period, ingestion of ~13g of protein immediately after exercise was necessary to maintain a net anabolic environment (i.e., positive protein balance) over the entire 24-h period in our healthy children.

During the early acute (i.e., 9 h) and prolonged (i.e., 24 h) recovery period, there was no measurable effect of protein ingestion on whole body protein synthesis or breakdown. This may be related in part to the relatively small differences in protein ingestion between conditions (~0.3 g/kg at the most) and/or the relatively short duration of the intervention (i.e., 24 h) in the present study. For example, chronic (i.e., 2 wk) ingestion of diets differing in protein intake by ~0.4-0.9 g·kg<sup>-1</sup>·d<sup>-1</sup> has been reported to measurably modify whole body rates of protein turnover as measured by oral [<sup>15</sup>N]glycine in adults (31). Moreover, lower habitual protein intake (by ~0.31 g/kg) in free-living young female gymnasts is associated with attenuated rates of whole body protein

breakdown relative to their non-training peers (7). Therefore, given the relatively small differences in protein intake between conditions, it is possible that if our intervention period persisted for a greater duration of time that rates of whole body protein turnover may eventually diverge between conditions. Alternatively, the sensitivity of oral [<sup>15</sup>N]glycine may have precluded our ability to detect differences in protein turnover with the relatively small acute differences in protein intake between conditions that other methodology, such as intravenous infusions, may have permitted; however, discrepancies between measured rates of whole body protein kinetics by oral [<sup>15</sup>N]glycine and <sup>13</sup>C]leucine infusion are more pronounced with measurements made in the fasted rather than fed state (14). Nevertheless, in general, whole body protein turnover (i.e., both synthesis and breakdown) is higher in children than adults and can be less sensitive to even large fluctuations in protein intake (19), which opens the possibility that even subtle changes in whole body protein synthesis and/or protein breakdown with exercise or nutrition could translate into physiologically more meaningful differences in WBPB due to these rapid rates of tissue remodeling.

In comparison to previously published whole body protein kinetics in healthy children, our rates of protein synthesis and protein breakdown calculated over 9 h appear to be greater than those previously measured in exercise-trained children over a similar time period but in the overnight fasted state (8, 33). In contrast, 24-h rates of whole body protein turnover in the present study were equivalent to those measured in healthy free-living children utilizing similar methods (7); this is likely due to the incorporation of a fed-state period in the previous (7) and present studies, which would be necessary to

elevate rates of protein turnover. In the present study, for example, rates of protein synthesis and protein breakdown as well as WBPB were greatest during the early 9-h exercise and recovery period, which is in agreement with the ability of exercise and protein feeding to increase whole body and muscle protein turnover in adults (11, 14). The overnight fasted period, on the other hand, is characterized by lower rates of turnover and a net catabolism of lean tissue, which likely contributed to the attenuation in protein turnover and the subsequent WBPB when measured over 24 h in the present study. The greater rates of protein synthesis and breakdown consonant with a positive net balance during the early acute recovery period highlights the importance of measuring protein metabolism in the fed state to elucidate the potential synergies between exercise and protein ingestion in children.

Arguably the most important variable for active children who would be experiencing concurrent growth in essentially all lean tissues would be WBPB, which would represent the sum of all anabolic and catabolic pathways. We observed that WBPB was positive during the early acute 9-h recovery period in all conditions, which is not surprising given that at least ~27 g (~0.7 g/kg) of protein was consumed within the breakfast and lunch meals independently of the postexercise beverage. As such, the positive protein balance over 9 h would be a reflection of the fed state anabolism that occurs with protein intake (27). In contrast, 24-h WBPB, which included both the daytime fed and overnight fasted periods, was markedly attenuated such that balance was lower than over 9 h and remained positive only during the HP intake condition. This suggests the fed-state gains in WBPB were generally counterbalanced by the overnight fasted state

losses, which is characteristic of the diurnal pattern of protein metabolism (27, 31) and highlights the importance of maximizing fed-state anabolism to sustain a net positive 24-h protein balance.

In the present study there was a dose response of protein ingestion on WBPB during the early 9-h exercise and recovery period. While it is unclear to what extent changes in WBPB reflect the metabolism within skeletal muscle in active, growing children, this dose response during the acute recovery period in the present study is congruent with previous observations in both young and older adults of a graded stimulation of postexercise muscle protein synthesis with ingested protein (30, 47). Interestingly, maintenance of a net positive WBPB over 24 h occurred only in the condition that consumed  $\sim 0.32$  g/kg of protein after exercise, which is greater than the relative dose of protein that is required to maximally stimulate muscle protein synthesis after exercise in young adults (~0.25 g/kg) (30). Given that skeletal muscle is a major storage reservoir for body amino acids and that the acute postexercise muscle anabolism that occurs with exogenous amino acid ingestion is additive to the cumulative 24-h response in adults (42), it is tempting to speculate the sustained positive WBPB over 24 h with HP was related in part to an optimal postexercise stimulation of muscle protein synthesis with the relatively higher protein ingestion. Indeed, a reasonable agreement between [<sup>15</sup>N]glycine-determined WBPB measured over 12 h and the exercise-induced increase in muscle protein synthesis in adults suggests these rates reflect, to some degree, changes in muscle protein metabolism (2, 29). However, the children in the present study would be experiencing normal somatic growth and, therefore, could be expected to have a positive 24-h WBPB with adequate protein and energy intake. As such, we cannot discount the possibility that the positive WBPB during HP was a reflection of the children consuming a protein intake that was similar to their habitual intake (i.e. ~1.49 vs. 1.56  $g \cdot kg^{-1} \cdot d^{-1}$ , respectively) and in line with recent revised recommendations for protein intake in children (15). In contrast, the neutral balance during CON (~1.16  $g \cdot kg^{-1} \cdot d^{-1}$ ) and LP (~1.35  $g \cdot kg^{-1} \cdot d^{-1}$ ) may have been related, in part, to a marginally lower (yet still adequate according to the current recommended dietary allowance of 0.95  $g \cdot kg^{-1} \cdot d^{-1}$ ) protein intake relative to their habitual dietary pattern, which could have resulted in an attenuated 24-h anabolic response that can occur during the early adaptation to a lower protein intake (34). Ultimately, substantiation of the potential positive synergies of exercise and protein ingestion on muscle protein metabolism in children and the relative importance of consuming said protein during the immediate postexercise period would need to be determined through longer-term studies, given the current limitations in measuring protein turnover in skeletal muscle of healthy children.

It has been highlighted previously that protein source, and not just protein quantity, should be considered when addressing the needs for optimal growth and development in children (35). In the present study, we provided milk proteins as the source of dietary nitrogen in the postexercise beverages given that they are complete, high-quality proteins that are generally associated with greater muscle remodeling and recovery after exercise in adults (32). For example, studies in adults have revealed that milk and associated dairy-based proteins (especially whey) support greater rates of muscle protein synthesis and net protein balance after exercise compared with plant-based

proteins such as soy (38, 46), which can ultimately translate into greater training-induced gains in muscle and lean body mass (20, 44). The greater anabolic effect of dairy compared with soy protein in adults occurs despite these sources having similarly high [albeit artificially truncated (37)] protein quality ratings according to the protein digestibility corrected amino acid score (32). Nevertheless, it is unclear whether protein quality may similarly affect protein anabolism in active, concurrently growing children. As such, it may be premature to suggest that similar elevations of postexercise net protein balance observed in the present study could occur with the ingestion of similar quantities of other protein sources that may be of lower quality (e.g., plant-based) given the complete lack of studies evaluating the effect of protein source, let alone protein quantity, on postexercise recovery in children. Therefore, we echo the sentiments of Rodriguez (35) that additional research is required on this important macronutrient to determine the optimal protein amount and/or composition for active children.

Our decision to utilize [ $^{15}$ N]glycine methodology to measure whole body protein metabolism, which has a long history of use in humans of all ages (14), was related to the ease with which protein kinetics can be measured over relatively long time frames (e.g., up to 24h) (19), the relatively low within-subject variability (16), and, because of the oral ingestion and urinary endpoint analysis, its feasible and ethical application in healthy children. In addition, although urinary nitrogen excretion can account for ~80-85% of all nitrogen loss in active adults (39, 40), we also utilized the measured exercise-induced change in body mass with average sweat nitrogen content (1) to estimate sweat nitrogen excretion, which can contribute ~10% of nitrogen loss in active adults (39) and, if not

accounted for, would subsequently increase the apparent net protein balance (1); this approach, in contrast to previous studies in active children that only considered urinary nitrogen excretion (7, 8, 33), would result in a conservative estimate of WBPB in our study in the absence of the technically and logistically challenging determination of all potential routes of nitrogen loss (e.g., fecal, integumentary, hair, etc.). As such, our study was able to assess the changes in whole body protein synthesis, breakdown, and net protein balance over periods encompassing both the exercise and early recovery (i.e., 9 h) period up to, and including, the entire 24-h day to determine how protein ingestion after exercise modulates these variables in a single cohort of healthy children. The potential limitation to oral tracers, such as [<sup>15</sup>N]glycine, is they represent the net sum of all nitrogen metabolism in the body (e.g., within muscle, splanchnic bed, etc.) and generally have a lower time resolution given the need to adequately collect the metabolic endproduct, which based on differences in pool size is  $\sim 9$  h and  $\sim 24$  h for urinary  $[^{15}N]$ ammonia and  $[^{15}N]$ urea, respectively (19). In addition, the noninvasive study design precluded our ability to measure other potential biological effectors of protein metabolism such as circulating insulin, which can suppress whole body protein breakdown (3) and, provided there was a differential response between conditions, may have also contributed to the changes in WBPB observed herein. Nevertheless, other stable isotope methodologies are available to study whole body protein metabolism such as the primed constant  $[^{13}C]$  leucine infusion (45), which has been utilized successfully in research in children (13) and is arguably the current gold standard methodology (45). Despite the disadvantage of requiring repeated breath and blood samples that is not always feasible in children, the advantages of  $[^{13}C]$  leucine infusion include its preferential metabolism within skeletal muscle, the ability to measure changes in circulating amino acid and insulin concentrations, and the greater time resolution over which changes in protein turnover can be measured. For example, utilization of non-steady-state kinetics can permit the measurement of changes in leucine oxidation, leucine rate of appearance (marker of protein breakdown) and nonoxidative leucine disposal (marker of protein synthesis) over 20-30min intervals (6), which makes it ideally suited to determine the effect of a single nutrition intervention (e.g., protein-containing beverage similar to that used in the present study) on acute protein metabolism. Therefore, given the need to better understand population-specific protein requirements (35), future studies in active children should endeavour, where possible, to utilize a variety of tracer methodologies to more accurately determine the effect of nutrition on the early (e.g., 0-4 h) and later (e.g., 6-24 h) postexercise recovery periods. Additionally, inclusion of non-exercise days would help advance our understanding of the potential interactive effects of physical activity and nutrition on protein metabolism in children.

In summary, we report here the first study to address the effects of graded postexercise protein ingestion on whole body protein metabolism in active healthy children. Elevated rates of protein turnover during the early exercise and recovery period are congruent with an increased remodeling of lean body tissues that is well-characterized in adult populations (11). However, despite the presence of an ingested protein doseresponse over the early exercise and 9-h recovery period between conditions, a sustained 24-h net positive WBPB was only observed with HP, suggesting a potential threshold is required to obtain a benefit of postexercise protein ingestion over the entire day in healthy children. Future studies should evaluate whether the acute elevation of a positive WBPB over 24 h observed in the present study would be sustained over more chronic periods of weeks to months, which ultimately would be a prerequisite for the remodeling and growth of lean tissue in active healthy children. Moreover, utilization of alternate stable isotope methodologies (e.g., [<sup>13</sup>C]leucine infusion) that may have a greater sensitivity and permit the determination of other biological effects of protein metabolism (e.g., blood amino acid and insulin concentrations) would be helpful in confirming the conclusions reached in this preliminary study around the importance of postactivity protein ingestion to enhance WBPB in children. Finally, leveraging the present results to pediatric populations whose growth may be impaired by chronic disease represents a fruitful area of further study.

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# 5.9. DISCLOSURES

Elizabeth Offord-Cavin is currently employed by Nestle Research Centre, which funded this study. When the study was completed, Daniel Moore was also employed by Nestle Research Centre. Dr. Moore is now employed by the University of Toronto.

#### **5.10. REFERENCES**

- 1. Alexiou D, Anagnostopoulos A and Papadatos C. Total free amino acids, ammonia, and protein in the sweat of children. *Am J Clin Nutr* 32: 750-752, 1979.
- Areta JL, Burke LM, Ross ML, Camera DM, West DW, Broad EM, Jeacocke NA, Moore DR, Stellingwerff T, Phillips SM, Hawley JA and Coffey VG. Timing and distribution of protein ingestion during prolonged recovery from resistance exercise alters myofibrillar protein synthesis. *J Physiol* 591: 2319-2331, 2013.
- 3. Arslanian SA and Kalhan SC. Protein turnover during puberty in normal children. *Am J Physiol* 270:E79-84, 1996.
- Baxter-Jones AD, Eisenmann JC, Mirwald RL, Faulkner RA and Bailey DA. The influence of physical activity on lean mass accrual during adolescence: a longitudinal analysis. *J Appl Physiol* 105: 734-741, 2008.
- Boirie Y, Dangin M, Gachon P, Vasson MP, Maubois JL and Beaufrere B. Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proc Natl Acad Sci U S A* 94: 14930-14935, 1997.
- Boisseau N, Persaud C, Jackson AA and Poortmans JR. Training does not affect protein turnover in pre- and early pubertal female gymnasts. *Eur J Appl Physiol* 94: 262-267, 2005.
- 8. Bolster DR, Pikosky MA, McCarthy LM and Rodriguez NR. Exercise affects protein utilization in healthy children. *J Nutr* 131: 2659-2663, 2001.

- Borsheim E, Tipton KD, Wolf SE and Wolfe RR. Essential amino acids and muscle protein recovery from resistance exercise. *Am J Physiol Endocrinol Metab* 283: E648-E657, 2002.
- Bundle MW, Hoyt RW and Weyand PG. High-speed running performance: a new approach to assessment and prediction. *J Appl Physiol (1985 )* 95: 1955-1962, 2003.
- Burd NA, Tang JE, Moore DR and Phillips SM. Exercise training and protein metabolism: influences of contraction, protein intake, and sex-based differences. J Appl Physiol 106: 1692-1701, 2009.
- Burd NA, West DW, Moore DR, Atherton PJ, Staples AW, Prior T, Tang JE, Rennie MJ, Baker SK and Phillips SM. Enhanced amino acid sensitivity of myofibrillar protein synthesis persists for up to 24 h after resistance exercise in young men. *J Nutr* 141: 568-573, 2011.
- Darmaun D and Mauras N. Use of stable isotopes to assess protein and amino acid metabolism in children and adolescents: a brief review. *Horm Res* 64 Suppl 3: 32-37, 2005.
- Duggleby SL and Waterlow JC. The end-product method of measuring wholebody protein turnover: a review of published results and a comparison with those obtained by leucine infusion. *Br J Nutr* 94: 141-153, 2005.
- Elango R, Humayun MA, Ball RO and Pencharz PB. Protein requirement of healthy school-age children determined by the indicator amino acid oxidation method. *Am J Clin Nutr* 94: 1545-1552, 2011.

- Fern EB, Garlick PJ, Sheppard HG and Fern M. The precision of measuring the rate of whole-body nitrogen flux and protein synthesis in man with a single dose of [15N]-glycine. *Hum Nutr Clin Nutr* 38: 63-73, 1984.
- Gattas V, Barrera GA, Riumallo JS and Uauy R. Protein-energy requirements of prepubertal school-age boys determined by using the nitrogen-balance response to a mixed-protein diet. *Am J Clin Nutr* 52: 1037-1042, 1990.
- Goulding A, Taylor RW, Grant AM, Jones S, Taylor BJ and Williams SM. Relationships of appendicular LMI and total body LMI to bone mass and physical activity levels in a birth cohort of New Zealand five-year olds. *Bone* 45: 455-459, 2009.
- Grove G and Jackson AA. Measurement of protein turnover in normal man using the end-product method with oral [15N]glycine: comparison of single-dose and intermittent-dose regimens. *Br J Nutr* 74: 491-507, 1995.
- Hartman JW, Tang JE, Wilkinson SB, Tarnopolsky MA, Lawrence RL, Fullerton AV and Phillips SM. Consumption of fat-free fluid milk after resistance exercise promotes greater lean mass accretion than does consumption of soy or carbohydrate in young, novice, male weightlifters. *Am J Clin Nutr* 86: 373-381, 2007.
- 21. Jacob JA and Nair MK. Protein and Micronutrient Supplementation in Complementing Pubertal Growth. *Indian J Pediatr* 2011.
- Joint WHO/FAO/UNU Expert Consultation. Energy and protein requirements. Tech. Rep. Ser. Geneva: World Health Organization, 1985, vol. 724.

- 21b. Joint WHO/FAO/UNU Expert Consultation. Protein and amino acid requirements in human nutrition. Tech Rep Ser. Geneva: World Health Organization, 2007, vol. 935.
- Kuczmarski RJ, Ogden CL, Guo SS, Grummer-Strawn LM, Flegal KM, Mei Z, Wei R, Curtin LR, Roche AF and Johnson CL. 2000 CDC Growth Charts for the United States: methods and development. *Vital Health Stat 11* 1-190, 2002.
- Levenhagen DK, Carr C, Carlson MG, Maron DJ, Borel MJ and Flakoll PJ. Postexercise protein intake enhances whole-body and leg protein accretion in humans. *Med Sci Sports Exerc* 34: 828-837, 2002.
- Levenhagen DK, Gresham JD, Carlson MG, Maron DJ, Borel MJ and Flakoll PJ. Postexercise nutrient intake timing in humans is critical to recovery of leg glucose and protein homeostasis. *Am J Physiol Endocrinol Metab* 280: E982-E993, 2001.
- 25. Lunn WR, Pasiakos SM, Colletto MR, Karfonta KE, Carbone JW, Anderson JM and Rodriguez NR. Chocolate milk and endurance exercise recovery: protein balance, glycogen, and performance. *Med Sci Sports Exerc* 44: 682-691, 2012.
- Miller, L. and Houghton, J. A. The micro-Kjeldahl determination of the nitrogen content of amino acids and proteins. J.Biol.Chem. 159, 373-383. 1945.
- Millward DJ, Price GM, Pacy PJ, Quevedo RM and Halliday D. The nutritional sensitivity of the diurnal cycling of body protein enables protein deposition to be measured in subjects at nitrogen equilibrium. *Clin Nutr* 10: 239-244, 1991.

- Mirwald RL, Baxter-Jones AD, Bailey DA and Beunen GP. An assessment of maturity from anthropometric measurements. *Med Sci Sports Exerc* 34: 689-694, 2002.
- 29. Moore DR, Areta J, Coffey VG, Stellingwerff T, Phillips SM, Burke LM, Cleroux M, Godin JP and Hawley JA. Daytime pattern of postexercise protein intake affects whole-body protein turnover in resistance-trained males. *Nutr Metab* (Lond) 9: 91, 2012.
- Moore DR, Robinson MJ, Fry JL, Tang JE, Glover EI, Wilkinson SB, Prior T, Tarnopolsky MA and Phillips SM. Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. *Am J Clin Nutr* 89: 161-168, 2009.
- Pacy PJ, Price GM, Halliday D, Quevedo MR and Millward DJ. Nitrogen homeostasis in man: the diurnal responses of protein synthesis and degradation and amino acid oxidation to diets with increasing protein intakes. *Clin Sci (Lond)* 86: 103-116, 1994.
- 32. Phillips SM, Tang JE and Moore DR. The role of milk- and soy-based protein in support of muscle protein synthesis and muscle protein accretion in young and elderly persons. *J Am Coll Nutr* 28: 343-354, 2009.
- Pikosky M, Faigenbaum A, Westcott W and Rodriguez N. Effects of resistance training on protein utilization in healthy children. *Med Sci Sports Exerc* 34: 820-827, 2002.
- 34. Quevedo MR, Price GM, Halliday D, Pacy PJ and Millward DJ. Nitrogen homoeostasis in man: diurnal changes in nitrogen excretion, leucine oxidation and
whole body leucine kinetics during a reduction from a high to a moderate protein intake. *Clin Sci (Lond)* 86: 185-193, 1994.

- 35. **Rodriguez NR**. Optimal quantity and composition of protein for growing children. *J Am Coll Nutr* 24: 150S-154S, 2005.
- 36. Roy BD, Luttmer K, Bosman MJ and Tarnopolsky MA. The influence of postexercise macronutrient intake on energy balance and protein metabolism in active females participating in endurance training. *Int J Sport Nutr Exerc Metab* 12: 172-188, 2002.
- Schaafsma G. The protein digestibility-corrected amino acid score. J Nutr 130: 1865S-1867S, 2000.
- 38. Tang JE, Moore DR, Kujbida GW, Tarnopolsky MA and Phillips SM. Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein synthesis at rest and following resistance exercise in young men. J Appl Physiol 107: 987-992, 2009.
- Tarnopolsky MA, Atkinson SA, MacDougall JD, Chesley A, Phillips S and Schwarcz HP. Evaluation of protein requirements for trained strength athletes. J Appl Physiol 73: 1986-1995, 1992.
- Tarnopolsky MA, MacDougall JD and Atkinson SA. Influence of protein intake and training status on nitrogen balance and lean body mass. *J Appl Physiol* 64: 187-193, 1988.

- Timmons BW, Tarnopolsky MA, Snider DP and Bar-Or O. Immunological changes in response to exercise: influence of age, puberty, and gender. *Med Sci Sports Exerc* 38: 293-304, 2006.
- 42. Tipton KD, Borsheim E, Wolf SE, Sanford AP and Wolfe RR. Acute response of net muscle protein balance reflects 24-h balance after exercise and amino acid ingestion. *Am J Physiol Endocrinol Metab* 284: E76-E89, 2003.
- 43. Tobias JH, Steer CD, Mattocks CG, Riddoch C and Ness AR. Habitual levels of physical activity influence bone mass in 11-year-old children from the United Kingdom: findings from a large population-based cohort. *J Bone Miner Res* 22: 101-109, 2007.
- 44. Volek JS, Volk BM, Gomez AL, Kunces LJ, Kupchak BR, Freidenreich DJ, Aristizabal JC, Saenz C, Dunn-Lewis C, Ballard KD, Quann EE, Kawiecki DL, Flanagan SD, Comstock BA, Fragala MS, Earp JE, Fernandez ML, Bruno RS, Ptolemy AS, Kellogg MD, Maresh CM and Kraemer WJ. Whey protein supplementation during resistance training augments lean body mass. J Am Coll Nutr 32: 122-135, 2013.
- 45. Wagenmakers AJ. Tracers to investigate protein and amino acid metabolism in human subjects. *Proc Nutr Soc* 58: 987-1000, 1999.
- 46. Wilkinson SB, Tarnopolsky MA, Macdonald MJ, Macdonald JR, Armstrong D and Phillips SM. Consumption of fluid skim milk promotes greater muscle protein accretion after resistance exercise than does consumption of an isonitrogenous and isoenergetic soy-protein beverage. *Am J Clin Nutr* 85: 1031-1040, 2007.

47. Yang Y, Breen L, Burd NA, Hector AJ, Churchward-Venne TA, Josse AR, Tarnopolsky MA and Phillips SM. Resistance exercise enhances myofibrillar protein synthesis with graded intakes of whey protein in older men. *Br J Nutr* 1-9, 2012.

# CHAPTER 6 : Dose-Response and Timing of Post-Activity Protein Ingestion on Whole Body Protein Balance and Leucine Metabolism in Children

## 6.1. FOREWORD

For this study, **Objectives #3** and **#4** of this thesis were addressed. This study used the  $[1-^{13}C]$ leucine infusion technique in healthy, active children to assess how postexercise protein intake alters whole body Leu<sub>BAL</sub>. This study demonstrated that the postexercise ingestion of protein acutely increased whole body Leu<sub>BAL</sub> in healthy children in a dose-dependent manner over 3 h following exercise. This study also investigated whether the timing of protein intake after exercise (immediate vs. delayed) had implications on WBPB. Although larger protein intakes (~0.22 g·kg<sup>-1</sup> body mass) are required to attain a net anabolic environment over an acute 6-h exercise and recovery period, ingestion of ~0.35 g·kg<sup>-1</sup> may not necessarily be sufficient to induce a positive WBPB over 24-h. Instead, a periodized protein intake with multiple protein feedings during post-exercise recovery may be more important than the absolute amount of protein ingested.

The authors are the following: Kimberly A. Volterman, Daniel R. Moore, Peter Breithaupt, Elizabeth Offord-Cavin, and Brian W. Timmons. The corresponding author is Dr. Brian W. Timmons. Daniel R. Moore, Elizabeth Offord-Cavin and Brian W. Timmons designed the study. Kimberly A. Volterman and Peter Breithaupt carried out the study. Kimberly A. Volterman and Daniel R. Moore conducted laboratory analyses. Kimberly A. Volterman performed the statistical analyses, interpreted the data, and wrote the manuscript with assistance from Daniel R. Moore and Brian W. Timmons.

# 6.2. ABSTRACT

Post-exercise protein ingestion acutely increases net protein balance early in recovery in an apparent dose-dependent manner in healthy children. Utilization of alternate stable isotope methodologies (e.g.,  $[^{13}C]$  leucine infusion) that have a greater sensitivity are necessary to confirm this dose-response relationship. In addition, a greater understanding of the importance of post-activity protein ingestion to enhance whole body protein balance (WBPB) in children is necessary. Moreover, no study has specifically investigated the optimal timing (i.e., immediately post-exercise) of post-exercise protein ingestion in order to maximize WBPB in children. Thirty-five active children (26 males; 9-13 yrs old) underwent a 5-day adaptation diet with a targeted protein ingestion of 0.95  $g \cdot kg^{-1} \cdot d^{-1}$ . On a subsequent intervention day they consumed an oral dose of [<sup>15</sup>N]glycine and had two indwelling catheters inserted for constant infusion of  $[^{13}C]$  leucine and associated blood samples, prior to performing a bout of exercise  $(3 \times 20 \text{ min of cycling})$ . Immediately after exercise, participants consumed an isoenergetic mixed-macronutrient beverage containing a variable amount of protein [0, 5, 10 and 15 g for control (CONT), low-protein (LP), moderate-protein (MP) and high-protein (HP), respectively] enriched with  $[^{2}H_{3}]$  leucine to a level of 4 % of beverage leucine content. Whole body net leucine balance (Leu<sub>BAL</sub>) was significantly different between CONT and all protein conditions (P < 0.001). One-way ANOVA showed a main effect for condition for absolute and relative Leu<sub>BAL</sub> (P < 0.001 for both): HP > MP > LP > CONT (all P < 0.01). Over 24 h, there were no differences between conditions for protein turnover (P = 0.64), synthesis (P =0.77), or breakdown (P = 0.64) as calculated by the harmonic mean of urinary [<sup>15</sup>N]ammonia and [<sup>15</sup>N]urea. WBPB demonstrated a main effect of condition (P < 0.05), with LP being greater than HP (P < 0.05). 24 h WBPB in LP was significantly greater than zero (P < 0.05). Whole body net Leu<sub>BAL</sub> was increased acutely during 3 h of recovery by the post-exercise ingestion of protein in a dose-dependent manner. However, a net positive WBPB was sustained over 24 h only with consumption of protein both immediately and at 3 h (i.e., 5 and 10 g, respectively) during recovery.

**Clinical trial registration #:** NCT01598935

# 6.3. INTRODUCTION

Childhood is characterized by periods of normal and accelerated growth; this is evident in the normal growth velocity of ~ 5 cm and ~ 3 kg per year up to ~ 10 years of age, which may then increase up to 3-fold during the early pubertal growth spurt (Tanner and Whitehouse 1976). Two important contributors to optimal growth and development during childhood are proper nutrition (Rodriguez 2005) and exercise (Tobias et al. 2007). Dietary protein, in particular, is important as it provides the substrates to support the remodeling and growth of lean tissues (i.e., muscle) during this time (Rodriguez 2005). Unfortunately, we know very little about the interactive effects of protein and exercise during the pediatric years.

Due to the rapid rates of tissue remodeling and higher rates of whole body protein turnover (both protein synthesis and protein breakdown) during periods of growth, children have an increased basal need for dietary protein above that of adults (Meyer et al. 2007). Currently, however, the recommendations for dietary protein in children (ages 9-13 years) are similar to that in healthy adults (0.95 vs. 0.80 g·kg<sup>-1</sup>·d<sup>-1</sup>, respectively) and are assumed to be adequate to ensure a positive nitrogen balance (N<sub>BAL</sub>) for optimal growth in all children (World Health Organization et al. 2007). As exercise provides a stimulus for muscle and bone growth, and can increase the use of amino acids as fuels, it is unclear if these recommendations are appropriate for active children. Moreover, these general daily recommendations provide no guidance with respect to requirements to optimize protein metabolism following a single bout of acute exercise.

We have previously investigated the cumulative effects of exercise and nutrition on whole body protein metabolism in healthy, active children and demonstrated that postexercise protein ingestion induced a dose-dependent increase in whole body protein balance (WBPB) over 9 h following a single bout of cycling exercise (Moore et al. 2014b). Despite the early dose-response, an intake of ~13 g (or ~0.32 g  $\cdot$  kg<sup>-1</sup>) of protein immediately following exercise was required to maintain a positive WBPB over an entire 24-h period. Interestingly, this dose is greater than the relative dose of protein to maximally stimulate MPS after exercise in young adults (~0.25 g·kg<sup>-1</sup>) (Moore et al. 2009). Although we previously demonstrated that the consumption of varying amounts of protein following exercise had little effect on whole body protein synthesis and breakdown (Moore et al. 2014b), it is possible that the sensitivity of the oral [<sup>15</sup>N]glycine used to measure WBPB may have precluded the detection of differences in protein turnover with the relatively small acute differences in protein intake between conditions. Therefore, utilization of alternate stable isotope methodologies (e.g.,  $[^{13}C]$  leucine infusion) that may have a greater sensitivity would be helpful in further understanding the importance of post-exercise protein ingestion to enhance WBPB in children.

Another key issue unresolved in the pediatric literature is the importance of timing of protein intake relative to exercise. In adults, delaying protein intake by ~3 h attenuates the post-exercise recovery process (Levenhagen et al. 2001). In contrast, consuming a dietary source of amino acids immediately after exercise results in a greater increase in MPS and muscle protein balance that is additive to the cumulative 24-h response (Tipton et al. 2003), indicating protein accretion occurring during the early recovery period is not

compensated for later in the day. When performed chronically, this ultimately translates into greater muscle growth and adaptations with training (Hartman et al. 2007). This adult literature demonstrates the critical importance of early post-exercise protein ingestion for optimizing muscle recovery. While these findings are likely to translate to the pediatric population, there is currently no data available on the optimal timing (i.e., immediately post-exercise) of protein ingestion following exercise in order to maximize WBPB in children (Meyer et al. 2007).

A better understanding of pediatric-specific protein requirements for the postexercise period is critical for providing seminal nutritional advice to active children, so as to support proper recovery from exercise and the growth of muscle and lean tissue. Therefore, the primary objective of this study was to examine the effects of ingesting variable amounts of protein post-exercise on changes in whole body leucine balance (Leu<sub>BAL</sub>) as measured by a primed constant infusion of  $[1-^{13}C]$ leucine over a 3-h period. The secondary objectives of this study were to determine postprandial changes in whole body leucine kinetics after a single mixed-protein meal with varying levels of protein and whether the timing of protein intake in relation to exercise (i.e., immediate vs. delayed) influenced WBPB and nitrogen balance (N<sub>BAL</sub>) in children.

## 6.4. METHODS

*Participants.* 36 children (26 males) volunteered to participate in the study; one female participant withdrew from the study prior to completion due to a refusal to eat the required controlled study diet. All participants were healthy and recreationally active as

determined by medical and activity questionnaire. Additional inclusion criteria required participants to be free of any existing medical condition, between the ages of 10-12 years, within 2 years from peak height velocity (YPHV) (Mirwald et al. 2002), and have a minimum aerobic fitness of 35 ml·kg<sup>-1</sup>·min<sup>-1</sup> as assessed by the McMaster All-Out Progressive Continuous Cycling Protocol. Participants were excluded based on the following criteria: current use of medication, food allergy to milk proteins (e.g., whey or casein), and current participation or having participated in another nutritional-clinical trial within 2 months of the current study. This study was approved by the Faculty of Health Sciences/Hamilton Health Sciences Research Ethics Board. Each participant provided written informed assent and written informed consent was obtained from each parent prior to enrolment in the study.

*General overview.* Participants were enrolled in the study for a total duration of  $\sim$ 3 wk whereby they reported to the laboratory on 3 separate occasions: a preliminary visit, a familiarization visit, and an exercise intervention visit. Participants were first provided with a 5-d energy and macronutrient-matched controlled diet to allow their body to adapt to a protein intake of 0.95 g protein·kg<sup>-1</sup>·d<sup>-1</sup> (the DRI for protein). Following this adaptation phase, participants completed the intervention phase. The intervention visits were conducted in a randomized, double-blind, parallel four group design. Participants were randomly allocated to one of the four different parallel intervention groups (control, CONT; low protein, LP; moderate protein, MP; high protein, HP). During the exercise intervention visit, participants continued to consume the controlled diet but also performed a bout of exercise in the morning. Participants orally ingested an isoenergetic

milk-based protein beverage containing a variable amount of protein (at a dose of 0, 5, 10 or 15 g) with additional carbohydrate (CHO) within 15 min (beverage 1; B1) and a beverage containing reciprocal amounts of protein at 4 h (beverage 2; B2) following exercise completion. To assess our primary objective, changes in whole body leucine kinetics were measured over 3 h after exercise and ingestion of B1 by a primed constant infusion of [1-<sup>13</sup>C]leucine on the exercise intervention day. Participants also ingested an oral [<sup>15</sup>N]glycine tracer (to measure whole body protein turnover) on the exercise intervention day, and collected all urine over the subsequent 24-h period (to measure 24-h N<sub>BAL</sub> and WBPB).

*Preliminary visit.* Participants completed a preliminary session during which height (Harpenden wall-mounted Stadiometer), body mass (BWB-800, Tanita Corp., Japan), percent body fat (% BF) and lean body mass (InBody520 bioelectrical impedance analyzer; Biospace Co., California, USA for both), body mass index (BMI), chronological age and maturity offset were determined. Maturity offset was calculated as YPHV (Mirwald et al. 2002). To measure aerobic fitness, peak oxygen uptake (VO<sub>2peak</sub>) was then assessed on a cycle ergometer (Lode Corival, The Netherlands), as previously described (Timmons et al. 2006). Participants performed each of their subsequent exercise sessions on the same cycle ergometer as their aerobic fitness test. Physical characteristics of the participants are presented in **Table 6.1**.

	CONT	LP	MP	HP
N (males)	10 (7)	8 (6)	9 (7)	8 (6)
Age (y)	12.4 (1.2)	12.4 (0.9)	12.8 (1.0)	12.1 (1.2)
Height (cm)	154.2 (11.5)	156.5 (10.6)	159.6 (9.7)	155.9 (12.2)
Height (percentile)	58.1 (28.9)	68.8 (32.7)	63.7 (35.5)	70.3 (20.9)
Body mass (kg)	41.8 (9.6)	44.2 (9.3)	46.8 (9.8)	48.2 (14.7)
Body mass (percentile)	40.8 (24.1)	51.8 (30.1)	54.0 (33.3)	63.4 (26.4)
BMI (kg·m <sup>-2</sup> )	17.4 (1.7)	17.9 (2.5)	18.2 (2.0)	19.5 (3.6)
BMI (percentile)	34.8 (20.6)	45.0 (33.5)	45.8 (28.7)	57.6 (30.6)
Lean body mass (kg)	36.9 (8.5)	38.0 (8.0)	41.5 (10.2)	39.7 (10.6)
Body fat (percent) <sup>1</sup>	11.8 (4.7)	14.2 (5.6)	11.5 (5.3)	16.6 (10.4)
Maturity offset <sup>2</sup>	1.0 (1.1)	1.0 (1.3)	0.6 (1.2)	1.1 (1.2)
VO <sub>2peak</sub> (ml·kg <sup>-1</sup> ·min <sup>-1</sup> )	44.1 (5.3)	44.7 (7.0)	45.3 (7.2)	40.5 (5.3)
RMR (kcal·d <sup>-1</sup> )	1455.1 (288.3)	1292.4 (183.4)	1433.1 (185.0)	1453.6 (258.8)
RMR x activity (kcal·d <sup>-1</sup> )	2182.7 (432.5)	2088.6 (275.1)	2149.7 (277.4)	2180.4 (388.1)

## Table 6.1. Participant characteristics.

BMI, body mass index; VO<sub>2peak</sub>, peak oxygen uptake; RMR, resting metabolic rate. Data are presented as mean (SD). <sup>1</sup> Determined using bioelectrical impedance analysis as described (Specker and Vukovich 2007). <sup>2</sup> Maturity offset defined as the difference between chronological age and estimated age of peak height velocity, the latter of which was calculated as previously described (Mirwald et al. 2002). Percentile rankings relative to 2000 CDC growth charts for the United States (Kuczmarski et al. 2002).

*Familiarization visit.* The purpose of this visit was to: 1) determine individual resting metabolic rate (RMR); 2) acquire baseline blood samples; 3) familiarize the participants with the exercise protocol and equipment; 4) confirm appropriate exercise intensities; and 5) ensure participants could successfully complete the exercise. Participants reported to the lab (~0730 h) in an overnight fasted condition. Participants then rested in a quiet room for 10 min while individual RMR was assessed using a calibrated metabolic cart (Vmax 29, SensorMedics, Yorba Linda, CA, U.S.A.) and an appropriately sized canopy. Participants then consumed a small liquid meal (Boost Meal Replacement, Nestle Canada Inc., North York, Ont. Canada) as a light breakfast as well as a minimum of 5 ml·kg<sup>-1</sup> of water and rested comfortably for 1 h prior to beginning the exercise protocol (using the same exercise as during the exercise intervention visit, described below). Upon completion of the familiarization session, participants were randomized into groups (CONT, LP, MP, or HP). To characterize habitual physical activity levels, participants were then asked to wear an accelerometer (ActiGraph GT1M or GT3X; AtiGraph, Pensacola, Florida) for 3 d. Data were analyzed using a custommade Microsoft Excel-based Visual Basic data reduction program (Microsoft Corp, Redmond, Washington) to assess total and moderate-to-vigorous physical activity (MVPA) levels, as previously reported (Obeid et al. 2014) using the cut points developed by Evenson et al. (2008).

*Exercise intervention visit.* For an overview of the exercise intervention visit, see **Figure 6.1**. Participants reported to the laboratory (~0730 h) after an overnight fast, voided their bladders and provided a spot urine sample. The urine sample was collected

and analyzed for background [<sup>15</sup>N]urea and ammonia enrichment. Any urine produced while in the laboratory was collected, and an aliquot taken for the 6-h exercise and acute recovery period (details on urine collection are described in a subsequent section). The remaining urine was stored at 4°C, and was subsequently combined with the urine collected outside of the laboratory on this day. Following the spot urine sample, participants consumed a small breakfast consisting of ~24% and ~22% of total daily energy and protein intake, respectively, and at least 5 mL·kg<sup>-1</sup> body weight of water, including 2 mg·kg<sup>-1</sup> body weight of [<sup>15</sup>N]glycine dissolved in the beverage provided.

Thirty min prior to beginning the exercise protocol, a venous catheter was placed in the antecubital vein of one arm for blood sampling and was kept patent with periodic flushing with 2-3 mL of saline. A second venous catheter was inserted in the contralateral arm for a priming dose of  $[1^{-13}C]$ leucine (7.6  $\mu$ mol·kg<sup>-1</sup>, 99 atoms %; Cambridge Isotopes) and NaH<sup>13</sup>CO<sub>3</sub> (2.35  $\mu$ mol·kg<sup>-1</sup>) prior to the initiation of the constant infusion of  $[1^{-13}C]$ leucine (7.6  $\mu$ mol·kg<sup>-1</sup>·h<sup>-1</sup>). Body weight was measured prior to exercise and during the rest period between exercise blocks and participants were provided with water to ensure adequate hydration (i.e., 100 mg body weight change = 100 mL fluid). Participants then began the exercise protocol, which consisted of three blocks of 20 min of exercise separated by 5 min of rest between blocks. Each exercise block consisted of 20 min of cycling at 50% of their previously determined VO<sub>2peak</sub>, with 10-s sprints at the workload corresponding to 100% VO<sub>2peak</sub> interspersed every 110-s between min 5 and 15 of exercise.



# Figure 6.1. General overview of exercise intervention visit.

Upon completing the exercise, participants rested comfortably for the remainder of the infusion. At 15 min post-exercise, a blood (10 mL) and breath sample were taken prior to the ingestion of the randomly assigned experimental beverage (B1). Participants were then instructed to consume the beverage within 5 min. B1 was provided as a single 250 ml serving that contained 0 g (CONT), 5 g (LP), 10 g (MP), or 15 g (HP) of bovine skim milk protein (subsequently containing both whey and casein protein fractions in a ratio ~1:4, respectively). Beverages were isoenergetic (~140 kcal) and provided a variable amount of carbohydrate (sucrose). Beverage characteristics are provided in **Table 6.2**.

Participants then rested comfortably in the lab for 3 h and additional breath and blood samples were collected at 30, 60, 90, 120 and 180 min after ingestion of B1 (i.e., 45, 75, 105, 135, and 195 min after exercise completion) to determine fed state leucine kinetics following a single meal, as described below. After the final samples were collected, the infusion was complete and the catheters were removed. At 225 min after B1 (i.e., 240 min after exercise), participants voided their bladders completely and the second intervention beverage (B2) was consumed and a lunch was provided. Again, participants were instructed to consume the beverage within 5 min. B2 was also provided as a single 250 ml serving and, depending on group assignment, contained reciprocal amounts of protein to B1, which provided a combined macronutrient and energy total of 15 g of protein, 55 g of CHO, and 280 kcal of energy over the 4-h post-exercise period (see **Table 6.2**). Participants were then allowed to return home, were provided with the remainder of the daily pre-packaged controlled diet and instructed to maintain habitual

levels of physical activity. They were provided with a container to collect all urine produced during the remainder of the day until the first urination the following morning (inclusive), and instructed to store this at 4°C; this urine was combined with the urine collected during the 6-h in lab period (i.e., exercise and 4-h recovery) and an aliquot was taken for the 24-h sample.

	B1		B2	
	PRO	СНО	PRO	СНО
CONT	0 g	35 g	15 g	20 g
LP	5 g	30 g	10 g	25 g
MP	10 g	25 g	5 g	30 g
HP	15 g	20 g	0 g	35 g

 Table 6.2. Composition of experimental beverages.

B1, Beverage 1; B2, Beverage 2; PRO, Protein; CHO, Carbohydrate. Primary outcome determined by B1. B2 used to maintain constant daily protein intake and to investigate the impact of protein timing on whole body protein balance (WBPB) and nitrogen balance (N<sub>BAL</sub>). Thus, total nutrition over 4 h after activity provided by the experimental beverages was 15 g of PRO, 55 g of CHO, and 280 kcal of energy. This is roughly equivalent to 250 mL of skim milk, 25 g granola bar, and 100 g of fat-free yogurt (~14.5 g PRO, ~42 g CHO, and ~270 kcal energy).

*Beverage preparation.* All beverages were developed and provided by Nestec in powder form. Beverages were reconstituted in deionized water to a fixed volume of 250 mL the evening prior to the trials and kept chilled at 4°C until ingestion. B1 was also enriched with  $[^{2}H_{3}]$ leucine to a level of 4% of total leucine content of the beverage (assuming milk protein is ~10% leucine) to provide an estimate of exogenous leucine appearance from the dietary amino acids.

*Controlled diet.* During the adaptation and intervention phases of the trial, participants were provided with a controlled diet providing 0.95 g protein·kg<sup>-1</sup>·d<sup>-1</sup>. Energy intake was calculated using the habitual activity levels of each participant (measured by accelerometer) and was varied throughout the trial to ensure energy balance was approximately neutral. For dietary compliance, all food was provided to the participants at their home in prepackaged containers for each day of the study and participants/guardians confirmed the ingestion of each food item during the day by selecting the item from a provided checklist upon eating. Participants/guardians were instructed that no food or drink (except water) other than what was provided was to be ingested during the controlled diet period. All food containers were kept and returned to the laboratory to ensure consumption, and any food item that was uneaten during the controlled diet period (and subsequently returned) was recorded and its nitrogen content was subtracted from the daily nitrogen intake.

*Breath samples.* For each breath sample, total VCO<sub>2</sub> was measured by indirect calorimetry over 10 min. Over this same sampling period, breath was collected directly from the breathing line into a 10 mL evacuated tube for determining breath  ${}^{13}CO_2/{}^{12}CO_2$ 

ratio by gas chromatography combustion-isotope ratio mass spectrometry (IRMS; BreathMat Plus, Finnigan MAT GmbH) by Metabolic Solutions Incorporated (Nashua, New Hampshire, USA).

*Blood samples.* Blood samples were obtained from an antecubital vein via an indwelling catheter according to the trial schedule (**Figure 6.1**). At each blood collection time point, 10 mL of whole blood was collected into a chilled EDTA-treated vacutainer and immediately placed on ice. Within 20 min of sampling, blood was centrifuged at 2,000×g for 20 min at 4°C to separate plasma. A 500-µl aliquot was then collected and immediately frozen at -80°C prior to shipment to Nestlé Research Center (NRC) for analysis of plasma and amino acid concentrations by amino acid analyzer, according to standard NRC protocols (J. Vuichoud, personal communication). The remaining plasma was also immediately frozen at -80°C and stored prior to metabolic and enrichment analysis. Plasma glucose was analyzed using a colorimetric assay (Cayman Chemical Company, Ann Arbor, MI, USA); plasma insulin was measured by enzyme-linked immunoassay (Biosource, Medicorp, PQ, Canada). Isotopic enrichment of plasma  $\alpha$ -[<sup>13</sup>C]ketoisocaproate acid and [<sup>2</sup>H<sub>3</sub>]leucine were measured by GC-MS (GC model 6890; MSD model 5973 network, Agilent Technologies).

In order to gain further understanding of the physiological changes to protein synthesis and breakdown in response to a single meal, measures of the rates of: leucine appearance (Leu  $R_a$ ; µmol·kg<sup>-1</sup>·min<sup>-1</sup>), and disappearance (Leu  $R_d$ ; µmol·kg<sup>-1</sup>·min<sup>-1</sup>) from the peripheral circulation; exogenous (dietary) (Exo  $R_a$ ; µmol·kg<sup>-1</sup>·min<sup>-1</sup>) and endogenous (Endo  $R_a$ ; µmol·kg<sup>-1</sup>·min<sup>-1</sup>) leucine appearance; and non-oxidative leucine disposal

(NOLD;  $\mu$ mol·kg<sup>-1</sup>·min<sup>-1</sup>) were then calculated using the oral and IV labeled leucine tracers ([<sup>2</sup>H<sub>3</sub>]leucine and [1-<sup>13</sup>C]leucine, respectively). Measurements taken immediately prior to beverage consumption (representing time 0 min) were calculated as static measures, with all points after 0 min determined using non-steady-state equations (developed by Proietto *et al.* (1987)) described previously by Boirie *et al.* (1996). Endo  $R_a$  was used as an estimate of whole body protein breakdown, whereas NOLD was used to estimate whole body protein synthesis.

Total Leu  $R_a$  in the free amino acid pool was calculated as:

Leu 
$$R_a = \text{Exo } R_a + \text{Endo } R_a$$
.

Given the non-steady state condition, Leu  $R_a$  was measured by the dilution of the IV tracer in venous blood, and was determined from the mean plasma leucine concentration (both labeled and unlabeled) between two given time points (C(*t*)), the difference in plasma [1-<sup>13</sup>C]leucine enrichment for the IV tracer as a function of time (dEi<sub>IV</sub>/d*t*), and the mean enrichment of the plasma [1-<sup>13</sup>C]leucine between two time points (Ei<sub>IV</sub>(*t*)) (Boirie et al. 1996). As proposed by Boirie *et al.* (1996), a correction factor of the pool size for instant mixing was applied to the volume of distribution in order to calculate the volume of leucine that was effectively mixed in the body (pV = 0.125 l·kg<sup>-1</sup>, as justified by Boirie *et al.*). Leu  $R_a$  was, therefore, be represented by:

Leu 
$$R_a = [F_{IV} - [pV \times C(t) \times dEi_{IV}/dt]] / Ei_{IV}(t)$$

Exo R<sub>a</sub> was then calculated from the mean plasma enrichment of  $[^{2}H_{3}]$ leucine (Ei<sub>PO</sub>(*t*)), the time difference in plasma  $[^{2}H_{3}]$ leucine enrichment between two points

 $(dEi_{PO}/dt)$ , and the leucine enrichment of the total dietary leucine (diet Leu Ei) using the specific equation developed by Proietto *et al.* (1987):

Exo 
$$R_a = [Leu R_a \times Ei_{PO}(t) + [pV \times C(t) \times dEi_{PO}/dt]] / diet Leu Ei$$

Finally, to estimate whole body protein breakdown, Endo  $R_a$  was calculated as:

Endo 
$$R_a$$
 = Leu  $R_a$  - Exo  $R_a$  - F<sub>IV</sub>

As leucine has one of two fates: oxidation (Leu<sub>Ox</sub>; to urea and CO<sub>2</sub>) or NOLD (an estimate of protein synthesis) (Darmaun and Mauras 2005), the rate of total Leu  $R_d$  represents the sum of these two pathways:

Leu 
$$R_d$$
 = Leu<sub>Ox</sub> + NOLD.

Leu<sub>Ox</sub> was calculated from the product of <sup>13</sup>CO<sub>2</sub> enrichment in expired CO<sub>2</sub> and average CO<sub>2</sub> production, using the fractional bicarbonate retention factor of 0.80 for the fed state and plasma, as previously described (Boirie et al. 1996). Plasma  $\alpha$ -[<sup>13</sup>C]ketoisocaproate acid enrichment was chosen as the precursor as it is a good reflection of the immediate intracellular precursor for the irreversible decarboxylation of leucine (Matthews et al. 1982). A retention factor of 0.8 for the fed state was applied in order to account for CO<sub>2</sub> lost between the oxidation site and its appearance in the breath, due to the fixation of bicarbonate (Wagenmakers 1999). Unlike the calculations for Leu *R<sub>a</sub>*, Boirie *et al.* (1996) demonstrated that the determination of Leu<sub>Ox</sub> is not dependent on the existence of a steady state after ingesting a single meal.

Applying the same correction factor of the pool size for instant mixing (pV) as was used for Leu  $R_a$ , and considering the difference in plasma leucine concentration between two given points in time (dC/dt), Leu  $R_d$  was calculated as (Boirie et al. 1996):

Leu 
$$R_d$$
 = Leu  $R_a - (pV \times dC/dt)$ 

The determination of leucine (both endogenous and exogenous) utilized for protein synthesis was then represented by the equation (Boirie et al. 1996):

NOLD = Leu 
$$R_d$$
 – Leu<sub>Ox</sub>

The primary outcome of whole body leucine balance (Leu<sub>BAL</sub>) over 3-h after exercise and B1 was then calculated as the difference in total leucine intake (accounting for the  $[1-^{13}C]$ leucine IV infusion, dietary beverage leucine content, assuming 10% leucine content of milk protein, and oral  $[^{2}H_{3}]$ leucine tracer) and the integrated area under the curve (AUC) of total Leu<sub>OX</sub> (Boirie et al. 1996). This calculation functions under the assumption that all of the leucine administered was absorbed effectively (Boirie et al. 1996).

*Urine samples*. All urine samples collected were measured to the nearest mL by graduated cylinder and the volume recorded. Samples were pooled accordingly (as previously described) and aliquots ( $3 \times 3$  mL for metabolite concentrations and isotopic enrichment analysis) were collected and frozen at -80°C prior to analysis. The concentration of the major nitrogen-containing metabolites urea and creatinine were determined colorimetrically by commercially available kits (Quantichrom, Bioassay Systems, USA), as an estimate of urinary nitrogen excretion (N<sub>EX</sub>). Total nitrogen intake (N<sub>IN</sub>) and N<sub>EX</sub> [using previously published values of fecal and miscellaneous nitrogen losses for children consuming a 1.2 g protein·kg<sup>-1</sup>·d<sup>-1</sup> diet (Gattas et al. 1990)] were used to calculate nitrogen balance (N<sub>BAL</sub> = N<sub>IN</sub> - N<sub>EX</sub>). In addition, The [<sup>15</sup>N] enrichments (i.e. ratio of tracer:trace, t:Tr) of urinary ammonia (at baseline, 6 h, and 24 h) and urea (at

baseline and 24 h) were determined in duplicate by isotope ratio mass spectrometry by Metabolic Solutions Incorporated (Nashua, New Hampshire, USA) to determine whole body measures of nitrogen turnover (Q), protein synthesis (S), protein breakdown (B) and net protein balance (WBPB), as previously described (Moore et al. 2014b).

*Statistics.* Based on the expected graded stimulation of protein balance typically observed in adults (Moore et al. 2009) and the dose-dependent increase in WBPB previously demonstrated in children (Moore et al. 2014b), we planned a priori comparisons between HP vs. CONT, MP vs. CONT and LP vs. CONT at 3 h following B1 consumption using independent *t*-tests with  $\alpha = 0.05$ , or non-parametric alternative if necessary (i.e., Wilcoxon). However, due to the exploratory nature of this trial in the absence of any previous pediatric studies and the uncertainty about the extent of similarities between children and adults, Leu<sub>BAL</sub> was also submitted to a one-way analysis of variance (ANOVA; condition). This allowed us to obtain comparisons between all beverage conditions that were not examined in the *a priori* analyses and, thus, further understand the dose-response relationship. Group differences in the controlled diet, habitual physical activity, and the diet on the exercise intervention day were examined using a one-way ANOVA (condition). Changes in the AUC for the amino acid concentrations and leucine kinetic parameters (e.g., Leu  $R_a$ , Leu  $R_d$ , Exo  $R_a$ , Endo  $R_a$ , Leu<sub>OX</sub> and NOLD) were analyzed using a one-way (condition) ANOVA. Two-way ANOVAs (condition  $\times$  time) were used to measure time-dependent changes in plasma amino acid concentrations, whole body leucine kinetics, and plasma insulin and glucose over 3 h after exercise and ingestion of B1. Six-h and 24-h measures of whole body Q, S,

B and WBPB measured by [<sup>15</sup>N]ammonia end-product method, as well as 6-h and 24-h measures of N<sub>BAL</sub> were also measured using two-way ANOVAs (condition × time). 24-h measures of whole body Q, S, B and WBPB measured by using the harmonic mean approach were analyzed using one-way (condition) ANOVA. Differences between means for significant main effects or interactions were determined using a Holm-Sidak post-hoc test. To determine if 6-h and 24-h WBPB and N<sub>BAL</sub> were significantly different from zero, a paired *t*-test was performed for each condition. Pearson product-moment correlation coefficients were determined for Leu<sub>BAL</sub> and leucine and protein intake over 3 h, for WBPB (determined by [<sup>15</sup>N]ammonia end-product method) and energy and protein intake over both 6 and 24 h, and for N<sub>BAL</sub> and protein intake over both 6 and 24 h. Statistical significance was set at P < 0.05 and all data are expressed as mean ± SD unless otherwise indicated. For secondary/exploratory analyses, *P* values between 0.05 and 0.1 were considered trends.

## 6.5. RESULTS

*Beverage protein intake.* Absolute protein intake from B1 was 0, 5, 10 and 15 g for CONT, LP, MP and HP, respectively. This resulted in a relative intake of 0,  $0.12 \pm 0.02$ ,  $0.22 \pm 0.04$ , and  $0.33 \pm 0.08$  g protein·kg<sup>-1</sup> for CONT, LP, MP, and HP, respectively. B2 provided 15, 10, 5, and 0 g of protein, resulting in a relative protein intake of  $0.37 \pm 0.08$ ,  $0.24 \pm 0.05$ ,  $0.11 \pm 0.02$ , and  $0 \text{ g·kg}^{-1}$  for CONT, LP, MP and HP, respectively. Throughout the 4-h post-exercise period, B1 and B2 combined for an

absolute protein intake of 15 g and a relative total protein intake of  $0.35 \pm 0.07$  g·kg<sup>-1</sup>; there was no significant difference between groups (P = 0.56).

*Macronutrient intake and habitual physical activity.* As there were no group differences for diet during the 5-day adaptation phase [either absolute (P = 0.74 - 0.91) or relative to body mass (P = 0.11 - 0.66)], or on the intervention day [either absolute (P = 0.50 - 0.81) or relative to body mass (P = 0.62 - 0.75)], total sample means  $\pm$  SD are reported in **Table 6.3**. There were no group differences for total habitual physical activity (P = 0.74) or MVPA (P = 0.27); total sample means  $\pm$  SD are 207.9  $\pm$  58.4 min·d<sup>-1</sup> and 62.7  $\pm$  28.1 min·d<sup>-1</sup>, respectively.

*Whole body leucine balance.* Treatment differences between HP and CONT, MP and CONT and LP and CONT are provided in **Table 6.4**. All conditions were significantly different compared with CONT (P < 0.001). Exploratory analyses of the primary outcome using one-way ANOVA (**Figure 6.2**) showed a main effect for condition for absolute Leu<sub>BAL</sub> (mg) (P < 0.001), where HP (1070 ± 146 mg) > MP (514 ± 181 mg) > LP (245 ± 61 mg) > CONT (-128 ± 93 mg) (all P < 0.001). When expressed relatively to body mass, Leu<sub>BAL</sub> (mg·kg<sup>-1</sup>) remained significantly different between conditions (P < 0.001), where again, HP (24.2 ± 8.2 mg·kg<sup>-1</sup>) > MP (11.6 ± 4.3 mg·kg<sup>-1</sup>) > LP (5.7 ± 1.9 mg·kg<sup>-1</sup>) > CONT (-3.0 ± 1.7 mg·kg<sup>-1</sup>) (all P < 0.01). Relative Leu<sub>BAL</sub> was significantly greater than zero for HP, MP and LP, and significantly less than zero for CONT (all P < 0.001).

Table 6.3.	Macronutrient intake across the 5-d dietary adaptation period and on the
exercise in	itervention day.

	Energy	PRO	СНО	Fat	
Dietary Adaptation					
Absolute	2003 (421)	40.6 (10.6)	313.4 (60.9)	70.6 (14.6)	
Relative	45.3 (7.1)	0.9 (0.1)	7.1 (1.2)	1.6 (0.2)	
Intervention Da	ау				
Absolute	2196 (441)	54.3 (12.3)	384.8 (78.2)	55.0 (19.3)	
Relative	49.4 (6.7)	1.2 (0.1)	8.7 (1.7)	1.2 (0.3)	

PRO, protein; CHO, carbohydrate. Data are presented as total sample (N = 35) means (SD) for absolute energy (kcal), PRO (g), CHO (g), and fat (g), as well as relative energy (kcal·kg<sup>-1</sup>), PRO (g·kg<sup>-1</sup>), CHO (g·kg<sup>-1</sup>), and fat (g·kg<sup>-1</sup>).

Table 6.4. Differences in relative and absolute leucine balance between HP and CONT, MP and CONT, and LP and CONT at 3 h following consumption of the experimental beverage.

	HP - CONT	MP - CONT	LP - CONT
Relative (mg·kg <sup>-1</sup> )	27.2*	14.5*	8.7*
	[22.7, 31.7]	[11.4, 17.6]	[6.9, 10.5]
Absolute (mg)	1198.3*	642.5*	38.3*
	[1073.5, 1323.1]	[505.5, 779.6]	[292.1, 454.3]

CONT, Control condition; LP, Low-protein condition; MP, moderate-protein condition; HP, High-protein condition. Data are presented as mean [95% confidence interval]. \*significant different between conditions (P < 0.001).

## Figure 6.2. Leucine balance over 3 h.

(A) Leucine balance over 3 h following exercise and consumption of one of four experimental beverages: CON, Control; LP, Low-protein; MP, Moderate-protein; HP, High-protein. (B) Leucine balance over 3 h expressed relative to beverage protein intake. Linear correlation (collapsed across all conditions): r = 0.97, P < 0.001. (C) Leucine balance over 3 h expressed relative to total leucine intake. Linear correlation (collapsed across all conditions): r = 0.97, P < 0.001. (C) Leucine balance over 3 h expressed relative to total leucine intake. Linear correlation (collapsed across all conditions): r = 0.97, P < 0.001. \*Significantly different from zero, P < 0.001. \*\*\*Main effect of condition, HP > MP > LP > CONT (P < 0.001).



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*Plasma amino acid concentrations.* The AUCs for plasma amino acid concentrations are presented in **Table 6.5**. The AUC for plasma leucine, branched-chain amino acid (BCAA), and essential amino acid concentrations demonstrated a main effect for condition (P < 0.001); HP > MP > LP > CONT (all P < 0.001). Total amino acid AUC was also significantly different between conditions (P < 0.001); with, HP = MP (P = 0.299) > LP > CONT (P < 0.001 for others).

*Whole body leucine kinetics.* The AUC of whole body leucine kinetic data are also shown in **Table 6.5**. All variables demonstrated a main effect for condition (P < 0.001 for all), except Endo  $R_a$  (P = 0.75). Total Leu  $R_a$ , Exo  $R_a$ , and Endo  $R_a$  over 3 h of recovery are represented graphically in **Figure 6.3**. Total Leu  $R_d$ , Leu<sub>OX</sub>, and NOLD over 3 h of recovery are represented graphically in **Figure 6.4**.

Total Leu  $R_a$  demonstrated a main effect for condition (P < 0.001), time (P < 0.001), and condition × time interaction (P < 0.001). HP was significantly greater than MP (P < 0.05), LP (P = 0.001) and CONT (P < 0.001). MP and LP were significantly different than CONT (P < 0.001), with no difference between MP and LP (P = 0.72). Total Leu  $R_a$  was elevated from baseline at 60 min post-beverage (P < 0.001). The values decreased throughout recovery and returned to baseline by 120 min post-beverage.

Exo  $R_a$  showed a main effect of condition (P < 0.001; HP > MP > LP > CONT), time (P < 0.001), and condition × time interaction (P < 0.001). Exo  $R_a$  increased following beverage consumption to a peak at 60 min. Values continued to decrease throughout recovery, but did not return to pre-beverage values. Endo  $R_a$  did not differ between conditions (main effect, P = 0.28), but did show a main effect of time (P < 0.001), and condition × time interaction (P < 0.01). Endo  $R_a$  was reduced from baseline to 60 min post-beverage (P < 0.001), then increased throughout recovery and returned to pre-beverage values by 120 min post-beverage.

Leu  $R_d$  showed a main effect of condition (P < 0.001), time (P < 0.001), and group × time interaction (P < 0.001). HP was significantly greater than MP (P = 0.034), LP (P = 0.001) and CONT (P < 0.001). MP and LP were significantly greater than CONT (P < 0.001), with no difference between the MP and LP (P = 0.72). Leu  $R_d$  was elevated from baseline 60 min post-beverage (P < 0.001). The values decreased throughout recovery and returned to pre-beverage values by 120 min post-beverage.

Leu<sub>OX</sub> demonstrated a main effect of condition (P < 0.001; HP = MP > LP > CONT), time (P < 0.001), and group × time interaction (P < 0.001). Leu<sub>OX</sub> increased from pre-beverage to a peak at 60 min post-beverage. Values then decreased throughout recovery and returned to pre-beverage values by 180 min post-beverage.

NOLD demonstrated a main effect of condition (P < 0.001) and time (P < 0.01). There was no group × time interaction (P = 0.25). HP was significantly greater than MP (P = 0.01) and CONT (P < 0.001). There was a trend for HP to be greater than LP (P = 0.07). MP and LP were greater than CONT (P < 0.001), with no difference between MP and LP (P = 1.00). There was a trend for NOLD to increase from baseline at 60 min postbeverage (P = 0.09), but returned to pre-beverage values by 90 min post-beverage.

	CONT	LP	MP	HP
Plasma leucine	16 (2) <sup>a</sup>	23 (1) <sup>b</sup>	30 (2) <sup>c</sup>	36 (4) <sup>d</sup>
BCAA	49 (5) <sup>a</sup>	67 (3) <sup>b</sup>	89 (5) <sup>c</sup>	106 (12) <sup>d</sup>
EAA	122 (9) <sup>a</sup>	153 (9) <sup>b</sup>	193 (7) <sup>c</sup>	212 (21) <sup>d</sup>
Total AA	390 (28) <sup>a</sup>	457 (26) <sup>b</sup>	525 (26) <sup>c</sup>	553 (37) <sup>c</sup>
Total Leu $R_a$	359 (24) <sup>a</sup>	476 (115) <sup>b</sup>	500 (56) <sup>b</sup>	545 (72) <sup>b</sup>
Endo $R_a$	336 (24)	368 (102)	339 (59)	344 (65)
Exo $R_a$	$0(0)^{a}$	85 (20) <sup>b</sup>	138 (31) <sup>b,c</sup>	179 (83) <sup>c</sup>
Total Leu $R_d$	340 (23) <sup>a</sup>	451 (106) <sup>b</sup>	473 (53) <sup>b</sup>	517 (69) <sup>b</sup>
Leu <sub>OX</sub>	69 (12) <sup>a</sup>	95 (17)* <sup>a</sup>	132 (28) <sup>b</sup>	125 (22) <sup>b</sup>
NOLD	290 (29) <sup>a</sup>	380 (115) <sup>a</sup>	367 (62) <sup>a</sup>	419 (71) <sup>b</sup>

Table 6.5. AUC of amino acid concentrations and leucine kinetic data.

AUC measured over 3 h of recovery after exercise and consumption of four different beverages: Control, CONT; Low-protein, LP; Moderate-protein, MP; High-protein, HP. BCAA, branched-chain amino acids; EAA, essential amino acids; AA, amino acids. Values are presented as mean (SD) in mmol·1<sup>-1</sup> for amino acid concentrations and in  $\mu$ mol·kg<sup>-1</sup> for leucine kinetic data. Values with different letters are significantly different from each other (within that parameter). \*Trend for significant difference from control (*P* = 0.07).

## Figure 6.3. Time course of leucine appearance.

(A) Total leucine rate of appearance ( $R_a$ ); (B) exogenous leucine  $R_a$ ; and (C) endogenous leucine  $R_a$  over 3 h following exercise and the consumption of one of four experimental beverages: Control, CONT; Low-protein, LP; Moderate-protein, MP; High-protein, HP. Exogenous leucine  $R_a$  represents the plasma appearance of leucine from both the intravenous and dietary sources, while endogenous leucine  $R_a$  represents the leucine that appears in the plasma as a result of protein breakdown. Time 0 represents a baseline value taken 15 min following cessation of exercise, and immediately prior to beverage consumption. Mean values for each time point are presented. Error bars were not included for clarity.





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## Figure 6.4. Time course of leucine disappearance.

(A) Total leucine rate of disappearance ( $R_d$ ); (B) total leucine oxidaiton; and (C) total non-oxidative leucine disposal over 3 h following exercise and the consumption of one of four experimental beverages: Control, CONT; Low-protein, LP; Moderate-protein, MP; High-protein, HP. Total leucine oxidation represents the leucine that was deaminated and utilized as a source of fuel while total NOLD is an estimate of the leucine used for protein synthesis. Time 0 represents a baseline value taken 15 min following cessation of exercise, and immediately prior to beverage consumption. Mean values for each time point are presented. Error bars were not included for clarity.


*Plasma glucose and insulin.* There was no significant difference in plasma insulin AUC between conditions (group mean:  $4242 \pm 1597 \ \mu U \cdot mL^{-1}$ , P = 0.53). Two-way ANOVA (condition × time) for plasma insulin concentrations demonstrated no main effect for condition (P = 0.17), but a main effect of time (P < 0.001). Plasma insulin concentrations were markedly increased from pre-beverage values ( $19.0 \pm 9.1 \ \mu U \cdot mL^{-1}$ ) to a peak concentration at 30 min post-beverage ( $73.5 \pm 29.9 \ \mu U \cdot mL^{-1}$ , P < 0.001). Values returned to pre-drink levels 60 min post-beverage ( $23.7 \pm 14.9 \ \mu U \cdot mL^{-1}$ ), and continued to decline throughout recovery. By 180 min post-beverage, values were significant lower than pre-beverage ( $6.4 \pm 2.9 \ \mu U \cdot mL^{-1}$ , P < 0.01).

There was no significant difference in plasma glucose AUC between conditions (group mean:  $13344 \pm 1255 \ \mu U \cdot m L^{-1}$ , P = 0.22). Two-way ANOVA for plasma glucose concentration demonstrated a main effect for condition (P = 0.007) and time (P < 0.001), as well as a condition × time interaction (P < 0.001). Plasma glucose was significantly different in HP compared with LP (P = 0.021) and CONT (P = 0.018), with no difference between the LP and CONT. MP was not different than any other group. Plasma glucose concentrations increased from pre-beverage values ( $81.8 \pm 10.4 \text{ mg} \cdot \text{dL}^{-1}$ ) to a peak concentration at 30 min post-beverage ( $91.1 \pm 19.6 \ \mu U \cdot m L^{-1}$ , P = 0.003). Values decreased below pre-beverage levels 60 min post-beverage ( $67.2 \pm 10.6 \ \text{mg} \cdot \text{dL}^{-1}$ ), and remained stable throughout the remainder of recovery.

Whole body protein metabolism by  $[^{15}N]$ ammonia end product enrichment. Whole body Q was not different (main effect for condition, P = 0.50; interaction, P = 0.86) between conditions over 6 h (group mean:  $49.6 \pm 14.1 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ) and 24 h (group mean:  $27.7 \pm 8.7 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ ). However, there was a significant effect of time (main effect, P < 0.001) with rates of Q being ~ 44% lower after 24 h compared with the early 6 h period. There was no difference between conditions for S (main effect for condition, P = 0.29; interaction, P = 0.90); however, B demonstrated a trend for a main effect of condition (P = 0.07). Both S and B had a main effect for time (P < 0.001) with rates over 24 h (group means: 249.4 ± 89.2 and 239.5 ± 96.4 mg \cdot \text{kg}^{-1} \cdot \text{h}^{-1} respectively) being ~52% and ~49% less than 6 h (group means:  $120.2 \pm 50.4$  and  $122.2 \pm 56.5 \text{ mg} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ , respectively), respectively.

There was a main effect of condition (P < 0.01) for WBPB, with significant differences at both 6 h (P < 0.01) and 24 h (P < 0.05) (**Figure 6.5**). At 6 h, HP and MP were greater than CONT. There was also a trend (P = 0.05) for HP to be greater than LP. At 24 h, however, LP was greater than HP (P < 0.05). Regardless of condition, WBPB was greater during the 6-h period compared with the 24-h period (main effect for time, P < 0.01). At 6 h, WBPB was greater than zero for HP and MP (P < 0.01 for both), but not for LP (P = 0.40). There was a trend for CONT to be significantly less than zero (P = 0.09). At 24 h, WBPB was not different from zero for CONT (P = 0.54) or MP (P = 0.45). There was a trend for LP to be greater than zero (P = 0.08), and a trend for HP to be less than zero (P = 0.09).

When collapsed across conditions, there was no relationship between the WBPB over 9 h (r = -0.09, P = 0.60) or 24 h (r = 0.15, P = 0.38) and energy intake (data not shown). There were linear relationships between WBPB and protein intake over 6 h (r = 0.77, P < 0.001) and 24 h (r = 0.40, P < 0.05; Figure 6.5, *B* and *C*, respectively).

Whole body 24 h protein metabolism by harmonic mean of [<sup>15</sup>N]ammonia and [<sup>15</sup>N]urea end product enrichment. Similar to rates calculated by the [<sup>15</sup>N]ammonia endproduct method, there were no differences between conditions for Q (P = 0.64), S (P = 0.77), or B (P = 0.64) calculated by the harmonic mean of urinary [<sup>15</sup>N]ammonia and [<sup>15</sup>N]urea (**Table 6.6**). WBPB demonstrated a main effect of condition (P < 0.05), with LP being greater than HP (P < 0.05). WBPB at 24 h was not significantly different from zero for HP (P = 0.14), MP (P = 0.15), and CONT (P = 0.77); however, 24-h WBPB in LP was significantly greater than zero (P < 0.05).

Whole body  $N_{BAL}$  as measured by the difference between dietary nitrogen intake and urinary and miscellaneous nitrogen losses.  $N_{BAL}$  demonstrated a main effect for condition (P < 0.05), no main effect for time (P = 0.53), and a group × time interaction (P < 0.01). At 6 h,  $N_{BAL}$  was significantly different between conditions (P < 0.001); HP (1.6 ± 1.1 g) was significantly greater than LP ( $0.24 \pm 0.58$  g; P < 0.05) and CONT (-0.45 ± 0.99 g; P < 0.001), and MP ( $0.95 \pm 0.78$  g) was significantly greater than CONT (P =0.01). At 24 h,  $N_{BAL}$  was also different between conditions (P = 0.03; CONT = -0.32 ± 2.2 g, LP = 1.8 ± 1.8 g, MP = 1.1 ± 1.3 g, HP = -1.3 ± 3.2); where LP was significantly greater than HP (P < 0.05). 6-h  $N_{BAL}$  was not significantly different from zero for CONT (P = 0.19) or LP (P = 0.29), but was significantly different from zero for CONT (P = 0.01) for both). 24-h  $N_{BAL}$  was not significantly different from zero for CONT (P = 0.66) or HP (P = 0.28). 24-h  $N_{BAL}$  was, however, greater than zero for LP and MP (P < 0.05for both).

# Figure 6.5. WBPB over 6 and 24 h.

(A) Whole body protein balance (WBPB) over 6 and 24 h calculated using urinary [<sup>15</sup>N]ammonia end-product enrichment. Individual points are presented for each participant, with group means demonstrated by a horizontal line. (B) WBPB over 6 h expressed relative to protein intake. Linear correlation (collapsed across all conditions): r = 0.77, P < 0.001; line of best fit: y = 128.0x - 44.1, *x*-intercept = 0.34 g·kg<sup>-1</sup>·6 h<sup>-1</sup>. (C) WBPB relative to protein intake over 24 h expressed relative to protein intake. Linear correlation (collapsed across all conditions): r = 0.40, P < 0.05; line of best fit: 52.1x - 65.2, *x*-intercept = 1.25 g·kg<sup>-1</sup>·24 h<sup>-1</sup>. \*Significantly different from zero, P < 0.001. #Trend for significant difference from zero, P = 0.09. \*\*\*Main effect of time, P < 0.01.



	CONT	LP	MP	HP
$Q (g N \cdot kg^{-1} \cdot d^{-1})$	0.63 (0.12)	0.60 (0.16)	0.57 (0.05)	0.62 (0.12)
$S(g\cdot kg^{-1}\cdot d^{-1})$	2.72 (0.58)	2.48 (1.02)	2.40 (0.42)	2.42 (0.70)
$B\left(g\cdot kg^{-1}\cdot d^{-1}\right)$	2.76 (0.86)	2.48 (1.04)	2.32 (0.33)	2.70 (0.81)
WBPB $(g \cdot kg^{-1} \cdot d^{-1})$	-0.03 (0.43) <sup>a,b</sup>	0.22 (0.24)* <sup>a</sup>	0.09 (0.16) <sup>a,b</sup>	-0.27 (0.46) <sup>b</sup>

Table 6.6. 24 h whole body protein metabolism.

Whole body nitrogen turnover (Q), protein synthesis (S), protein breakdown (B), and net protein balance (WBPB) calculated using the harmonic mean of [<sup>15</sup>N]ammonia and [<sup>15</sup>N]urea as the end-product for four different beverages: Control, CONT; Low-protein, LP; Moderate-protein, MP; High-protein, HP. \*Significantly different from zero for WBPB, P < 0.05.

# 6.6. **DISCUSSION**

During the acute (~3 h) post-prandial recovery period after an acute bout of cycling exercise in healthy active children, LeuBAL is negative in the absence of postexercise protein ingestion but demonstrates a dose-dependent response following the ingestion of a single mixed-protein meal in the form of milk-protein. This novel finding was made possible by the use of the primed constant  $[1-^{13}C]$  leucine infusion technique, which we employed for the first time in children. As leucine is preferentially metabolized in skeletal muscle (Brosnan and Brosnan 2006, Monirujjaman and Ferdouse 2014), it can be argued that the changes in leucine metabolism that were observed reflect, to some degree, the metabolism within skeletal muscle. As such, the dose-response in  $Leu_{BAL}$ during the acute 3-h recovery period is in general agreement with previous observations in the adult literature with the ingestion of protein inducing a graded stimulation of postexercise MPS (Moore et al. 2009, Witard et al. 2014). Moreover, the net positive Leu<sub>BAL</sub> following consumption of a milk-protein beverage over 3 h of recovery is in agreement with the observation in adults of a positive Leu<sub>BAL</sub> during the first 3-4 h after consumption of 30 g of protein (Boirie et al. 1996).

Using leucine kinetics we observed that the addition of protein to a CHO beverage only stimulated whole body protein synthesis (as assessed by NOLD) in the HP conditions (15 g protein), as the other protein conditions were not statistically different from a CHO only beverage (CONT). On the other hand, the addition of milk-protein to a CHO only beverage had no measureable effect on whole body protein breakdown (as assessed by Endo  $R_a$ ). Previous studies in adults (Boirie et al. 1996, Dangin et al. 2001b) have similarly demonstrated that the administration of protein (either 30 g or 0.31 g·kg<sup>-1</sup>, respectively) stimulated NOLD. Although the study by Boirie *et al.* (1996) used a considerably different amount protein in absolute terms (30 g of protein compared with 15 g used in the HP condition of the present study), it is possible that when considered in relation to body mass (not reported by Boirie *et al.* (1996)), the relative protein intake would be similar to that in the present study (~0.33 g·kg<sup>-1</sup>). Boirie *et al.* (1996) also demonstrated no measureable inhibition of Endo  $R_a$ ; however, Dangin et al. (2001b) did see a reduction with casein, which would account for ~80% of the protein source in the present study. It is possible that, in the present study, the lack of difference between conditions could be a result of the combination of CHO and protein and the similar insulinogenic responses between conditions (see below for further discussion).

The enhancement in whole body Leu<sub>BAL</sub> in response to post-exercise protein ingestion was not reflected in differences in the response of plasma insulin or glucose between the groups over the 3-h monitoring period. In the present study, protein was provided in a mixed meal where the effects of CHO on the insulin and glucose response would also be present. As the experimental beverages in the present study were isoenergetic, there was an inverse relationship (r = -0.61, P < 0.001) between the relative amount of CHO and protein in the post-exercise beverage, with relative CHO contents ranging from  $0.87 \pm 0.18$  g·kg<sup>-1</sup> in the CONT beverage to  $0.44 \pm 0.11$  g·kg<sup>-1</sup> in the HP beverage. As both CHO and amino acids (e.g., leucine) appear to modulate insulinemia and glucose use by skeletal muscle, and that the combination of CHO and amino acids can be more insulinogenic than either alone (van Loon et al. 2000c), the lack of difference between plasma insulin and plasma glucose responses between conditions is not altogether surprising considering the relatively small difference in protein and CHO intake between conditions, as well as the inverse relationship between CHO and protein content in the drinks. Given the association between insulin and the inhibition of the increase in protein breakdown normally seen following exercise (Gelfand and Barrett 1987, Denne et al. 1991, Koopman et al. 2007b, Greenhaff et al. 2008), this could also explain the lack of effect on protein breakdown between conditions. The stimulation in protein synthesis, however, appears less dependent on the elevation of plasma insulin and more dependent on hyperaminoacidemia (Fujita et al. 2006).

In contrast to the non-response seen in plasma insulin and glucose, a greater protein intake resulted in higher plasma concentrations of leucine, BCAAs, and essential amino acids (**Table 6.5**). While the present study is limited in its ability to determine the underlying mechanism(s) responsible for the enhanced protein metabolism, it is possible that the increase in protein synthesis observed in the HP condition was, in part, related to the presence of certain amino acids. For example, in adults, essential amino acids are particularly effective stimulators of MPS (Paddon-Jones 2006), with increased concentrations of essential amino acids in the blood resulting in enhanced rates of MPS (Biolo et al. 1997, Volpi et al. 1998, 2000, Bohé et al. 2003). The presence of BCAAs (namely leucine) have been shown to activate key signaling pathways that regulate protein metabolism (Wu 2009) via the phosphorylation of key proteins involved in signal transduction at the level of mRNA translation (Liu et al. 2001, Proud 2004, Karlsson et al. 2004). These changes in MPS and tissue sensitivity (Elia and Livesey 1983) have shown

to occur even in the absence of an increase in circulating insulin concentrations. Therefore, similar to adults, hyperaminoacidemia (hyperleucinemia) in the post-exercise period may also have the ability to function as key nutrient-signaling molecules that produce anabolic effects on protein metabolism in children.

Though it is possible that the present study was underpowered to detect subtle differences between each of the groups (partial eta squared for NOLD was 0.329), it is also possible that a threshold exists for the beneficial effects of amino acids on the regulation of protein synthesis. In adults, such a threshold has been shown; the stimulation of whole body protein synthesis requires an increase in plasma aminoacidemia at least twice as high as baseline levels (Tessari et al. 1987, Castellino et al. 1987, Giordano et al. 1996). Nonetheless, it appears that compared with the consumption of CHO alone, the co-ingestion of protein synthesis rather than an inhibition of protein breakdown, which ultimately lends to beneficial effects on protein balance.

Due to ethical and practical limitations in the study design, both  $Leu_{BAL}$  and associated leucine kinetics were only monitored over 3 h following exercise and experimental beverage consumption. It is possible that the  $Leu_{BAL}$  measured over this 3-h period in the LP condition is a good reflection of the true balance, as the  $Leu_{OX}$  values appear to return to baseline (or close to). However, it was not possible to capture the complete course of oxidation during this 3-h period for the higher protein intakes (MP and HP) (see **Figure 6.4**). In adults, following consumption of 30 g of whey protein (Boirie et al. 1996) Leu<sub>OX</sub> did not return to baseline until approximately 7 h of recovery.

Thus, utilization of a longer post-exercise recovery period in the present study may have allowed for a more accurate determination of Leu<sub>BAL</sub>. Though the present study demonstrated a dose-dependent response in whole body Leu<sub>BAL</sub>, it remains unclear whether a true dose-dependent response is present. For example, exploratory analyses of the relationship between relative leucine intake and relative Leu<sub>OX</sub> revealed that a segmental linear regression better explains the data than a simple linear regression (P <0.01) (see Appendix VI); the inflection point in  $Leu_{OX}$  was found to occur at 33.85  $mg \cdot kg^{-1}$  [95% confidence interval: 24.90 - 42.80  $mg \cdot kg^{-1}$ ] of ingested leucine. The plateau in <sup>13</sup>CO<sub>2</sub> appearance in breath with excess leucine intakes, particularly when coupled with a continued rise in amino acid concentration, may represent an upper limit in the metabolic capacity to oxidize/catabolize a particular amino acid (in this case, leucine) and/or to dispose of the excess (Pencharz et al. 2008). The finding that  $Leu_{OX}$ appears to plateau after the ingestion of  $\sim 34 \text{ mg} \cdot \text{kg}^{-1}$  of dietary leucine suggests that the linear Leu<sub>BAL</sub> may be an artifact of the plateau in Leu<sub>OX</sub> that occurs, and the limited time frame (~3 h) over which Leu<sub>OX</sub> was measured. Again, this would have had the biggest effect on the HP condition as the children in this group received an average of  $\sim 41 \text{ mg} \cdot \text{kg}^{-1}$ <sup>1</sup> of leucine between the experimental beverage and leucine infusion, which is above the level at which  $Leu_{OX}$  plateaued. Therefore, the inability to capture the full oxidation in the present study would have lead to an underestimation of leucine lost from the body and, combined with the presence of a plateau in Leu<sub>OX</sub>, may have resulted in an overestimate of Leu<sub>BAL</sub> that is amplified at higher protein intakes (i.e., MP and, especially, HP).

Another limitation of the present study that should be acknowledged is the timing of the baseline samples. In contrast to previous studies (Boirie et al. 1996) where the baseline samples were taken at isotopic plateau of the intravenous tracer, the baseline values in the present study were represented by a single measurement taken following exercise and prior to the consumption of the experimental beverage. Though it is not possible to confirm that our participants were at true isotopic steady state prior to ingesting the post-exercise beverage, it is likely that a steady state was achieved considering that 1) the participants had been infused for > 120 min prior to the first blood sample, which should be sufficient to obtain isotopic steady state with a primed, constant infusion, and 2) there was no difference between baseline (time 0 min) and 30 min postbeverage in the CONT condition, which would suggest that in the absence of ingesting dietary amino acids the participants were likely at (or very close to) isotopic steady state.

As a secondary objective of this study, we used the [<sup>15</sup>N]glycine technique to give an adjacent measure of WBPB. Although we have acknowledged previously the limitations to this technique (Moore et al. 2014b), the use of [<sup>15</sup>N]glycine allowed us to determine measures over a 24-h period that would not have been ethically feasible using the [1-<sup>13</sup>C]leucine infusion. Similar to our previous findings (Moore et al. 2014b), we found that rates of protein synthesis and breakdown (as well as WBPB) were greatest during the 6-h exercise and recovery period, which is in line with the adult literature demonstrating an increase in whole body and muscle protein turnover in response to exercise and protein feeding (Duggleby and Waterlow 2005, Burd et al. 2009). We also found that regardless of the dose, post-exercise protein ingestion had little effect on whole body protein synthesis or breakdown during the early acute (i.e., 6 h) and prolonged (i.e., 24 h) recovery period. Although the present study did not observe the same early acute dose-dependent response in WBPB between experimental conditions as was shown previously (Moore et al. 2014b), HP and MP were greater than CONT, with a trend for HP to be greater than LP. This finding may, in part, be a result of *1*) the relatively smaller differences in protein ingestion between conditions (ranging between ~0.10 g·kg<sup>-1</sup> to ~0.33 g·kg<sup>-1</sup> at the most); *2*) the shorter duration (6 h) of the observation period; *3*) smaller group sizes (e.g., N=8-10 per group in the present study vs. N=12 in the previous (Moore et al. 2014b); and *4*) the difference in design between the present (e.g., group) and previous (e.g., crossover) (Moore et al. 2014b) studies. Nonetheless, the linear relationship in **Figure 6.5B** suggests that, to some degree, a dose-response exists between protein intake and WBPB in the acute recovery period.

In the present study, we also examined whether the timing of protein intake after exercise (i.e., immediate vs. delayed) impacted WBPB. It was demonstrated that although larger protein intakes (~0.22-0.33 g·kg<sup>-1</sup> body mass) are required to attain a net anabolic environment over an acute 6-h exercise and recovery period, consumption of a total of 15 g of protein in the post-exercise period was not necessarily sufficient to induce a positive WBPB over the entire 24-h period in our healthy children. Instead, maintenance of a net positive WBPB and N<sub>BAL</sub> over 24-h occurred only in the LP condition, where participants consumed ~0.12 g·kg<sup>-1</sup> of protein immediately after exercise, and then consumed and additional ~0.24 g·kg<sup>-1</sup> 3 h later. Though the other condition (MP) that received protein both immediately (~0.22 g·kg<sup>-1</sup>) and again at 3 h (~0.11 g·kg<sup>-1</sup>) following exercise did not have a WBPB that was statistically different from zero, they were able to attain a net positive  $N_{BAL}$ . Interestingly, both conditions (CONT and HP) that received protein only once during the recovery period were not able to induce a positive WBPB nor  $N_{BAL}$  over the 24-h period. It is possible, therefore, that the enhanced WBPB and  $N_{BAL}$  in the present study are due to the incorporation of a second fed-state period and may reflect the fed-state anabolism that occurs with protein intake (Millward et al. 1991). Thus, periodized protein intake in the post-exercise recovery period may play a more important role in enhancing WBPB than consuming large amounts of protein at one time following exercise. As the MP and HP groups had the highest  $N_{BAL}$  following exercise, whether ingestion of a second protein dose of the same amount 3 h later would have lead to a larger cumulative response over 24 h remains unknown.

# 6.7. CONCLUSION

In conclusion, whole body Leu<sub>BAL</sub> was acutely increased by the post-exercise ingestion of protein in a dose-dependent manner. Moreover, the addition of protein to a post-exercise CHO beverage was necessary to attain a positive whole body Leu<sub>BAL</sub> during the early (3 h) recovery period. Over 3 h of recovery from exercise, the addition of protein to a CHO beverage had no effect on whole body protein breakdown; however, larger amounts of protein (~0.33 g·kg<sup>-1</sup>) stimulated NOLD (an estimate of whole body protein synthesis). In order to sustain a 24-h net positive WBPB, a periodized protein

intake with multiple protein feedings during recovery may be required, suggesting that there are possible additive effects of protein consumption.

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# 6.9. REFERENCES

Biolo, G., Tipton, K.D., Klein, S., and Wolfe, R.R. 1997. An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein. *Am. J. Physiol.* **273**(1 Pt 1): E122–129.

Bohé, J., Low, A., Wolfe, R.R., and Rennie, M.J. 2003. Human muscle protein synthesis is modulated by extracellular, not intramuscular amino acid availability: a dose-response study. *J. Physiol.* **552**(Pt 1): 315–324.

Boirie, Y., Gachon, P., Corny, S., Fauquant, J., Maubois, J.L., and Beaufrère, B. 1996. Acute postprandial changes in leucine metabolism as assessed with an intrinsically labeled milk protein. *Am. J. Physiol.* **271**(6 Pt 1): E1083–1091.

Brosnan, J.T., and Brosnan, M.E. 2006. Branched-chain amino acids: enzyme and substrate regulation. *J. Nutr.* **136**(1 Suppl): 207S–11S.

Burd, N.A., Tang, J.E., Moore, D.R., and Phillips, S.M. 2009. Exercise training and protein metabolism: influences of contraction, protein intake, and sex-based differences. *J. Appl. Physiol.* **106**(5): 1692–1701.

Castellino, P., Luzi, L., Simonson, D.C., Haymond, M., and DeFronzo, R.A. 1987. Effect of insulin and plasma amino acid concentrations on leucine metabolism in man. Role of substrate availability on estimates of whole body protein synthesis. *J. Clin. Invest.* **80**(6): 1784–1793.

Dangin, M., Boirie, Y., Garcia-Rodenas, C., Gachon, P., Fauquant, J., Callier, P., Ballèvre, O., and Beaufrère, B. 2001. The digestion rate of protein is an independent regulating factor of postprandial protein retention. *Am. J. Physiol. Endocrinol. Metab.* **280**(2): E340–348.

Darmaun, D., and Mauras, N. 2005. Use of stable isotopes to assess protein and amino acid metabolism in children and adolescents: a brief review. *Horm. Res.* **64 Suppl 3**: 32–37.

Denne, S.C., Liechty, E.A., Liu, Y.M., Brechtel, G., and Baron, A.D. 1991. Proteolysis in skeletal muscle and whole body in response to euglycemic hyperinsulinemia in normal adults. *Am. J. Physiol.* **261**(6 Pt 1): E809–814.

Duggleby, S.L., and Waterlow, J.C. 2005. The end-product method of measuring wholebody protein turnover: a review of published results and a comparison with those obtained by leucine infusion. *Br. J. Nutr.* **94**(2): 141–153. Elia, M., and Livesey, G. 1983. Effects of ingested steak and infused leucine on forelimb metabolism in man and the fate of the carbon skeletons and amino groups of branchedchain amino acids. *Clin. Sci. Lond. Engl.* 1979 **64**(5): 517–526.

Evenson, K.R., Catellier, D.J., Gill, K., Ondrak, K.S., and McMurray, R.G. 2008. Calibration of two objective measures of physical activity for children. *J. Sports Sci.* **26**(14): 1557–1565.

Fujita, S., Rasmussen, B.B., Cadenas, J.G., Grady, J.J., and Volpi, E. 2006. Effect of insulin on human skeletal muscle protein synthesis is modulated by insulin-induced changes in muscle blood flow and amino acid availability. *Am. J. Physiol. Endocrinol. Metab.* **291**(4): E745–754.

Gattas, V., Barrera, G.A., Riumallo, J.S., and Uauy, R. 1990. Protein-energy requirements of prepubertal school-age boys determined by using the nitrogen-balance response to a mixed-protein diet. *Am. J. Clin. Nutr.* **52**(6): 1037–1042.

Gelfand, R.A., and Barrett, E.J. 1987. Effect of physiologic hyperinsulinemia on skeletal muscle protein synthesis and breakdown in man. *J. Clin. Invest.* **80**(1): 1–6.

Giordano, M., Castellino, P., and DeFronzo, R.A. 1996. Differential responsiveness of protein synthesis and degradation to amino acid availability in humans. *Diabetes*. **45**(4): 393–399.

Greenhaff, P.L., Karagounis, L.G., Peirce, N., Simpson, E.J., Hazell, M., Layfield, R., Wackerhage, H., Smith, K., Atherton, P., Selby, A., and Rennie, M.J. 2008. Disassociation between the effects of amino acids and insulin on signaling, ubiquitin ligases, and protein turnover in human muscle. *Am. J. Physiol. Endocrinol. Metab.* **295**(3): E595–604.

Hartman, J.W., Tang, J.E., Wilkinson, S.B., Tarnopolsky, M.A., Lawrence, R.L., Fullerton, A.V., and Phillips, S.M. 2007. Consumption of fat-free fluid milk after resistance exercise promotes greater lean mass accretion than does consumption of soy or carbohydrate in young, novice, male weightlifters. *Am. J. Clin. Nutr.* **86**(2): 373–381.

Karlsson, H.K.R., Nilsson, P.-A., Nilsson, J., Chibalin, A.V., Zierath, J.R., and Blomstrand, E. 2004. Branched-chain amino acids increase p70S6k phosphorylation in human skeletal muscle after resistance exercise. *Am. J. Physiol. Endocrinol. Metab.* **287**(1): E1–7.

Koopman, R., Saris, W.H.M., Wagenmakers, A.J.M., and van Loon, L.J.C. 2007. Nutritional interventions to promote post-exercise muscle protein synthesis. *Sports Med.* **37**(10): 895–906.

Kuczmarski, R.J., Ogden, C.L., Guo, S.S., Grummer-Strawn, L.M., Flegal, K.M., Mei, Z., Wei, R., Curtin, L.R., Roche, A.F., and Johnson, C.L. 2002. 2000 CDC Growth Charts for the United States: methods and development. *Vital Health Stat.* 11. (246): 1–190.

Levenhagen, D.K., Gresham, J.D., Carlson, M.G., Maron, D.J., Borel, M.J., and Flakoll, P.J. 2001. Postexercise nutrient intake timing in humans is critical to recovery of leg glucose and protein homeostasis. *Am. J. Physiol. Endocrinol. Metab.* **280**(6): E982–993.

Liu, Z., Jahn, L.A., Long, W., Fryburg, D.A., Wei, L., and Barrett, E.J. 2001. Branched chain amino acids activate messenger ribonucleic acid translation regulatory proteins in human skeletal muscle, and glucocorticoids blunt this action. *J. Clin. Endocrinol. Metab.* **86**(5): 2136–2143.

van Loon, L.J., Saris, W.H., Verhagen, H., and Wagenmakers, A.J. 2000. Plasma insulin responses after ingestion of different amino acid or protein mixtures with carbohydrate. *Am. J. Clin. Nutr.* **72**(1): 96–105.

Matthews, D.E., Schwarz, H.P., Yang, R.D., Motil, K.J., Young, V.R., and Bier, D.M. 1982. Relationship of plasma leucine and alpha-ketoisocaproate during a L-[1-13C]leucine infusion in man: a method for measuring human intracellular leucine tracer enrichment. *Metabolism*. **31**(11): 1105–1112.

Meyer, F., O'Connor, H., Shirreffs, S.M., and International Association of Athletics Federations. 2007. Nutrition for the young athlete. *J. Sports Sci.* **25 Suppl 1**: S73–82.

Millward, D.J., Price, G.M., Pacy, P.J., Quevedo, R.M., and Halliday, D. 1991. The nutritional sensitivity of the diurnal cycling of body protein enables protein deposition to be measured in subjects at nitrogen equilibrium. *Clin. Nutr. Edinb. Scotl.* **10**(5): 239–244.

Mirwald, R.L., Baxter-Jones, A.D.G., Bailey, D.A., and Beunen, G.P. 2002. An assessment of maturity from anthropometric measurements. *Med. Sci. Sports Exerc.* **34**(4): 689–694.

Monirujjaman, M., and Ferdouse, A. 2014. Metabolic and physiological roles of branched-chain amino acids. *Adv. Molec. Biol.* 2014: 6 pages.

Moore, D.R., Robinson, M.J., Fry, J.L., Tang, J.E., Glover, E.I., Wilkinson, S.B., Prior, T., Tarnopolsky, M.A., and Phillips, S.M. 2009. Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. *Am. J. Clin. Nutr.* **89**(1): 161–168.

Moore, D.R., Volterman, K.A., Obeid, J., Offord, E.A., and Timmons, B.W. 2014. Postexercise protein ingestion increases whole body net protein balance in healthy children. *J. Appl. Physiol.* **117**(12): 1493-501. Obeid, J., Nguyen, T., Walker, R.G., Gillis, L.J., and Timmons, B.W. 2014. Circulating endothelial cells in children: role of fitness, activity, and adiposity. *Med. Sci. Sports Exerc.* **46**(10): 1974–1980.

Paddon-Jones, D. 2006. Interplay of stress and physical inactivity on muscle loss: Nutritional countermeasures. J. Nutr. **136**(8): 2123–2126.

Pencharz, P.B., Elango, R., and Ball, R.O. 2008. An approach to defining the upper safe limits of amino acid intake. *J. Nutr.* **138**(10): 1996S–2002S.

Proietto, J., Rohner-Jeanrenaud, F., Ionescu, E., Terrettaz, J., Sauter, J.F., and Jeanrenaud, B. 1987. Non-steady-state measurement of glucose turnover in rats by using a one-compartment model. *Am. J. Physiol.* **252**(1 Pt 1): E77–84.

Proud, C.G. 2004. mTOR-mediated regulation of translation factors by amino acids. *Biochem. Biophys. Res. Commun.* **313**(2): 429–436.

Rodriguez, N.R. 2005. Optimal quantity and composition of protein for growing children. *J. Am. Coll. Nutr.* **24**(2): 1508–1548.

Specker, B., and Vukovich, M. 2007. Evidence for an interaction between exercise and nutrition for improved bone health during growth. *Med. Sport Sci.* **51**: 50–63.

Tanner, J.M., and Whitehouse, R.H. 1976. Clinical longitudinal standards for height, weight, height velocity, weight velocity, and stages of puberty. *Arch. Dis. Child.* **51**(3): 170–179.

Tessari, P., Inchiostro, S., Biolo, G., Trevisan, R., Fantin, G., Marescotti, M.C., Iori, E., Tiengo, A., and Crepaldi, G. 1987. Differential effects of hyperinsulinemia and hyperaminoacidemia on leucine-carbon metabolism in vivo. Evidence for distinct mechanisms in regulation of net amino acid deposition. *J. Clin. Invest.* **79**(4): 1062–1069.

Timmons, B.W., Tarnopolsky, M.A., Snider, D.P., and Bar-Or, O. 2006. Immunological changes in response to exercise: influence of age, puberty, and gender. *Med. Sci. Sports Exerc.* **38**(2): 293–304.

Tipton, K.D., Borsheim, E., Wolf, S.E., Sanford, A.P., and Wolfe, R.R. 2003. Acute response of net muscle protein balance reflects 24-h balance after exercise and amino acid ingestion. *Am. J. Physiol. Endocrinol. Metab.* **284**(1): E76–89.

Tobias, J.H., Steer, C.D., Mattocks, C.G., Riddoch, C., and Ness, A.R. 2007. Habitual levels of physical activity influence bone mass in 11-year-old children from the United

Kingdom: findings from a large population-based cohort. J. Bone Miner. Res. 22(1): 101–109.

Volpi, E., Ferrando, A.A., Yeckel, C.W., Tipton, K.D., and Wolfe, R.R. 1998. Exogenous amino acids stimulate net muscle protein synthesis in the elderly. *J. Clin. Invest.* **101**(9): 2000–2007.

Volpi, E., Mittendorfer, B., Rasmussen, B.B., and Wolfe, R.R. 2000. The response of muscle protein anabolism to combined hyperaminoacidemia and glucose-induced hyperinsulinemia is impaired in the elderly. *J. Clin. Endocrinol. Metab.* **85**(12): 4481–4490.

Wagenmakers, A.J. 1999. Tracers to investigate protein and amino acid metabolism in human subjects. *Proc. Nutr.* Soc. **58**(4): 987–1000.

Witard, O.C., Jackman, S.R., Breen, L., Smith, K., Selby, A., and Tipton, K.D. 2014. Myofibrillar muscle protein synthesis rates subsequent to a meal in response to increasing doses of whey protein at rest and after resistance exercise. *Am. J. Clin. Nutr.* **99**(1): 86–95.

World Health Organization, Food and Agriculture Organization of the United Nations, and United Nations University. 2007. Protein and amino acid requirements in human nutrition. Report of a joint FAO/WHO/UNU expert consultation. *World Health Organ. Tech. Rep. Ser.* 935:1-265, back cover.

Wu, G. 2009. Amino acids: metabolism, functions, and nutrition. *Amino Acids*. **37**(1): 1–17.

# **CHAPTER 7 : General Discussion and Conclusion**

To optimally support youth's developing bodies, exercise and nutrition play complementary roles. Recommendations for how to optimize whole body protein retention following exercise in children is limited to date and is reliant on adult guidelines, which do not take into consideration the complex process of concurrent growth in the majority of all lean tissues. This thesis aimed to gain a better understanding of the combined effects of protein and exercise on protein metabolism at the whole body level, using pediatric-specific studies, in order to provide evidence regarding the quantity and timing of post-exercise protein ingestion to help children optimize recovery from exercise, while maintaining growth of lean tissue.

## 7.1. HIGHLIGHTS OF RESULTS

## 7.1.1. Skim milk as a whole-food protein-containing recovery beverage

**Chapter 4** was designed to establish the general importance of a whole-food protein-containing beverage (i.e., SM) in the post-exercise period to stimulate whole body protein synthesis and, as a result, create an anabolic environment (**Chapter 4**). This study demonstrated that consuming SM immediately following exercise increased both whole body Q and protein synthesis, with a lesser effect on protein breakdown, compared with two beverages commonly consumed in the post-exercise period (water and a CES). As a result, WBPB was less negative in SM compared with water or CES.

In this study, children received  $\sim 0.40 \text{ g} \cdot \text{kg}^{-1}$  body mass of protein following

exercise, which is a relative dose well above that which has shown to maximally stimulate post-exercise MPS in young adults (~0.25 g·kg<sup>-1</sup> body mass) (Moore et al. 2009). As children are experiencing higher rates of tissue remodeling compared with adults, it is possible that they require a larger relative protein dose to meet these needs. Interestingly, however, despite this relatively large post-exercise protein intake, the majority of children (~68%) in the SM group still experienced a negative WBPB.

It is possible that the negative balance experienced in Chapter 4 was, in part, due to the decision to use a 16-h observation period. Since our participants performed the exercise in the afternoon, the majority of post-exercise recovery period took place in the post-prandial and overnight fasted state. According to the studies by Bolster et al. (2001) and Pikosky et al. (2002) who examined overnight whole body protein metabolism in healthy 8-10 year old boys and girls using the <sup>15</sup>N-glycine methodology, overnight protein balance in healthy children is not necessarily negative. However, as the study by Pikosky et al. (2002) did not account for estimates of fecal and miscellaneous N losses, it is possible that their measure of N<sub>BAL</sub> was overestimated. Moreover, both of these studies examined the basal rates of protein metabolism with the absence of an exercise stimulus in the measurement period. A discrepancy between overnight fasted and daily net protein balance in previous studies (Bolster et al. 2001, Pikosky et al. 2002) could be due to an enhanced anabolic sensitivity during the daily fed state induced by exercise, an effect previously reported in exercising adults (Burd et al. 2011). Thus, the anabolic effects of exercise in children may be underestimated when protein metabolism is measured in the fasted state. Despite the elevated rate of protein turnover in the SM group, it is possible that the lack of additional feeding periods resulted in an insufficient stimulation of protein synthesis to offset the fasted losses that were experienced. Therefore, in order to further understand the combined effects of exercise and protein on protein metabolism in active children, the studies in **Chapters 5** and **6** were designed to have participants perform the exercise session in the morning and utilized a full 24-h examination period with recovery occurring throughout the remainder of the day and following overnight period.

From a practical perspective, a number of children and youth commonly perform exercise sessions after school hours or in the evening, similar to the exercise in Chapter 4. Given the potential importance of consuming protein at multiple times throughout the post-exercise period that was demonstrated in Chapter 6, and considering the relationship between increased availability of exogenous amino acids and stimulation of protein synthesis (even in the rested state) (Wolfe 2002, Bohé et al. 2003), it is possible that protein intake throughout the overnight recovery could have enhanced WBPB relative to the immediate feeding. Unfortunately, this would have little practical applicability (Beelen et al. 2010). Instead, providing a second source of dietary protein following exercise and prior to sleep could help to further enhance protein synthesis during overnight recovery in addition to feeding in the immediate post-exercise period (Beelen et al. 2008, Res et al. 2012). Using a study design similar to that in Chapter 6, but with the exercise occurring in the evening as opposed to the morning, future research should investigate the ability of the distribution of protein administration following an evening exercise session to enhance WBPB during the overnight period. Moreover, it is also possible that a slowly digested protein immediately before bed could produce a more

sustained increase in plasma amino acid concentrations throughout the overnight period, which might have further benefits on protein synthesis. Therefore, future research should also investigate the administration of different sources of dietary protein following an evening exercise session on protein metabolism during the subsequent overnight period.

## 7.1.2. Dose-effect of protein intake on post-exercise protein metabolism

While **Chapter 4** demonstrated the applied benefit of consuming a whole-food protein-containing beverage in the immediate post-exercise period, due to the compositional differences in the experimental beverages used, it was not possible to determine the benefits of protein, *per se.* As such, **Chapters 5** and **6** were designed using isoenergetic experimental beverages in order to determine the effects of different doses of protein to acutely enhance WBPB in both the early (e.g., 9 h and 6 h, respectively) and later (e.g., 24 h) exercise and recovery periods.

In **Chapter 5** we demonstrated that consuming varying amounts of protein following exercise had little effect on whole body protein synthesis and breakdown, yet induced a dose-dependent increase in WBPB during the early 9-h exercise and recovery period, with all experimental conditions being significantly greater than zero. Despite the presence of this apparent dose-response early in recovery, only participants who consumed the larger protein intake of the HP condition (~0.32 g·kg<sup>-1</sup>) were able to sustain a net anabolic environment (i.e., positive WBPB) over an entire 24-h period. This suggests there may be a threshold in order for healthy children to obtain a benefit of postexercise protein ingestion over the course of an entire day. Alternatively, as the study in **Chapter 5** only had three experimental conditions and did not demonstrate a plateau, it is possible that the dose needed to sustain anabolism lies between the intake of the MP ( $\sim 0.18 \text{ g} \cdot \text{kg}^{-1}$ ) and HP ( $\sim 0.32 \text{ g} \cdot \text{kg}^{-1}$ ) conditions.

In contrast to Chapter 5, however, when the children in Chapter 6 consumed a single ingestion of ~0.33 g·kg<sup>-1</sup> of protein immediately following exercise they tended to be in a net catabolic environment (i.e., negative WBPB) over the subsequent 24-h period. Though the possibility that these differences are a result of variations in the duration and modality of exercise (60 min of cycling in Chapter 6 compared with 45 min of alternating cycling and running in Chapter 5) cannot be discounted, it is not likely that these minor variations in exercise protocols would have a major impact on whole body protein metabolism over the course of 24 h. Although it must be acknowledged that the studies in this thesis utilized a variety of exercise protocols, each of the studies could be characterized as moderate-intensity aerobic exercise. For example, all exercise protocols consisted of 40-60 minutes of running or cycling between 50-60% VO<sub>2peak</sub> with heart rates between 70-85% of maximum heart rates. Moreover, it could be argued that the type of exercises chosen better mimics the natural exercise patterns of young children (i.e., team sports or free play) compared to more structured exercise protocols often used in the laboratory setting and, thus, could increase the generalizability of the findings. Therefore, it is possible that the discrepancies in the findings between **Chapters 5** and 6 are, in part, related other factors including the total dietary protein intake throughout the 24-h period.

The relative protein intake throughout an entire 24-h experimental period is of particular importance if energy or any amino acids are limiting, as this would lead to a concomitant increase in the oxidation of amino acids and general decrease in net protein anabolism (Elango et al. 2011). In Chapter 5, participants in the HP group had a total dietary protein intake of ~1.49 g·kg<sup>-1</sup> (including the experimental beverage) over the 24-h experimental session. In contrast, the HP group in Chapter 6 consumed  $\sim 1.20 \text{ g} \cdot \text{kg}^{-1}$ (including the experimental beverage) of dietary protein over 24 h. This resulted in the HP group of Chapter 6 having a 24-h protein intake closer to the CONT (1.16  $g \cdot kg^{-1}$ ) and even LP (1.35  $g \cdot kg^{-1}$ ) groups of Chapter 5, which both had a negative WBPB over the course of 24 h. Although these daily intakes are above the current RDA, recent recommendations have suggested an increase in the RDA for children (Elango et al. 2011). As seen in Figure 5.3C, the x-intercept would correspond to an estimated average requirement of ~1.23 g·kg<sup>-1</sup>·d<sup>-1</sup>. Similarly, as seen in Figure 6.5, the x-intercept would correspond to an estimated average requirement of  $\sim 1.25 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ . While it was not the intention of the present studies to re-assess the RDA in active children, nor was our design conducive to this goal, analysis of the 24-h WBPB findings relative to protein intake would generally concur with the suggestion that protein requirements may be elevated in active children. Importantly, the fact that the HP group in Chapter 6 consumed a 24-h habitual protein intake below the suggested x-intercept of both studies could help to explain the negative 24-h WBPB observed.

# 7.1.3. Acute effects of post-exercise protein intake on protein metabolism assessed by [1-<sup>13</sup>C]leucine infusion

Both Chapter 4 and Chapter 5 utilized the oral [<sup>15</sup>N]glycine end-product method to investigate the cumulative effects of exercise and protein ingestion on whole body protein metabolism. Given the relatively small differences in protein ingestion between conditions in both studies (~ranging between ~ $0.10 \text{ g} \cdot \text{kg}^{-1}$  to ~ $0.4 \text{ g} \cdot \text{kg}^{-1}$  at the most), the lack of sensitivity of the oral [<sup>15</sup>N]glycine may have precluded our ability to detect differences in protein turnover. Another consideration with the [<sup>15</sup>N]glycine methodology is the relatively long time-frame needed to accurately collect the metabolic end-product, given the specified time course of ammonia and urea excretion (Grove and Jackson Although this provides the benefit of allowing measures to occur over an 1995). extended period of time (e.g., as little as 9 h, and up to a full 24 h), this also provides some methodological restrictions. For example, given the ethical limitations to having children proceed for extended periods of time (at least  $\sim 7$  h) without eating, the children in both Chapters 4 and 5 received additional feedings over the course of the designed monitoring period, in supplement to their experimental beverage. While these additional feedings would have practical applicability and mimic the normal feeding patterns over the course of a day, it also limits the ability to investigate the isolated effects of a single nutritional intervention on post-exercise protein metabolism.

Currently, the arguable gold standard methodology to investigate whole-body protein metabolism is the primed, constant  $[1-^{13}C]$ leucine infusion method (Wagenmakers 1999). This methodology has greater sensitivity than the oral  $[^{15}N]$ glycine isotope, which

could allow for the detection of subtle, yet physiologically relevant, changes in whole body protein metabolism between relatively small acute differences in protein ingestion. Moreover, the [1-<sup>13</sup>C]leucine methodology has a better time resolution than the [<sup>15</sup>N]glycine methodology, and can measure protein turnover over 20-30 min intervals (Boirie et al. 1996). This makes it ideally suited to determine the acute effects of a single nutrition intervention on protein metabolism, without the confounding effects of additional feeding periods.

Given the need to better understand population-specific protein requirements (Rodriguez 2005), **Chapter 6** was designed to utilize, for the first time in children, the primed, constant [<sup>13</sup>C]leucine infusion technique to more accurately determine the effects of a single mixed-protein meal containing varying amounts of protein during the early (e.g., 3 h) post-exercise period. In this study we demonstrated that over 3 h of recovery from exercise, protein ingestion induced a dose-dependent response in whole body Leu<sub>BAL</sub>. Regardless of protein dose, protein ingestion was necessary to attain a net positive Leu<sub>BAL</sub> over this 3-h period.

In **Chapters 4** and **5**, additional biological effects that occurred in the body to contribute to the enhanced protein balance were not investigated. In contrast, blood samples were acquired in **Chapter 6** so as to examine changes in circulating amino acid, as well as insulin and glucose concentrations, over the same 3-h period that leucine kinetics were monitored. Although no significant differences were found in plasma insulin or glucose between the groups over the 3-h monitoring period, greater protein intakes resulted in greater plasma concentrations of leucine, branched-chain amino acids,

and essential amino acids. While the study was limited in its ability to determine the underlying mechanism(s) responsible for this enhanced protein balance, it is possible that, in children, the presence of BCAAs (namely leucine) in the post-exercise period have the ability to act independent of insulin to produce anabolic effects on protein metabolism (Elia and Livesey 1983), similar to what has been observed previously in adults.

## 7.1.4. Distribution of protein intake in the post-exercise period

Another critical issue, and one that might be arguably more important than protein quantity for enhancing WBPB, is the timing of protein intake relative to exercise (Moore et al. 2012, Areta et al. 2013). Chapter 6 was unique in that it had the secondary aim of addressing whether the timing of protein intake in relation to exercise (i.e., immediate vs. delayed) influenced the protein metabolic response in children. Despite all conditions receiving similar amounts of total protein ( $\sim 0.35 \text{ g} \cdot \text{kg}^{-1}$ ) from the two experimental beverages by 4 h after exercise, WBPB over the entire 24-h period differed between conditions. It appeared that the distribution of protein in relation to exercise, particularly the number of protein feedings, was more important than the absolute protein intake. For example, regardless of when the protein was consumed (either immediately or 4 h after exercise), both conditions that received 15 g of protein only once during the recovery period did not achieve a positive WBPB or N<sub>BAL</sub> over the 24-h period. On the other hand, both conditions that received protein twice throughout the recovery period attained a positive N<sub>BAL</sub> over the 24-h period, with one group also achieving a positive WBPB. These findings suggest that even if active children and youth consume enough protein throughout the day in their habitual diet, the distribution of the protein intake may have significant implications for their ability to effectively utilize the protein and attain the positive protein balance required for growth. Support for this theory has previously been demonstrated in free-living children where a greater distribution of animal protein throughout the day resulted in a greater  $N_{BAL}$  over a 3-d period (Barja et al. 1972).

## 7.2. METHODOLOGICAL ISSUES AND CONSIDERATIONS

### 7.2.1. Selecting an appropriate oral tracer for use with non-steady state equations

Studying protein metabolism after a single meal has practical implications for elucidating the potential synergies between exercise and protein ingestion in children, as it represents the normal feeding pattern following a bout of acute exercise. There are, however, a number of methodological consideration including the selection of an appropriate oral tracer and use of non-steady-state equations (Boirie et al. 1996). During a single protein meal, Boirie *et al.* (1996) concluded that the use of an intrinsically labeled protein is more appropriate than a free tracer added to protein. Unfortunately, due to a number of constraints the study in this thesis was forced to use free  $[^{2}H_{3}]$ leucine added to unlabeled milk-proteins, in combination with an IV infusion of  $[1-^{13}C]$ leucine.

One limitation to this approach is the assumption that the free  $[{}^{2}H_{3}]$ leucine provides a surrogate marker of dietary leucine appearance to model whole body kinetics. However, it is unknown if the proteins in the experimental beverage behaved identical to free labeled  $[{}^{2}H_{3}]$ leucine. **Appendix VII** shows the plasma appearance of leucine and both the intravenous and oral tracers. As the gastric emptying and gut absorption of free amino acids is rapid (Silk et al. 1979, Dangin et al. 2001b), it is likely that the peak in  $[^{2}H_{3}]$  leucine plasma enrichment had a more rapid absorption of the tracer than of the tracee. This is in line with the findings of Boirie et al. (1996) who discovered that the free leucine appeared in the plasma faster than the intrinsically labeled protein when ingesting whey proteins. As the present study used milk proteins, which contain both casein and whey, and provided this protein as a mixed meal with CHO, it is likely that the gastric emptying of the dietary protein was delayed even further compared with the free  $[^{2}H_{3}]$  leucine (Dangin et al. 2001b). This would likely have the greatest implications for the HP group, as the disconnect between the rapid appearance of a large quantity of free amino acids from the oral tracer and delayed ingestion the dietary protein from the experimental beverage would be magnified (see Appendix VIII). This is further evidenced in Figure 6.3; the sharp drop in Endo  $R_a$  at 60 min in HP compared with LP (P < 0.001) and CONT (P < 0.01) would be expected from the slower digestion of the larger protein intake, yet rapid uptake of the free  $[{}^{2}H_{3}]$  leucine.

Interestingly, the LP and MP conditions do not demonstrate the same initial drop in Endo  $R_a$ , and the time course of plasma leucine concentrations and [<sup>2</sup>H<sub>3</sub>]leucine enrichment demonstrate similar patterns (**Appendix VIII**). Moreover, both LP ( $r^2 = 0.96$ , P < 0.001) and MP ( $r^2 = 0.99$ , P < 0.001) demonstrate a strong linear and inverse relationship between the plasma enrichment of the oral and intravenous tracer (see **Appendix IX**). This relationship is similar to that shown in adults when whey protein was intrinsically labeled with [<sup>13</sup>C]leucine (Boirie et al. 1996), and would suggest that the dilution of the intravenous tracer is due to the appearance of the dietary leucine. Unfortunately, this relationship is weakened in the HP condition ( $r^2 = 0.58$ , P = 0.08) (**Appendix IX**). Together, these findings suggest that the approach of adding the free [<sup>2</sup>H<sub>3</sub>]leucine to the milk-protein beverage was more appropriate at lower protein contents.

## 7.2.2. Inter-individual variations in protein metabolism

Protein metabolism can differ significantly between individuals (Pencharz et al. 2008). The repeated measures design used in **Chapters 4** and **5** would have minimized any potential differences between groups due to inter-individual variability, and would increase the statistical confidence associated with the dose-response relationship (Pencharz et al. 2008). Unfortunately, participants in **Chapter 6** were randomized to a single experimental group and, as such, each participant only received 1 of the 4 possible protein intakes. In attempts to minimize differences between groups, participants were stratified by gender and lean body mass upon random allocation into groups. Although a within participant, repeated measures design would have strengthened the study, the lack of difference between experimental groups for any physical characteristics helps to support the idea that the groups were equally balanced and that the results seen were, in fact, due to the presence of protein. The homogeneity of the experimental groups is further supported by the fact that there were no statistical differences between leucine metabolism variables at baseline (time 0 min).

Although the groups in **Chapter 6** were balanced based on both body mass and lean body mass, the beverages were administered in a fixed volume with protein content

in absolute amounts (i.e., 0g, 5g, 10g or 15g in the CONT, LP, MP and HP conditions, respectively). Due to inter-individual differences in body mass within each condition, participants within the same group received a large variation in relative protein intake, which may have minimized the ability to detect differences between conditions given the relationship between relative protein intake and WBPB. Therefore, examining the *x*-intercept of the correlational data may provide more useful information of the relative quantity of protein required to achieve a positive WBPB in the acute post-exercise period, rather than assessing differences in WBPB between conditions.

# 7.2.3. Effects of sex and puberty on protein metabolism

Given the known sex- and growth-related differences in substrate utilization during exercise (Timmons et al. 2003, 2007a, 2007b), and the potential differences in protein metabolism (Haschke 1989, Beckett et al. 1997), **Chapter 4** examined the effects of puberty and sex on milk's ability to maintain WBPB. As a result, the study enrolled an equal number of boys and girls, as well as children of two distinct pubertal stages. Since pubertal status was based on the criteria of Tanner (Tanner 1955) (using pubic hair development for boys and breast development for girls), it was not possible to derive any information as to whether a child was entering a stage, leaving a stage, or how long they had been at a particular stage. Thus, the decision to include PEP and MLP children allowed us to assess two distinct pubertal groups and, arguably, bypass the potential inaccuracy of detecting differences around APHV.

Chapter 4 demonstrated that when consuming SM immediately following exercise, PEP girls were able to attain a positive WBPB over the 16-h recovery period, which is in contrast to the PEP boys and both the MLP groups who remained in a net negative WBPB. As a whole, the PEP children had an enhanced WBPB compared with the MLP children. Although it is possible that the MLP group required larger protein doses than those provided (i.e.,  $> 0.40 \text{ g} \cdot \text{kg}^{-1}$ ), this seems unlikely given the lack of relationship between beverage protein intake and WBPB in any of the groups (PEP girls,  $r^2 = 0.09, P = 0.51$ ; PEP boys,  $r^2 = 0.00, P = 0.89$ ; MLP girls,  $r^2 = 0.10, P = 0.60$ ; MLP boys,  $r^2 = 0.08$ , P = 0.55). One possibility for the pubertal difference in WBPB seen between the groups could be, in part, related to other aspects of the dietary intake. For example, there was a main effect of puberty (P < 0.05) for energy intake (kcal·kg<sup>-1</sup>) throughout the 16-h period, whereby PEP children consumed significantly more calories relative to body mass compared with the MLP group. Given the relationship between energy intake and protein metabolism (Calloway 1975), it is possible that if additional calories were provided, particularly in MLP youth, that group differences in WBPB would have been negated.

One limitation that must be acknowledged is the fact that the comparison of findings by sex and pubertal status were secondary outcomes. The primary outcome was related to rehydration (specifically, whole body fluid balance) (Volterman et al. 2014) and, therefore, sample size was based on the rehydration potential of the beverages, assumed from adult studies. As such, *a priori* power calculations were not performed for the protein measures. Part of the challenge when the study was designed was that no

previous literature had focused on the protein metabolic response to acute exercise and protein ingestion in children. Given the potentially misleading nature of retrospective power calculations, it would not be appropriate to calculate post-hoc power for these outcomes. As a result, it is possible that this study was insufficiently powered for several outcomes. For example, when examining the partial eta squared value as a marker of effect size for the sex and maturity factors in Chapter 4, effect sizes were large for protein turnover (0.72-0.96), small for protein synthesis (0.00-0.001), and small/medium for breakdown (0.03-0.09), suggesting that the study was likely insufficiently powered to fully detect differences by sex and maturity. Regardless, the fact that PEP girls were the only group to attain a positive WBPB cannot be discounted and demonstrates that there may, in fact, be important sex differences to consider when developing recommendations for protein intake in children and youth. Other studies have also suggested possible differences in daily protein needs between active boys and girls. For example, Aerenhouts et al. (2013) proposed that in order a reach a positive N<sub>BAL</sub>, even during periods of peak growth, a sufficient protein intake for girls and boys was 1.46 g·kg<sup>-1</sup>·day<sup>-1</sup> and 1.35 g·kg<sup>-1</sup>·day<sup>-1</sup>, respectively. Unfortunately, the current DRIs do not distinguish between sexes with respect to relative protein requirements. Further investigation into the potential sex differences in protein requirements using appropriately powered larger-scale studies is warranted.

In contrast to **Chapter 4**, the results of both sexes were pooled in both **Chapters 5** and **6**. Although these studies were not designed to assess potential sex differences in protein metabolism, the inclusion of both boys and girls cannot be overlooked.
Exploratory analysis in **Chapter 5** revealed that WBPB was not different between boys and girls ( $P \ge 0.30$ ). Additionally, secondary correlational analyses suggested that, independent of sex, WBPB over both 9 h (r = 0.14, P = 0.44) and 24 h (r = 0.20, P = 0.25) were not influenced by the biological age of the children (i.e. maturity offset). Therefore, within the constraints of the small study number (N = 13), the data were not influenced by sex or maturity *per se*. As with **Chapter 4**, the data presented in **Chapter 5** were secondary outcomes, with the primary outcome of the study being whole body fluid balance.

Exploratory analysis of the data from **Chapter 6** revealed that WBPB was not different between boys and girls at 6 h (P = 0.74); however, there was a trend for significance at 24 h (P = 0.07). When secondary correlational analyses were performed, it was observed that independent of sex, WBPB over 6 h (r = -0.12, P = 0.51) was not influenced by the biological age of the children (i.e. maturity offset). Interestingly, however, when controlling for sex, WBPB over 24 h (r = -0.34, P = 0.05) was significantly related to biological age. These findings lend to the importance of considering both biological age and sex when considering protein requirements for children. One major limitation in **Chapter 6** is that a small number of females (N= 9) were included in the study compared to males (N = 26). While the study was designed to recruit an even number of males and females, it was not possible to attain the target number of female participants within the recruitment time frame. In an attempt to minimize the impact of sex on between-group comparisons, participants were stratified based on sex when being randomized into study groups, which resulted in an equal

number of females across each condition (N = 3, 2, 2, and 2, for CON, LP, MP, and HP, respectively). Unfortunately, due to the constraints of the small number of females within each condition, the study was limited in its ability to detect statistical differences between genders across study conditions.

An important consideration with all three studies in this thesis is that study visits were not timed with menstrual cycles in our post-menarcheal females. In naturally cycling eumenorrhoeic women, the ovarian hormones (e.g., oestrogen and progesterone) fluctuate predictably between phases of the menstrual cycle, which can influence protein metabolism (Oosthuyse and Bosch 2012). Adult studies have demonstrated an increased catabolism in the luteal phase, both at rest (Lariviere et al. 1994, Kriengsinvos et al. 2004, Toth et al. 2006) and during exercise (Lamont et al. 1987, Bailey et al. 2000), which is generally a result of progesterone (Kriengsinyos et al. 2004). On the other hand, there is also evidence that oestrogen has a positive influence in decreasing protein oxidation (Hamadeh et al. 2005) both at rest and during exercise, which would lead to an enhanced protein balance. Therefore, protein requirements can vary across menstrual phases. The decision not to control for menstrual cycle in the studies within this thesis was made for two reasons: 1) in Chapters 4 and 5, the protein variables were secondary analyses and, as menstrual cycle did not affect the primary outcome of the study, it was not considered in the recruitment, and 2) many of the younger girls were pre-menarche at the time of testing or did not have regular cycles, making it exceedingly difficult to control for menstrual phase.

#### 7.3. PRACTICAL APPLICATIONS OF MILK AND MILK-BASED PROTEINS

Protein quality is an important factor to consider when addressing the needs for optimal growth and development in children (Rodriguez 2005). The studies in this thesis used SM and milk-based proteins as the source of dietary N in the post-exercise beverages, as they are complete, high-quality protein sources (i.e., PDCAAS > 1) (Schaafsma 2000). However, given the complete lack of data investigating protein source or quality on protein utilization in active, growing children, it remains to be determined whether other protein sources that may be of "lower quality" (e.g., plant-based) would have similar findings on post-exercise net protein balance as those observed in the present studies. This becomes of interest when considering the practical application of advising the consumption of bovine milk and milk-based proteins as a post-exercise recovery beverage to the general population. Despite the possible benefits to muscle and net protein balance, it is important to consider potential problems for individuals with lactose intolerance. It is estimated that more than 7 million Canadians experience some form of lactose intolerance, while some Asian populations can have prevalence rates of 90-100% (de Vrese et al. 2001). Therefore, future investigations should evaluate whether other protein sources are equally suitable to provide the same response for children who are not able to consume milk or its associated proteins.

Another practical consideration with using a nutrient-dense beverage such as milk as a post-exercise recovery beverage is the possibility that it may induce greater gastric disturbances compared with typical post-exercise beverages. The energy density of a solution is inversely proportional to the rate at which the solution is emptied from the stomach (McHugh and Moran 1979); therefore, the milk solution in **Chapter 4** would have emptied from the stomach at a slower rate than both the water and CES. As the energy density of a solution has a greater effect on gastric emptying than the components of energy-containing nutrients (Calbet and MacLean 1997), this would have less of an inter-beverage effect in **Chapters 5** and **6**, as the beverages used were isoenergetic. Although **Chapter 4** found no differences between groups with respect to subjective feelings including stomach fullness (data not shown), caution should be taken when consuming energy dense solutions like milk in fluid volumes that may induce gastric disturbances.

The use of milk and milk-based protein beverages in the post-exercise period can have benefits beyond whole body protein metabolism. Following an acute bout of exercise, the human body can incur significant metabolic demands. In addition to repairing/regenerating body proteins and skeletal muscle tissue, as well as restoring endogenous fuel stores (e.g., liver and muscle glycogen), another important property of a post-exercise recovery beverage is to promote rehydration. Previous pediatric research found that milk effectively induced a greater fluid balance and fraction of beverage retained in the body 2 h following exercise compared with water and a CES (Volterman et al. 2014). Recent evidence also demonstrated that the addition of protein to a postexercise CHO beverage favourably affected fluid retention in healthy, active children (Volterman et al. 2015). Thus, the combined beneficial effects on protein metabolism and rehydration suggest that milk and milk-based protein beverages may represent complete and suitable post-exercise recovery beverages for the active child.

# 7.4. LONG-TERM EFFECTS OF EXERCISE AND PROTEIN INGESTION ON PROTEIN METBAOLISM

The experiments in each of the studies in this thesis were performed exclusively following a single bout of exercise. While these acute studies (up to 24 h) provide initial evidence regarding the effects of post-exercise protein ingestion on whole body protein metabolism, it must be recognized that the effects following a single bout of exercise may be different from those observed over a prolonged period of time. For example, the adult literature has shown that although Leu<sub>OX</sub> increases following an acute bout of exercise, the contribution of this increase to total daily oxidation may be minimal (4-7%), with inconsequential effects on net body Leu<sub>BAL</sub> over 24-h (Forslund et al. 1999). El-Khoury et al. (1997) found that there was no impact on leucine homeostasis over a 24-h period (including two 90 min moderate exercise sessions, one during each of the fed and fasted periods) despite the initial exercise-induced increase in Leu<sub>OX</sub>. As a result, metabolic accommodations might occur over the 24-h period in an attempt to spare net leucine losses (Devlin et al. 1990). Therefore, although consumption of larger amounts of protein in the post-exercise period induced a positive net LeuBAL and WBPB in the acute postexercise period, it remains unclear whether these positive balances would be sufficient to translate into enhanced lean tissue remodeling or increased lean body mass over time. Future studies should examine whether acute elevations of WBPB (similar to those observed over 24 h in the present studies) can be sustained over periods of weeks to

months following chronic periods of exercise, a result which would ultimately be a prerequisite for the remodeling and growth of lean tissue in healthy, active children.

Another interesting question that cannot be answered by the present studies is the effect of protein ingestion on subsequent measures of exercise performance (e.g., strength and/or increased aerobic measures of oxidative capacity during exercise). The adult literature has provided evidence that CHO-protein co-ingestion in the post-exercise period has positive effects on subsequent exercise performance (Beelen et al. 2010), which could be beneficial for young athletes who are required to train or compete multiple times on the same or successive days – a phenomenon that is often experienced by children due to the nature of youth sports (i.e., tournament play) (Beelen et al. 2010). Although it is unlikely that changes in performance would be seen in the present studies given the relatively small differences between groups, it is possible that longitudinal effects on exercise performance may be seen. It would also be interesting to examine the relationship between changes in growth and lean body mass and subsequent effects on performance measures.

#### 7.5. KNOWLEDGE TRANSLATION

The role of protein in the diet and the potential usefulness of protein supplements have been of interest to athletes, coaches, and sport scientists for many years. Although children and youth are old enough to make decisions on foods they enjoy or dislike, they rely heavily on coaches and parents for nutritional advice, and on their parents to make and provide their meals. Despite the fact that most coaches have limited knowledge and training in nutrition, they are often in positions that require them to provide nutritional advice (Smith-Rockwell et al. 2001, Juzwiak and Ancona-Lopez 2004, Torres-McGehee et al. 2012). As a result, many coaches make inappropriate recommendations for protein intakes and weight gain for athletes (Shifflett et al. 2002). Consequently, there appears to be a large disconnect between an athlete's knowledge and current protein recommendations, especially in young athletes and those at the high school level (Shifflett et al. 2002), which puts these youth at risk of making inappropriate food choices that could ultimately affect their growth and performance (Jonnalagadda et al. 2001).

One problem surrounding the misconceptions of protein intake stems from the fact that even scientists often disagree on the amount of protein required for athletes (Phillips 2012), and, possibly more so in children and youth due to a general lack of understanding of protein metabolism. Another problem, however, is the disconnect that often exists when linking the scientific findings from the researchers to the end-users. For example, many athletes and coaches make decisions with little awareness of the scientific literature, and are of the belief that it is necessary to consume protein well above the RDA in order to obtain maximal gains in lean body mass (Jonnalagadda et al. 2001, Shifflett et al. 2002). While the findings of this thesis do generally support the suggestion for an increase in the RDA for [active] children (Elango et al. 2011), the increased requirement is likely still within the range that is normally being consumed by the diet alone (Petrie et al. 2004). For example, according to the Canadian Community Health Survey, usual daily protein intakes from food sources are 2.80 g·kg<sup>-1</sup>·d<sup>-1</sup> from 4 to 8 years of age, 1.91 g·kg<sup>-1</sup>·d<sup>-1</sup> for 9 to 13 year olds, and 1.64 g·kg<sup>-1</sup>·d<sup>-1</sup> for 14 to 18 year olds (Health Canada

2004). Similarly, all children in the present studies consumed an average habitual daily protein intake ranging between 1.56  $g \cdot kg^{-1} \cdot d^{-1}$  (**Chapter 5**) to 1.64  $g \cdot kg^{-1} \cdot d^{-1}$  (**Chapter 6**).

Given the fact that the majority of children in Ontario are likely to be consuming sufficient protein on a daily basis, it is not likely that these active children and youth require additional protein supplementation above and beyond their diet alone. Instead, children and youth may be able to benefit from consuming ~10-13g of high-quality dietary protein from whole-foods during the post-exercise period. It is possible that ~300-400 mL of bovine milk represents a good option for a protein-rich whole food following exercise. Other whole foods that contain high-quality protein (and are rich in leucine) include eggs and soy. Although further research is needed to confirm the impact of these protein sources on protein metabolism in the pediatric population, it is likely that they will also have beneficial effects on whole body protein anabolism.

What may, in fact, be more important for parents and coaches to consider, is the timing at which these young athletes consume their protein, particularly in relation to when they had their exercise session. This thesis provides insight that consuming protein in the immediate post-exercise period is important to enhance whole body protein metabolism through recovery; however, it may also be important to ensure that a child receives an additional source of protein within a few hours (i.e., 3-4 h) after completing the exercise in order to support the protein metabolic response over the course of a day (**Chapter 6**).

It is important that both young athletes and those individuals involved in advising young athletes receive accurate information and guidance for adequate protein intake. One means by which this could be accomplished is by creating pamphlets to provide to sporting organizations for distribution to their coaches. Another avenue could include small information facts in sport-specific magazines. Finally, academic institutions and laboratories can provide family-friendly resources on their websites, including short blurbs of their research findings using easy to read language. Therefore, in order to educate coaches, parents and children alike, future endeavours aimed to support appropriate knowledge translation to the greater community and to put the findings of this thesis to practical use, are needed.

#### 7.6. CONCLUSIONS

In conclusion, the studies in this thesis demonstrated the benefit of consuming a high-quality protein source such as SM or milk-based proteins in the post-exercise period in order to enhance WBPB in children and youth. Specifically, this thesis advanced our knowledge regarding the acute impact of protein ingestion on protein metabolism following a single bout of aerobic exercise in healthy active children by demonstrating: *1*) whole-protein beverages such as milk are more effective at enhancing WBPB compared with protein-free beverages typically consumed in the post-exercise period (i.e., CES and water); *2*) it is important to consider both biological age (i.e., pubertal stage) and sex when developing post-exercise protein recommendations for children and youth; *3*) post-exercise protein ingestion results in acute enhancements of net protein balance in children early in recovery in an apparent dose-dependent manner; *4*) total daily protein intake is an important consideration when making recommendations for the quantity of protein to be

consumed post-exercise; and *5*) the distribution of protein intake around exercise may be arguably more important than the quantity of protein consumed following exercise, with the consumption of multiple protein feedings in the post-exercise period having seemingly beneficial effects compared with a single feeding. Together, this information provides initial evidence upon which nutritional advice for active children can be built.

#### **CHAPTER 8 : References**

Aerenhouts, D., Van Cauwenberg, J., Poortmans, J.R., Hauspie, R., and Clarys, P. 2013. Influence of growth rate on nitrogen balance in adolescent sprint athletes. *Int. J. Sport Nutr. Exerc. Metab.* **23**(4): 409–417.

Aerenhouts, D., Hebbelinck, M., Poortmans, J.R., and Clarys, P. 2008. Nutritional habits of Flemish adolescent sprint athletes. *Int. J. Sport Nutr. Exerc. Metab.* **18**(5): 509–523.

Alexiou, D., Anagnostopoulos, A., and Papadatos, C. 1979. Total free amino acids, ammonia, and protein in the sweat of children. *Am. J. Clin. Nutr.* **32**(4): 750–752.

American College of Sports Medicine, Sawka, M.N., Burke, L.M., Eichner, E.R., Maughan, R.J., Montain, S.J., and Stachenfeld, N.S. 2007. American College of Sports Medicine position stand. Exercise and fluid replacement. *Med. Sci. Sports Exerc.* **39**(2): 377–390.

American Dietetic Association, Dietitians of Canada, American College of Sports Medicine, Rodriguez, N.R., Di Marco, N.M., and Langley, S. 2009. American College of Sports Medicine position stand. Nutrition and athletic performance. *Med. Sci. Sports Exerc.* **41**(3): 709–731.

Areta, J.L., Burke, L.M., Ross, M.L., Camera, D.M., West, D.W.D., Broad, E.M., Jeacocke, N.A., Moore, D.R., Stellingwerff, T., Phillips, S.M., Hawley, J.A., and Coffey, V.G. 2013. Timing and distribution of protein ingestion during prolonged recovery from resistance exercise alters myofibrillar protein synthesis. *J. Physiol.* **591**(Pt 9): 2319–2331.

Bailey, S.P., Zacher, C.M., and Mittleman, K.D. 2000. Effect of menstrual cycle phase on carbohydrate supplementation during prolonged exercise to fatigue. *J. Appl. Physiol.* **88**(2): 690–697.

Barja, I., Araya, H., Muñoz, P., Vega, L., Arteaga, A., and Tagle, M.A. 1972. Effect of spacing protein intake on nitrogen balance in normal children. *Am. J. Clin. Nutr.* **25**(5): 506–511.

Baxter-Jones, A.D.G., Eisenmann, J.C., Mirwald, R.L., Faulkner, R.A., and Bailey, D.A. 2008. The influence of physical activity on lean mass accrual during adolescence: a longitudinal analysis. *J. Appl. Physiol.* **105**(2): 734–741.

Beckett, P.R., Jahoor, F., and Copeland, K.C. 1997. The efficiency of dietary protein utilization is increased during puberty. *J. Clin. Endocrinol. Metab.* **82**(8): 2445–2449.

Beelen, M., Burke, L.M., Gibala, M.J., and van Loon L, J.C. 2010. Nutritional strategies to promote postexercise recovery. *Int. J. Sport Nutr. Exerc. Metab.* **20**(6): 515–532.

Beelen, M., Tieland, M., Gijsen, A.P., Vandereyt, H., Kies, A.K., Kuipers, H., Saris, W.H.M., Koopman, R., and van Loon, L.J.C. 2008. Coingestion of carbohydrate and protein hydrolysate stimulates muscle protein synthesis during exercise in young men, with no further increase during subsequent overnight recovery. *J. Nutr.* **138**(11): 2198–2204.

Berardi, J.M., Noreen, E.E., and Lemon, P.W. 2008. Recovery from a cycling time trial is enhanced with carbohydrate-protein supplementation vs. isoenergetic carbohydrate supplementation. *J. Int. Soc. Sports Nutr.* **5**: 24.

Berardi, J.M., Price, T.B., Noreen, E.E., and Lemon, P.W.R. 2006. Postexercise muscle glycogen recovery enhanced with a carbohydrate-protein supplement. *Med. Sci. Sports Exerc.* **38**(6): 1106–1113.

Berg, J.M., Tymoczko, J.L., and Stryer, L. 2002. The First Step in Amino Acid Degradation Is the Removal of Nitrogen. Biochemistry. 5<sup>th</sup> Edition. W H Freeman, New York.

Betts, J.A., and Williams, C. 2010. Short-term recovery from prolonged exercise: exploring the potential for protein ingestion to accentuate the benefits of carbohydrate supplements. *Sports Med.* **40**(11): 941–959.

Betts, J., Williams, C., Duffy, K., and Gunner, F. 2007. The influence of carbohydrate and protein ingestion during recovery from prolonged exercise on subsequent endurance performance. *J. Sports Sci.* **25**(13): 1449–1460.

Biolo, G., Declan Fleming, R.Y., and Wolfe, R.R. 1995a. Physiologic hyperinsulinemia stimulates protein synthesis and enhances transport of selected amino acids in human skeletal muscle. *J. Clin. Invest.* **95**(2): 811–819.

Biolo, G., Maggi, S.P., Williams, B.D., Tipton, K.D., and Wolfe, R.R. 1995b. Increased rates of muscle protein turnover and amino acid transport after resistance exercise in humans. *Am. J. Physiol.* **268**(3 Pt 1): E514–520.

Biolo, G., Tipton, K.D., Klein, S., and Wolfe, R.R. 1997. An abundant supply of amino acids enhances the metabolic effect of exercise on muscle protein. *Am. J. Physiol.* **273**(1 Pt 1): E122–129.

Biolo, G., Williams, B.D., Fleming, R.Y., and Wolfe, R.R. 1999. Insulin action on muscle protein kinetics and amino acid transport during recovery after resistance exercise. *Diabetes*. **48**(5): 949–957.

Blomstrand, E., Eliasson, J., Karlsson, H.K.R., and Köhnke, R. 2006. Branched-chain amino acids activate key enzymes in protein synthesis after physical exercise. *J. Nutr.* **136**(1 Suppl): 2698–738.

Bodamer, O.A., and Halliday, D. 2001. Uses of stable isotopes in clinical diagnosis and research in the paediatric population. *Arch. Dis. Child.* **84**(5): 444–448.

Bohé, J., Low, A., Wolfe, R.R., and Rennie, M.J. 2003. Human muscle protein synthesis is modulated by extracellular, not intramuscular amino acid availability: a dose-response study. *J. Physiol.* **552**(Pt 1): 315–324.

Boirie, Y., Dangin, M., Gachon, P., Vasson, M.P., Maubois, J.L., and Beaufrère, B. 1997. Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proc. Natl. Acad. Sci.* U. S. A. **94**(26): 14930–14935.

Boirie, Y., Gachon, P., Corny, S., Fauquant, J., Maubois, J.L., and Beaufrère, B. 1996. Acute postprandial changes in leucine metabolism as assessed with an intrinsically labeled milk protein. *Am. J. Physiol.* **271**(6 Pt 1): E1083–1091.

Boisseau, N., Persaud, C., Jackson, A.A., and Poortmans, J.R. 2005. Training does not affect protein turnover in pre- and early pubertal female gymnasts. *Eur. J. Appl. Physiol.* **94**(3): 262–267.

Boisseau, N., Vermorel, M., Rance, M., Duché, P., and Patureau-Mirand, P. 2007. Protein requirements in male adolescent soccer players. *Eur. J. Appl. Physiol.* **100**(1): 27–33.

Bolster, D.R., Pikosky, M.A., Gaine, P.C., Martin, W., Wolfe, R.R., Tipton, K.D., Maclean, D., Maresh, C.M., and Rodriguez, N.R. 2005. Dietary protein intake impacts human skeletal muscle protein fractional synthetic rates after endurance exercise. *Am. J. Physiol. Endocrinol. Metab.* **289**(4): E678–683.

Bolster, D.R., Pikosky, M.A., McCarthy, L.M., and Rodriguez, N.R. 2001. Exercise affects protein utilization in healthy children. *J. Nutr.* **131**(10): 2659–2663.

Børsheim, E., Cree, M.G., Tipton, K.D., Elliott, T.A., Aarsland, A., and Wolfe, R.R. 2004. Effect of carbohydrate intake on net muscle protein synthesis during recovery from resistance exercise. *J. Appl. Physiol.* **96**(2): 674–678.

Børsheim, E., Tipton, K.D., Wolf, S.E., and Wolfe, R.R. 2002. Essential amino acids and muscle protein recovery from resistance exercise. *Am. J. Physiol. Endocrinol. Metab.* **283**(4): E648–657.

Boye, J., Wijesinha-Bettoni, R., and Burlingame, B. 2012. Protein quality evaluation twenty years after the introduction of the protein digestibility corrected amino acid score method. *Br. J. Nutr.* **108 Suppl 2**: S183–211.

Breen, L., Philp, A., Witard, O.C., Jackman, S.R., Selby, A., Smith, K., Baar, K., and Tipton, K.D. 2011. The influence of carbohydrate-protein co-ingestion following endurance exercise on myofibrillar and mitochondrial protein synthesis. *J. Physiol.* **589**(Pt 16): 4011–4025.

Brosnan, J.T., and Brosnan, M.E. 2006. Branched-chain amino acids: enzyme and substrate regulation. *J. Nutr.* **136**(1 Suppl): 2078–11S.

Burd, N.A., Tang, J.E., Moore, D.R., and Phillips, S.M. 2009. Exercise training and protein metabolism: influences of contraction, protein intake, and sex-based differences. *J. Appl. Physiol.* 5 **106**(5): 1692–1701.

Burd, N.A., West, D.W.D., Moore, D.R., Atherton, P.J., Staples, A.W., Prior, T., Tang, J.E., Rennie, M.J., Baker, S.K., and Phillips, S.M. 2011. Enhanced amino acid sensitivity of myofibrillar protein synthesis persists for up to 24 h after resistance exercise in young men. *J. Nutr.* **141**(4): 568–573.

Burke, L.M., Kiens, B., and Ivy, J.L. 2004. Carbohydrates and fat for training and recovery. J. Sports Sci. **22**(1): 15–30. doi: 10.1080/0264041031000140527. Burke, L.M., Loucks, A.B., and Broad, N. 2006. Energy and carbohydrate for training and recovery. *J. Sports Sci.* **24**(7): 675–685.

Burke, L.M., Millet, G., Tarnopolsky, M.A., and International Association of Athletics Federations. 2007. Nutrition for distance events. *J. Sports Sci.* **25** Suppl 1: S29–38.

Burke, L.M., Winter, J.A., Cameron-Smith, D., Enslen, M., Farnfield, M., and Decombaz, J. 2012. Effect of intake of different dietary protein sources on plasma amino Acid profiles at rest and after exercise. *Int. J. Sport Nutr. Exerc. Metab.* **22**(6): 452–462.

Butte, N.F. 2000. Fat intake of children in relation to energy requirements. *Am. J. Clin. Nutr.* **72**(5 Suppl): 1246S–1252S.

Calbet, J.A., and MacLean, D.A. 1997. Role of caloric content on gastric emptying in humans. *J. Physiol.* **498 ( Pt 2)**: 553–559.

Calloway, D.H. 1975. Nitrogen balance of men with marginal intakes of protein and energy. J. Nutr. 105(7): 914–923.

Churchward-Venne, T.A., Burd, N.A., and Phillips, S.M. 2012. Nutritional regulation of muscle protein synthesis with resistance exercise: strategies to enhance anabolism. *Nutr. Metab.* 9(1): 40.

Cotugna, N., Vickery, C.E., and McBee, S. 2005. Sports Nutrition for Young Athletes. J. Sch. Nurs. **21**(6): 323–328.

Dangin, M., Boirie, Y., Garcia-Rodenas, C., Gachon, P., Fauquant, J., Callier, P., Ballèvre, O., and Beaufrère, B. 2001a. The digestion rate of protein is an independent regulating factor of postprandial protein retention. *Am. J. Physiol. Endocrinol. Metab.* **280**(2): E340–348.

Dangin, M., Boirie, Y., Garcia-Rodenas, C., Gachon, P., Fauquant, J., Callier, P., Ballèvre, O., and Beaufrère, B. 2001b. The digestion rate of protein is an independent regulating factor of postprandial protein retention. *Am. J. Physiol. Endocrinol. Metab.* **280**(2): E340–348.

Danner, D.J., Lemmon, S.K., Besharse, J.C., and Elsas, L.J. 1979. Purification and characterization of branched chain alpha-ketoacid dehydrogenase from bovine liver mitochondria. *J. Biol. Chem.* **254**(12): 5522–5526.

Darmaun, D., and Mauras, N. 2005. Use of stable isotopes to assess protein and amino acid metabolism in children and adolescents: a brief review. *Horm. Res.* **64 Suppl 3**: 32–37.

Darragh, A.J., and Hodgkinson, S.M. 2000. Quantifying the digestibility of dietary protein. *J. Nutr.* **130**(7): 1850S–6S.

Delistraty, D.A., Reisman, E.J., and Snipes, M. 1992. A physiological and nutritional profile of young female figure skaters. *J. Sports Med. Phys. Fitness.* **32**(2): 149–155.

Denne, S.C., Liechty, E.A., Liu, Y.M., Brechtel, G., and Baron, A.D. 1991. Proteolysis in skeletal muscle and whole body in response to euglycemic hyperinsulinemia in normal adults. *Am. J. Physiol.* **261**(6 Pt 1): E809–814.

Devlin, J.T., Brodsky, I., Scrimgeour, A., Fuller, S., and Bier, D.M. 1990. Amino acid metabolism after intense exercise. *Am. J. Physiol.* **258**(2 Pt 1): E249–255.

Drummond, M.J., and Rasmussen, B.B. 2008. Leucine-enriched nutrients and the regulation of mammalian target of rapamycin signalling and human skeletal muscle protein synthesis. *Curr. Opin. Clin. Nutr. Metab. Care.* **11**(3): 222–226.

Duggleby, S.L., and Waterlow, J.C. 2005. The end-product method of measuring wholebody protein turnover: a review of published results and a comparison with those obtained by leucine infusion. *Br. J. Nutr.* 94(2): 141–153.

Ebbeling, C.B., and Rodriguez, N.R. 1999. Effects of exercise combined with diet therapy on protein utilization in obese children. *Med. Sci. Sports Exerc.* **31**(3): 378–385.

Elango, R., Humayun, M.A., Ball, R.O., and Pencharz, P.B. 2011. Protein requirement of healthy school-age children determined by the indicator amino acid oxidation method. *Am. J. Clin. Nutr.* **94**(6): 1545–1552.

Elia, M., and Livesey, G. 1983. Effects of ingested steak and infused leucine on forelimb metabolism in man and the fate of the carbon skeletons and amino groups of branchedchain amino acids. *Clin. Sci. Lond. Engl.* 1979 **64**(5): 517–526.

Ellis, K.J. 1997. Body composition of a young, multiethnic, male population. *Am. J. Clin. Nutr.* **66**(6): 1323–1331.

Ellis, K.J., Abrams, S.A., and Wong, W.W. 1997. Body composition of a young, multiethnic female population. *Am. J. Clin. Nutr.* **65**(3): 724–731.

Faucette, N., Sallis, J.F., McKenzie, T., Alcaraz, J., Kolody, B., and Nugent, P. 1995. Comparison of fourth grade students' out-of-school physical activity levels and choices by gender: project SPARK. *J. Health Educ.* **26**(Suppl 2): S82-90.

Ferguson-Stegall, L., McCleave, E., Ding, Z., Doerner Iii, P.G., Liu, Y., Wang, B., Healy, M., Kleinert, M., Dessard, B., Lassiter, D.G., Kammer, L., and Ivy, J.L. 2011a. Aerobic exercise training adaptations are increased by postexercise carbohydrate-protein supplementation. *J. Nutr. Metab.* **2011**: 623182.

Ferguson-Stegall, L., McCleave, E.L., Ding, Z., Doerner, P.G., Wang, B., Liao, Y.-H., Kammer, L., Liu, Y., Hwang, J., Dessard, B.M., and Ivy, J.L. 2011b. Postexercise carbohydrate-protein supplementation improves subsequent exercise performance and intracellular signaling for protein synthesis. *J. Strength Cond. Res. Natl. Strength Cond. Assoc.* **25**(5): 1210–1224.

Fern, E.B., Garlick, P.J., McNurlan, M.A., and Waterlow, J.C. 1981. The excretion of isotope in urea and ammonia for estimating protein turnover in man with [15N]glycine. *Clin. Sci. (Lond).* **61**(2): 217–228.

Fern, E.B., Garlick, P.J., and Waterlow, J.C. 1985a. The concept of the single body pool of metabolic nitrogen in determining the rate of whole-body nitrogen turnover. *Hum. Nutr. Clin. Nutr.* **39**(2): 85–99.

Fern, E.B., Garlick, P.J., and Waterlow, J.C. 1985b. Apparent compartmentation of body nitrogen in one human subject: its consequences in measuring the rate of whole-body protein synthesis with 15N. *Clin. Sci. (Lond).* **68**(3): 271–282.

Forbes, G.B. 1981. Body composition in adolescence. Prog. Clin. Biol. Res. 61: 55-72.

Forslund, A.H., El-Khoury, A.E., Olsson, R.M., Sjödin, A.M., Hambraeus, L., and Young, V.R. 1999. Effect of protein intake and physical activity on 24-h pattern and rate of macronutrient utilization. *Am. J. Physiol.* **276**(5 Pt 1): E964–976.

Fryburg, D.A., Jahn, L.A., Hill, S.A., Oliveras, D.M., and Barrett, E.J. 1995. Insulin and insulin-like growth factor-I enhance human skeletal muscle protein anabolism during hyperaminoacidemia by different mechanisms. *J. Clin. Invest.* **96**(4): 1722–1729.

Fujita, S., Rasmussen, B.B., Cadenas, J.G., Grady, J.J., and Volpi, E. 2006. Effect of insulin on human skeletal muscle protein synthesis is modulated by insulin-induced changes in muscle blood flow and amino acid availability. *Am. J. Physiol. Endocrinol. Metab.* **291**(4): E745–754.

Gaine, P.C., Pikosky, M.A., Bolster, D.R., Martin, W.F., Maresh, C.M., and Rodriguez, N.R. 2007. Postexercise whole-body protein turnover response to three levels of protein intake. *Med. Sci. Sports Exerc.* **39**(3): 480–486.

Gaine, P.C., Pikosky, M.A., Martin, W.F., Bolster, D.R., Maresh, C.M., and Rodriguez, N.R. 2006. Level of dietary protein impacts whole body protein turnover in trained males at rest. *Metabolism*. **55**(4): 501–507.

Garlick, P.J. 2005. The role of leucine in the regulation of protein metabolism. *J. Nutr.* **135**(6 Suppl): 1553S–6S.

Gattas, V., Barrera, G.A., Riumallo, J.S., and Uauy, R. 1990. Protein-energy requirements of prepubertal school-age boys determined by using the nitrogen-balance response to a mixed-protein diet. *Am. J. Clin. Nutr.* **52**(6): 1037–1042.

Gelfand, R.A., and Barrett, E.J. 1987. Effect of physiologic hyperinsulinemia on skeletal muscle protein synthesis and breakdown in man. *J. Clin. Invest.* **80**(1): 1–6.

Gibala, M.J. 2007. Protein metabolism and endurance exercise. *Sports Med.* **37**(4-5): 337–340.

Gillis, L., Phillips, S., Atkinson, S.A., and Timmons, B.W. 2012. Milk combined with high intensity training improves body fat and protein balance in overweight children. *In* 19th European Congress on Obesity. Lyon, France.

Goforth, H.W., Laurent, D., Prusaczyk, W.K., Schneider, K.E., Petersen, K.F., and Shulman, G.I. 2003. Effects of depletion exercise and light training on muscle glycogen supercompensation in men. *Am. J. Physiol. Endocrinol. Metab.* **285**(6): E1304–1311.

Goulding, A., Taylor, R.W., Grant, A.M., Jones, S., Taylor, B.J., and Williams, S.M. 2009. Relationships of appendicular LMI and total body LMI to bone mass and physical activity levels in a birth cohort of New Zealand five-year olds. *Bone* **45**(3): 455–459.

Greenhaff, P.L., Karagounis, L.G., Peirce, N., Simpson, E.J., Hazell, M., Layfield, R., Wackerhage, H., Smith, K., Atherton, P., Selby, A., and Rennie, M.J. 2008. Disassociation between the effects of amino acids and insulin on signaling, ubiquitin ligases, and protein turnover in human muscle. *Am. J. Physiol. Endocrinol. Metab.* **295**(3): E595–604.

Grove, G., and Jackson, A.A. 1995. Measurement of protein turnover in normal man using the end-product method with oral [15N]glycine: comparison of single-dose and intermittent-dose regimens. *Br. J. Nutr.* 74(4): 491–507.

Hallal, P.C., Victora, C.G., Azevedo, M.R., and Wells, J.C.K. 2006. Adolescent physical activity and health: a systematic review. *Sports Med.* **36**(12): 1019–1030.

Hamadeh, M.J., Devries, M.C., and Tarnopolsky, M.A. 2005. Estrogen supplementation reduces whole body leucine and carbohydrate oxidation and increases lipid oxidation in men during endurance exercise. *J. Clin. Endocrinol. Metab.* **90**(6): 3592–3599.

Hartman, J.W., Moore, D.R., and Phillips, S.M. 2006. Resistance training reduces wholebody protein turnover and improves net protein retention in untrained young males. *Appl. Physiol. Nutr. Metab.* **31**(5): 557–564.

Hartman, J.W., Tang, J.E., Wilkinson, S.B., Tarnopolsky, M.A., Lawrence, R.L., Fullerton, A.V., and Phillips, S.M. 2007. Consumption of fat-free fluid milk after resistance exercise promotes greater lean mass accretion than does consumption of soy or carbohydrate in young, novice, male weightlifters. *Am. J. Clin. Nutr.* **86**(2): 373–381.

Haschke, F. 1989. Body composition during adolescence. *In* Body Composition Measurements in Infants and Children: Report of the 98th Ross Conference on Pediatric Research. Ross Laboratories, Columbus, OH. pp. 76–83.

Hawley, J.A., Burke, L.M., Phillips, S.M., and Spriet, L.L. 2011. Nutritional modulation of training-induced skeletal muscle adaptations. *J. Appl. Physiol.* **110**(3): 834–845.

Hawley, J.A., Tipton, K.D., and Millard-Stafford, M.L. 2006. Promoting training adaptations through nutritional interventions. *J. Sports Sci.* **24**(7): 709–721.

Health Canada. 2004. Energy and nutrient intakes of Ontario children and adults. *Canadian Community Health Survey Cycle 2.2 Ontario Nutrition Data Analysis Project.* 

Hillier, T.A., Fryburg, D.A., Jahn, L.A., and Barrett, E.J. 1998. Extreme hyperinsulinemia unmasks insulin's effect to stimulate protein synthesis in the human forearm. *Am. J. Physiol.* **274**(6 Pt 1): E1067–1074.

Horswill, C.A., Park, S.H., and Roemmich, J.N. 1990. Changes in the protein nutritional status of adolescent wrestlers. *Med. Sci. Sports Exerc.* **22**(5): 599–604.

Howarth, K.R., Moreau, N.A., Phillips, S.M., and Gibala, M.J. 2009. Coingestion of protein with carbohydrate during recovery from endurance exercise stimulates skeletal muscle protein synthesis in humans. *J. Appl. Physiol.* **106**(4): 1394–1402.

Howarth, K.R., Phillips, S.M., MacDonald, M.J., Richards, D., Moreau, N.A., and Gibala, M.J. 2010. Effect of glycogen availability on human skeletal muscle protein turnover during exercise and recovery. *J. Appl. Physiol.* **109**(2): 431–438.

Humayun, M.A., Elango, R., Ball, R.O., and Pencharz, P.B. 2007. Reevaluation of the protein requirement in young men with the indicator amino acid oxidation technique. Am. *J. Clin. Nutr.* **86**(4): 995–1002.

Huth, P.J., DiRienzo, D.B., and Miller, G.D. 2006. Major scientific advances with dairy foods in nutrition and health. *J. Dairy Sci.* **89**(4): 1207–1221.

Imura, K., and Okada, A. 1998. Amino acid metabolism in pediatric patients. Nutr. Burbank Los Angel. Cty. Calif **14**(1): 143–148.

Jacob, J.A., and Nair, M.K.C. 2012. Protein and micronutrient supplementation in complementing pubertal growth. *Indian J. Pediatr.* **79 Suppl 1**: S84–91.

Jeejeebhoy, K.N. 1986. Nutritional balance studies: indicators of human requirements or adaptive mechanisms. *J. Nutr.* **116**(10): 2061–2063.

Jentjens, R., and Jeukendrup, A. 2003. Determinants of post-exercise glycogen synthesis during short-term recovery. *Sports Med.* **33**(2): 117–144.

Jonnalagadda, S.S., Rosenbloom, C.A., and Skinner, R. 2001. Dietary practices, attitudes, and physiological status of collegiate freshman football players. *J. Strength Cond. Res.* **15**(4): 507–513.

Juzwiak, C.R., and Ancona-Lopez, F. 2004. Evaluation of nutrition knowledge and dietary recommendations by coaches of adolescent Brazilian athletes. *Int. J. Sport Nutr. Exerc. Metab.* **14**(2): 222–235.

Kaastra, B., Manders, R.J.F., Van Breda, E., Kies, A., Jeukendrup, A.E., Keizer, H.A., Kuipers, H., and Van Loon, L.J.C. 2006. Effects of increasing insulin secretion on acute postexercise blood glucose disposal. *Med. Sci. Sports Exerc.* **38**(2): 268–275.

Kabasakalis, A., Kalitsis, K., Nikolaidis, M.G., Tsalis, G., Kouretas, D., Loupos, D., and Mougios, V. 2009. Redox, iron, and nutritional status of children during swimming training. *J. Sci. Med. Sport Sports Med. Aust.* **12**(6): 691–696.

Kelder, S.H., Perry, C.L., Klepp, K.I., and Lytle, L.L. 1994. Longitudinal tracking of adolescent smoking, physical activity, and food choice behaviors. *Am. J. Public Health* **84**(7): 1121–1126.

el-Khoury, A.E., Forslund, A., Olsson, R., Branth, S., Sjödin, A., Andersson, A., Atkinson, A., Selvaraj, A., Hambraeus, L., and Young, V.R. 1997. Moderate exercise at energy balance does not affect 24-h leucine oxidation or nitrogen retention in healthy men. *Am. J. Physiol.* **273**(2 Pt 1): E394–407.

Koletzko, B., Demmelmair, H., Hartl, W., Kindermann, A., Koletzko, S., Sauerwald, T., and Szitanyi, P. 1998. The use of stable isotope techniques for nutritional and metabolic research in paediatrics. *Early Hum. Dev.* **53 Suppl**: S77–97.

Koopman, R., Beelen, M., Stellingwerff, T., Pennings, B., Saris, W.H.M., Kies, A.K., Kuipers, H., and van Loon, L.J.C. 2007a. Coingestion of carbohydrate with protein does not further augment postexercise muscle protein synthesis. *Am. J. Physiol. Endocrinol. Metab.* **293**(3): E833–842.

Koopman, R., Crombach, N., Gijsen, A.P., Walrand, S., Fauquant, J., Kies, A.K., Lemosquet, S., Saris, W.H.M., Boirie, Y., and van Loon, L.J.C. 2009. Ingestion of a protein hydrolysate is accompanied by an accelerated in vivo digestion and absorption rate when compared with its intact protein. *Am. J. Clin. Nutr.* **90**(1): 106–115.

Koopman, R., Saris, W.H.M., Wagenmakers, A.J.M., and van Loon, L.J.C. 2007b. Nutritional interventions to promote post-exercise muscle protein synthesis. *Sports Med.* **37**(10): 895–906.

Koopman, R., Wagenmakers, A.J.M., Manders, R.J.F., Zorenc, A.H.G., Senden, J.M.G., Gorselink, M., Keizer, H.A., and van Loon, L.J.C. 2005. Combined ingestion of protein and free leucine with carbohydrate increases postexercise muscle protein synthesis in vivo in male subjects. *Am. J. Physiol. Endocrinol. Metab.* **288**(4): E645–653.

Kriengsinyos, W., Wykes, L.J., Goonewardene, L.A., Ball, R.O., and Pencharz, P.B. 2004. Phase of menstrual cycle affects lysine requirement in healthy women. *Am. J. Physiol. Endocrinol. Metab.* **287**(3): E489–496.

Kumar, V., Atherton, P., Smith, K., and Rennie, M.J. 2009. Human muscle protein synthesis and breakdown during and after exercise. *J. Appl. Physiol.* 1985 **106**(6): 2026–2039.

Lamont, L.S., Lemon, P.W., and Bruot, B.C. 1987. Menstrual cycle and exercise effects on protein catabolism. *Med. Sci. Sports Exerc.* **19**(2): 106–110.

Lariviere, F., Moussalli, R., and Garrel, D.R. 1994. Increased leucine flux and leucine oxidation during the luteal phase of the menstrual cycle in women. *Am. J. Physiol.* **267**(3 Pt 1): E422–428.

Layman, D.K. 2003. The role of leucine in weight loss diets and glucose homeostasis. *J. Nutr.* **133**(1): 261S–267S.

Leick, L., Plomgaard, P., Grønløkke, L., Al-Abaiji, F., Wojtaszewski, J.F.P., and Pilegaard, H. 2010. Endurance exercise induces mRNA expression of oxidative enzymes in human skeletal muscle late in recovery. *Scand. J. Med. Sci. Sports* **20**(4): 593–599.

Lemon, P.W. 1996. Is increased dietary protein necessary or beneficial for individuals with a physically active lifestyle? *Nutr. Rev.* **54**(4 Pt 2): S169–175.

Lemon, P.W., and Mullin, J.P. 1980. Effect of initial muscle glycogen levels on protein catabolism during exercise. *J. Appl. Physiol.* **48**(4): 624–629.

Levenhagen, D.K., Carr, C., Carlson, M.G., Maron, D.J., Borel, M.J., and Flakoll, P.J. 2002. Postexercise protein intake enhances whole-body and leg protein accretion in humans. *Med. Sci. Sports Exerc.* **34**(5): 828–837.

Levenhagen, D.K., Gresham, J.D., Carlson, M.G., Maron, D.J., Borel, M.J., and Flakoll, P.J. 2001. Postexercise nutrient intake timing in humans is critical to recovery of leg glucose and protein homeostasis. *Am. J. Physiol. Endocrinol. Metab.* **280**(6): E982–993.

van Loon, L.J.C. 2014. Dietary protein as a trigger for metabolic adaptation. *In* The Encyclopaedia of Sports Medicine: An IOC Medical Commission Publication, Volume XIX, Sports Nutrition, Edited by Maughan RJ. Wiley-Blackwell, Oxford. pp. 147–155.

van Loon, L.J., Greenhaff, P.L., Constantin-Teodosiu, D., Saris, W.H., and Wagenmakers, A.J. 2001. The effects of increasing exercise intensity on muscle fuel utilisation in humans. *J. Physiol.* **536**(Pt 1): 295–304.

van Loon, L.J., Kruijshoop, M., Verhagen, H., Saris, W.H., and Wagenmakers, A.J. 2000a. Ingestion of protein hydrolysate and amino acid-carbohydrate mixtures increases postexercise plasma insulin responses in men. *J. Nutr.* **130**(10): 2508–2513.

van Loon, L.J., Saris, W.H., Kruijshoop, M., and Wagenmakers, A.J. 2000b. Maximizing postexercise muscle glycogen synthesis: carbohydrate supplementation and the application of amino acid or protein hydrolysate mixtures. *Am. J. Clin. Nutr.* **72**(1): 106–111.

van Loon, L.J., Saris, W.H., Verhagen, H., and Wagenmakers, A.J. 2000c. Plasma insulin responses after ingestion of different amino acid or protein mixtures with carbohydrate. *Am. J. Clin. Nutr.* **72**(1): 96–105.

Lunn, W.R., Pasiakos, S.M., Colletto, M.R., Karfonta, K.E., Carbone, J.W., Anderson, J.M., and Rodriguez, N.R. 2012. Chocolate milk and endurance exercise recovery: protein balance, glycogen, and performance. *Med. Sci. Sports Exerc.* **44**(4): 682–691.

Mager, D.R., Wykes, L.J., Ball, R.O., and Pencharz, P.B. 2003. Branched-chain amino acid requirements in school-aged children determined by indicator amino acid oxidation (IAAO). *J. Nutr.* **133**(11): 3540–3545.

Mahon, A.D., and Timmons, B.W. 2014. Application of stable isotope tracers in the study of exercise metabolism in children: a primer. *Pediatr. Exerc. Sci.* **26**(1): 3–10.

Matthews, D.E., Schwarz, H.P., Yang, R.D., Motil, K.J., Young, V.R., and Bier, D.M. 1982. Relationship of plasma leucine and alpha-ketoisocaproate during a L-[1-13C]leucine infusion in man: a method for measuring human intracellular leucine tracer enrichment. *Metabolism*. **31**(11): 1105–1112.

McHugh, P.R., and Moran, T.H. 1979. Calories and gastric emptying: a regulatory capacity with implications for feeding. *Am. J. Physiol.* **236**(5): R254–260.

McNurlan, M.A., and Garlick, P.J. 1980. Contribution of rat liver and gastrointestinal tract to whole-body protein synthesis in the rat. *Biochem. J.* **186**(1): 381–383.

Meakins, T.S., and Jackson, A.A. 1996. Salvage of exogenous urea nitrogen enhances nitrogen balance in normal men consuming marginally inadequate protein diets. *Clin. Sci. (Lond).* **90**(3): 215–225.

Meyer, F., O'Connor, H., Shirreffs, S.M., and International Association of Athletics Federations. 2007. Nutrition for the young athlete. *J. Sports Sci.* **25 Suppl 1**: S73–82.

Miller, S.L., Tipton, K.D., Chinkes, D.L., Wolf, S.E., and Wolfe, R.R. 2003. Independent and combined effects of amino acids and glucose after resistance exercise. *Med. Sci. Sports Exerc.* **35**(3): 449–455.

Millward, D.J. 2012. Amino acid scoring patterns for protein quality assessment. *Br. J. Nutr.* **108 Suppl 2**: S31–43.

Millward, D.J., Layman, D.K., Tomé, D., and Schaafsma, G. 2008. Protein quality assessment: impact of expanding understanding of protein and amino acid needs for optimal health. *Am. J. Clin. Nutr.* **87**(5): 1576S–1581S.

Monirujjaman, M., and Ferdouse, A. 2014. Metabolic and physiological roles of branched-chain amino acids. A *Adv. Molec. Biol.* 2014: 6 pages

Moore, D.R., Areta, J., Coffey, V.G., Stellingwerff, T., Phillips, S.M., Burke, L.M., Cléroux, M., Godin, J.-P., and Hawley, J.A. 2012. Daytime pattern of post-exercise protein intake affects whole-body protein turnover in resistance-trained males. *Nutr. Metab.* **9**(1): 91.

Moore, D.R., Camera, D.M., Areta, J.L., and Hawley, J.A. 2014. Beyond muscle hypertrophy: why dietary protein is important for endurance athletes. *Appl. Physiol. Nutr. Metab.* **39**(9): 987–997.

Moore, D.R., Robinson, M.J., Fry, J.L., Tang, J.E., Glover, E.I., Wilkinson, S.B., Prior, T., Tarnopolsky, M.A., and Phillips, S.M. 2009. Ingested protein dose response of muscle and albumin protein synthesis after resistance exercise in young men. *Am. J. Clin. Nutr.* **89**(1): 161–168.

Nakagawa, I., Takahashi, T., and Suzuki, T. 1961a. Amino acid requirements of children: Isoleucine and leucine. *J. Nutr.* **73**: 186–190.

Nakagawa, I., Takahashi, T., and Suzuki, T. 1961b. Amino acid requirements of children: Minimal needs of lysine and methionine based on nitrogen balance method. *J. Nutr.* **74**: 401–407.

Nakagawa, I., Takahashi, T., Suzuki, T., and Kobayashi, K. 1962. Amino acid requirements of children: Minimal needs of threonine, valine and phenylalanine based on nitrogen balance method. *J. Nutr.* **77**: 61–68.

Nakagawa, I., Takahashi, T., Suzuki, T., and Kobayashi, K. 1963. Amino acid requirements of children: Minimal needs of tryptophan, valine and phenylalanine based on nitrogen balance method. *J. Nutr.* **80**: 305–310.

Nakagawa, I., Takahashi, T., Suzuki, T., and Kobayashi, K. 1964. Amino acid requirements of children: Nitrogen balance at the minimal level of essential amino acids. *J. Nutr.* **83**: 115–118.

National Research Council. 2005. Dietary reference intakes for energy, carbohydrate, fiber, fat, fatty acids, cholesterol, protein, and amino acids (Macronutrients). The National Academy Press, Washington, DC.

Neubauer, O., Sabapathy, S., Ashton, K.J., Desbrow, B., Peake, J.M., Lazarus, R., Wessner, B., Cameron-Smith, D., Wagner, K.-H., Haseler, L.J., and Bulmer, A.C. 2014. Time course-dependent changes in the transcriptome of human skeletal muscle during recovery from endurance exercise: from inflammation to adaptive remodeling. *J. Appl. Physiol.* **116**(3): 274–287.

Nieman, P. 2002. Psychosocial aspects of physical activity. *Paediatr. Child Health* 7(5): 309–312.

Nuttall, F.Q., Mooradian, A.D., Gannon, M.C., Billington, C., and Krezowski, P. 1984. Effect of protein ingestion on the glucose and insulin response to a standardized oral glucose load. *Diabetes Care* **7**(5): 465–470.

Oosthuyse, T., and Bosch, A.N. 2012. The Effect of the Menstrual Cycle on Exercise Metabolism. *Sports Med.* **40**(3): 207–227.

Pacy, P.J., Price, G.M., Halliday, D., Quevedo, M.R., and Millward, D.J. 1994. Nitrogen homeostasis in man: the diurnal responses of protein synthesis and degradation and amino acid oxidation to diets with increasing protein intakes. *Clin. Sci. (Lond).* **86**(1): 103–116.

Paddon-Jones, D. 2006. Interplay of stress and physical inactivity on muscle loss: Nutritional countermeasures. *J. Nutr.* **136**(8): 2123–2126.

Pallotta, J.A., and Kennedy, P.J. 1968. Response of plasma insulin and growth hormone to carbohydrate and protein feeding. *Metabolism*. **17**(10): 901–908.

Papadopoulou, S.K., Papadopoulou, S.D., and Gallos, G.K. 2002. Macro- and micronutrient intake of adolescent Greek female volleyball players. Int. J. Sport Nutr. Exerc. Metab. **12**(1): 73–80.

ParticipATION. 2014. The Biggest Risk is Keeping Kids Indoors: The ParticipACTION report card on the physical activity of children and youth. Toronto, ON.

Pasiakos, S.M., McLellan, T.M., and Lieberman, H.R. 2015. The effects of protein supplements on muscle mass, strength, and aerobic and anaerobic power in healthy adults: a systematic review. *Sports Med.* **45**(1): 111–131.

Pencharz, P.B., Elango, R., and Ball, R.O. 2008. An approach to defining the upper safe limits of amino acid intake. *J. Nutr.* **138**(10): 1996S–2002S.

Pennings, B., Boirie, Y., Senden, J.M.G., Gijsen, A.P., Kuipers, H., and van Loon, L.J.C. 2011. Whey protein stimulates postprandial muscle protein accretion more effectively than do casein and casein hydrolysate in older men. *Am. J. Clin. Nutr.* **93**(5): 997–1005.

Petrie, H.J., Stover, E.A., and Horswill, C.A. 2004. Nutritional concerns for the child and adolescent competitor. *Nutrition* **20**(7–8): 620–631.

Phillips, S.M. 2012. Dietary protein requirements and adaptive advantages in athletes. Br *J. Nutr.* **108**(Suppl 2): S158–67.

Phillips, S.M., Hartman, J.W., and Wilkinson, S.B. 2005. Dietary protein to support anabolism with resistance exercise in young men. *J. Am. Coll. Nutr.* **24**(2): 134S–139S.

Phillips, S.M., and Van Loon, L.J.C. 2011. Dietary protein for athletes: from requirements to optimum adaptation. *J. Sports Sci.* **29 Suppl 1**: S29–38.

Phillips, S.M., Tang, J.E., and Moore, D.R. 2009. The role of milk- and soy-based protein in support of muscle protein synthesis and muscle protein accretion in young and elderly persons. *J. Am. Coll. Nutr.* **28**(4): 343–354.

Phillips, S.M., Tipton, K.D., Aarsland, A., Wolf, S.E., and Wolfe, R.R. 1997. Mixed muscle protein synthesis and breakdown after resistance exercise in humans. *Am. J. Physiol.* **273**(1 Pt 1): E99–107.

Pike, R., and Brown, M. 1984. Nutrition. An integrated approach, 3rd ed. MacMillan Publishing Co, New York.

Pikosky, M., Faigenbaum, A., Westcott, W., and Rodriguez, N. 2002. Effects of resistance training on protein utilization in healthy children. *Med. Sci. Sports Exerc.* **34**(5): 820–827.

Pitkanen, H.T., Nykanen, T., Knuutinen, J., Lahti, K., Keinanen, O., Alen, M., Komi, P.V., and Mero, A.A. 2003. Free amino acid pool and muscle protein balance after resistance exercise. *Med. Sci. Sports Exerc.* **35**(5): 784–792.

Pitts, R.F., and Pilkington, L.A. 1966. The relation between plasma concentrations of glutamine and glycine and utilization of their nitrogens as sources of urinary ammonia. *J. Clin. Invest.* **45**(1): 86–93.

Rabinowitz, D., Merimee, T.J., Maffezzoli, R., and Burgess, J.A. 1966. Patterns of hormonal release after glucose, protein, and glucose plus protein. *Lancet* **2**(7461): 454–456.

Rand, W.M., Pellett, P.L., and Young, V.R. 2003. Meta-analysis of nitrogen balance studies for estimating protein requirements in healthy adults. *Am. J. Clin. Nutr.* **77**(1): 109–127.

Rand, W.M., Young, V.R., and Scrimshaw, N.S. 1976. Change of urinary nitrogen excretion in response to low-protein diets in adults. *Am. J. Clin. Nutr.* **29**(6): 639–644.

Rasmussen, B.B., Tipton, K.D., Miller, S.L., Wolf, S.E., and Wolfe, R.R. 2000. An oral essential amino acid-carbohydrate supplement enhances muscle protein anabolism after resistance exercise. *J. Appl. Physiol.* **88**(2): 386–392.

Rauch, F., Bailey, D.A., Baxter-Jones, A., Mirwald, R., and Faulkner, R. 2004. The "muscle-bone unit" during the pubertal growth spurt. *Bone* **34**(5): 771–775.

Reeds, P.J., and Garlick, P.J. 1984. Nutrition and protein turnover in man. *Adv. Nutr. Res.* **6**: 93–138.

Rees, J.M., and Christine, M.T. (1989). Nutritional influences on physical growth and behaviour in adolescence. *In* Biology of adolescent behaviour and development. Sage Publications., Thousand Oaks, CA. pp. 195-222.

Rennie, M.J., and Tipton, K.D. 2000. Protein and amino acid metabolism during and after exercise and the effects of nutrition. *Annu. Rev. Nutr.* **20**: 457–483.

Res, P.T., Groen, B., Pennings, B., Beelen, M., Wallis, G.A., Gijsen, A.P., Senden, J.M.G., and VAN Loon, L.J.C. 2012. Protein ingestion before sleep improves postexercise overnight recovery. *Med. Sci. Sports Exerc.* **44**(8): 1560–1569.

Rivera-Brown, A.M., and Frontera, W.R. 2012. Principles of exercise physiology: responses to acute exercise and long-term adaptations to training. PM R 4(11): 797–804.

Robinson, M.M., Turner, S.M., Hellerstein, M.K., Hamilton, K.L., and Miller, B.F. 2011. Long-term synthesis rates of skeletal muscle DNA and protein are higher during aerobic training in older humans than in sedentary young subjects but are not altered by protein supplementation. *FASEB J.* **25**(9): 3240–3249.

Rodriguez, N.R. 2005. Optimal quantity and composition of protein for growing children. *J. Am. Coll. Nutr.* **24**(2): 1508–1548.

Rogol, A.D., Clark, P.A., and Roemmich, J.N. 2000. Growth and pubertal development in children and adolescents: effects of diet and physical activity. *Am. J. Clin. Nutr.* **72**(2 Suppl): 521S–8S.

Romijn, J.A., Coyle, E.F., Sidossis, L.S., Gastaldelli, A., Horowitz, J.F., Endert, E., and Wolfe, R.R. 1993. Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration. *Am. J. Physiol.* **265**(3 Pt 1): E380–391.

Rooyackers, O.E., and Nair, K.S. 1997. Hormonal regulation of human muscle protein metabolism. *Annu. Rev. Nutr.* **17**: 457–485.

Rowlands, D.S., Thorp, R.M., Rossler, K., Graham, D.F., and Rockell, M.J. 2007. Effect of protein-rich feeding on recovery after intense exercise. *Int. J. Sport Nutr. Exerc. Metab.* **17**(6): 521–543.

Schaafsma, G. 2000. The protein digestibility-corrected amino acid score. J. Nutr. 130(7): 1865S–7S.

Schaafsma, G. 2005. The Protein Digestibility-Corrected Amino Acid Score (PDCAAS)-a concept for describing protein quality in foods and food ingredients: a critical review. *J. AOAC Int.* **88**(3): 988–994.

Schoenfeld, B.J., Aragon, A.A., and Krieger, J.W. 2013. The effect of protein timing on muscle strength and hypertrophy: a meta-analysis. *J. Int. Soc. Sports Nutr.* **10**(1): 53. doi: 10.1186/1550-2783-10-53.

Shields, M. 2005. Measured Obesity: Overweight Canadian children and adolescents. *In* ISSN. Statistics Canada, Analytic Studies and Reports.

Shifflett, B., Timm, C., and Kahanov, L. 2002. Understanding of athletes' nutritional needs among athletes, coaches, and athletic trainers. *Res. Q. Exerc. Sport* **73**(3): 357–362.

Shirreffs, S.M., Watson, P., and Maughan, R.J. 2007. Milk as an effective post-exercise rehydration drink. *Br. J. Nutr.* **98**(1): 173–180.

Sigal, R.J., Kenny, G.P., Wasserman, D.H., Castaneda-Sceppa, C., and White, R.D. 2006. Physical activity/exercise and type 2 diabetes: a consensus statement from the American Diabetes Association. *Diabetes Care* **29**(6): 1433–1438.

Silk, D.B., Chung, Y.C., Berger, K.L., Conley, K., Beigler, M., Sleisenger, M.H., Spiller, G.A., and Kim, Y.S. 1979. Comparison of oral feeding of peptide and amino acid meals to normal human subjects. *Gut* **20**(4): 291–299.

Smith-Rockwell, M., Nickols-Richardson, S.M., and Thye, F.W. 2001. Nutrition knowledge, opinions, and practices of coaches and athletic trainers at a division 1 university. *Int. J. Sport Nutr. Exerc. Metab.* **11**(2): 174–185.

Spodaryk, K. 2002. Iron metabolism in boys involved in intensive physical training. *Physiol. Behav.* **75**(1-2): 201–206.

Steen, S.N. 1996a. Timely statement of The American Dietetic Association: nutrition guidance for child athletes in organized sports. *J. Am. Diet. Assoc.* **96**(6): 610–611.

Steen, S.N. 1996b. Timely statement of The American Dietetic Association: nutrition guidance for adolescent athletes in organized sports. *J. Am. Diet. Assoc.* **96**(6): 611–612.

Tang, J.E., Moore, D.R., Kujbida, G.W., Tarnopolsky, M.A., and Phillips, S.M. 2009. Ingestion of whey hydrolysate, casein, or soy protein isolate: effects on mixed muscle protein synthesis at rest and following resistance exercise in young men. *J. Appl. Physiol.* **107**(3): 987–992.

Tang, J.E., and Phillips, S.M. 2009. Maximizing muscle protein anabolism: the role of protein quality. *Curr. Opin. Clin. Nutr. Metab. Care* **12**(1): 66–71.

Tanner, J. 1955. Growth at Adolescence. Charles C. Thomas, Springfield, IL.

Tanner, J.M., and Whitehouse, R.H. 1976. Clinical longitudinal standards for height, weight, height velocity, weight velocity, and stages of puberty. *Arch. Dis. Child.* **51**(3): 170–179.

Tarnopolsky, M. 2004. Protein requirements for endurance athletes. *Nutr.* **20**(7-8): 662–668.

Tarnopolsky, M.A., Atkinson, S.A., MacDougall, J.D., Chesley, A., Phillips, S., and Schwarcz, H.P. 1992. Evaluation of protein requirements for trained strength athletes. *J. Appl. Physiol.* **73**(5): 1986–1995.

Tarnopolsky, M.A., MacDougall, J.D., and Atkinson, S.A. 1988. Influence of protein intake and training status on nitrogen balance and lean body mass. *J. Appl. Physiol.* **64**(1): 187–193.

Thomson, J.S., Ali, A., and Rowlands, D.S. 2011. Leucine-protein supplemented recovery feeding enhances subsequent cycling performance in well-trained men. *Appl. Physiol. Nutr. Metab.* **36**(2): 242–253.

Timmons, B.W., Bar-Or, O., and Riddell, M.C. 2003. Oxidation rate of exogenous carbohydrate during exercise is higher in boys than in men. *J. Appl. Physiol.* **94**(1): 278–284.

Timmons, B.W., Bar-Or, O., and Riddell, M.C. 2007a. Influence of age and pubertal status on substrate utilization during exercise with and without carbohydrate intake in healthy boys. *Appl. Physiol. Nutr. Metab.* **32**(3): 416–425.

Timmons, B.W., Bar-Or, O., and Riddell, M.C. 2007b. Energy substrate utilization during prolonged exercise with and without carbohydrate intake in preadolescent and adolescent girls. *J. Appl. Physiol.* **103**(3): 995–1000.

Tipton, K.D., Borsheim, E., Wolf, S.E., Sanford, A.P., and Wolfe, R.R. 2003. Acute response of net muscle protein balance reflects 24-h balance after exercise and amino acid ingestion. *Am. J. Physiol. Endocrinol. Metab.* **284**(1): E76–89.

Tipton, K.D., Ferrando, A.A., Phillips, S.M., Doyle, D., and Wolfe, R.R. 1999a. Postexercise net protein synthesis in human muscle from orally administered amino acids. *Am. J. Physiol.* **276**(4 Pt 1): E628–634.

Tipton, K.D., Gurkin, B.E., Matin, S., and Wolfe, R.R. 1999b. Nonessential amino acids are not necessary to stimulate net muscle protein synthesis in healthy volunteers. *J. Nutr. Biochem.* **10**(2): 89–95.

Tipton, K.D., and Wolfe, R.R. 2004. Protein and amino acids for athletes. J. Sports Sci. **22**(1): 65–79.

Tobias, J.H., Steer, C.D., Mattocks, C.G., Riddoch, C., and Ness, A.R. 2007. Habitual levels of physical activity influence bone mass in 11-year-old children from the United Kingdom: findings from a large population-based cohort. J. *Bone Miner. Res.* **22**(1): 101–109.

Tomé, D., and Bos, C. 2000. Dietary protein and nitrogen utilization. J. Nutr. 130(7): 18688–73S.

Torres-McGehee, T.M., Pritchett, K.L., Zippel, D., Minton, D.M., Cellamare, A., and Sibilia, M. 2012. Sports nutrition knowledge among collegiate athletes, coaches, athletic trainers, and strength and conditioning specialists. *J. Athl. Train.* **47**(2): 205–211.

Torun, B. 2005. Energy requirements of children and adolescents. *Public Health Nutr.* **8**(7A): 968–993.

Toth, M.J., Sites, C.K., Matthews, D.E., and Casson, P.R. 2006. Ovarian suppression with gonadotropin-releasing hormone agonist reduces whole body protein turnover in women. *Am. J. Physiol. Endocrinol. Metab.* **291**(3): E483–490.

Volpi, E., Ferrando, A.A., Yeckel, C.W., Tipton, K.D., and Wolfe, R.R. 1998. Exogenous amino acids stimulate net muscle protein synthesis in the elderly. *J. Clin. Invest.* **101**(9): 2000–2007.

Volpi, E., Mittendorfer, B., Rasmussen, B.B., and Wolfe, R.R. 2000. The response of muscle protein anabolism to combined hyperaminoacidemia and glucose-induced hyperinsulinemia is impaired in the elderly. *J. Clin. Endocrinol. Metab.* **85**(12): 4481–4490.

Volterman, K.A., Obeid, J., Wilk, B., and Timmons, B.W. 2014. Effect of milk consumption on rehydration in youth following exercise in the heat. *Appl. Physiol. Nutr. Metab.* **39**(11): 1257–1264.

Volterman, K.A., Moore, D.R., Obeid, J., Offord, E.A., and Timmons, B.W. 2015. The effect of post-exercise milk protein intake on rehydration of children. *Pediatr. Exerc. Sci.* [Epub ahead of print].

de Vrese, M., Stegelmann, A., Richter, B., Fenselau, S., Laue, C., and Schrezenmeir, J. 2001. Probiotics--compensation for lactase insufficiency. *Am. J. Clin. Nutr.* **73**(2 Suppl): 4218–4298.

Wagenmakers, A.J. 1998. Muscle amino acid metabolism at rest and during exercise: role in human physiology and metabolism. *Exerc. Sport Sci. Rev.* **26**: 287–314.

Wagenmakers, A.J. 1999. Tracers to investigate protein and amino acid metabolism in human subjects. *Proc. Nutr. Soc.* **58**(4): 987–1000.

Waterlow, J.C. 1984. Protein turnover with special reference to man. *Q. J. Exp. Physiol.* **69**(3): 409–438.

West, D.W.D., Burd, N.A., Coffey, V.G., Baker, S.K., Burke, L.M., Hawley, J.A., Moore, D.R., Stellingwerff, T., and Phillips, S.M. 2011. Rapid aminoacidemia enhances myofibrillar protein synthesis and anabolic intramuscular signaling responses after resistance exercise. *Am. J. Clin. Nutr.* **94**(3): 795–803.

Wilkinson, S.B., Phillips, S.M., Atherton, P.J., Patel, R., Yarasheski, K.E., Tarnopolsky, M.A., and Rennie, M.J. 2008. Differential effects of resistance and endurance exercise in the fed state on signalling molecule phosphorylation and protein synthesis in human muscle. *J. Physiol.* **586**(Pt 15): 3701–3717.

Wilkinson, S.B., Tarnopolsky, M.A., Macdonald, M.J., Macdonald, J.R., Armstrong, D., and Phillips, S.M. 2007. Consumption of fluid skim milk promotes greater muscle protein accretion after resistance exercise than does consumption of an isonitrogenous and isoenergetic soy-protein beverage. *Am. J. Clin. Nutr.* **85**(4): 1031–1040.

Witard, O.C., Jackman, S.R., Breen, L., Smith, K., Selby, A., and Tipton, K.D. 2014. Myofibrillar muscle protein synthesis rates subsequent to a meal in response to increasing doses of whey protein at rest and after resistance exercise. *Am. J. Clin. Nutr.* **99**(1): 86–95.

Wolfe, R.R. 2002. Regulation of muscle protein by amino acids. J. Nutr. **132**(10): 3219S–24S.

Wolfe, R.R., and Chinkes, D.L. 2005. Isotope Tracers in Metabolic Research. John Wiley & Sons, Inc., Hoboken, NJ.

Wu, G. 2009. Amino acids: metabolism, functions, and nutrition. *Amino Acids* **37**(1): 1–17.

Ziegler, P.J., Khoo, C.S., Kris-Etherton, P.M., Jonnalagadda, S.S., Sherr, B., and Nelson, J.A. 1998. Nutritional status of nationally ranked junior US figure skaters. *J. Am. Diet. Assoc.* **98**(7): 809–811.

### APPENDICES

Non-Essential	Essential	Conditionally	Precursors of conditionally		
		Essential	essential		
Alanine	Histidine	Arginine <sup>b</sup>	Glutamine/glutamate, aspartate		
Asparagine	Isoleucine <sup>a</sup>	Cysteine <sup>b</sup>	Methionine, serine		
Aspartate	Leucine <sup>a,b</sup>	Glutamine <sup>b</sup>	Glutamic acid/ammonia		
Glutamate	Lysine	Glycine	Serine, choline		
Serine	Methionine	Proline <sup>b</sup>	Glutamate		
	Phenylalanine	Tyrosine	Phenylalanine		
	Threonine				
	Tryptophan <sup>b</sup>				
	Valine <sup>a</sup>				

APPENDIX I: List of amino acids found within	in b	l within	body	lv i	protein	s.
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Non-essential amino acids can be made *de novo* by the human body, either by modifying other amino acids or by combining CHO precursors and assimilated N (National Research Council 2005). Essential amino acids cannot be synthesized by the body (or are synthesized in insufficient quantities, as is the case for histidine) and must therefore be obtained from dietary sources (National Research Council 2005). If the diet is insufficient in any of the essential amino acids, the synthesis of proteins and other important biological substances are affected. As a result, the body's proteins (e.g., lean tissue) will be degraded in order to obtain the amino acid(s) required. Conditionally essential amino acids are defined as requiring a dietary source when endogenous synthesis cannot meet metabolic needs. <sup>a</sup>Branched-chain amino acids (BCAAs) are a

special subset of essential amino acids which have unique physiologic and metabolic characteristics (Monirujjaman and Ferdouse 2014). <sup>b</sup>Functional amino acids have specific benefits for stimulating the protein anabolic pathways and optimizing metabolism (Wu 2009).

## **APPENDIX II:** Adult studies examining the effects of postexercise protein ingestion on MPS following endurance or high-intensity sprint exercise.

When amino acids and/or protein are ingested following endurance-type exercise, the rates of MPS are increased. When small (10 g) amounts of protein were consumed immediately and 30 min following a single bout of endurance exercise, the rates of myofibrillar protein synthesis were enhanced (Breen et al. 2011). It appears, however, that  $\sim 20$  g of protein ingestion has minimal effects on enhancing the rates of mitochondrial protein synthesis following endurance exercise, a finding that is in contrast to the up-regulation seen following the intake of exogenous amino acids at rest (Bohé et al. 2003). Despite the apparent lack of stimulation following exercise, a number of chronic (i.e., 4-6 weeks) studies have shown that ingesting protein in the post-exercise period enhances aerobic capacity in both young (Ferguson-Stegall et al. 2011a) and older (Robinson et al. 2011) adults. In light of these findings, and given that the peak upregulation of endurance exercise-specific gene expression occurs between 10 to 24 h post-exercise, the effects of protein ingestion may have implications beyond the acute post-exercise period; in order to detect worthwhile training adaptations, repeated bouts of exercise may be required (Leick et al. 2010, Neubauer et al. 2014). Reprinted from Open Access source. Moore, D. R., Camera, D. M., Areta, J. L., and Hawley, J. A. (2014). Beyond muscle hypertrophy: why dietary protein is important for endurance athletes. *Appl Physiol Nutr Metab* **39**(9), 987-997.

Study		<sup>V̇</sup> O <sub>2max</sub>		Nutritional						
(reference)	Subjects	(mL/(kg·min))	Exercise stimulus	intervention	Protein type	Control condition	Outcome	MPS ES* (95% CI)		
Mixed muscle and myofibrillar FSR										
Breen et al. 2011	10 males, endurance trained	66.5±5.1	1.5 h cycling at ~70% VO <sub>2max</sub>	10.2 g protein and 25.4 g CHO at 0 and 0.5 h of recovery	Whey	25.2 g CHO at 0 and 0.5 h of recovery	Myofibrillar FSR (0–4 h recovery)	1.25 (0.25–2.15)		
Camera et al. (unpublished)†	8 males, recreationally active	46.7±4.4	8×5 reps at 80% 1RM, 0.5 h cycling at 70% VO <sub>2max</sub>	25 g protein at 0 h of recovery	Whey	Fasted (no nutrition)	Myofibrillar FSR (1–4 h recovery)	0.88 (-0.19-1.85)		
Coffey et al. 2011	8 males, trained	51.3±5.6	10x6-s maximal sprints, 54 s recovery	24 g protein, 4.8 g leucine, 50 g CHO	Whey	Fasted (no nutrition)	Myofibrillar FSR (0–4 h recovery)	0.99 (-0.10-1.97)		
Harber et al. 2010	8 males, recreationally active	~53	1 h cycling at ~70% VO <sub>2max</sub>	~27 g protein, 62 g CHO, and ~2 g fat at 0 and 1 h of recovery	Milk (20% whey, 80% casein)	Fasted (no nutrition)	Mixed muscle FSR (2–6 h recovery)	0.49 (-0.53-1.46)		
Howarth et al. 2009	6 males, recreationally active	48.9±3.3	2 h variable intensity cycling at 50%–80% VO <sub>2max</sub>	0.4 g/(kg·h) protein and 1.2 g/(kg·h) CHO (at 15-min intervals) from 0-3 h recovery	Whey	1.6 g/(kg·h) CHO (at 15-min intervals) from 0-3 h recovery	Mixed muscle FSR (0-4 h recovery)	1.51 (0.13–2.65)		
Lunn et al. 2012	8 males, recreationally	53.1±1.6	45 min treadmill at 65% VO <sub>2max</sub>	16 g protein/58 g CHO at 0 h of recovery	Milk (20% whey, 80% casein)	74 g CHO at 0 h of recovery	Mixed muscle FSR (0-3 h recovery)	0.79 (-0.27-1.76)		
	acuve						Mean ES (95% CI)	0.95 (0.53-1.38)		
Mitochondrial frac	tional synthetic	rate								
Breen et al. 2011	10 males, endurance trained	66.5±5.1	1.5 h cycling at ~70% VO <sub>2max</sub>	10.2 g protein and 25.4 g CHO at 0 and 0.5 h of recovery	Whey	25.2 g CHO at 0 and 0.5 h of recovery	Mitochondrial FSR (0–4 h recovery)	-0.15 (-1.02-0.74)		
Camera et al. (unpublished)†	8 males, recreationally active	46.7±4.4	8×5 reps at 80% 1RM, 0.5 h cycling at 70% VO <sub>2max</sub>	25 g protein at 0 h of recovery	Whey	Fasted (no nutrition)	Mitochondrial FSR (1–4 h recovery)	0.34 (-0.67-1.31)		
Coffey et al. 2011	8 males, trained	51.3±5.6	10x6-s maximal sprints, 54 s of recovery	24 g protein, 4.8 g leucine,	Whey	Fasted (no nutrition)	Mitochondrial FSR (0-4 h recovery)	0.08 (-0.91-1.05)		
				50 g CHO			Mean ES (95% CI)	0.07 (-0.48-0.62)		

Note: 1RM, 1-repetition maximum; CI, confidence interval; CHO, carbohydrate; FSR, fractional synthetic rate; VO<sub>2max</sub>, maximum aerobic capacity.

\*MPS ES: muscle protein synthesis effect size of protein ingestion relative to control condition.

† D.M. Camera, D.W.D. West, S.M. Phillips, T. Rerecich, T. Stellingwerff, J.A. Hawley, and V.G. Coffey. Unpublished.
### **APPENDIX III:** Several assumptions in the [<sup>13</sup>C]leucine model

Absence of isotopic effect Absence of any metabolic effect of infused tracer The fraction of <sup>13</sup>CO<sub>2</sub> produced that is recovered in breath is known Absence of tracer 'recycling' The fate of the tracer (even though labelled on a single carbon) reflects that of the entire molecule Plasma 'pool' reflects intracellular leucine pool

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Leucine oxidation relative to leucine intake over 3 hours collapsed across all conditions. Preferred model is segmental linear relationship (P < 0.01 compared with linear correlation):  $r^2 = 0.68$ ; line of best fit < BP (breakpoint): y = 7.2X - 0.31, BP at x = 33.85 mg·kg<sup>-1</sup> [95% confidence interval for BP = 24.90 to 42.80 mg·kg<sup>-1</sup>], slope > BP = 0.

# APPENDIX VII: Plasma appearance of leucine, oral $[^{2}H_{3}]$ leucine tracer and intravenous $[^{13}C]$ leucine tracer.

(A) Plasma leucine concentration; (B) plasma  $[^{2}H_{3}]$ leucine enrichment; and (C) plasma  $[^{13}C]$ leucine enrichment over 3 hours following exercise and the consumption of one of four experimental beverages: Control, CONT; Low-protein, LP; Moderate-protein, MP; and High-protein, HP.  $[^{2}H_{3}]$ leucine was provided from the oral tracer, while  $[^{13}C]$ leucine was provided from the intravenous tracer. Time 0 represents a baseline value taken 15 minutes following cessation of exercise, and immediately prior to beverage consumption. Mean values for each time point are presented. Error bars were not included for clarity.



# **APPENDIX VIII:** Time course of plasma leucine concentrations and plasma leucine enrichment of oral [<sup>2</sup>H<sub>3</sub>]leucine tracer.

Comparison of the time course of plasma leucine concentration and plasma  $[^{2}H_{3}]$ leucine enrichment over 3 hours following exercise and the consumption of one of four experimental beverages: (A) Control, CONT; (B) Low-protein, LP; (C) Moderate-protein, MP; and (D) High-protein, HP. Each beverage was enriched with  $[^{2}H_{3}]$ leucine to a level of 4 % of beverage leucine content. Time 0 represents a baseline value taken 15 minutes following cessation of exercise, and immediately prior to beverage consumption. Mean values for each time point are presented. Error bars were not included for clarity.



## **APPENDIX IX:** Relationship between plasma leucine enrichment of oral [<sup>2</sup>H<sub>3</sub>]leucine and intravenous [<sup>13</sup>C]leucine tracers.

Relationship between the plasma enrichment of  $[{}^{2}H_{3}]$ leucine from the oral tracer and  $[{}^{13}C]$ leucine from the intravenous tracer over 3 hours following exercise and the consumption of one of four experimental beverages: (A) Control, CONT; (B) Low-protein, LP; (C) Moderate-protein, MP; and (D) High-protein, HP. Mean values for each time point (0, 30, 60, 90, 120, and 180 min) are presented, with both x and y error bars.

