Impact of Protein Intake on Protein Metabolism and Exercise Performance in Endurance-Trained Males

by

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A thesis submitted in conformity with the requirements for the degree of Master of Science in Exercise Science

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Abstract:

**Background:** Current recommendations for athletes consider dietary protein requirements that maintain nitrogen (i.e. protein) balance rather than an optimal dosage to enhance metabolism and exercise performance. **Purpose:** The primary objective of this study was to determine how differing protein intakes alter protein metabolism and exercise performance. **Methods:** Using a double blind randomized crossover design, 10 male runners completed 3 trials involving 4 days of controlled training and ingestion of either 0.94 (LOW), 1.20 (MOD) or 1.83 g protein/kg body weight/d (HIGH). Whole body protein metabolism and exercise performance were assessed. **Results:** Whole body net protein balance displayed a relative dose-response (HIGH > MOD > LOW, \(P < 0.05\)). Inferential statistics revealed that 1.83 g protein/kg provides notable benefits in exercise performance compared to intakes of 0.94 g/kg or 1.2 g/kg. **Conclusion:** These data suggest that consuming protein towards the high end of recommendations (1.2-2g/kg) better maintains metabolism and exercise performance.
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**List of Abbreviations**

BCAAs - Branched-Chain Amino Acids  
EAAs - Essential Amino Acids  
EAR - Estimated Average Requirement  
FFM - Fat-Free Mass  
FM - Fat Mass  
FP – Follicular Phase  
IAAO- Indicator Amino Acid Oxidation  
LP – Luteal Phase  
MPS - Muscle Protein Synthesis  
MVC - Maximum Voluntary Isometric Contraction  
NBAL - Nitrogen Balance  
NIN - Nitrogen Intake  
NOUT - Nitrogen Excretion  
PAR-Q – Physical Activity Readiness Questionnaire  
RDA - Recommended Dietary Allowance  
REE - Resting Energy Expenditure  
SD – Standardized Differences  
TT - Time Trial

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Chapter 1. Literature Review

1.1 Introduction

It is the evidenced-based consensus in the exercise science community that proteins play an important role in both exercise performance and recovery (Beleen et al., 2010; Howarth et al., 2009; Phillips, Moore & Tang, 2007). Proteins play many important structural and mechanical roles in living organisms. At the biochemical level, they consist of polymer chains made up of a series of 20 different amino acids linked together by peptide bonds. ‘The sequence of the polymer chain of amino acids in the protein determines its structure, physical and chemical properties and function. Understanding of protein structures and function is of particular relevance in exercise science in general and endurance training in particular because different proteins serve different cellular functions within the human body acting as enzymes, antibodies, cell receptors, hormones and structural proteins. Additionally, individual amino acids can also be cleaved from the protein and provide a source of energy to the body’s cells.

1.1.1 Protein as Building Blocks of Body Tissue

In addition to fat and water, the human body is comprised of protein. The building blocks for this protein and for the peptide synthesis process are amino acids. Body muscles, such as those connected to the musculoskeletal system, are able to contract because of the force generated and exerted from motor proteins. The degradation of old proteins into individual amino acids and the reutilization of these amino acids to synthesize new proteins, a process called protein turnover, is important for the maintenance of an adequate quantity and quality of body proteins. Protein turnover will be discussed in greater detail in subsequent sections.
1.1.2 Protein as a Fuel Source

As a fuel source, proteins contain 4 kilocalories (17 kJ) of energy per gram. To provide energy to the cell complete proteins are cleaved into individual amino acids which can then be oxidized in the citric acid cycle to generate ATP. A portion of energy being generated within the human body is provided through oxidation of amino acids at any given time. However, the percentage of fuel sourced from amino acids relative to other macronutrients is dependent on a number of factors including exercise and nutrition, which will be discussed in further detail below. There are two ways in which amino acids can ultimately provide as a fuel source. The first pathway is through direct oxidation in the citric acid cycle and the other is by being converted into glucose through the process of gluconeogenesis for subsequent use in glycolysis or oxidation. Amino acid oxidation is a constant process which is elevated when the body is not obtaining adequate fuel from carbohydrate or fat sources, such as during prolonged exercise and/or fasting (Graham et al., 1995; Wagenmakers, et al., 1991).

High levels of protein intake that exceed the rate at which amino acids can be incorporated into body tissue also upregulates key enzymes involved in oxidation of these amino acids (Brosnan, 2003; Bowtell et al., 1998). Moderate-intensity exercise and low glycogen availability (Lemon & Mullin, 1980) increase rates of amino acid oxidation, particularly of the branched chain amino acids (BCAAs) (Rennie et al., 1981). Given that oxidized amino acids are not available to participate in protein synthesis, the greater amino acid oxidation during, particularly prolonged and/or higher intensity, exercise may ultimately impact the nutritional requirements during endurance training; these concepts will be discussed in detail below.
1.1.3 Dietary Protein

Protein is an essential nutrient to the human body as it is the only dietary source of nitrogen. In free living humans, amino acids are obtained through the consumption of foods containing protein. Of the 20 amino acids, nine are essential. These essential amino acids (EAAs), which cannot be directly synthesized by the body, are required to stimulate synthesis of new tissues and can come from dietary protein and/or crystalline amino acids (Tipton, Gurkin et al., 1999; Tipton, Ferrando et al., 1999). Sources of dietary protein that contain all non-essential and essential amino acids in adequate compositions necessary for physiological needs are termed complete proteins or whole proteins. In sufficient quantities, foods such as most meat products, milk products, eggs, soy, and fish are sources of complete protein (Matthews et al., 1982). These food sources can also be imitated through mirrored compositions of crystalline amino acids to stimulate protein synthesis in the same manner (Tipton, Gurkin et al., 1999; Volpi et al., 2003). Likewise, body proteins are made up of complete proteins and the synthesis of new tissue requires all amino acids. Remaining amino acids not incorporated into body proteins are oxidized in the citric acid cycle (Brosnan, 2003; Bowtell et al., 1998). Dietary protein needs for humans are largely based on overall energy intake, stage of life, physical activity and the presence of illness or injury (Phillips, Chevalier & Leidy, 2016). For the general adult population, Health Canada has set the Recommended Dietary Allowance (RDA) of protein intake at 0.8g/kg (Health Canada, 2006). However, these minimum requirements may not be suitable for those engaged in chronic exercise such as endurance athletes.
1.2 Protein Turnover

Protein is the main building block of tissue within the human body and it is in a state of continuous metabolic flux known as protein turnover (Figure 1) (Tarnopolsky, 2004). This is an energy requiring process that represents about 20% of an adult’s metabolic rate (Schutz, 2011). After digestive breakdown of proteins into amino acids and their subsequent absorption, they are then reassembled and sequenced into specific proteins to make up tissue in the body in a process known as protein synthesis. Alternately, these bodily proteins can then be broken down for use in other tissues and/or to be used as a fuel source, as previously discussed. Within the body, muscle protein synthesis and muscle protein breakdown continually occur; however, the rates at which they occur is influenced by many factors. If protein synthesis is occurring at a greater rate than protein breakdown this leads to net positive protein balance and anabolism. If protein breakdown is occurring at a greater rate than protein synthesis this leads to negative net balance and catabolism. Measured overall net effects on whole body protein turnover are a reflection of the turnover rates within the different protein pools (e.g. muscle, organs, etc.) of the body which vary in size and rates of turnover (Waterlow, 1995).

![Figure 1. Protein turnover and amino acid flux. (Rasmussen & Phillips, 2003)](image-url)
1.2.1 Protein Synthesis

Protein synthesis fluctuates throughout the day depending on influences such as nutrition and exercise. Due to reductions in the amino acid pool, protein synthesis occurs at a reduced rate in the fasted-state compared to the fed state (Rennie & Tipton, 2000; Rasmussen, Wolfe & Volpi, 2002). In the fasted state (such as after an overnight fast), free amino acids are largely provided via endogenous sources that have been broken down and released into the blood stream (Biolo et al., 1997) allowing the body to recycle its own sources of amino acids. With this greater rate of protein breakdown over protein synthesis, protein turnover is in a net negative balance (Phillips et al., 1997). Protein turnover becomes positive in the fed state, primarily due to an amino acid-induced stimulation of protein synthesis, which occurs with the ingestion or infusion of an amino acid-containing meal (Biolo et al., 1997; Aguirre, van Loon & Baar, 2013). Both intracellular amino acids and extracellular amino acids (i.e. within the blood) are now said to be in the free amino acid pool. Increases in this pool serve as the main facilitator of whole body protein synthesis (Arnal et al., 1987) and, therefore, consumption of dietary proteins allows the body to build and repair tissues.

Skeletal muscle is highly plastic tissue and responses to exercise stimuli must relate to changes in protein synthesis, which has shown to be greater with increasing intensities of exercise (Wilkinson et al., 2008; Di Donato et al., 2014). Following exercise, there is an increase in protein synthesis that has been proposed as a “catching up” mechanism. This mechanism is suggested to compensate for the depression of protein synthesis and increase in breakdown observed during exercise (Rennie et al., 1981). The increase in synthesis rates, which can have an overall net anabolic effect with the ingestion of dietary protein (Levenhagen et al., 2002),
contributes to adaptations (e.g. improved oxidative capacity, muscle hypertrophy, etc.) to induced physiological demands of exercise training (Atherton & Smith, 2012; Mascher et al., 2011; Di Donato et al., 2014).

Protein synthesis specifically induced by endurance exercise has shown to be elevated for up to ~28 h following activity (Di Donato et al., 2014). Di Donato et al. (2014) found that over a 24–28 h post-endurance exercise period, mitochondrial protein synthesis was significantly greater after a high intensity bout of training (cycling at 60% Watts$_{\text{max}}$) compared to a low intensity bout (cycling at 30% Watts$_{\text{max}}$). Over 0.5–4.5 h protein synthesis was increased equivalently during recovery, but remained elevated at 24–28 h post exercise only after high intensity exercise. Therefore, this increase in synthesis rates following exercise with the ingestion of protein can lead to a positive net balance resulting in greater myofibrillar and mitochondrial turnover as well as greater mitochondrial protein content. When repeated over time this could translate into subsequent improvements in strength and power generation (Frontera et al., 1988), and greater muscle oxidative capacity and resistance to fatigue (Hawley, 2002; Di Donato et al., 2014), although endurance training studies with targeted post-exercise recovery nutrition are currently lacking.

1.2.2 Protein Breakdown

The body is able to add to the free amino acid pool through degradation of its own tissue. The process of degradation is also known as protein breakdown and there are three major systems which carry out this action: ubiquitin-proteosome pathway, the lysosomal systems, and the calpain systems (Lecker et al., 1999; Belcastro, Shewchuk, & Raj, 1998). Protein breakdown of
endogenous tissue produces single amino acids and smaller polypeptides through the hydrolysis of peptide bonds. The rate of this process can be influenced by pH and temperature, but is largely dependent on the activation of protease cellular enzymes which catalyze the reaction. Once cleaved from the tissue, the free amino acids can either be used in the synthesis of other proteins, serve as a fuel source via oxygenation or gluconeogenesis, or be converted into citric acid cycle intermediates (Wagenmakers, 1998). Protein breakdown provides an essential quality control mechanism to eliminate abnormally folded or damaged proteins that have arisen by faulty mutations, biosynthetic errors, damage by oxygen radicals, or denaturation (Lecker et al., 1999).

Similar to synthesis, breakdown is elevated by exercise. This has been shown in both whole body protein breakdown measures (Wolfe et al., 1982; Phillips et al., 1993) as well as muscle protein breakdown measures (Rennie et al., 1981). Even small accelerations in degradation, if sustained, can result in a marked loss of tissue mass that may have important implications for endurance athletes, specifically concerning muscle tissue (Lecker et al., 1999).

In addition to the need for amino acids as a fuel source in endurance exercise, muscle proteins are subject to damage via free radicals, which also increases muscle protein breakdown rates (Davies et al., 1982). This muscle remodeling is essential to break down damaged protein and resynthesize new functional proteins. Thus, the stimulation of protein synthesis can replace oxidative losses as well as provide qualitative changes in muscle protein fractions (e.g. increased concentration of energy producing mitochondrial proteins) and the ultimate adaptive response to endurance training (e.g. more fatigue resistance).
Due to both the elevation in breakdown as well as a reduction in synthesis, previous reports have shown that protein turnover net balance is reduced when glycogen stores are low (Howarth et al., 2010; Van Hall, Saltin & Wagenmakers, 1999). This is particularly true for the BCAAs which is noteworthy considering they are a major component of muscle tissue (Ferrando et al., 1995). There is no uptake of BCAAs at rest, during exercise the uptake is substantial (13-16 µmol BCAAs/min/kg dry muscle) (Howarth et al., 2010) and low glycogen levels further exaggerate this effect (Wagenmakers et al., 1991; Jackman et al., 1997). This result of breakdown being most amplified in the state of low glycogen storage can be induced by either inadequate carbohydrate intake or, of relevance to endurance athletes, from glycogen depleting exercise such as intense and/or prolonged training.

1.2.3 Amino Acid Oxidation during Exercise

Although the dominant substrates catabolized for energy during exercise are carbohydrates and fat, exercise also increases the total rate of amino acid oxidation as a function to spare muscle glycogen and increase potential for prolonged high metabolic rates (Hawley & Hopkins, 1995). The contribution of amino acid oxidation to energy is approximately 3-10% depending on certain exercise factors and nutrition status (Tarnopolsky, 2004; Gibala, 2001). Notable influences are intensity and duration; both of which increase this rate of oxidation through increases in overall energy demands and through glycogen depletion (Rennie et al., 1981). Following degradation, amino acids subsequently travel to the liver for gluconeogenesis and/or are deaminated and oxidized within muscle mitochondria as a fuel source (Tarnopolsky, 2004). This has recovery and potential subsequent performance implications for endurance athletes as these amino acids are now unavailable to be recycled or participate in the recovery process. Kato, Suzuki, Bannai
and Moore (2016) estimated an amino acid oxidation total of ~14g or the equivalent of ~0.2g/kg/d over a 20 km run and point out that this rate would be greater alongside inadequate carbohydrate intake. Replacing this loss through dietary means needs to be considered in addition to needs for recovery of damaged tissue and generation of new tissue.

1.3 Protein Requirements for Endurance Athletes

Current protein intake recommendations for athletes stand at 1.2-2.0 g/kg body weight/d (ACSM, 2016). This recommendation is largely based on research examining protein intakes required to achieve nitrogen balance (NBAL), such as that which was conducted by Meredith et al. (1989). This study tested 3 different protein intakes (0.6, 0.9 and 1.2 g/kg/d) on nitrogen balance in 12 endurance trained adults. The training was reported to be an average of 9.9 h/week, was uncontrolled, and the participants were asked to maintain normal activity in their habitual environment (cycling, running, rowing, and/or calisthenics). Their results demonstrated a mean protein requirement needed to maintain nitrogen balance of 0.94 g/kg/d, putting their RDA at 1.26 g/kg/d. However, these recommendations, as well as those found in studies of similar design, have been questioned given that there is little direct link to performance (Phillips, 2006; Phillips, Moore & Tang, 2007; Phillips, Chevalier & Leidy, 2016). In other words, they merely encompass a minimal requirement to offset deficiency rather than an optimal dosage to maximize recovery and improvements in performance. More recently, Kato et al. (2016) have demonstrated a need for much greater requirements in this population. This group studied a range of protein intakes (0.2–2.8g/kg/d) in 6 endurance trained men after a 20km training session within a 3-day controlled training period using the indicator amino acid oxidation (IAAO) method. This method measures amino acid oxidation, with the reciprocal being whole body
protein synthesis, suggesting that the intake determined may be more likely to maximize recovery. The mean protein requirement suggested by Kato et al. (2016) was 1.65g/kg/d and the RDA was 1.83 g/kg/d. The discrepancy in findings between these two studies is likely primarily a result of the difference in methodologies used in measurement of protein turnover with the IAAO method suggested to be more accurate and allowing for a more precise requirement due to the greater number of intakes that can be tested compared to controlled nitrogen balance studies (Zello et al., 1995; Elango, Ball, & Pencharz, 2008).

It is important to note that higher protein intakes above the RDA have also demonstrated effectiveness in reducing frequency of illness, including upper respiratory tract infections, particularly during periods of intensified training (Gleeson, 2007). This is suggested to be a result of improvements in immune function (Witard et al., 2014; Witard et al., 2012). These additional benefits not only improve performance adaptations but also would result in less time away from training due illness.

1.3.1 Performance as an Indicator of Protein Requirements

For an endurance athlete, the primary goal of adequate protein consumption is not at a fundamental level, nitrogen balance, but rather to be able to support increased protein synthesis to improve performance. Therefore, it would be valuable for research to not only focus on measurements of metabolic processes but also on measures of performance. However, unfortunately it has remained rather unusual for measures of performance to be included in combination with research analyzing protein metabolism. This is a limitation as regardless of whether or not greater protein is required to achieve nitrogen balance, protein intakes above the
current recommendation could be beneficial and desirable if performance is enhanced. Direct measurements such as those of strength, power and resistance to fatigue could potentially be used to demonstrate changes in performance.

Of the few studies that have used performance measurements, there appears to be a delay (i.e. >24 h) in which performance differences can be observed with differing protein intakes. This may be related to the mRNA response to endurance exercise having a peak at 8-12 h post-exercise (Yang et al., 2005) and adaptive protein synthesis being a cumulative process augmented by recovery periods between bouts (Hood et al., 2006). Rowlands et al. (2008) showed that a less negative nitrogen balance is correlated with better performance after 4 days of controlled training in endurance trained cyclists. The protein enriched diet, which allowed for the less negative nitrogen balance, provided 0.8 g/kg fat free mass per hour (averaging 1.9 g/kg/d for their sample) while the non-enriched condition provided 0.12 g/kg fat free mass per hour (averaging 1.2 g/kg/d). The greater protein intake had no impact on the 15-h performance, but showed enhancements (4.1%) in the 60-h subsequent performance tests.

Some research has shown next day performance benefits in tests of endurance time to exhaustion; however, this is only when greater protein intakes are given with supplemental carbohydrate (compared to supplemental carbohydrate alone) (Berardi et al., 2006; Saunders, Kane & Todd 2004; Zawadski, Yaspelkis & Ivy, 1992; Niles et al., 2001). This is attributed to enhanced glycogen resynthesis. Protein intakes above 0.8 g/kg/d given without supplemental carbohydrate have consistently shown no next day performance benefits (cycling sprint power) (Rowlands et al., 2007; Rowlands & Wadsworth, 2011). However, a less negative nitrogen
balance is correlated with greater rates of recovery, notably through reductions in plasma
creatine kinase and later onset tiredness and muscle soreness (Rowlands et al., 2008; Rowlands
et al., 2007; Rowlands & Wadsworth, 2011; Millard-Stafford et al., 2005). This, along with the
findings of Rowlands et al. (2008), provides evidence that greater intakes of protein than
currently recommended could contribute to greater subsequent performance ability after multiple
days. According to Rowlands et al. (2008) this intake for performance benefits is 1.2 g/kg;
however, no other intakes were tested. It seems that numerous exposures are required to produce
a summative and detectable response and that future research should focus on testing
performance after a longer period, beyond the next day. This is also supported by the findings of
a meta-analysis by Pasiakos et al. (2014) who determined that only when protein
supplementation continued for extended periods, were performance benefits for strength, peak
power output and total work performed evident.

A range of protein intakes is yet to be examined to determine a more precise requirement for this
population to enhance performance and/or mitigate performance decrements between controlled
training sessions. The study by Rowlands et al. (2008) only compared two intakes with the
higher intake (average 1.9 g/kg/d) resulting in an ergogenic effect over the lower intake (average
0.8 g/kg/d). The study by Meredith et al. (1989) compared the effects of multiple protein intakes
(0.6, 1.9 and 1.2 g/kg/d) on whole body protein turnover but did not include any measures of
performance.
**1.3.2 Research Gaps and Conclusion**

Protein is a key dietary component for any athlete seeking the adaptations required to enhance performance. With there being additional benefits to protein intakes above the RDA (Witard et al., 2014; Witard et al., 2012; Rowlands et al., 2008; Rowlands et al., 2007; Rowlands & Wadsworth, 2011; Millard-Stafford et al., 2005) as well as a point at which, if exceeded, additional protein does not provide any benefits (Brosnan, 2003; Bowtell et al., 1998), a definition of an optimal requirement could be facilitated (Millward et al., 1998).

The key gaps that currently remain within the literature pertaining to dietary protein needs for endurance exercise include:

1. Current recommendations only take into account minimal requirements rather than an optimal dosage to enhance recovery and improvements in performance.

2. The important practical objective related to dietary protein intake of performance has rarely been measured concurrently with protein metabolism.
Chapter 2. Study Proposal

2.1 Introduction

The purpose of this study was to determine the effects of different protein doses on performance and recovery during a period of controlled training characterized by repeated days of high volume exercise. Dietary intake was controlled with participants receiving a specific amount of protein in differing quantities: LOW (0.94 g protein /kg/d), MOD (1.20 g /kg/d) and HIGH (1.83 g/kg/d). The LOW protein intake represented the current estimated average requirement (EAR) to maintain nitrogen balance for endurance athletes (Meredith et al., 1989). The MOD protein intake represented the RDA for endurance trained men (Meredith et al., 1989) and the current lower limit of the recommended protein intake for endurance athletes advised by the American College of Sports Medicine (ACSM, 2000). The HIGH protein intake represented that which was determined to be adequate using the indicator amino acid oxidation (IAAO) method by Kato et al. (2016).

2.2 Hypothesis

It was hypothesized that following a period of controlled training, the high protein group would experience the least negative whole body protein balance. Furthermore, based on the link between protein metabolism and performance, it was expected that this less negative balance would lead to an attenuation in performance decrements as a result of frequent training in strength, jump measures and 5km run trial time. This hypothesis was based primarily on the recently published study mentioned above using the contemporary IAAO technique (Kato et al.,
The results of this study suggest that the intake provided in the high group will maximize whole body protein synthesis. By doing so, enhanced adaptive tissue remodeling associated with up-regulation of endurance-exercise specific gene expression (Yang et al., 2005; Hawley et al. 2007) and amino-acid stimulated protein synthesis (Howarth et al., 2009) can occur in an optimal capacity. Particularly, due to the comparable nature of the test to the training, it was expected that the mitigations in performance decrements would be most pronounced in the 5km time trial performance test.

2.3 Phases of Research

The proposed study was divided into a preliminary session and three separate intervention trials. It was conducted as a double-blind, randomized 3-way crossover design. The preliminary session required one visit and each intervention trial required 11 days of total monitoring with 6 total visits. A 7-day washout period was required between each trial. During each of the trials, participants received one of the described protein dosages for their 4 days of intensified training.

Phase I- Introduction and consent

Phase 1 consisted of a single laboratory visit lasting approximately 1.5 h and had two primary objectives. First, it served to provide a comprehensive oral introduction to ensure participants were properly informed of the study protocol, their duties as participants and any associated risks and benefits. Subsequent to the detailed explanation of the study and answering of all of the participants’ questions, those who wished to participate in the study were given the consent document to read and sign. Other documents that were completed during this phase include: the Physical Activity Readiness Questionnaire (PAR-Q+) (Appendix C) to ensure the participants
were physically suited to perform the study protocol safely and a self-reported training log to ensure they were currently active enough to be considered ‘trained’ (Shepherd, 2015).

**Phase II- Fitness Assessment**

Phase 2 consisted of a single laboratory visit lasting approximately 5 h in length. The objectives of this phase was to obtain general anthropometric measurements (body weight, height and composition), resting energy expenditure (REE), and baseline physical performance tests (maximal aerobic power (VO2peak), vertical jump impulse and peak force, maximum voluntary contraction (MVC) and 5 km time trial (TT)).

**Phase III – Experimental Trials**

There were 3 experimental trials, each including performance testing before and after the 4-day period of controlled training. Each trial consisted of a total of 11 days. Days 1 and 2 consisted of voluntary exercise, day 3 was a rest day and pre-training performance testing will be completed on day 4. Participants then rested on days 5 and 6 prior to commencing the training protocol through days 7 to 10 in which one of the 3 protein dosages were given. Post training performance testing was done on day 11. An in-depth review of the experimental trial methodology is provided in the Methodology section.
2.4 Knowledge Translation

The data obtained from this study will provide novel insight into the effects of differing protein dosages on whole body protein metabolism, recovery and physical performance, which will be important in the development of guidelines and recommendations for endurance exercisers.

Upon study completion and data analysis/interpretation, the results will be communicated to the scientific community through non-peer-reviewed publications, peer-reviewed journal(s), and conference presentations. Table 1 shows the projected timeline to complete each phase of the research process.

| Table 1. Project timeline |
|---------------------------------|------------------|-------------------|
| **Stage**                       | **Start Date**   | **End Date**      |
| Ethics Submission and Approval  | January 2016     | May 2016          |
| Participant Recruitment         | May 2016         | June, 2016        |
| Proposal Defense                | September 2016   | September 2016    |
| Data Collection (Phases I-III)  | June 2016        | December 2016     |
| Data Analysis                   | Jan. 2017        | April 2017        |
| Thesis Writing and Submission   | May 2017         | August 2017       |
Chapter 3. Methodology

3.1 Introduction

The following will provide detailed information on the research design, participants, materials, testing procedures, analytical methods and statistical analyses. Figure 2 provides a schematic representation of the sequence of research execution. All participant testing and analysis was conducted at the Goldring Centre for High Performance Sport within the University of Toronto.

3.2 Participants and Study Design

Based on former studies (Pacy et al., 1994; Meredith et al., 1989), the effect size in whole body protein net balance we expect to detect is 0.6, with 3 repeated measurements, \( \alpha = 0.05 \) and a power of 0.80, the a priori power calculation using G*power3 (Faul et al., 2007) suggested a total sample size of 24 (i.e. 8 participants * 3 repeated measures was required). Thus, N=8 provided sufficient power to detect differences in whole body protein net balance. In order to account for a potential 10% dropout and noncompliance, we recruited N=11 participants. The sample consisted of healthy endurance trained males between the ages of 18 and 50 yr. Trained was defined as a recent (over the past 4 weeks) running history of \( \geq 45 \) km/week. Those who run less than this benchmark were not considered eligible to participate. Participation was completely voluntary and participants were free to withdraw from the study at any time without penalty. Other criteria for exclusion included regular tobacco use or use of anabolic drugs (eg. growth hormone, testosterone, etc.). Please see Table 2 for further inclusion/exclusion criteria information. Participants considered safe to engage in the study were determined by the PAR-Q+.
Table 2. Inclusion and exclusion criteria

<table>
<thead>
<tr>
<th>Inclusion</th>
<th>Exclusion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td>Regular tobacco use</td>
</tr>
<tr>
<td>Healthy</td>
<td>Illicit drug use (e.g. growth hormone, testosterone, etc.)</td>
</tr>
<tr>
<td>Age of 18-50 years</td>
<td>Inability to meet health and physical activity guidelines according to the PAR-Q+</td>
</tr>
<tr>
<td>Recent running history &gt;45km or 4.5 hours/week</td>
<td></td>
</tr>
<tr>
<td>VO\textsubscript{2}max at least “very good” based on standards by Shvartz &amp; Reibold*</td>
<td></td>
</tr>
<tr>
<td>Ability to cover 10 km in less than 60 min after the VO\textsubscript{2}peak test and 5 km Time trial in session 2</td>
<td></td>
</tr>
</tbody>
</table>

* VO\textsubscript{2}max of $\geq 57$ ml/kg/min (18-24 y), $\geq 54$ ml/kg/min (25-29 y), $\geq 52$ ml/kg/min (30-34 y), $\geq 49$ ml/kg/min (35-39 y), $\geq 47$ ml/kg/min (40-44 y), $\geq 44$ ml/kg/min (45-50 y) according to age (Shvartz & Reibold, 1990)

3.3 Participant Consent, Explanation of Study and Familiarization

Individuals interested in participating in the study were scheduled for session 1 to complete the requirements of phase 1. During this initial meeting, the study was explained in detail, a training log retrospective of the last 4 weeks was completed to ensure adequate training load to meet our definition of trained and the PAR-Q was completed to confirm eligibility to participate.

Participants were given the opportunity to ask any questions pertaining to the study. All who qualified and agreed to participate in the study were required to sign a detailed consent form and were booked for session 2 to undergo the requirements of phase 2. They were given an accelerometer to wear and a dietary log to complete for 3 days prior to their session 2 date. The data collected from these tools were used to assess typical activity patterns, energy expenditure and food intake.

At session 2, participants arrived at the lab in the morning after a minimum of 7 hours of fasting (i.e. no food or fluid intake with the exception of water). The following assessments were then completed:
**Resting Energy Expenditure (REE):** The participant’s REE was measured using open circuit indirect calorimetry (GA-300, iWorxSystems Inc.). REE was calculated from oxygen consumption (VO₂) and carbon dioxide production (VCO₂) using the abbreviated Weir equation (WEIR, 1949). This data was used to provide an estimation of energy requirements for the controlled diet.

**Body Composition:** Following completion of the REE assessment, the participants underwent body composition measurement via air displacement plethysmography and a correction for internal volumes (Bod Pod, COSMED USA Inc., Chicago, IL). This method measures body mass (weight) using a calibrated scale, and volume by sitting inside the Bod Pod to determine body density. Body density can then be used to estimate fat mass (FM) and fat-free mass (FFM).

**VO₂peak:** Following body composition analysis the participants warmed up on the treadmill before engaging in a VO₂peak test. This test required participants to wear a heart rate monitor and aerobic fitness was assessed by measurement of respiratory gas exchange throughout a ramp protocol. Participants performed the graded exercise test to exhaustion. VO₂peak was determined via open-circuit spirometry on a metabolic cart (GA-300, iWorxSystems Inc.). A constant running speed was maintained while treadmill incline (starting at 0%) was increased by 2% every 2 minutes. VO₂peak was be defined as the oxygen consumption at which the participant’s RER is 1.15, HR (beats/min) of the age-predicted maximum, and/or the point at which the participant was unable to continue running (volitional fatigue).
5-Km Time Trial (5kmTT): Following an appropriate rest (however long the participant chose/until they were ready) after the VO₂peak test, participants ran 5 km at a 0° slope as fast as possible. This test was performed within the lab on a powered treadmill (LifeFitness 9500HR, Brunswick and Co.). Participants were free to adjust the speed as desired using the buttons on the treadmill. No information was given on heart rate, speed, or time; the participants were only able to see the distance they had covered. Water was provided for participants to consume ad libitum during the entire test. This test is comparable to the training of the participants. It has a high specificity to the improvements in performance desired by this population and also has a high degree of reliability (Russell et al., 2004).

Maximum Voluntary Contraction (MVC): After completion of the 5kmTT, participants rested before performing 3 MVC strength tests. The MVC requires the participants to sit in a chair-like apparatus with their right leg secured to an immovable arm in a bent 90° position by straps on the lower leg. The participants were then asked to perform a maximal isometric contraction of their knee extensors by attempting to straighten their leg against the lower pad of the machine for ~5 seconds. Participants were allowed three 5-second attempts with 1 min of rest between attempts and the maximal strength of their right knee extensors was determined through force output signals recorded using PowerLab with LabChart Pro v.8.0.5 (ADInstruments Inc., Colorado Springs, CO, USA). This strength test provided a measure of changes in muscle fatigue as defined as an exercise-induced reduction in maximal voluntary force (Place et al., 2007) as well as differences in muscle damage (Clarkson & Hubal, 2002).
Jump Impulse and Peak Force (Jump): Jump impulse and peak force were then assessed via a vertical jump test (using AMTI NetForce software) on force platforms. Participants were directed on how to perform a countermovement jump to achieve maximal jump height. They were then given 3 jumps to attain maximal jump height separated by 1 minute of recovery. Jump impulse and peak force exerted on the force platform during the takeoff phase of the jump was assessed. This test was used to measure differences in neuromuscular fatigue (Gathercole, Sporer & Stellingwerff, 2015) associated with the concentric power production of the leg extensors (Arteaga et al., 2000).

The 5kmTT, MVC and jump tests in session 2 are to familiarize the participant with the tests they were expected to perform during phase 3. In order to familiarize the participants with the training protocol as well, they ran 10 km at a self-selected pace on a treadmill in the lab. Participants were then be scheduled for sessions 3, 4 and 5 to undergo the experimental trials of phase 3.

3.4 Training Periods and Performance Assessments

Phase 3 involved the performance tests (MVC, 5kmTT and Jump) as described previously on days 4 and 11 as well as the training protocol on days 7 through 10. This allowed for a comparison of the described measurements before and after the controlled training. Training consisted of 20km, 5km, 10km, 20km respectively each training day. These training volumes were completed at the runner’s convenience. An HR and GPS monitor (M400, Polar Electro, Kempele, Finland) was provided for participants to wear for 5 minutes in the morning before breakfast to record morning heart rate and during each training session. This allowed the researcher to control and measure the quantity and intensity of each training bout. Participants
were asked to refrain from all other exercise during the day with the exception of normal daily activities (eg. commuting, shopping, etc.). The 3 sessions involved in phase 3 were separated by a minimum of 7 days. For a detailed schematic please see Figure 2.

The exercise load (20km, 5km, 10km, 20km running) was selected to provide a stimulus that would presumably enhance mitochondrial protein synthesis (Harber et al., 2010; Wilkinson et al., 2008) and induce elevations in post exercise muscle protein synthesis sensitivity to feeding (Harber et al., 2010). It is also reflective of the habitual training of trained distance runners aiming to augment aerobic adaptations and improve their performance.

Figure 2. Study overview
3.4.1 Stable Isotopes

On day 3 (of one of these sessions, randomly selected), day 7, and day 10 of each session, whole body protein metabolism for 24 hours was assessed using the oral $[^{15}\text{N}]$ glycine method. Day 3 of the randomly selected session allowed determination of protein balance on a rest day (refraining from structured exercise). Measurement on day 7 and day 10 allowed a comparison of resting metabolism to training day (20 km run) metabolism during each trial. The principle behind this method is that the isotopically labelled nitrogen, which was ingested in the morning of the metabolic trial day, was diluted by non-labelled nitrogen derived from de novo synthesis (which arose from endogenous protein degradation). The enrichment of $[^{15}\text{N}]$ in urinary ammonia and urea was used to calculate whole body nitrogen turnover over 12h (fed period; $[^{15}\text{N}]$ammonia) and 24h (harmonic mean of $[^{15}\text{N}]$ammonia and urea) (Duggleby & Waterlow, 2005). Subsequently, on day 8 and day 9, nitrogen balance for 24 hours was estimated by measuring 24-h urinary nitrogen excretion and approximating other losses based on past data in similar populations (Consolazio et al., 1963; Calloway, Odell, & Margen, 1971). In order to account for potential variation in nitrogen loss through sweat (Consolazio et al., 1963), participants were required to weigh themselves before and after the runs and record how much fluid they consumed (100g = 100ml of body water).

Whole-body turnover ($Q$) could then be calculated using the formula:

$$Q = \frac{N_{\text{IN}}}{N_{\text{OUT}}} \text{ or } Q = S + N_{\text{OUT}} = B + N_{\text{IN}}$$

$N_{\text{OUT}}$ (Nitrogen Excretion) is the $^{15}$N enrichment of urea. Assuming the N metabolic pool is in steady-state, then the flux (i.e. $Q$) equals the amount of N leaving the pool ($S + N_{\text{OUT}}$, where $S$ is synthesis and $N_{\text{OUT}}$ is urinary N excretion) and equals the amount of N entering the pool ($B +$...
\[ N_{IN}, \text{ where } B \text{ is breakdown and } N_{IN} \text{ is intake}. \] \[ N_{OUT} \text{ and } N_{IN} \text{ could be measured and therefore } S \text{ and } B \text{ could be calculated. Whole body protein synthesis and breakdown were calculated after determination of total nitrogen intake and excretion (i.e. from the major nitrogen-containing metabolites and estimated average sweat and miscellaneous losses). Miscellaneous nitrogen excretion including that of sweat, fecal and other losses was estimated using a miscellaneous to urinary loss ratio determined from the data of Tarnopolsky, MacDougall & Atkinson (1988) who measured urine, sweat and fecal nitrogen losses in a trained endurance running population consuming 1.7 g/kg/d of protein.}

\section*{3.5 Controlled Diet and Test Drinks}

Participants consumed the controlled diet providing 0.8 g/kg/d of protein, 6-9 g carbohydrate/kg/d and an energy intake to cover 1.6x their REE calculated from the indirect calorimetry in session 2 and their estimated exercise-induced energy expenditure.

In order to increase the protein intake to the target levels and maintain the double-blind nature of the trial, test drinks consisting of a complete profile of crystalline amino acids were provided to increase the protein intake by 0.14 g/kg/d (LOW), 0.40 g/kg/d (MOD) or 1.03 g/kg/d (HIGH) to meet the needs of the tested dosages. Participants were instructed to not consume anything other than water, outside of the prepared meals and snacks, and were required to complete a dietary checklist to indicate that they had consumed the provided meals. The test drinks were provided in powdered form and mixed/dissolved in water by the participant. Participants took 3 test-drinks per day separated by at least 3 hours; they consumed 1 drink immediately after exercise and the other 2 drinks between two of the main meals of the day (e.g. mid-afternoon and before bed).
3.6 Data Analysis

All protein turnover variables were analyzed using a two-way (pre-post * diet) repeated measures ANOVA. Differences between means for significant main effects or interactions were determined using a Holm-Sidak *post-hoc* test. The relationship between protein intake and all variables was analyzed using bilinear regression analysis and biphasic linear regression analysis according to the situation. Statistical significance is established at P < 0.05, and all data were expressed as means ± SD.

3.7 Precision of Estimation and Statistical Inference.

Adopting recommendations for progressive inferential statistics published in Medicine and Science in Sports and Exercise (Hopkins et al., 2009) and elsewhere (Cohen, 1994; Schmidt & Hunter, 1997), performance data was analyzed by magnitude-based inference (Hopkins, 2004). This approach was taken due to the fact that, in traditional statistics based on the null hypothesis and a P-value, the real-world importance of an effect (e.g. performance benefit) may be present and clinically meaningful but could be misinterpreted as a non-significant effect by these classic statistical approaches (Hopkins et al., 2009). This more intuitive approach to inferences is based on where the confidence interval lies in relation to threshold values for substantial effects rather than the null value (Batterham & Hopkins, 2006). Probabilistic determination that effects are greater than, less than, or equivalent to the defined practically or mechanistically quantitative thresholds are provided with this method.

Estimated precision was set at 95% confidence interval. After standardization, probabilistic inferences about the true value for outcomes except performance were qualified using the
standardized difference (adopted from the Cohen effect size d: mean difference/appropriate SD). Standardized difference thresholds were as follows: very small = 0.01-0.2 (Sawilowsky, 2009), Small = 0.2–0.5, moderate = 0.5-0.8, large = >0.8 (Cohen, 1988). Probability thresholds were obtained from the $t$ distribution with likelihoods ordered into cutoffs and inferred as follows: almost certainly not <0.5%, very unlikely = 0.5%–5%, unlikely = 5%–25%, possibly = 25%–75%, likely = 75%–95%, very likely = 95%–99%, and almost certain >99% (Hopkins et al., 2009).

The smallest worthwhile effect for qualitative inferences of the MVC and Jump measures was set at 0.2 based on the small standardized difference threshold of Cohen, 1988. The smallest worthwhile effect for qualitative inferences of the 5kmTT was set at 0.16. This is based on the smallest worthwhile difference of 4 seconds determined for this test by Stevens et al. (2015) divided by the pooled between subjects SD.
Chapter 4. Results

4.1 Participants

A total of 11 endurance trained male participants were recruited for the study. All participants met our definition of trained as determined in their activity history report taken in session 1 and in the fitness assessment in session 2. All participants completed all sessions with the exception of one participant who voluntarily withdrew from the study after session 3 (Trial #1) due to upcoming time conflicts. Participant characteristics are provided in Table 3. Characteristics and results do not include those of the participant who withdrew.

Table 3 Participant characteristics

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value (mean ± SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>31.5±7.6</td>
</tr>
<tr>
<td>Height, cm</td>
<td>180.3±8.3</td>
</tr>
<tr>
<td>Body weight, kg</td>
<td>73.7±7.5</td>
</tr>
<tr>
<td>FFM, kg</td>
<td>65.9±6.8</td>
</tr>
<tr>
<td>Percent Body Fat, %</td>
<td>10.3±5.3</td>
</tr>
<tr>
<td>VO$_{2peak}$, ml/kg/min</td>
<td>64.7±7.9</td>
</tr>
<tr>
<td>Prior 4 week running history, km/wk</td>
<td>62.3±29.7</td>
</tr>
</tbody>
</table>
4.2 Whole Body Protein Metabolism

Whole body protein turnover parameters of synthesis, breakdown and net balance are shown in Figure 3.

4.2.1 Whole Body Protein Synthesis

Synthesis (g/kg/d) results are shown in Figure 3. Mean differences between MOD and LOW was -1.33±0.43 g/kg/d on training day 1 and +1.09±0.92 g/kg/d on training day 4. Mean differences between HIGH and MOD was +1.69±1.70 g/kg/d on day 1 and +1.47±1.85 g/kg/d on day 4. Mean differences between HIGH and LOW was +0.37±1.27 g/kg/d on day 1 and +2.56±2.76 g/kg/d on day 4. The test of significance demonstrated no clear effect (P>0.05) on synthesis rates (g/kg/d) across time or within groups.

Figure 3. Whole body protein synthesis rates on days 1 and 4 of training. No significant differences (P>0.05).
4.2.2 Whole Body Protein Breakdown

Breakdown (g/kg/d) results are shown in Figure 4. Mean differences between MOD and LOW was -1.47±0.26 g/kg/d on day 1 and +0.91±1.03 g/kg/d on day 4. Mean differences between HIGH and MOD was +1.34±1.24 g/kg/d on day 1 and +1.18±1.41 g/kg/d on day 4. Mean differences between the HIGH and LOW was +0.13±0.98 g/kg/d on day 1 and +2.09±2.44 g/kg/d on day 4. The test of significance demonstrated no clear effect (P>0.05) on breakdown rates (g/kg/d) across time or within groups.

![Breakdown Graph](image)

**Figure 4.** Whole body protein breakdown rates on days 1 and 4 of training. No significant differences (P>0.05).

4.2.3 Whole Body Protein Net Balance

Net balance (g/kg/d) results are shown in Figure 5. HIGH demonstrated the most positive net balance (mean of 0.21±0.65 g/kg/d) and MOD was less negative (mean of -0.02±0.52 g/kg/d) than LOW (mean of -0.18±0.28 g/kg/d). A significant difference was found in net balance between all 3 conditions (LOW-MOD, P=0.03; LOW-HIGH, P=0.01; MOD-HIGH, P=0.02) and significant differences were found on day 1 (LOW-MOD, P=0.05; LOW-HIGH, P=0.01; MOD-
HIGH, P=0.01) and day 4 (LOW-HIGH, P=0.04).

Net balance day 1 to day 4 had mean increases of 0.07±0.12, 0.10±0.29, 0.07±0.10 g/kg/d for LOW, MOD and HIGH respectively, but no significant effect (P>0.05) of time was demonstrated.

Figure 5. Capital letters indicate main effect of condition (P<0.05); * indicates different from LOW within Day (P<0.05); † indicates different from MOD within Day. Whole body net protein balance rates within groups on days 1 and 4 of training. Significant differences (P<0.05) were found between all groups without the effect of time. Significant differences (P<0.05) were also found between all groups on day 1 and between groups LOW and HIGH on day 4.
4.3 Performance

4.3.1 MVC

MVC performance outcomes are summarized in Table 4. HIGH had improved MVC performance (mean of +0.40 N/kg or ~2.6%) from pre to post testing, whereas LOW resulted in a mean decrease in MVC performance (mean of -0.87 N/kg or ~ -5.6%) putting the standard difference at 1.27 g N/kg (ES=0.57±0.77) for HIGH over LOW. MOD also showed a decrease in MVC performance from pre to post (mean of -0.58 N/kg or ~ -3.7%) with a standard difference of 0.98 N/kg (ES=0.42±0.70) for HIGH over MOD. There was a standard difference of 0.29 N/kg (ES=0.12±0.68) for MOD over LOW. For mean pre to post performance outcomes and graphic depictions of mean differences please see Appendix A.

4.3.2 5kmTT

Inferential 5kmTT performance outcomes are summarized in Table 4. LOW and MOD had pre to post increases in mean time trial time (decrease in performance) of +7.8 seconds (0.7%) and +8.4 seconds (0.8%) respectively. The standard difference for MOD over LOW is -0.84 seconds (ES= -0.02±0.60). HIGH had a mean pre to post reduction in time trial time (increase in performance) of -7.8 seconds (-0.7%). The standard difference for HIGH over MOD is -16.0 seconds (ES=-0.26±0.50) and the standard difference for HIGH over LOW is -15.2 seconds (ES=-0.24±0.25). For mean pre to post performance outcomes and graphic depictions of mean differences please see Appendix A.
Analysis of pre-performance data (irrespective of treatment) indicated no significant differences [trial 1 vs. trial 2 (P=0.70); trial 1 vs. trial 3 (P=0.06); trial 2 vs. trial 3 (P=0.93)]. This demonstrates that there was unlikely to be an order or learning effect between trials, suggesting that participants did not require further familiarization.

**4.3.3 Jump**

Jump performance outcomes are summarized in Table 4. There are no clear effects on jump impulse or peak force performance within the three protein conditions. The mean differences in impulse pre to post performance are -0.08 kg.m/s (-2.4%), -0.28 kg.m/s (-8.3%) and -0.18 kg.m/s (-5.3%) for LOW, MOD and HIGH respectively. The standard differences are: -0.20 kg.m/s (ES=-0.13±0.31) for MOD over LOW, 0.10 kg.m/s (ES=0.10±0.27) for HIGH over MOD, and -0.10 kg.m/s (ES=-0.11±0.31) for HIGH over LOW. The mean differences in peak force pre to post performance are -0.21 N/kg (-0.9%), -0.45 N/kg (-2.0%) and -0.33 N/kg (-1.5%) for LOW, MOD and HIGH respectively. The standard differences are: -0.25 N/kg (ES=-0.08±0.32) for MOD over LOW, 0.12 N/kg (ES=0.07±0.38) for HIGH over MOD, and -0.13 N/kg (ES=-0.06±0.21) for HIGH over LOW. For mean pre to post performance outcomes and graphic depictions of mean differences please see Appendix A.
<table>
<thead>
<tr>
<th>Outcome</th>
<th>MOD - LOW</th>
<th></th>
<th>Probability of a substantial effect</th>
<th>HIGH - MOD</th>
<th></th>
<th>Probability of a substantial effect</th>
<th>HIGH - LOW</th>
<th></th>
<th>Probability of a substantial effect</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ES;CI</td>
<td>Qualitative inference</td>
<td>ES;CI</td>
<td>Qualitative inference</td>
<td>ES;CI</td>
<td>Qualitative inference</td>
<td>ES;CI</td>
<td>Qualitative inference</td>
<td>ES;CI</td>
</tr>
<tr>
<td>MVC</td>
<td>0.12±0.68</td>
<td>Possible beneficial very small effect</td>
<td>39% 0.42±0.70</td>
<td>Likely beneficial small effect</td>
<td>77% 0.57±0.77</td>
<td>Likely beneficial moderate effect</td>
<td>87%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5kmTT</td>
<td>-0.02±0.60</td>
<td>Unlikely effect</td>
<td>23% -0.26±0.50</td>
<td>Possible beneficial small effect</td>
<td>69% -0.24±0.25</td>
<td>Likely beneficial small effect</td>
<td>79%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jump Impulse</td>
<td>-0.13±0.31</td>
<td>Very unlikely effect</td>
<td>1% 0.10±0.27</td>
<td>Unlikely effect</td>
<td>18% -0.11±0.31</td>
<td>Very unlikely effect</td>
<td>2%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Jump Peak Force</td>
<td>0.08±0.32</td>
<td>Unlikely effect</td>
<td>18% 0.07±0.38</td>
<td>Unlikely effect</td>
<td>20% -0.06±0.21</td>
<td>Very unlikely effect</td>
<td>1%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Standardized difference thresholds were as follows: very small = 0.01–0.2 (Sawilowsky, 2009), Small = 0.2–0.5, moderate = 0.5–0.8, large = >0.8 (Cohen, 1988)

Probability thresholds were obtained from the $t$ distribution with likelihoods ordered into cutoffs and inferred as follows: almost certainly not <0.5%, very unlikely = 0.5%–5%, unlikely = 5%–25%, possibly = 25%–75%, likely = 75%–95%, very likely = 95%–99%, and almost certain >99% (Hopkins et al., 2009)
Chapter 5. Discussion

The aim of the present study was to determine the effects of different protein dosages, being 1.83 g/kg/d (HIGH), 1.2 g/kg/d (MOD) and 0.94 g/kg/d (LOW) of daily protein intake, on performance and recovery during a period of controlled endurance training. This data shows for the first time that over four days of controlled variable distance exercise, 1.83 g/kg has marked benefits for performance (MVC and 5kmTT) and improvements in whole body net protein balance over 1.2 and 0.94 g/kg in trained male adult endurance exercisers.

The HIGH condition had the most positive net balance in protein turnover values as it was the only group that demonstrated a positive overall net balance. This group also had the greatest retention of performance in both the right quadricep MVC and the 5km time trial performance tests. Thus, these data suggest that the typical intake of this population (1.2-1.6 g/kg/d) (Tarnopolsky, 2004) as well as the low end of the current ACSM recommendation for endurance athletes (1.2-2.0 g/kg/d) (ACSM, 2016) may be suboptimal for recovery and performance. The RDA of 1.83 g/kg suggested by Kato et al. (2016) may be closer to that needed for this population to best adapt to or sustain performance during a typical endurance training regimen.

The following sections contextualize the results considering previous studies that have determined protein requirements using various techniques to measure protein turnover and performance variables. Suggestions for avenues of future research are proposed in light of a discussion of the strengths, limitations, and implications of the present study.
5.1 Changes in Protein Metabolism

Nitrogen balance (NBAL) is a classic method used to determine the protein requirements of humans. The technique involves quantifying all the protein that enters the body (diet or intravenous) and all the nitrogen that is excreted. Because the body excretes nitrogenous compounds rather than whole proteins and proteins are approximately 16% nitrogen, NBAL involves measurement of total nitrogen intake ($N_{IN}$) and total nitrogen excretion ($N_{OUT}$ = urine + feces + sweat + miscellaneous, i.e., menstrual loss, hair, semen, and skin). NBAL is deemed to be positive during net anabolism and negative if a person is losing more protein than he or she is taking in.

According to international health organizations “energy requirements change with activity and lifestyle [but] protein requirements do not” (World Health Organization, 1985). However, it has been demonstrated that amino acid oxidation rates are elevated during exercise, especially as glycogen becomes depleted such as during prolonged (>1.5 hr) endurance training (Graham et al., 1995; Wagenmakers, et al., 1991; Lemon & Mullin, 1980). Also, protein is needed not only to replace these losses but to also provide the building blocks to repair damaged tissue and generate new tissue that serve as the adaptations to the training stimuli. Essential amino acids (EAAs) cannot be synthesized by the body, meaning they must be obtained through dietary sources or in crystalline amino acid form, such as those given within this study, as they in particular are required to stimulate synthesis of this new tissues (Tipton, Gurkin et al., 1999; Tipton, Ferrando et al., 1999). Past studies have indeed shown that synthesis rates are increased following endurance training for as long as 24-28 h (Wilkinson et al., 2008; Di Donato et al., 2014).
Our study supports the hypothesis that dietary protein needs are increased by physical activity such as endurance exercise as demonstrated by the less negative net protein balance with the increasing protein intakes. Furthermore, the overall net balance with the LOW protein intake was negative despite being slightly higher (additional 0.14 g/kg) than the RDA suggested for the general population (Health Canada, 2006), further highlighting the inadequacy of this intake for athletic populations. The additional amino acids are posited to be required to replace oxidative losses that occur secondary to exercise-induced increases in breakdown. In addition, these amino acids may also support increased rates of protein synthesis to repair and generate new tissue during recovery. As supported by our performance data (benefits in quadriceps strength and 5kmTT speed), these processes ultimately serve as the adaptations that are ultimately of paramount importance to the endurance athlete.

Whole body protein metabolism was assessed using the oral $[^{15}\text{N}]$ glycine method. This method allows whole body protein turnover (synthesis and breakdown) to be calculated using measures and estimates of nitrogen intake and excretion. By measuring $[^{15}\text{N}]$ urea and ammonia enrichment after the continuous administration of $[^{15}\text{N}]$ glycine over a controlled training period, protein turnover rates were determined over days rather than hours. This allows integration of rest, exercise and recovery from exercise, the fed state and the fasted state, as well as daily activities and sleeping. Moreover, unlike invasive muscle biopsies that can cause muscle soreness and discomfort during the hours to days after the procedure, the noninvasive nature of our oral tracer would have little to no discernible impact on the athletes’ metabolism and thus represent an ideal methodology when assessing parallel measures of performance. The data from this measurement suggests that whole body net protein balance becomes less negative with each
increase in protein intake used in our study as LOW was negative (-0.18±0.28 g/kg/d), MOD was neutral (-0.02±0.52 g/kg/d) and HIGH was positive (+0.21±0.65 g/kg/d).

Despite the robust differences in net balance, there were no significant differences found in rates of protein synthesis or protein breakdown. Past studies have demonstrated that it is likely that increases in rates of protein synthesis are more of a driving factor than reductions in breakdown on changes in net balance (Koopman et al., 2004; Levenhagen et al., 2001). Meredith et al. (1989) found significantly lower synthesis rates at protein intakes of 0.6 g/kg/d compared to 1.2 g/kg/d. However, synthesis rates cannot be attributed to the total difference in net balance. It is likely that subtle changes in both synthesis and breakdown are translating into the observable effects on net balance. It is also important to note that these subtle differences in synthesis, breakdown and net balance following exercise at the whole body level have shown in past data to be magnified at the muscle level (Levenhagen et al., 2002) supporting the suggestion that these processes are contributing to recovery and adaptation of this tissue. Thus, maximizing whole body net balance would be ideal as this would ensure most/all relevant protein pools are enhanced and protein synthesis is presumably maximized, the latter of which has been suggested to be the main recovery goal for athletes (Phillips & van Loon, 2011).

In addition to this study, there are three others which have suggested that protein intakes at or just below 1.0 g/kg/d are not adequate to meet the needs of most men and women with this type of exercise. Phillips et al. (1993) examined the effect of the then Canadian recommended daily protein intake of 0.8 g/kg/d on NBAL in endurance-trained men and women. The participants in this study were trained runners and had a reported running frequency of 4.4±1.4 days/wk for
males and 5.0±1.2 days/wk for females. Their activity during the study was uncontrolled but was within the realm of their habitual activity according to their training logs. Their reported weekly mileage was 50.3±22.3 km for the male participants and 43.3±11.0 km the female participants, which is just below what our participants were asked to do within 4 days (55 km). Following the 10-d adaptation period, participants were found to be in negative NBAL, which would be consistent with our athletes on the LOW diet. Lamont, Patel and Kalhan (1990) studied male and female endurance trained cyclists and runners consuming protein at 1.0 g/kg/d. Training was not controlled but participants were asked to rest 3 days prior to metabolic measurement as the purpose of the study was to understand protein metabolism kinetics in a resting state for this population. At the time of measurement, participants were found to be in slightly negative NBAL (women, −0.22 g N/d; men, −3.95 g N/d). Similar to our study, Meredith et al. (1989) who measured NBAL in male trained adults had their participants consume three different protein intakes (0.61, 0.92, and 1.21 g/kg/d). Participants had a 2-40 year regular training history of cycling, running, rowing and/or calisthenics. Training during the study was not controlled but participants were asked to maintain their habitual activity in their habitual environment. The results found a mean protein intake for a zero NBAL of 0.94 g/kg/d and an RDA of 1.26 g/kg/d. These above studies’ protein intake recommendations were assessing the minimal intake of protein required to offset deficiency (i.e. a zero NBAL) rather than an optimal dosage for eliciting adaptive improvements in performance. Meredith et al.’s (1989) findings are the basis for the ACSMs lower end of protein intake recommendations (ACSM, 2016) and were the support for the previous more specific recommendations of 1.2-1.4 g/kg in 2009 (ACSM, 2009).
Two studies, one by Butterfield and Calloway (1983) and the other by Todd, Butterfield & Calloway (1984), proposed that protein requirements are less in active individuals based on their finding that nitrogen retention was improved in this population. In both studies, participants were housed for 108 days and their daily controlled training consisted of 1 hr on a treadmill and 1 hr on a cycle ergometer at an intensity calculated to result in a combined energy expenditure of 15% of total daily energy needs. Butterfield and Calloway (1983) state that “apparent slight retention of N even with the marginal protein intake suggests that tissue is being laid down under these circumstances”. However, this is not a basis that the protein intakes are maximizing the utilization of protein for tissue to adapt to the training. Indeed, Young et al. (1987) have demonstrated that participants who are given insufficient leucine intakes of 7 and 14 mg/kg/d are able to maintain leucine balance by reducing rates of protein synthesis whereas with 30 mg/kg/d they were in balance at higher rates of synthesis. This suggests that part of the way the body compensates for lower intakes is by reducing synthesis rates which could be affecting the potential for muscle growth and other adaptations to exercise. In other words, this instead may be evidence of a shift in the hierarchy of amino acid–requiring processes toward muscle protein synthesis getting a “greater share” of circulating amino acids in both fasted and fed states. This may also be occurring in the studies which only found slight increases in protein intake to be needed such as 0.46 g/kg above the RDA for sedentary individuals suggested by Meredith et al. (1989). It is arguable that athletes are seeking maximal benefits from their training rather than just accommodation of their body to meet hierarchical needs.

With the above studies in mind, which base their recommendations on that which is needed to achieve a net zero nitrogen balance, it brings forth the consideration that the primary goal of
adequate protein consumption is not at a fundamental level, protein balance, but rather to be able to support increased protein retention and utilization to procure greatest adaptation potential. For endurance athletes wishing to have protein tissue adequately repaired and turned over, a positive net protein balance is the desired goal. This is presumably because of the periodic stimulation of protein synthesis, which, if it is to support the net gain of new proteins or enhanced remodeling/repair, could require net extra amino acids (Philips, Moore & Tang 2007; Phillips, 2006; Phillips, 2004; Rennie & Tipton, 2000; Rennie et al., 2004). Adequate protein intake could be partially defined as maximizing the increase in protein synthesis that occurs after exercise (Carraro et al., 1990; Miller et al., 2005; Sheffield-Moore et al., 2004) as well as balancing the losses of BCAAs which are oxidized to an appreciable extent during endurance exercise (Lamont, McCollough & Kalhan, 1999; Lamont, McCollough & Kalhan, 2001; McKenzie et al. 2000). These could also be said to be primary goals of an endurance athlete aspiring to maximize their performance improvement from training.

If a proposed protein requirement were to be based on a net zero protein balance alone, our data would suggest that requirements for this population would be closer to Meredith et al.’s (1989) RDA of 1.26g/kg/d as our 1.2 g/kg/d intake resulted in a mean net balance just below zero (-0.02 g N/kg/d). However, at a zero net balance it is possible that physiological (mal)adaptations may be present (such as curtailing of other important processes) in addition to the demonstrated reduced synthesis rates (Young et al., 1987). Past studies have also shown benefits of higher intakes such as improved immune function (Gleeson, 2007; Witard et al., 2014; Witard et al., 2012). In other words, this could be an indicator that the body is sacrificing amino acids for the tissue repair process over other relevant processes and that a zero net balance is an inappropriate
index for the basis of a suggested requirement.

The IAAO method of measuring protein turnover has been posited to provide a better indication of the true protein intake needs for optimization (i.e. maximization of the body’s use of protein for tissue turnover, repair and development) (Zello et al., 1995; Elango, Ball, & Pencharz, 2008). This method measures amino acid oxidation, with the reciprocal being whole body protein synthesis, suggesting that the intake determined would optimize recovery. Indeed, Levenhagen et al. (2002) demonstrated that muscle protein synthesis displays a greater magnitude of change compared to whole body protein synthesis, suggesting that all tissue is similarly being stimulated with protein ingestion after exercise. Therefore, these intakes would presumably allow the body the ability to use dietary protein for performance enhancement rather than just for maintaining basic physiological functioning. The protein intake required to maximize synthesis rates would be less likely to result in a downgrading of the use of protein for any physiologically relevant processes. Considering the inherent problems associated with the nitrogen balance method (Pencharz & Bell, 2003), many have hypothesized that the protein requirements it provides are underestimated (Pencharz, Elango & Ball 2008; Tarnopolsky, 2004; Phillips, Chevalier & Leidy, 2016; Lamont, 2012; Phillips, Moore & Tang, 2007). The IAAO method on the other hand is suggested to be more accurate (Pencharz, Elango & Ball, 2008; Pencharz & Ball, 2003) and allows for a greater number of test protein intakes to be performed within a given participant to find a more precise requirement (Elango, Ball & Pencharz, 2012; Elango et al., 2009). As further support, the Institute of Medicine encourages this method for the determination of amino acid requirements (Institute of Medicine, 2005). This method could not be used with our participants due to impracticalities in a “free living” situation. However, our reasoning for our
HIGH protein dose is based on the findings of Kato et al. (2016) who used the IAAO method to determine this intake to be what is required in this population to maximize synthesis rates.

Another important note is that we used crystalline amino acids that reflected the amino acid composition of egg whites which could have influenced our results. This amino acid profile is considered “high quality” (i.e., PDCAAS >1) due to its high EAA content. These amino acids in particular are the primary drivers of protein synthesis (Tipton, Ferrando et al., 1999, Tipton, Gurkin et al., 1999). Dietary protein in other forms could lead to differences in support of maximal rates of synthesis after exercise and ultimately adaptation to training (Tang et al. 2009). This has implications for more varied diets which consist of more or exclusively protein from plant based sources. These sources of protein are typically lower in EAAs and therefore may require a greater overall protein intake to achieve maximal synthesis rates (Phillips, 2006). Thus, inasmuch as the protein quality influenced the present results, dietary protein quality may have varied effects on protein metabolism and our choice of protein for our test drinks would further support maximal synthesis rates. However, vegetarian or vegan athletes may require a greater protein intake that utilized within the present study to obtain similar metabolic and performance benefits due to the generally lower protein quality of their plant-based diets.

5.2 Performance

This study is the first of its kind to examine the effects of multiple protein intakes to enhance performance and/or mitigate performance decrements in a trained endurance running population during controlled training. The studies that have shown that active individuals adapt to less than adequate protein intakes by lowering nitrogen excretion (Butterfield & Calloway, 1984; Todd,
Butterfield & Calloway, 1984) indicate that there is no apparent relationship between nitrogen balance and physical activity. Despite these earlier results, our study suggests that there is a likely compromise of some physiologically relevant processes in our trained population as performance (MVC and 5kmTT) was not retained with the MOD condition as well as it was with the HIGH condition. These performance decrements will be discussed in detail below.

5.2.1 5kmTT

This test was selected because it has a high specificity to the improvements in performance desired by this population as it simulates endurance competition. It also has a high degree of reliability (Russell et al., 2004; Stevens et al., 2015;). A meta-analysis by Laursen et al. (2007) has shown that time trial tests are less variable than their often-used counterpart being time-to-exhaustion tests for endurance performance testing.

Stevens et al. (2015) have suggested that for trained runners who can run a 5km time trial within 17-23 minutes, a 4s difference is meaningful and worthwhile. All of our participants’ time trial results fell within this range with the exception of one participant whose time trial was completed in 16.65 minutes. The difference from pre to post performance between HIGH and LOW and between HIGH and MOD was 15.6 seconds and 16.2 seconds respectively suggesting a meaningful difference outside of the typical variability suggested by Stevens et al. (2015). It is also of note that the HIGH treatment was the only group to improve their time trial time from pre to post. This is a novel finding as no past studies have analyzed time trial performance in relation to differing protein intakes alone. Millard-Stanford et al. (2005) compared a carbohydrate plus protein recovery drink to carbohydrates alone following a 21km run plus
treadmill run to fatigue at 90% VO2max. Diet was not controlled but instructions outside of the test drinks were provided (55% carbohydrate, 15% protein, 30% fat). No significant differences in 5kmTT performance were found. Our study used a training regime more specific to how this population trains (i.e. multiple days of variable distance running). Also, as speculated by the authors in the article, it is unlikely that 24 hours was long enough to result in a detectable difference. This will be discussed in further detail below. Our study incorporated 4 days of training between pre and post testing, which the performance (MVC and 5kmTT) would suggest allowed sufficient time for the benefits of the additional protein to be realized. Both factors, being high relevance and sufficient training time, add precedence to our novel and important finding in the results of this test.

5.2.2 MVC

The MVC test may be a biomarker of changes in muscle damage as defined as an exercise-induced reduction in maximal voluntary isometric force (Place et al., 2007; Clarkson & Hubal, 2002). This test has been used in runners in past studies to assess recovery of muscle function (Green et al., 2008; Etheridge, Philp & Watt, 2008) and it does so by directly measuring leg extensor strength. The direct results of this measurement are relevant to this population as increases in leg extensor strength have shown to contribute to positive effects on running performance by enhancing running economy (Vikmoen et al., 2016; Støren et al., 2008; Sedano et al., 2013).

Mean performance in the MVC test from pre to post testing was better retained in MOD over LOW and improved from pre to post only in HIGH. This is in line with our hypothesis that the
HIGH protein intake would demonstrate the greatest performance benefit related to this group having the least negative nitrogen balance. This is consistent with previous findings showing that a less negative nitrogen balance is correlated with greater rates of recovery, notably through reductions in plasma creatine kinase and later onset tiredness and muscle soreness (Rowlands et al., 2008; Rowlands et al., 2007; Rowlands & Wadsworth, 2011; Millard-Stafford et al., 2005). The reductions in performance from pre to post testing are suggested to be a result of increased rates of muscle protein breakdown from the induced training (Rennie et al., 1981). These broken down amino acids would subsequently be used for oxidation (Tarnopolsky, 2004) and, if not replaced via adequate dietary amino acids, would result in less usable tissue for subsequent performance (Lecker et al., 1999). Increases in muscle protein synthesis following the training via provision of adequate amino acids would expectedly result in not only the repair of damaged proteins, but the synthesis of new functional myofibrillar protein leading to greater strength improvements/retention. This likely played a role in the strength improvements seen in the HIGH group.

Counter to our results, Rowlands and Wadsworth (2011) found reductions in perceived leg strength after high protein feeding (0.7 g/kg/h) for 4 hours comparatively to a low protein control group (0.1 g/kg/h) following 2 days of controlled training. This study was done in 12 endurance trained female cyclists and performance testing was completed on day 2 (as part of the controlled training) and day 4. Perceptions of leg strength were taken via a visual analog scale. Ethridge, Philp & Watt (2008) tested the effects of protein supplementation compared to a calorie free water placebo. It was found that although delayed onset muscle soreness is still present 72 hours after a downhill running bout, MVC performance had returned to pre-testing levels. They state,
“although this appears contradictory, it may indicate that participants’ perceptions of pain are not a strong indicator of the decline in muscle function”. In other words, perceived pain may not accurately reflect muscular damage and (or) may follow a different time course response. This could explain the discrepancy between our findings and Rowlands and Wadsworth’s (2011). The participants in Rowlands and Wadsworth (2011) may have perceived their leg strength to be lower due to the feeling of soreness, but their actual strength may have been recovered. This argument is also supported by the findings of a systematic review by Pasiakos, Lieberman & McLellan (2014) who determined little evidence to support a relationship between changes in markers of muscle damage or soreness and recovery of muscle function with protein supplementation within a 24 hour period. However, they found that when protein supplementation continued for extended periods, indirect markers of muscle damage (i.e. MVC) appear to be reduced. Given LOW, MOD and HIGH were in negative, neutral and positive net balance, respectively, these findings that a less positive nitrogen balance leads to leg strength performance reductions are also supported by Nelson et al. (2012) and Lunn et al. (2012). Both of these studies determined that ergogenic effects associated with protein supplementation on physical performance are only evident over several days of training when compared to participants who are in less positive balance.

5.2.3 Jump

Jump performance using force plate software has been shown to be an indicator of neuromuscular fatigue (Gathercole, Sporer, & Stellingwerff, 2015) associated with power production of the leg extensors (Arteaga et al., 2000). This test is of practical importance to athletes as performance can be impaired by exercise-induced muscle damage resulting in
neuromuscular fatigue (Bryne, Twist & Eston, 2004). Both neuromuscular fatigue and muscle damage have been observed to increase the physiological demand of endurance exercise (Bryne, Twist & Eston, 2004). We observed no clear difference in performance within the different protein intakes. A high variation in jump impulse and peak force in each condition indicates that individual responses to the intervention contributed to the unclear performance outcome.

Jumping metrics have not been used as a measure of performance in this population to compare protein intakes in the past. A potential reason for the lack of mean difference between groups in the present study could be the fact that performance in this test has previously shown to be strongly related to the fiber-type profile of the muscle with a more type II dominated musculature resulting in greater improvements in jump performance (Mero, Jaakkola & Komi, 1991). This fiber type experiences increases in hypertrophy to a much larger degree following resistance training relative to endurance exercise (Farup, Sørensen & Kjølhede, 2014). Differences in jumping performance have been seen within 24 hours following just 1 bout of resistance exercise (West et al., 2017) and to a greater degree after 14 weeks of resistance training (Andersen et al., 2005) in a resistance trained population.

In general, endurance trained participants display a greater proportion of type I muscle fibers (Gollnick et al., 1972; Costill et al., 1979; Harber et al., 2004) and endurance training elicits less change to these muscle fibers relative to resistance training, particularly in a trained population (Trappe et al., 2006). Therefore, a longer training intervention may be required to detect noticeable differences.
5.2.4 Performance Implications

Other studies that have examined the effect of different protein intakes on performance measured repeated-sprint mean power in endurance cyclists after one training session and within only 24 hours (Rowlands et al., 2007; Rowlands & Wadsworth, 2011; Rowlands et al., 2008). Rowlands et al. (2008) tested performance 24 h and 60 h post training and showed that performance differences were not detectable at 24 h, but were at 60 h. This may be related to the time needed to generate new tissue in order to bring about a detectable difference. Our results, although on different performance measures, support the construct that there is an effect after multiple days (Pasiakos, Lieberman & McLellan, 2014).

The present study’s performance data also expand on Rowlands et al.’s (2008) findings related to the effect of different dietary protein intakes on performance benefits. This group compared two intakes with the higher intake (average 1.9 g/kg/d) resulting in a protection against performance deterioration over the lower intake (average 0.8 g/kg/d). The HIGH protein dosage in the present study was based on the RDA determined through the IAAO methodology by Kato et al. (2016). This intake is posited to allow amino-acid stimulated protein synthesis to occur at an optimal capacity and, thus, lead to physiological adaptations that provide improvements in performance. The benefits demonstrated in the MVC and 5kmTT tests in the HIGH intake over the other treatments in our study support this.

Reviews on protein requirements for exercising individuals by Phillips, Moore & Tang (2007), Phillips and van Loon (2011) and Tarnopolsky (2004) have suggested that NBAL is insufficient to determine an optimal intake. Proposedly, using signs of physiological adaptation along with
differences in measured metabolism are more accurate presumptions for distinction of a protein intake recommendation on with this method. In this study, differences in metabolism were found and signs of physiological adaptations were measured via performance, the latter of which is arguably the most relevant outcome for this athletic population. Of the three intakes tested, 1.83 g/kg would be most optimal for an endurance exerciser to consume based on these findings.

5.3 Strengths and Limitations

The participants’ food intake and exercise were rigorously controlled within the realm of free-living practicality in this study. This gives further precedence to the findings as it reduces the “noise” of other influences to determine the true relationships between the variables being tested and measured. However, due to some inherent flaws of the modality of protein metabolism measurements and common issues that come with controlling free living humans, this study is not without its limitations.

Although there was no clear distinction of differences in breakdown and synthesis, there is evidence to suggest that protein synthesis is the primary driver of increases in net balance following endurance exercise (Koopman et al., 2004; Levenhagen et al., 2001). It is not surprising that there was a lack of detectable difference in synthesis and breakdown in our study as it was powered for net balance, which is arguably the more important factor that suggests protein retention for tissue development. Net balance is a function of synthesis and breakdown rates and subtle changes in both can produce differences in net balance. It is also important to note that whole body protein turnover is less sensitive but that changes are magnified at the muscle level in this population (Levenhagen et al., 2002). Future studies should include more
participants and study kinetics at both the whole body and muscle level to further determine the magnitude of effects that synthesis and breakdown have on net balance in this group.

The length of the training period is critical to produce detectable differences in performance as a result of differing protein intakes. Not only did our study measure metabolism alongside effects on performance, which is a unique feature of the study design, but the training stimulus had reason to be long enough to elicit noticeable changes according to prior research (Rowlands et al., 2008). This is a strength when compared to past studies which looked at nitrogen balance alone (Phillips et al., 1993; Lamont, Patel & Kalhan, 1990; Butterfeild & Calloway, 1984; Todd, Butterfield & Calloway, 1984; Meredith et al., 1989) and those that took performance measurements too soon to elicit detectable results (Rowlands et al., 2007; Rowlands & Wadsworth, 2011). However, it could also be observed as a limitation that it was not longer. An even longer period of controlled training may have provided greater divergence between groups giving better insight into the differences in both protein turnover and performance that could be expected over weeks, months and years of training for this population.

It is important to note that our results and the results of past studies using this method to measure protein turnover may not be an accurate representation of actual net balance due to potential inaccuracies in measurement. This, as well as the fact that training was not controlled are indicated as limitations by Meredith et al.’s (1989) determined RDA for this population. Against this background, our use of estimation of nitrogen losses could be viewed as a limitation and contributed to an underestimation of actual net balance (Phillips, Moore, & Tang, 2007; Phillips & Loon, 2011; Tarnopolsky, 2004). We measured urinary output which accounts for ≥75% of
total losses of nitrogen (Tarnopolsky, MacDougall & Atkinson 1988) leaving ≤25% of our losses participant to possible errors in estimation. Part of this ≤25% includes nitrogen losses in sweat, which may represent a significant route of nitrogen loss in active populations (Calloway, Odell & Margen, 1971). Differences in sweat rates of our participants may be seen as a limitation as this could impact our error in nitrogen excretion estimation. For example, participants generally trained outdoors and therefore could be under the influence of ambient temperature, which could have induced variations in sweat, and thus nitrogen, losses. However, to account for this possibility, participants were asked to weigh themselves before and after their training and to indicate how much water they had to drink during to estimate sweat loss. No significant differences were found between protein intake groups for sweat losses, suggesting that the amount of error within groups was the same (Appendix A). Thus, while absolute net balance may have been subject to a small systematic bias it would not invalidate our between group differences. All of our excretion estimates were based on previously established values determined on the population used in this study (Meredith et al., 1989). The magnitude of the differences between groups should be looked at as a reliable indicator of actual differences due to same assumptions made within groups. As this method of measurement has been postulated to underestimate requirements (Pencharz, Elango & Ball, 2008; Tarnopolsky, 2004; Phillips, Chevalier & Leidy, 2016; Lamont, 2012; Phillips, Moore, & Tang, 2007), performance along with measured differences in net balance between groups are likely more accurate presumptions to base the distinction of an optimal intake.

Finally, the limitation of how the participants chose to break up their runs may have influenced their whole body net balance. Running the entire 20 km in one bout may be suggested to have
increased glycogen store losses over the period of the run since it has been determined that glycogen can be depleted within 90-120 minutes of exercise when there are no carbohydrates supplied exogenously (Coyle & Coggan, 1984). Since amino acid oxidation rates increase as glycogen stores become depleted (Lemon & Mullin, 1980; Wagenmakers et al., 1991; Jackman et al., 1997), this running distance could have resulted in a greater total amino acid oxidation. This would increase protein needs following the run to replace these losses compared to multiple shorter runs which would have given the opportunity to recover glycogen stores. A total of 9 out of 60 (15%) of the 20km training sessions were run differently from other 20km training sessions among the participants which could cause an increase in estimation error when comparing these training sessions to the others. Tarnopolsky, MacDougall & Atkinson (1988) suggest that endurance participants consuming a similar protein intake (1.7 g/kg/d) and running a similar distance to our participants have a total N excretion of 23.46 g/d. If we use Kato et al.’s (2016) estimated amino acid oxidation total being ~14g over a 20km run, this converts to 2.25 g N. These studies combined suggest N excretion from a 20km run would account for approximately 9.6% of total N excretion in a day. Therefore, nitrogen losses from a 20 km run likely made up <10% of total losses throughout the day regardless of how the run was broken up. Also, to reduce the risk of glycogen depletion and therefore differences in amino acid oxidation rates, participants were fed 6-8g/kg of carbohydrate per day as part of their controlled diet. This falls within the ACSM recommendation of 6-10 g/kg/d said to meet the needs of “high quality and at high intensity” endurance training lasting 1-3 hours (ACSM, 2016). All participants’ 20 km runs were completed within this time frame.
5.4 Avenues for Future Research

With this data as a background, many more avenues for future research are presented involving alterations to the population, training and nutrition. The following are next steps for future research.

As past research has demonstrated metabolic differences between trained and untrained populations, particularly in skeletal muscle (Carter et al., 2001), it would be beneficial to repeat a similar study in an untrained population. It has been shown that when untrained individuals are started on a new exercise regimen, especially one which is strenuous and of long duration, there is a transient period of increased N loss (Molé & Johnson, 1971; Gontzea, Sutzesco & Dumitrache, 1962). McKenzie et al. (2000) determined that total BCAA oxidation is higher during and away from exercise in untrained participants, implying that more amino acids are being used as a fuel source (Tarnopolsky, 2004). Therefore, it seems that there is a protein sparing effect of being endurance trained both during and away from exercise. Furthermore, studies have shown muscle hypertrophy as an initial adaptation to endurance training, including running and cycling, in untrained individuals (Konopka & Harber, 2014). It is likely that untrained individuals’ dietary protein needs would be greater to both provide the amino acid building blocks to generate new tissue as well as to replace the extra losses compared to those who are trained. This would be in order to retain both a positive net balance and performance since performance has been shown to be reduced over multiple days when individuals are in negative NBAL (Lunn, Nelson & Rowlands, 2008).

Many studies have determined sex differences in metabolic fuel selection during endurance
activity (McKenzie et al., 2000; Tarnopolsky et al., 1990; Phillips et al., 1993). Women use proportionately more endogenous lipid and less carbohydrate and protein (Riddell et al., 2003) during endurance exercise than do men. Taking an average of 5 studies, a review by Tarnopolsky (2004) found a mean in endurance trained women of 2.1% of their fuel sourced from amino acids whereas 5.5% of fuel is sourced from amino acids in endurance trained men during training sessions that averaged an intensity of 67% VO2max. With this being said, as protein breakdown losses would be reduced, it is possible that intake requirements for women to optimize adaptations to their training is less than that of men’s. Studies that include women and men find a reduced NBAL in the men during periods of endurance training (Phillips et al., 1993; Lamont, Patel & Kalhan, 1990; McKenzie et al., 2000). However, women also have the added variable of the menstrual cycle with the phases influencing protein metabolism in different ways. Specifically, studies have found amino acid oxidation to be greater in the luteal phase (LP) compared with the follicular phase (FP) at rest (Kriengsinyos et al., 2004; Lariviere, Moussalli & Garrel, 1994; Toth et al., 2006). Bailey, Zacher and Mittleman (2000) as well as Lamont, Lemon and Bruot (1987) have reported that protein catabolism is greater in the LP due to the effects of progesterone as it appears to increase amino acid oxidation (Kriengsinyos et al., 2004), while estrogen, which is higher in the FP has been shown to reduce protein catabolism (Hamadeh, Devries & Tarnopolsky, 2005). In addition to this, performance differences have been seen depending on menstrual cycle phase (Oosthuyse & Bosch, 2010) with improved endurance time trial performance in the FP (Oosthuyse, Bosch & Jackson, 2005; Campbell, Angus & Febbraio, 2001). Therefore, phase of menstrual cycle would need to be taken into consideration when examining protein metabolism and performance. Differences in requirements between the phases should be examined.
The effects of differing intensities, durations and nutritional strategies on protein metabolism and requirements for optimization of adaptation should also be compared. With increasing duration, glycogen stores would be depleted resulting in increased amino acid oxidation rates (Graham et al., 1995; Wagenmakers, et al., 1991; Lemon & Mullin, 1980). Increasing intensity has shown to proportionally increase amino acid oxidation (Lemon et al., 1982). Therefore, training periods with longer durations and (or) greater intensities would likely increase protein intake requirements to both retain a positive nitrogen balance as well as performance. Nutritionally, “training low”, which involves training with low carbohydrate availability, is becoming increasingly common. It is speculated to be a potential means of stimulating increased mitochondrial protein bio-genesis to improve resistance to fatigue (ACSM, 2016; Jeukendrup, 2017). With amino acids being the required building blocks for this desired process, protein intake requirements should also be studied in this nutritional state. Since this dietary strategy involves periodized low glycogen availability or glycogen depletion prior to training (ACSM, 2016; Jeukendrup, 2017) it is likely that amino acid oxidation rates would be higher both during (Wagenmakers et al., 1991; Jackman et al., 1997) and away from (Manninen, 2004) training. With this, it would be posited that dietary protein intake requirements would be higher during these times.

5.5 Conclusion

The hypothesis that dietary protein needs are increased by endurance exercise is supported. The additional dietary protein is suggested to be required to replace losses as a result of increased breakdown rates and to stimulate synthesis to repair and generate new tissue. Previously determined intakes do not consider an optimal dosage to support what endurance exercisers are
seeking, which is arguably improvements in performance. Our data suggests that a protein intake of 1.83 g/kg enhances whole body net protein balance and provides small but notable benefits in exercise performance compared to intakes of 0.94 g/kg or 1.2 g/kg. The IAAO method used to determine a robust breakpoint of what would be optimal to maximize rates of whole body synthesis and, thus, adaptation to the induced demands of exercise are supported in the performance improvements found in the group consuming the amount of protein determined by this method.

These data suggest that endurance exercisers who consume dietary protein towards the high end of current recommendations by the ACSM (1.2-2g/kg) would better maintain protein metabolism and exercise performance as compared to previous endurance-trained specific recommendations (i.e. 1.2-1.4 g/kg).
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Appendices
Appendix

A. STATISTICAL RESULTS AND RAW DATA
Performance Mean Differences, Standard Differences and Effect Sizes

MVC

Mean Difference Pre-Post (N/kg)

STD Diff

ES
5kmTT

Mean Difference Pre-Post (min)

STD Diff (min)

ES
Jump

**Impulse**

**Mean Difference Pre-Post (kg.m/s)**

- High
- Mod
- Low

**STD Diff**

- H - L
- H - M
- M - L

**ES**

- H
- H - M
- M - L

**Peak Force**

**Mean Difference Pre-Post (N/kg)**

- High
- Mod
- Low

**STD Diff (N/kg)**

- H - L
- H - M
- M - L

**ES**

- H
- H - M
- M - L
## Participants’ 20km Run Splits

<table>
<thead>
<tr>
<th>Participant</th>
<th>Intake</th>
<th>Day 7</th>
<th>Day 10</th>
</tr>
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<tbody>
<tr>
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# Participant Sweat Losses

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</table>
Appendix

B. CONSENT AND ASSENT FORMS
Consent Form

**Title of Research Project:**

- Impact of the protein intake on the amino acid and protein metabolism in the endurance-trained adults during intensified training period

**Investigators**

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**Purpose of the Research:**

Protein is an essential nutrient that we need to eat enough of in our diet to maintain important bodily functions and to recover from exercise. In a recent study, we determined how much protein is needed in healthy, endurance-trained adults after a single 20 km run, which appears to be slightly more than what is currently recommended to be consumed (i.e. the recommended daily allowance or “RDA”) according to world nutrition consensus statements. However, it still remains to be determined what impact consuming the RDA for protein compared to our new estimated minimum intake has on the athletic performance and protein metabolism over repeated days of exercise. A better understanding of the specific importance for protein in male adult endurance athletes is needed to ensure that they are meeting the body’s protein needs, including that for optimal recovery from exercise.

**Description of the Research:**

If you decide to enter this study, we will assess your physical performance and mood state before and after 4 days of high volume training while consuming three levels of dietary protein to determine the influence of the habitual protein intake on athletic performance. Protein metabolism will be assessed through the 4 days training period. This study will involve a total of 5 separate sessions including today’s information session (Session 1: Information; Session 2: Fitness Assessment and Familiarization; Sessions 3, 4 and 5: Intervention; see Figure.1 and below for details). All the sessions will be separate but will be completed over a period spanning approximately 2 to 6 months.
In the event that you agree to participate in the study, the remainder of this session will serve to assess your habitual physical activity levels and general health through the completion of two questionnaires. Following the completion of the questionnaires, we will provide you with an accelerometer (a device used to measure normal activity patterns and energy expenditure), dietary log and instructions of dietary log to record 3 days of your habitual food intake. For the 3 days prior to returning to the Iovate/Muscletech Exercise Metabolism and Sports Science Lab (IM lab) at the Goldring Centre for High Performance Sport (GC) at the University of Toronto for Session 2, you will be required to wear the accelerometer as well as record your normal food intake.

Session 2: Fitness assessment and familiarization
You will return to the IM Lab in the morning after at least 7h of an overnight fast (i.e. no food or drink intake, with the exception of water). First, you will be required to breathe into a mouthpiece or mask for 30 minutes, which will allow us to assess how much oxygen you use during rest as an estimate of your body’s normal (resting) energy expenditure. After completion of the resting energy expenditure assessment, you will consume a carbohydrate energy drink prior to undergoing body composition testing to assess the amount of fat and fat-free mass you have using non-invasive method of the Bod Pod, which is commonly used for individuals of all ages and sizes. The Bod Pod will involve you having...
to provide your body weight on a standard scale prior to entering an air tight, pressure controlled pod-like device (with a small window) that is slightly larger than your body. This "pod" will tell us how much volume your body has, which will allow us to determine your body density to approximate your fat mass and fat-free mass (i.e. total amount of muscle, bone, organs, etc.). The time spent in the "pod" will only be a few minutes and will require you to wear a tight-fitting bathing suit and swimmer’s cap. This method is completely safe but may make you anxious if you are uncomfortable with small spaces.

Upon completion of the body composition measures, and following a warm-up, you will engage in a 12-minute treadmill-based fitness assessment. You will be required to wear a heart rate monitor on your chest and breathe into a mouthpiece or mask, which will allow us to assess how much oxygen you use during a treadmill exercise test as an estimate of your fitness level. Although the mask or mouthpiece may be uncomfortable, it will not cause any pain. This treadmill test will require you to begin running on a treadmill at a comfortable pace after which the work rate will increase at a constant rate; this will be done by maintaining the treadmill speed but increasing the treadmill inclination (i.e. you will feel like you are starting to run uphill). The test will finish when you reach a point in which you can no longer continue (volitional fatigue), which should occur within approximately 12-minutes.

Once the treadmill-based fitness assessment has been completed, you will be given an opportunity to rest prior to performing a 5-Km Time trial (5kmTT) to familiarize yourself with this test. This exercise bout will involve running at a race pace for 5 km. You will be required to run 5 km as fast as you can. You can adjust your running speed during the time trial. During this test you will not be given any feedback on your performance other than your distance traveled. This test will simulate your performance in a standard 5-km road race, for example.

After completion of the 5kmTT, you will be given an opportunity to rest prior to performing the other exercise test that will be conducted twice on the subsequent trials. These exercise tests will measure your muscle strength (maximum voluntary contraction; MVC), vertical jump, and muscle soreness. The maximal strength test will require you to sit on a chair-like apparatus with your leg secured to an immovable arm in a bent 90° position by straps on your thigh and lower leg. You will then be asked to perform a maximal voluntary contraction (MVC) in which you attempt to straighten your leg against the lower pad of the machine for ~5 seconds. The machine will limit the movement of your leg but will be able to measure how much force your leg can produce (i.e. measure your MVC). You will be asked to perform 3 MVC tests separated by ~1 min of rest each. This test will be performed on days 2 and day 9 of sessions 3 and 4 (see below for details). We will also test your peak power be determining how high you can jump (vertical jump) while standing on a force platform in the lab. For this test you will jump 3 separate times separated by ~60s rest on days 2 and day 9 of sessions 3 and 4.

As new or unaccustomed exercise can be associated with some mild muscle stiffness or soreness, your subjective ratings of muscle soreness (quadriceps and
gastrocnemius/soleus) will be collected using a validated visual analogue scale. Muscle soreness will be rated with a visual analog scale that incorporated a 100-mm line, with 0 indicating no pain and 100 representing extremely painful. You will be asked to mark their perceived soreness on the 100-mm line when the knee joint was forced to be flexed and extended by an investigator and when an investigator palpates your calf muscle and thigh muscle.

Finally, you will be required to run 10 km on a treadmill at self-selected pace for familiarization with 20 km run. You will engage in a total of approx. 20 km on session 2.

**Session 3, 4, 5: Training session**

See details in Table 1.

### Table 1. Contents of Session 3, 4, 5

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</table>

(■): Single randomly selected session of three sessions
*: POMS: Profile of Mood Status
#: DALDA: Daily Analysis of Life Demand of Athlete

**Day 1, 2: Monitoring days**

You will record a dietary log for 2 days. Training will not be controlled or restricted for these two days.

**Day 3: Rest day and basal protein metabolism (one randomly selected session)**

On day 3, you will be required to refrain from any exercise during the day with the exception of normal daily activities (e.g. commuting, shopping and normal daily living). In
addition, you will be required to keep a dietary log. On day 3 of one of the sessions, we will measure how your body breaks down and rebuilds proteins in your body throughout your usual daily life. To do this, you will be required to provide a spot urine in the morning. After this you will consume a drink containing 2 mg/kg of $^{15}$Nglycine* dissolved in water before breakfast. To fully understand how much protein the body is making, we will have to collect all of the urine you produce during the day. Therefore, we will give you two special containers: one to collect urine that you produce during the day up to and including just before you go to bed and the other to collect what you produce during the night including the first urination on the following day. You will store the urine at 4 °C and will either drop it off at the lab or we will pick it up at your home the following day.

*: $^{15}$Nglycine is an amino acid (building block of protein) that is slightly heavier than most of the naturally occurring $^{14}$Nglycine in the body: this is called a stable isotope. Your body contains $^{15}$Nglycine naturally, just at a very low level. By providing this stable isotope, we will be able to measure how the glycine is being used in the body. $^{15}$Nglycine is safe and non-radioactive and will allow us to measure how much protein your body is making.

**Day 4:** Blood sampling (one of the sessions) & Pre-Performance test
You will report to the GC in the morning after an overnight fast (i.e. having refrained from breakfast). Of one of the sessions (randomly selected), your blood (~10 ml) will be drawn. A small needle will be inserted into a forearm vein by a trained phlebotomist and a single blood sample (~10 ml) will be taken into a special, evacuated tube. The discomfort of this procedure is transient and is very similar to having an injection by a needle or when donating blood. The phlebotomist will make every effort to minimize the discomfort and take the sample as quickly as possible. Topical anesthetics will be applied to the phlebotomy sites to minimize needle injection pain if desired. Upon removal of the needle any discomfort should subside. In this experiment the blood taken is ~10 ml, which is approximately ~2% of the blood removed during a donation to a blood bank. It is not enough to affect physical performance or health in any way and your body will naturally replace this blood loss over the following days. After each blood sample has been taken, pressure will be placed on the site in order to minimize bleeding and facilitate healing. This type of blood sampling is a common medical practice and involves few risks if proper precautions are taken. The needles are single-use and sterile and the site of the sample collection will be thoroughly cleaned and sterilized prior to the sample being taken; however, there is a theoretical risk of infection. There is a chance of internal bleeding if adequate pressure is not maintained upon removal of the needle. This may cause some minor discomfort and could result in bruising/skin discoloration which could last up to a few weeks. In very rare occasions, trauma to the vessel wall could result in the formation of a small blood clot, which could travel through the bloodstream and become lodged in a smaller vessel. However, we have never experienced such a complication after several thousand blood draws.

After completion of blood sampling, your body composition will be measured using the Bodpod. After this you will consume a protein-free breakfast that will contain carbohydrate that will provide a standardized amount of energy for the following performance tests. After taking the breakfast, you will be required to fill out a questionnaire that will assess
how you feel that day and your general mood. The questionnaire will consist of 65 questions. Once the mood assessment has been completed, you will perform the performance tests above (i.e. 5kmTT, and MVC, vertical jump test and muscle soreness) in the same order as session 2. No pain medication (e.g. Tylenol, Advil, aspirin) are to be taken on the Performance Days.

**Day 5, 6: 2 days adaption period**
You will consume a controlled diet in the form of commercially available, pre-packaged foods that will be as similar as possible to your normal dietary intake. During this time you will not be allowed to consume anything other than water outside of the prepared meals. You will be required to refrain from any exercise during the day with the exception of any normal daily activities (e.g. commuting, shopping, etc.). Your activity during this time will be monitored by accelerometer.

**Day 7-10: 4 days controlled training and diet**
On days 7-10 you will continue to consume the controlled diet and wear the accelerometer. You will be required to wear a HR monitor on your chest for 5 minutes in the morning before eating breakfast to record morning HR. You will also be required to run 20 km (Day7), 5 km (Day8), 10 km (Day9), 20 km (Day10). In addition, you will consume 3 additional test drinks per day that will add a variable amount of protein (in the form of crystalline amino acids, the building blocks of protein) to your diet. The test drinks will be provided in powdered form and will contain free amino acids, carbohydrates, fat, sweetener and flavouring. You will dissolve the test drink with water by yourself and will consume 3 test-drinks per day, which will be separated by at least 3 hours and preferable be consumed in between each of the main meals of the day. The meal and test drinks will provide adequate energy and carbohydrates for endurance-trained subjects. You will be required to consume one of three test drinks (i.e. Low, Mod, or High), but will be blinded to which test drink you are provided. Test drinks and controlled-diet meals will be delivered to you by the investigators.

You will be required to complete a total training volume of 20 km (Day7), 5km (Day8), 10 km (Day9), 20km (day10), which will be completed according to your convenience and will be replicated on each day. During the designated training, you will be required to wear the accelerometer HR monitor watch with GPS on your chest; this will allow us to measure the quantity and intensity of your training bouts. Before and after the 20 km run, you will record your body weight to estimate the volume of sweat lost during exercise. This information will be recorded in a training diary.

During this time you will not be allowed to consume anything other than water outside of the prepared meals. If you take any medication, you will record it in the dietary checklist we provide. You will be required to refrain from any exercise during the day with the exception of any normal daily activities (e.g. commuting, shopping, etc.) and the daily training. You will be required to complete daily mood state questionnaires at the end of the day.
Day 7-10: Protein metabolism (24h urine collection)
On the first and last day of training we will measure how your body breaks down and rebuilds protein in your body, which are normal processes that may be increased by exercise and can be influenced by your dietary intake. To do this, you will be required to provide a spot urine on the mornings of days 7 and 10. After this you will consume a drink containing 2 mg/kg of [15N]glycine dissolved in water before breakfast. To fully understand how much protein the body is making, we will have to collect all of the urine you produce during the day. Therefore, we will give you two special containers: one to collect urine that you produce during the day up to and including just before you go to bed and the other to collect what you produce during the night including the first urination on the following day. On the second and third day of training, we will measure your protein balance (protein synthesis – protein breakdown). To this end, you will be required to collect all the urine you produced into a single container. You will store the urine at 4 °C and will either drop it off at the lab or we will pick it up at your home the following day.

Day 11 Blood sampling & post Performance test
The procedure will be the same as Day 4 of session 3. You will report to the GC in the morning after an overnight fast (i.e. having refrained from breakfast). After completion of blood sampling, you will consume a protein-free breakfast, have your body composition measured, fill out the questionnaire and then perform the physical performance tests (MVC, vertical jump test, muscle soreness and 5 km TT) with the accelerometer and the HR monitor in the GC. Any concomitant medications are not allowed.

These 3 sessions will be separated by a minimum of 7 days. Throughout session 3, 4, 5, you will be required to wear an accelerometer for the entire day to estimate your usual daily energy expenditure. This small device will be worn on your waist and will measure your activity levels. Mood state questionnaires will also be required to be completed at the end of each day.

Potential Harms:
• The VO2Max test will finish when you reach a point in which you can no longer continue (volitional fatigue). Therefore, there is a risk of falling, although handrails are provided on the treadmill and you will be monitored closely by an investigator during the whole test.
• There is a risk that the training for 4 consecutive days (which may be an increase from your habitual training) may lead to an increased risk of infection (e.g. developing a common cold) and/or an overuse injury (e.g. Plantar fasciitis, Shin splints, Achilles tendonitis, Iliotibial band (ITB) friction syndrome, and Runner’s knee, etc.). To minimize these risks, you should ensure that you get enough rest when not performing the prescribed training, get enough sleep (at least 7 hours), and that you consume the entire diet provided to you in the study.
• There is a theoretical risk that the blood sampling procedure may lead to infection: however, this will be minimized by cleansing the area to be sampled and using sterile equipment.

• There is a small risk that the blood sampling may lead to local discolouration and bruising at the site of the needle; this risk will be minimized by applying pressure after the procedure.

• There is a theoretical risk that a small clot might form within your blood vessel after the procedure, which in the worst case scenario could lead to a complication like a stroke; however this has never happened in our experience.

• As you will run 20 km, 5 km, 10 km, 20 km run for 4 consecutive days (which may be an increase from your habitual training) you may experience increased feelings of tiredness from usual and your mood state may change (e.g. you may feel increased tension, depression, anger, fatigue, and confusion). Thus, there is a theoretical risk that academic/work performance could be mildly affected by the study. To help reduce this risk you should ensure you get proper rest (e.g. at least 7 hours of sleep) when you are not exercising. In addition, we can arrange the date of each session according to your preferred schedule (e.g. participants would have the opportunity to avoid busy times at work or school, such as exams). However, any changes to your mood state are likely to be related to how tired you feel and therefore once you complete the intensified training you should return to your pre-study state.

• As you may perform 4 days of training that have a greater volume than what you do normally, your muscles may become mildly sore and this may persist for up to 7 days after you complete the exercise. However, this is a normal part of how your body responds to changes in training (you may have already experienced this in your normal training) and therefore this soreness is only temporary. If it persists for greater than 7 days you should report this immediately to a study investigator.

• While this study does not cause harm we recognize that the length of the trial day, the number of days required to complete the study, and travel to the University of Toronto (a total of 8 visits are necessary, including this introduction session) might pose an inconvenience to you.

• If you are uncomfortable with small spaces then you may experience some anxiety during the short time when you are in the Bodpod.

• The test drink that you will consume during the consecutive 4 days will have a distinct taste that some people find unpleasant. Additional flavouring will be provided to help improve the taste. In addition, a small protein-free cookie will be provided to consume after the drink, which will remove most, if not all, of any potential unpleasant after taste of the test drink.

• The discomfort of blood sampling is transient and is very similar to having an injection by a needle, or when donating blood. Upon removal of the needle any discomfort should subside. However, topical anesthetics could be applied to phlebotomy sites to minimize needle injection pain, if you want.

Potential Benefits:
• You will be given personal testing results (i.e. VO2Max, Body composition, resting
energy expenditure and results of performance tests)
• Your participation will contribute to the scientific understanding of protein requirements for endurance-trained young adults, which is essential for optimal health.

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

The data produced from this study will be stored in a secure, locked location. Only members of the research team will have access to the data. This could include external research team members. Following completion of the research study the data will be kept five years following the study completion then destroyed as required by University of Toronto policy. Published study results will not reveal your identity.

Reimbursement:
• The proposed compensation is meant to adequately reimburse you for any costs incurred (e.g. parking and a small post-study meal) and to provide a token gift of appreciation for your effort. A total of $1150 will be provided as a compensation, after the completion of all sessions.

Should you choose to withdraw from the study for personal reasons, your reimbursement will be pro-rated at the following rate: session 2: $50; 1 training session (contains additional blood withdrawal and additional measurement of 24h protein metabolism): $400; 2 training sessions4, 5: $350 each for a total of $1150 (note: each of session 3, 4 and 5 will be further prorated based on percentage of trial commenced).

Participation:
• It is your choice to take part in this study. You can stop at any time. You will be compensated for all trials that you partake in.
• Participation in this study without meeting the minimum fitness level can put you at an increased risk for physical injuries. Therefore, you will be required to meet minimum standards on the fitness and performance tests as well as during the familiarization session.
• Any data collected and analyzed up to your decision to stop will remain in the study database.
• New information that we get while we are doing this study may affect your decision to take part in this study. If this happens, we will tell you about this new information. And we will ask you again if you still want to be in the study.
**Conflict of Interest:**

- This study is sponsored by Ajinomoto co., Inc. which produces crystalline amino acids used in this study. The sponsor can access the data which is coded with a study identification number and does not contain the participants’ personal information. The results might be utilized for the company’s business.
- Hiroyuki KATO is an employee of Ajinomoto co., Inc.

**Declaration of Helsinki**

- This study will be conducted in accordance with the Declaration of Helsinki.
Consent:

By signing this form, I agree that:
1) You have explained this study to me. You have answered all my questions.
2) You have explained the possible harms and benefits (if any) of this study.
3) I understand that I have the right to refuse to participate in the study. I also understand that have the right to withdraw from the study at any time without penalty and that any data analyzed during my participation will remain in the study database.
4) I am free now, and in the future, to ask questions about the study.
5) I understand that no personal information about myself will be given to anyone or be published without first asking my permission.
6) I have also been provided the study timeline and been given demonstrations of all the measures to be used.
7) I agree, or consent, that I __________________ may take part in this study.

______________________________  _________________________
Printed Name of participant     Participant’s signature & date

______________________________  _________________________
Printed Name of person who explained consent  Signature of Person who explained consent & date

If you have any questions about this study, please call Dr. Daniel Moore at 416-946-4088 or by email at dr.moore@utoronto.ca

If you have questions about your rights as a subject in a study or if you experience injuries related with this study protocol, please contact either of the investigators or the ethics review board at ethics.review@utoronto.ca or 416 946 3273.

The research study you are participating in may be reviewed for quality assurance to make sure that the required laws and guidelines are followed. If chosen, (a) representative(s) of the Human Research Ethics Program (HREP) may access study-related data and/or consent materials as part of the review. All information accessed by the HREP will be upheld to the same level of confidentiality that has been stated by the research team.
Appendix

C. ELICITATION TOOLS
Habitual Food Intake Record

INSTRUCTIONS FOR KEEPING YOUR THREE DAY FOOD RECORD

1. Record EVERYTHING that you eat and drink for the 72h before your trial.

2. Record EXACT AMOUNTS when possible. Record WEIGHT of each food item if available (e.g. meat weight; 6 oz of grilled salmon, etc.). Household measuring cups or spoons can also be used to estimate portions (e.g. ½ cup of plain oatmeal, dry; 1 cup cooked pasta, etc). 

3. Record BRAND NAMES, if known (e.g. Nature Value Harvest Bar, Oats ’N Honey).

4. If eating out, record foods eaten as accurately as possible, including the NAME OF The ESTABLISHMENT and the SPECIFIC FOOD ITEM ORDERED.

5. Always specify METHOD OF PREPARATION. Examples include: baked, broiled, fried, breaded, sautéed, etc.

6. Describe all foods as fully as possible. For example: 3 oz. baked chicken thigh, no skin.

7. List ALL INGREDIENTS for sandwiches, casseroles, and other mixed dishes. Example: Peanut butter sandwich – 2 pieces whole wheat bread, 1 ½ Tbsp. creamy peanut butter.

8. For accuracy, it is best to record each meal or snack immediately after it is eaten. Be sure to include drinks (water, coffee, tea, soda, etc.)

9. Include all ADDITIONS to food at the table, such as salt, sugar, or milk. Record each addition on a separate line.

10. Record the time of day in which each item of food was eaten.

Example:

<table>
<thead>
<tr>
<th>Time</th>
<th>Food</th>
<th>Preparation</th>
<th>Quantity (ml, g, cup, tsp, tbsp)</th>
<th>Quantity Left Over (ml, g, cup, tsp, tbsp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6:15 pm</td>
<td>Skinless chicken breast</td>
<td>Baked</td>
<td>200 g</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Diana’s BBQ sauce</td>
<td>n/a</td>
<td>3 tbsp</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Broccoli</td>
<td>Steamed</td>
<td>½ cup</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Brown rice</td>
<td>Boiled</td>
<td>½ cup</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Water</td>
<td>n/a</td>
<td>2 cups</td>
<td>0</td>
</tr>
<tr>
<td>Time</td>
<td>Food</td>
<td>Preparation</td>
<td>Quantity</td>
<td>Quantity Left Over</td>
</tr>
<tr>
<td>------</td>
<td>------</td>
<td>-------------</td>
<td>----------</td>
<td>-------------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>How did you cook it</em></td>
<td><em>(ml, g, cup, tsp, tbsp)</em></td>
<td><em>(ml, g, cup, tsp, tbsp)</em></td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>What did you add to it</em></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For Office Use Only
Initials Entered ____
Initials Proof ____
<table>
<thead>
<tr>
<th>Time</th>
<th>Food</th>
<th>Preparation</th>
<th>Quantity</th>
<th>Quantity Left Over</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>How did you cook it</td>
<td>(ml, g, cup, tsp, tbsp)</td>
<td>(ml, g, cup, tsp, tbsp)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>What did you add to it</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

For Office Use Only

Initials Entered _____
PAR-Q+

The Physical Activity Readiness Questionnaire for Everyone

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

SECTION 1 - GENERAL HEALTH

Please read the 7 questions below carefully and answer each one honestly. Check YES or NO.

1. Has your doctor ever said that you have a heart condition OR high blood pressure? ☐ ☐

2. Do you feel pain in your chest at rest, during your daily activities of living, OR when you do physical activity? ☐ ☐

3. Do you lose balance because of dizziness OR have you lost consciousness in the last 12 months? Please answer NO if your dizziness was associated with over breathing (including during vigorous exercise). ☐ ☐

4. Have you ever been diagnosed with another chronic medical condition (other than heart disease or high blood pressure)? ☐ ☐

5. Are you currently taking prescribed medications for a chronic medical condition? ☐ ☐

6. Do you have a bone or joint problem that could be made worse by becoming more physically active? Please answer NO if you had a joint problem in the past, but it does not limit your current ability to be physically active. For example, knee, ankle, shoulder or other. ☐ ☐

7. Has your doctor ever said that you should only do medically supervised physical activity? ☐ ☐

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

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

Start becoming much more physically active – start slowly and build up gradually.

- Follow the Canadian Physical Activity Guidelines for your age (www.csep.ca/guidelines).
- You may take part in a health and fitness appraisal.
- If you have any further questions, contact a qualified exercise professional such as a CSEP Certified Exercise Physiologist” (CSEP-CEP) or CSEP Certified Personal Trainer” (CSEP-CPT).
- If you are over the age of 45 yrs. and NOT accustomed to regular vigorous physical activity, please consult a qualified exercise professional (CSEP-CEP) before engaging in maximal effort exercise.

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

Delay becoming more active if:

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

Please read the questions below carefully and answer each one honestly: check YES or NO.

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

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

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

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

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

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

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

Delay becoming more active if:

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

SECTION 3 - DECLARATION

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

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

NAME _______________________________ DATE __________________

SIGNATURE __________________________ WITNESS __________________________

SIGNATURE OF PARENT/GUARDIAN/CARE PROVIDER __________________________

For more information, please contact:
Canadian Society for Exercise Physiology
www.csep.ca

KEY REFERENCES

The PAR-Q+ was created using the evidence-based AGREE process (1) by the PAR-Q+Collaboration chaired by Dr. Darren E. R. Warburton with Dr. Norman Gledhill, Dr. Veronica Jamnik, and Dr. Donald C. McKenzie (2). Production of this document has been made possible through financial contributions from the Public Health Agency of Canada and the BC Ministry of Health Services. The views expressed herein do not necessarily represent the views of the Public Health Agency of Canada or BC Ministry of Health Services.
## Study Training Record

### Training Schedule

**Day 7** Date: ____________

**Morning heart rate (5 mins)**  HR  bpm  Time (HH:MM)  :  ~  :  

**Exercise (a total of 20 km)**

<table>
<thead>
<tr>
<th>PRE</th>
<th>Start time (HH:MM)</th>
<th>End time (HH:MM)</th>
<th>POST</th>
<th>Drink volume (ml)</th>
<th>Mileage (km)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg or lbs)</td>
<td></td>
<td></td>
<td>Body weight (kg or lbs)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1st run</td>
<td>:</td>
<td>:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd run (if any)</td>
<td>:</td>
<td>:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd run (if any)</td>
<td>:</td>
<td>:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Urine collection

<table>
<thead>
<tr>
<th>Bottle</th>
<th>Time</th>
<th>Instruction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base</td>
<td>:</td>
<td>Morning pee before intake <strong>Glycine</strong></td>
</tr>
<tr>
<td>FED</td>
<td>:</td>
<td>All urine must be collected into “FED” bottle. Keep the bottle cool.</td>
</tr>
<tr>
<td></td>
<td>:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>:</td>
<td>Last pee before going to bed.</td>
</tr>
</tbody>
</table>

DALDA _______  Check ☐
Day 8 Date: __________

**Morning heart rate (5 mins)**

<table>
<thead>
<tr>
<th>HR</th>
<th>bpm</th>
<th>Time (HH:MM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>~</td>
</tr>
</tbody>
</table>

**Exercise (a total of 5 km)**

<table>
<thead>
<tr>
<th></th>
<th>PRE</th>
<th>Start time (HH:MM)</th>
<th>End time (HH:MM)</th>
<th>POST</th>
<th>Drink volume (ml)</th>
<th>Mileage (km)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st run</td>
<td>Body weight (kg or lbs)</td>
<td>:</td>
<td>:</td>
<td>Body weight (kg or lbs)</td>
<td>:</td>
<td>:</td>
</tr>
<tr>
<td>2nd run (if any)</td>
<td>:</td>
<td>:</td>
<td>:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Urine collection**

<table>
<thead>
<tr>
<th>Bottle</th>
<th>Time</th>
<th>Instruction</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAST</td>
<td>:</td>
<td>Morning pee</td>
</tr>
<tr>
<td>Day8</td>
<td>:</td>
<td>All urine up to tomorrow morning must be collected into “Day8” bottle. Keep the bottle cool.</td>
</tr>
<tr>
<td></td>
<td>:</td>
<td>Last pee before going to bed.</td>
</tr>
</tbody>
</table>

DALDA ________ Check ☐
**Day 9**

**Date:**

**Morning heart rate (5 mins)**  
HR bpm Time (HH:MM) :

---

**Exercise (a total of 10 km)**

<table>
<thead>
<tr>
<th></th>
<th>PRE Body weight (kg or lbs)</th>
<th>Start time (HH:MM)</th>
<th>End time (HH:MM)</th>
<th>POST Body weight (kg or lbs)</th>
<th>Drink volume (ml)</th>
<th>Mileage (km)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st run</td>
<td></td>
<td>:</td>
<td>:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd run (if any)</td>
<td></td>
<td>:</td>
<td>:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd run (if any)</td>
<td></td>
<td>:</td>
<td>:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

---

**Urine collection**

<table>
<thead>
<tr>
<th>Bottle</th>
<th>Time</th>
<th>Instruction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Day 8</td>
<td>:</td>
<td>Morning pee</td>
</tr>
<tr>
<td>Day 9</td>
<td>:</td>
<td>All urine up to tomorrow morning must be collected into “Day 9” bottle. Keep the bottle cool.</td>
</tr>
<tr>
<td></td>
<td>:</td>
<td></td>
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<td>:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>:</td>
<td>Last pee before going to bed.</td>
</tr>
</tbody>
</table>

---

**DALDA**

Check [ ]
Day 10  Date: 

Morning heart rate (5 mins)  HR  bpm  Time (HH:MM) :  ~ : 

Exercise (a total of 20 km)

<table>
<thead>
<tr>
<th></th>
<th>PRE Body weight (kg or lbs)</th>
<th>Start time (HH:MM)</th>
<th>End time (HH:MM)</th>
<th>POST Body weight (kg or lbs)</th>
<th>Drink volume (ml)</th>
<th>Mileage (km)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st run</td>
<td></td>
<td>:</td>
<td>:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2nd run (if any)</td>
<td></td>
<td>:</td>
<td>:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd run (if any)</td>
<td></td>
<td>:</td>
<td>:</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Urine collection

<table>
<thead>
<tr>
<th>Bottle</th>
<th>Time</th>
<th>Instruction</th>
</tr>
</thead>
<tbody>
<tr>
<td>Base</td>
<td>:</td>
<td>Morning pee before intake <strong>Glycine</strong></td>
</tr>
<tr>
<td>FED</td>
<td>:</td>
<td>All urine must be collected into “FED” bottle. Keep the bottle cool.</td>
</tr>
<tr>
<td></td>
<td>:</td>
<td></td>
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<tr>
<td></td>
<td>:</td>
<td></td>
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<tr>
<td></td>
<td>:</td>
<td></td>
</tr>
<tr>
<td></td>
<td>:</td>
<td>Last pee before going to bed.</td>
</tr>
</tbody>
</table>

DALDA ______  Check  ☐
Day 11  Date: ___________

Morning heart rate (5 mins)  HR  bpm  Time (HH:MM)  ~  

Urine collection

<table>
<thead>
<tr>
<th>Bottle</th>
<th>Time</th>
<th>Instruction</th>
</tr>
</thead>
<tbody>
<tr>
<td>FAST</td>
<td>:</td>
<td>Morning pee</td>
</tr>
</tbody>
</table>
Urine Collection Instructions

Urine collection Procedure (Day7, Day11)

- **BASE**
  - On the day of the collection, collect the first morning urine void into "**PRE**" bottle.

- **DAY**
  - All the urination before going to bed, should be collected and pooled.
  - The urine sample will be collected into the 1 L container and transferred the "**DAY**" 3 L jug.
  - The 3 L jug will be kept in the refrigerator until the investigators collects it.

- **NIGHT**
  - Anytime the participant needs to urinate while at home (up until 07.00h, or before the first meal of the day the following morning), the urine sample will be collected into the 1 L container and transferred the 3 L jug.
Habitual Training Questionnaire

**Preliminary assessment**

Date:___________________________ subject ID:___________________________

Birthday (dd/mo/yr): ___/____/______ Age (yr):___________

Height (cm or inch):______________ Weight (kg or lbs):___________

**Training log of previous month**

Weekly training volume (time (h) or running distance (km))

Last week ___________________________

Last 2nd week ___________________________

Last 3rd week ___________________________

Last 4th week ___________________________

Maximum mileage per week (in the last 6 months) ____________km / wk

**Recent records (on the previous 1 year)**

(example; Date: 2014/11/26 Distance: 10 km Time: 0:38:00)

Date: ______ Distance: _______ km time: ______ -

Date: ______ Distance: _______ km time: ______ -

Date: ______ Distance: _______ km time: ______ -

**Others**

Regular Tabaco use : Yes / No
Anabolic drugs (e.g. growth hormone, testosterone, etc.) use : Yes / No