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A Measure of Dietary Protein Requirement in Endurance Trained Women

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Abstract

Purpose: Inference from dietary surveys and experimental models suggest that the female endurance athlete dietary protein requirement is 15-20% less than their male counterparts, but to date empirical measurement of the habitual protein requirement has not been undertaken. **Methods:** 72-h nitrogen balance (NBAL) was determined in 10 female cyclists training $10.8 \text{ h} \cdot \text{w}^{-1}$ (SD: 2.82), following two habituated protein intakes: a) a diet representing normal habitual intake (NH) (Protein: $85 \text{ g} \cdot \text{d}^{-1}$ Energy: $9078 \text{ kJ} \cdot \text{d}^{-1}$), b) an isocaloric high-protein diet (HP) (Protein: $166 \text{ g} \cdot \text{d}^{-1}$, Energy: $8909 \text{ kJ} \cdot \text{d}^{-1}$). Total 72-h nitrogen intake was determined from Leco total combustion analysis from samples of the ingested food items, while total loss was determined from micro-kjeldahl analysis of total 72-h urine, urea nitrogen concentration of regional resting and exercise sweat sampling, and literature-based estimates of fecal and miscellaneous nitrogen losses. Habituated protein requirement was estimated by the mean regression of the two estimates of 72-h nitrogen balance vs. nitrogen intake. **Results:** Mean (SD) 24-h dietary protein intake during the 72-h sampling period was NH: 85 (11g), HP: 166 (19g). Mean 24-h urinary nitrogen during the NH and HP blocks were $13.19 (2.39 \text{ g} \cdot \text{d}^{-1})$ and $21.53 (3.94 \text{ g} \cdot \text{d}^{-1})$ respectively. Sweat urea nitrogen excretion was NH: $0.33 (0.08 \text{ g} \cdot \text{d}^{-1})$ and HP: $0.54 (0.12 \text{ g} \cdot \text{d}^{-1})$. Normal habitual and high-protein intakes resulted in a mean negative and positive nitrogen balance, respectively (mean \pm SD) (NH: -0.59 ± 1.64 , HP: 2.69 ± 3.09). Estimated mean protein requirement to achieve NBAL was calculated to be $1.63 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ (95% confidence interval: 1.14–3.77). **Conclusions:** Our data shows that the dietary protein requirement for well-trained females taking part in daily moderate intensity and duration endurance training is within the range of measured requirement for similarly trained men and suggests that the current estimated range of protein requirement for females may be inadequate.

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The Massey University Human Ethics Committee: (Southern A, Application 10/76) approved the testing procedure and written consent was obtained from all participants prior to commencing the study.

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1. Introduction

The daily protein requirement for endurance athletes undertaking intensive high-volume training is greater than the recommended dietary allowance for the general population. Adequate diet plays a central role in optimising the benefits of training by enabling sustained training load and promoting adaptive responses during recovery. While carbohydrate and fat provide almost the entire caloric requirement of exercise, amino acid oxidation contributes a small but appreciable amount to energy expenditure. Optimal protein intake serves to replace the oxidative loss of essential amino acids and support increased protein turnover during exercise, while inadequate protein intake may lead to a net catabolic state and suboptimal metabolic responses during training and recovery (Phillips, Moore et al. 2007). A daily protein intake in excess of metabolic requirement results in oxidative amino acid losses to maintain physiologic concentration within body amino acid pools. A less favourable energy supply from amino acid oxidation may be mitigated by the correct dietary approach, particularly by replenishing glycogen stores through adequate carbohydrate intake. In males, the state of glycogen depletion is related to increased amino acid oxidation (Lemon and Mullin 1980). Females however, rely less on carbohydrate as an energy substrate, which appears to lower net protein utilisation compared to males (Tarnopolsky 2004). Despite evidence to suggest females require less dietary protein than fitness matched males, no attempt has been made to determine habituated protein balance and set a recommended range of protein intake for female athletes undergoing daily endurance training.

Nitrogen balance (NBAL) is a commonly used method to determine dietary protein requirement (Tarnopolsky, MacDougall et al. 1988). The technique is used to

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calculate the dietary protein intake required to achieve zero nitrogen balance from a correlation of two or more nitrogen balance periods with adapted protein intake. Nitrogen balance in male endurance athletes was used to calculate a mean daily protein requirement of $1.37 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, and an upper intake of $1.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ was recommended to settle individual variability (Tarnopolsky, MacDougall et al. 1988). Subsequently, nitrogen balance in elite male cyclists showed a daily intake as high as $1.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ may be needed to achieve balance (Brouns, Saris et al. 1989). To date, gender comparison has provided a basis for current estimates of female protein requirement. Females consistently express lower net protein catabolism (Lamont, Patel et al. 1990) and amino acid oxidation (McKenzie, Phillips et al. 2000) than males during exercise. Females also exhibit a more favourable nitrogen balance and oxidise less leucine compared to males while consuming the same relative protein intake (Phillips, Atkinson et al. 1993). Furthermore, an intake of $1.0 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ of protein was inadequate to achieve NBAL in females (Lamont, Patel et al. 1990). Recently, unadapted nitrogen balance in male and female cyclists estimated the female protein requirement to be 0.65 of that for males ($1.28 - 1.98 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) (Rowlands, Rossler et al. 2008; Rowlands and Wadsworth 2010). While these findings provide noteworthy gender differences, the methodologies did not set out to determine adapted protein balance in females. They do, however, provide insight that the calculated male requirement likely exceeds the female one. Current estimates are inferred from these and other findings and suggest the female protein requirement to be ~15-20% below that for males.

In the present study, we set out to determine habituated protein requirement in endurance trained female cyclists by measuring nitrogen balance during two levels

of adapted dietary protein intakes. We recruited ten well-trained female cyclists and adapted an experimental protocol used previously to determine protein requirement for males (Tarnopolsky, MacDougall et al. 1988). Nitrogen balance was measured in the mid-follicular phase to control for the hormone mediated adjustments in exercise metabolism. Our findings provide further evidence that endurance athletes require more dietary protein than sedentary individuals, and indicate that in some cases females require a similar relative protein intake to males.

2. Literature review

For athletes, diet plays a critical role in optimising the benefits of training and potentiating competitive performance. Endurance athletes typically undergo sustained periods of high-volume and/or high-intensity training, resulting in an increased dietary requirement of carbohydrate, fat, and protein. While carbohydrate and fat largely supply the caloric demand of exercise, dietary protein supports the complex amino acid requirements involved in muscle protein turnover, metabolic enzyme regulation, and contractile energy demand, while promoting the adaptive process of chronic training. Inadequate dietary protein may compromise training adaptation and exercise capacity by suppressing non-essential physiological pathways in which amino acids are involved. Conversely, excessive protein intake, while not known to be harmful, may compromise adequate carbohydrate intake, a primary factor in maintaining training volume and/or intensity. Measured dietary requirements for athletes are important and empowering recommendations in potentiating competitive performance.

Amino acid oxidation during endurance exercise is a primary contributor to the elevated protein requirement of endurance athletes, and increases in proportion with exercise duration, intensity, and level of glycogen depletion (Phillips and Phillips 2006). It is well documented that metabolic responses during exercise differ between men and women (Tarnopolsky 2000). Males rely greater on carbohydrate as an energy source during exercise, leading to more protein being oxidised, and may result in a higher dietary protein requirement than women (Tarnopolsky 2004). A landmark calculation of dietary protein requirement shows that male endurance athletes undergoing daily training have a greater requirement compared to sedentary

individuals (Tarnopolsky, MacDougall et al. 1988); however, no work has been conducted thus far to determine the same requirement in females, despite evidence of a gender difference (Phillips, Atkinson et al. 1993; McKenzie, Phillips et al. 2000; Rowlands and Wadsworth 2010).

2. 1. The metabolic demand of protein

2. 1. 1. The purine nucleotide cycle

Amino acids are utilised for the formation of enzymes involved in adenosine triphosphate (ATP) synthesis. ATP and adenosine monophosphate (AMP) + inorganic phosphate (Pi) are produced from two adenosine diphosphate (ADP) molecules in a reaction catalysed by adenylate kinase. The rise in AMP stimulates the purine nucleotide cycle. AMP deaminase catalyses the formation of inosine monophosphate (IMP) and ammonia from AMP. The amino-acid aspartate is then utilised in the formation of the tricarboxylic acid (TCA) cycle intermediate fumarate via the enzyme adenylosuccinate synthetase and the presence of energy yielding guanosine triphosphate (GTP). In this way, ATP demand is satisfied through the up-regulation of oxidative metabolism in the state of increased energetic stress, characterised by increased AMP concentration. The liberation of ammonia from the purine nucleotide cycle stimulates the glycolytic enzyme phosphofructokinase, further stimulating oxidative pathways via glycolysis (Hargreaves and Thompson 1999; Brooks, Fahey et al. 2005).

2. 1. 2. Amino acids and oxidative metabolism

In skeletal muscle, amino acids contribute to oxidative energy demand and serve to support the oxidative pathways through formation of TCA cycle intermediates. Of the
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20 amino acids, eight (alanine, asparagine, aspartate, glutamate, isoleucine, leucine, lysine, and valine) can be oxidised by skeletal muscle, providing energy for contraction. During exercise, the branched-chain amino acids (BCAA) (isoleucine, leucine and valine) are preferentially used (Smith and Rennie 1996). The pathway for amino acid oxidation begins with a transamination reaction, whereby the N group of the BCAA is removed to form an oxidisable carbon skeleton (keto-acid), a process catalysed by the branched-chain amino-acid transferase enzyme (Afring, Block et al. 1986). The BCAA is then transaminated with alpha-ketoglutarate to form glutamate and the original BCAA's corresponding keto-acid. In the case of leucine, the resulting keto-acid forms the oxidisable substrate acetyl-CoA via the branched-chain keto-acid dehydrogenase (BCKAD) enzyme (Boyer and Odessey 1991).

The transamination of glutamate with pyruvate is the primary pathway of TCA cycle intermediate expansion. During the initial stages (~10-min) of moderate intensity exercise, the glycolytic pathway raises pyruvate concentration. The amino-acid glutamate will undergo a near equilibrium, reversible transamination reaction with pyruvate forming alanine and alpha-ketoglutarate. Hence, the concentration of glutamate in exercising skeletal muscle falls by as much as 70%, with a corresponding rise of 50-60% in alanine. During the initial stages (<30-min) of exercise, alanine is released in great quantity into the circulation (van Hall, van der Vusse et al. 1995). Felig and Wahren (1971) suggest a link between glucose derived pyruvate formation and the production and release of alanine from the exercising muscle. Alanine is taken up by the liver where it is converted to glucose via gluconeogenesis, a pathway thought to regulate blood glucose concentration at the onset of exercise.

In the liver, glutamate may undergo oxidative deamination with water and NAD^+ to its corresponding keto-acid, alpha-ketoglutarate. The deamination of glutamate results in three outcomes. Firstly, the amine group containing unwanted nitrogen from the original amino acid can be removed from the circulation through the formation of ammonia and synthesis of urea. Secondly, the formation of the TCA cycle intermediate alpha-ketoglutarate ensures the integrity of oxidative ATP synthesis. Thirdly, the ATP yielding substrate NADH^+ is formed and utilised to yield energy through oxidative phosphorylation (Brooks, Fahey et al. 2005). Ultimately, amino acid oxidation drains the body amino acid pool of essential components creating the need for dietary replenishment (Tarnopolsky 2004).

2. 1. 3. BCAA oxidation during exercise

During exercise, amino acid oxidation contributes 1-6% of the energy requirement (Tarnopolsky 2004). The percentage contribution and resultant loss of those amino acids from the body pool is dependent on training status, exercise intensity and duration, level of glycogen depletion, and gender (Tarnopolsky 2004). During prolonged, high-intensity exercise, stored muscle and liver glycogen provides high rates of oxidisable substrate via glycolysis. Due to the limited storage capacity of glycogen and the high rate of flux through the glycolytic pathway, the level of stored glycogen is the determining factor in maintaining high-intensity exercise. The relationship between carbohydrate utilisation and amino acid oxidation is well documented (Tarnopolsky 2000). High rates of glycogen use during intense exercise increases amino acid oxidation (Wagenmakers, Beckers et al. 1991), while the state of glycogen depleted is inversely related to amino acid oxidation (Lemon and Mullin 1980).

Leucine is an essential amino acid and one of three branched-chain amino acids. Unlike other amino acids, which can be glucogenic and ketogenic, leucine is purely ketogenic, therefore the fate of leucine catabolism is always oxidation (Brooks, Fahey et al. 2005). For this reason, leucine is of interest when measuring protein as an energy substrate. In skeletal muscle, leucine content (9%) is higher than any other amino acid (Layman 2002), and is preferentially oxidised in exercising skeletal muscle (Smith and Rennie 1996). During exercise, there is a relative decrease from rest in leucine oxidation of between 1.5 and 5% (Lamont, Patel et al. 1990; Phillips, Atkinson et al. 1993; McKenzie, Phillips et al. 2000), however, the ~10-fold increase in energy expenditure during exercise increases the total amount of amino acid oxidation (Tarnopolsky 2004).

The oxidation of leucine begins with transamination and the formation of a keto-acid. The keto-acid then enters a series of reactions to form acetyl-CoA, an intermediate in the TCA cycle. The enzyme branched chain keto-acid dehydrogenase (BCKAD) is the rate-limiting enzyme in the pathway of leucine catabolism. More specifically, the level of BCKAD enzyme activation (via dephosphorylation) is known to be the rate limiting step in leucine oxidation and is up-regulated from rest (5-8%) to exercise (20-25%) (Phillips, Atkinson et al. 1993).

2. 1. 4. Skeletal muscle protein turnover

For athletes, protein balance is a dynamic interaction between exercise-induced net protein turnover and dietary protein intake. Skeletal muscle protein turnover increases immediately after endurance exercise (Bolster, Picosky et al. 2005; Rodriguez, Vislocky et al. 2007), and is typically expressed in relation to protein synthesis (fractional synthetic rate), protein breakdown (fractional breakdown rate),

and the net difference between the two. At modest dietary protein levels, endurance exercise results in net negative protein balance (Rodriguez, Vislocky et al. 2007). Provision of adequate or plentiful dietary protein in exercising individuals increases plasma essential amino acids and significantly improves net protein balance post-exercise. Primarily, a reduction in fractional breakdown rate and subsequent attenuation of the need for synthesis (Bolster, Picosky et al. 2005).

2. 2. Gender differences in metabolism

For researchers to accurately determine gender differences during exercise, careful consideration is given to suitably matching male and female participants. Traditionally, a form fitness matching is used based on training status by assessing current training load and training history. Males and females differ in body composition; therefore, measured differences must take this factor into account. Females have an inherently higher fat mass than males, therefore it is more useful to base findings relative to fat-free mass rather than body weight (Tarnopolsky 2008). Although males elicit a higher VO_{2max} relative to body weight than fitness matched females, the difference is largely nullified if expressed relative to fat-free mass. Despite these challenges, research has provided the evidence to cement certain gender difference in common theory.

During exercise, it is established that females utilise more lipid, rely less on carbohydrate, and have lower amino acid oxidation than males (Tarnopolsky, MacDougall et al. 1990; Phillips, Atkinson et al. 1993; McKenzie, Phillips et al. 2000). Initial gender comparisons of energy substrate examined indirect calorimetry via the respiratory exchange ratio (RER) (Tarnopolsky, MacDougall et al. 1990; McKenzie, A measure of dietary protein requirement in endurance trained women

Phillips et al. 2000). RER is a criterion measure of the contribution of fat and carbohydrate to whole body energy expenditure through breath by breath analysis of the oxygen to carbon dioxide ratio. Females consistently display a lower RER than males, which indicates fat as a more dominant fuel source. Furthermore, research has consistently reported attenuated muscle glycogen depletion, less leucine oxidation (McKenzie, Phillips et al. 2000), and lower urea nitrogen excretion (Tarnopolsky, MacDougall et al. 1990) in females. Men seem to have a greater ability to carbohydrate load than women (Tarnopolsky, Atkinson et al. 1995), although female athletes do respond favourably to carbohydrate loading when consuming high amounts of carbohydrate ($>8 \text{ gCHO} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$) (Tarnopolsky, Atkinson et al. 1995), and when carbohydrate loading in the luteal phase of the menstrual cycle (Nicklas, Hackney et al. 1989; McCracken, Ainsworth et al. 1994). The underlying basis for the gender differences in substrate utilisation during exercise appear to be partly related to hormonal profile. The ovarian hormones estradiol and progesterone appear to regulate metabolic responses and are thought to be responsible for the mediation of substrate use between menstrual phases. The role of estradiol in particular has been studied at length (Ruby, Robergs et al. 1997; Ashley, Kramer et al. 2000; Carter, McKenzie et al. 2001).

2. 2. 1. Amino acid oxidation during exercise

Research has accurately determined that male athletes oxidise more leucine both at rest and during endurance exercise than females (Phillips, Atkinson et al. 1993; McKenzie, Phillips et al. 2000). Researchers have commonly used nitrogen balance to determine whole-body protein turnover and dietary protein requirement. The use

of stable isotope infusion of a single amino acid, usually leucine L-1[¹³C], is a popular method of determining amino acid oxidation rates.

The first study conducted to compare the effect of gender on exercise protein catabolism was performed by Tarnopolsky et al. (1990). In this study, six males and six females, matched for training status and VO_{2max} relative to fat free mass, completed a 15.5km treadmill run at 65% VO_{2max} . To measure the effect of exercise on amino acid oxidation, 24-hour urine collections made on a rest day were compared with those made on the exercise day. At rest, there was no meaningful difference in urinary urea excretion between genders when expressed in relative or absolute terms. However, during the exercise day, males excreted significantly more urea than females in both absolute and relative terms. In fact, females exhibited no meaningful increase in urinary urea excretion on the exercise day, indicating minimal exercise induced catabolic amino acid losses. The percentage contribution of protein oxidation to energy expenditure was calculated to be 0.3% for females and 9.1% for males. The gender difference in urea excretion coincided with greater exercising RER and glycogen depletion in males. Researchers calculated that males oxidised ~40% more carbohydrate during exercise, and concluded that the greater glycogen depletion in males lead to greater amino acid oxidation during exercise (Tarnopolsky, MacDougall et al. 1990).

Similar results were reported by McKenzie et al. (2000), who investigated the gender difference in percent BCKAD activation and leucine oxidation as a result of endurance training using the leucine L-1[¹³C] stable isotope tracer. Six untrained males and females performed 90-min of cycle ergometry before a 38-d training program, and were retested post training at the same intensity relative to VO_{2peak} and

the same absolute work output (W) as in the pre-training test. Training resulted in the attenuation of leucine oxidation and BCKAD percent activation in male and females during exercise. Leucine oxidation in the female cohort was only half that measured in the males before training and this difference was extended to the post-training tests. All participants experienced an increase in fitness due to training characterised by reduced fat mass, greater reliance on fat as an energy substrate, and a reduction in plasma lactate concentration and rate of glycogen utilisation for the same absolute exercise intensity. Females expressed lower RER values than the males pre and post-training in both relative and absolute terms indicating that females utilise more lipid than males (McKenzie, Phillips et al. 2000). Since the rate of glycogen depletion correlates well with the percentage increase in BCKAD activation, it is possible that leucine oxidation is attenuated due to training; however the increase in mitochondrial volume due to training and subsequent increase in total volume of BCKAD may serve to mask the true mechanism of unchanged leucine oxidation rates after endurance training (McKenzie, Phillips et al. 2000). Furthermore, Phillips et al. (1993) investigated leucine oxidation and flux in 6 trained male and female participants exercising for 90-min at 65% $\text{VO}_{2\text{max}}$ (Phillips, Atkinson et al. 1993). Participants were infused prior to exercise with the leucine L-1[^{13}C] stable isotope tracer for 3.75-h at a rate of $1 \text{ mg}\cdot\text{kg}^{-1}\cdot\text{h}^{-1}$. The leucine oxidation rate significantly increased in both genders from rest to exercise, with males showing a significantly greater increase compared to females (Phillips, Atkinson et al. 1993).

2. 2. 2. Effect of the menstrual cycle

In eumenorrheic females, the menstrual cycle follows a pattern of secretion of four hormones: estradiol (E), progesterone (P), follicle stimulating hormone (FSH), and

luteinizing hormone (LH). The metabolic influence of estradiol and progesterone are of particular interest to researchers. Not only are the ovarian hormones partly responsible for the gender differences in metabolism, they alter metabolism throughout the cycle in eumenorrheic women. Hence, when conducting research on females, there is a need to control hormonal factors by testing in a given menstrual phase. The mid-follicular phase is commonly used in research to mitigate any hormonal effects on measured variables. Estradiol and progesterone are basal in the early-follicular phase. Estradiol steadily rises throughout the follicular phase and a surge in secretion occurs late in the follicular phase. A testing window (days 4-11 after first day on menses) is often adopted to avoid the surge in estradiol in the late-follicular phase. Furthermore, hormone interaction is likely to alter metabolic responses between the luteal and follicular phases. Due to the profound effect of the ovarian hormones, experimental observation are often expressed relative to the menstrual phase in which the testing is conducted.

During the luteal phase, estradiol and progesterone are in high concentration and may act antagonistically on substrate use (Oosthuyse and Bosch 2010). There is evidence that high circulating estradiol concentrations enhances lipolysis through the stimulation of growth hormone release and that progesterone acts antagonistically on growth hormone (Faria, Bekenstein et al. 1992). Furthermore, it is proposed that estradiol enhances the effect of the catecholamines on lipolysis (Benoit, Valette et al. 1982), possibly leading to the sparing of muscle glycogen (Oosthuyse and Bosch 2010). Several investigators have confirmed the lipolytic effect of estradiol on substrate utilisation via indirect calorimetry using the RER (Hackney, McCracken-Compton et al. 1994; Campbell, Angus et al. 2001; Zderic, Coggan et al. 2001).

During the late-follicular phase, RER is consistently lower, indicating a greater contribution of lipid to energy expenditure, and affirms the proposed enhancement of estradiol on lipolysis, although there is some conflicting evidence (Horton, Miller et al. 2002; Devries, Hamadeh et al. 2006). Measures of muscle glycogen use from muscle biopsies suggests glycogen is spared in the luteal compared to the early-follicular phase (Hackney 1999; Zderic, Coggan et al. 2001), and it is proposed that this is primarily due to the effect of estradiol (Oosthuyse and Bosch 2010). Estimated muscle glycogen utilisation may be lowest during the late-follicular phase when estradiol is high and progesterone is low. It is unclear as to whether muscle glycogen utilisation is lower in the luteal compared to the mid-follicular phase. High circulating progesterone may override the glycogen sparing effects of estradiol in the luteal phase; however, there are conflicting observations (Hackney 1999; Zderic, Coggan et al. 2001; D'Eon, Sharoff et al. 2002). Muscle biopsy studies have consistently estimated that muscle glycogen storage is potentiated during the luteal phase (Nicklas, Hackney et al. 1989; McLay, Thomson et al. 2007). Estradiol is proposed to influence muscle glycogen storage by increasing insulin sensitivity (van Pelt, Gozansky et al. 2003). Although estradiol has been shown to have no effect on insulin during times of high exogenous glucose ingestion (Cooper, Sites et al. 2007). Despite conflicting findings, it is generally accepted that high circulating estradiol enhances lipid utilisation by promoting lipolysis, and as a result, glycogen is spared.

2. 2. 3. Amino acid oxidation

Amino acid oxidation at rest is consistently found to be greatest during the luteal phase (Lamont, Lemon et al. 1987; Lariviere, Moussalli et al. 1994; Kriengsinyos, Wykes et al. 2004; Toth, Sites et al. 2006). Lamont et al. (1987) measured nitrogen

balance in females exercising at 70% $\text{VO}_{2\text{max}}$, the balance value was higher during the luteal phase compared to menses, indicating greater amino acid oxidation in the luteal phase (Lamont, Lemon et al. 1987). In another study using amino acid tracers, progesterone was responsible for increased phenylalanine oxidation at rest and during exercise during the luteal phase when compared to the follicular phase (Kriengsinyos, Wykes et al. 2004). A recent review states that progesterone is likely responsible for increased amino acid catabolism and that estradiol may reduce it (Oosthuyse and Bosch 2010). Estradiol supplementation in recreationally active men found a 16% reduction in leucine oxidation at rest and during exercise (Hamadeh, Devries et al. 2005). It is suggested that it would be useful to investigate protein catabolism during exercise in the late-follicular phase in eumenorrheic women to verify the effect of reduced protein oxidation measured in males during estradiol supplementation (Oosthuyse and Bosch 2010). In summary, it is recognised that protein catabolism varies according to menstrual phase, possibly leading to corresponding fluctuations in dietary protein requirement (Oosthuyse and Bosch 2010).

2. 3. The measurement of dietary protein requirement

Within the body exists several distinct but interconnected amino acid pools. The blood is the free amino acid pool that maintains equilibrium of amino acid concentration with two major stores of body protein- the liver and skeletal muscle, and acts as the transporter for the removal of nitrogenous compounds resulting from amino acid turnover and catabolism. To support the metabolic processes needed to satisfy the energetic stress of exercise, there is a rapid exchange of several amino

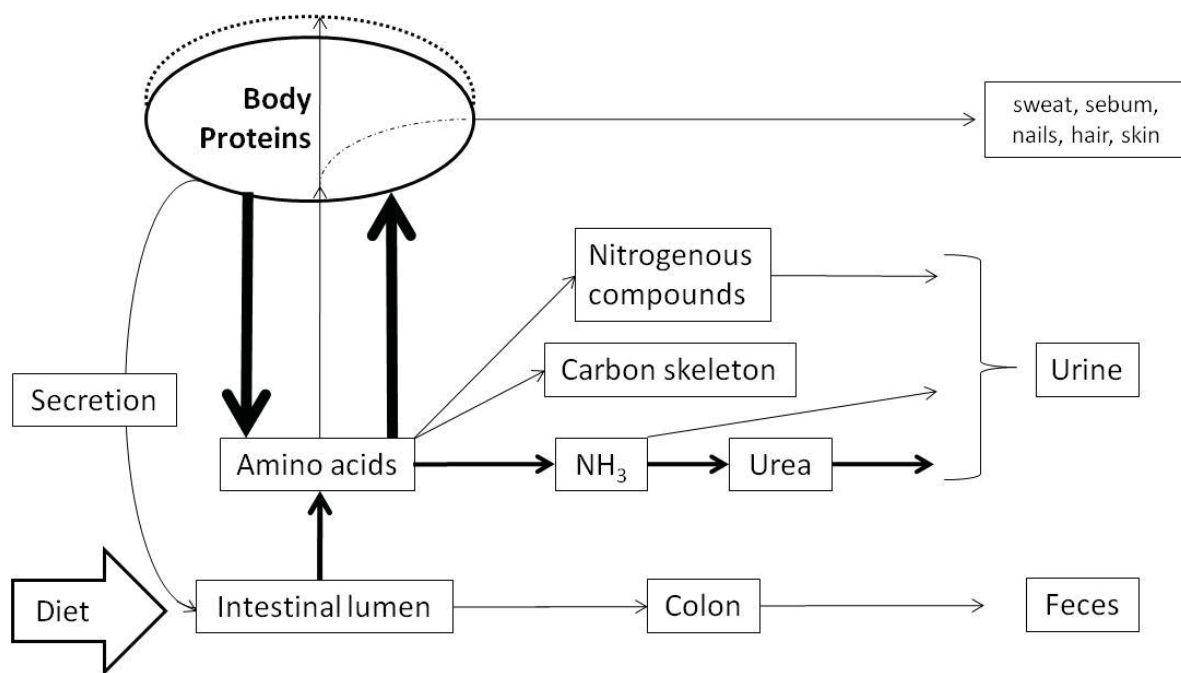
acids between the liver and skeletal muscle. In the liver, the nitrogenous constituents of amino acids are separated from the oxidisable carbon skeletons and removed from the circulation via the urine in the form of the efficient nitrogen carrier, urea.

2. 3. 1. The urea cycle

The primary function of the urea cycle is to produce a biologically favorable nitrogen carrier from more toxic nitrogen species, such as ammonia. During conversion of amino acids to an oxidisable substrate, the amine nitrogen is used to form other amino or keto acids. Eventually, ammonia is irreversibly produced and converted to urea in the urea cycle. In the liver, oxidative deamination of glutamate creates ammonia by removing the amine group to form intermediates involved in oxidative metabolism. The urea cycle is primarily a function of the liver, and utilises the intermediate ornithine and the amino-acid aspartate to produce urea. The molecular structure of urea is such that two nitrogen atoms are required to enter the cycle for its formation. Firstly, two ATP molecules are required to create carbamoyl phosphate in a reaction with CO_2 and the nitrogen donor, ammonium. Carbamoyl phosphate then reacts with ornithine, liberating phosphate and forming citruline. The second nitrogen terminal of the urea molecule is provided by the amino-acid aspartate, which reacts with citruline in another ATP requiring reaction to form arginosuccinate. The enzyme arginosuccinase then catalyses the formation of arginine and the TCA cycle intermediate fumarate. In the presence of water, the R group of arginine is removed creating urea and ornithine. Ornithine can begin the cycle again by receiving nitrogen from ammonium (Brooks, Fahey et al. 2005). Urea is then released into the blood and removed from the circulation by the kidneys during the formation of urine. Urea is also released in small quantity in sweat.

2. 3. 2. Nitrogen balance methodology

Nitrogen balance is a validated technique in the assessment of nitrogen loss (Bingham 2003), and a commonly used method for determining protein requirement (Tarnopolsky, MacDougall et al. 1988; Phillips, Atkinson et al. 1993). The basis of this methodology is the comparison of amino acid nitrogen intake from the diet with nitrogen output in the urine, sweat, feces and other miscellaneous sources (skin, semen, menstrual loss, hair) (Tarnopolsky 2004). Positive nitrogen balance (intake exceeds excretion) indicates amino acid retention and expansion of the whole-body amino acid pool while negative balance relates to net protein catabolism and loss from the whole-body amino acid pool (Tarnopolsky 2004). Urinary nitrogen excretion in the form of urea, ammonia and creatinine is the primary mode of nitrogen removal from the body, accounting for 84.4% of total nitrogen excretion (Calloway, Odell et al. 1970). Urinary urea excretion correlates well with prolonged constant nitrogen intake (Bingham and Cummings 1985). Nitrogen loss from the sweat accounts for a small but significant proportion of total nitrogen excretion, especially when assessing athletes (Hargreaves and Thompson 1999; Brooks, Fahey et al. 2005). Figure 1 illustrates the routes and contribution to excretion of protein derived nitrogen.



1988). Nitrogen intake is correlated with two calculations of nitrogen balance and a regression line is extrapolated to estimate an equivalent level of mean protein intake that would achieve theoretical nitrogen balance in the participant pool. In this case, a protein intake of $1.37 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ was estimated to achieve nitrogen balance and was used to set a window of dietary protein intake of $1.4\text{-}1.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ for male endurance athletes. The range was set by researchers using one standard deviation (SD) above the mean instead of the typical 2 SD to set the upper level of $1.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ and cited the inherent overestimation of nitrogen balance during high dietary protein intake (Hegsted 1975) as the rationale for their approach.

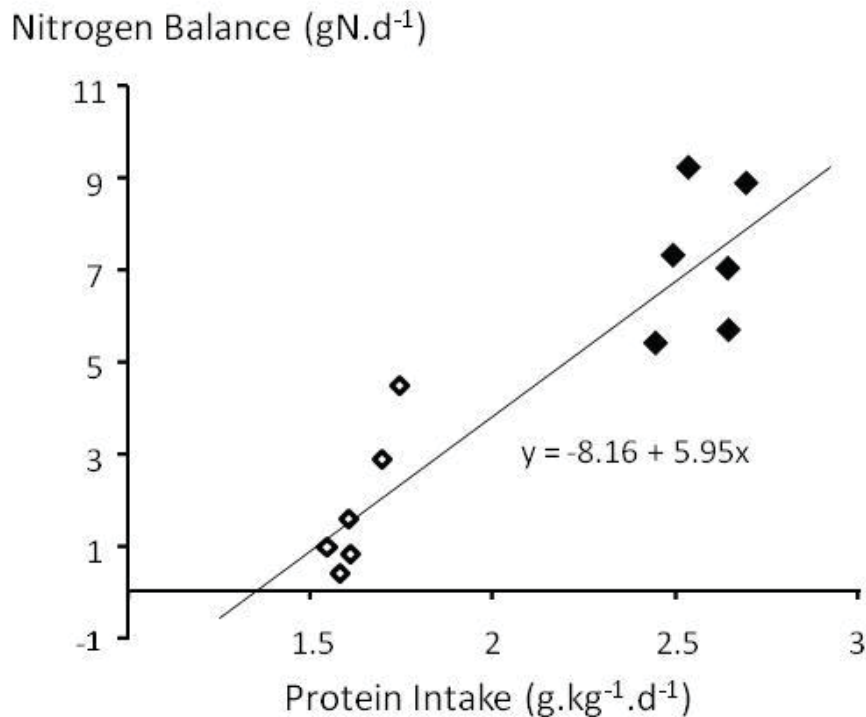


Figure 2. Predicted dietary protein requirement for male endurance athletes.

Adapted from Tarnopolsky et al. (1988)

2. 3. 4. Exercise and resting sweat nitrogen measurement

Sweat nitrogen in the form of urea, creatinine, and ammonia represents a small significant proportion of total nitrogen loss. It is essential that sweat nitrogen be measured to accurately assess nitrogen loss in balance studies. Nitrogen concentration in the sweat has been shown to increase during periods of increased protein intake (Tarnopolsky, MacDougall et al. 1988), and is dependent on exercise ambient temperature (Dolny and Lemon 1988), heat acclimatisation (Ashworth and Harrower 1967), glycogen status (Lemon and Mullin 1980), and dietary protein intake (Calloway, Odell et al. 1970). During exercise, sweat nitrogen loss can account for 100-400 mg·h⁻¹ (Calloway, Odell et al. 1970; Lemon, Yarasheski et al. 1986), and has been measured to contain a similar urea concentration (~200 mg·L⁻¹) as urine (Liappis and Hungerland 1972).

The quantification of sweat nitrogen excretion can be accurately measured using the whole-body washdown or regional collection techniques (Colombani, Späti et al. 1997). The whole-body washdown technique is the criterion measurement for sweat nitrogen analysis (Lemon, Yarasheski et al. 1986), and requires clothing to be rinsed several times with a known quantity (2-4 L) of distilled water in a closed circulation shower (Lemon and Yarasheski 1985; Lemon, Yarasheski et al. 1986). The regional sweat collection method is carried out by fixing gauze pads on various sites over the body (upper/lower back, mid-chest, stomach, thigh) (Lemon, Yarasheski et al. 1986).

There have been two landmark studies conducted to evaluate and determine the validity of regional collections using the whole-body washdown technique as the criterion measure (Lemon, Yarasheski et al. 1986; Colombani, Späti et al. 1997). Colombani et al. (1997) evaluated the regional collection method by comparing urea

and ammonia sweat nitrogen excretion from the upper and lower back, mid-chest, stomach, and thigh to those measured using the whole-body washdown technique during moderate intensity treadmill running. Regional collection from the thigh most accurately resembled the washdown rate for urea; however, ammonia from the thigh was significantly higher than whole-body washdown. When all regional sites were aggregated, the estimate of total urea and ammonia rate of excretion was very similar to the washdown value (Colombani, Späti et al. 1997).

Lemon et al. (1986) aimed to validate the regional collection procedure by comparing urea-N excretion from the whole-body washdown technique to five regional sites (upper and lower back, mid chest, stomach and thigh) at three exercise intensities (low: 42% $\text{VO}_{2\text{max}}$, mod: 55%, high: 67%) (Lemon, Yarasheski et al. 1986). The urea-nitrogen concentration of all of the regional samples tended to overestimate the whole-body washdown values at moderate and high intensities, however the values measured from the mid-chest were the only significantly different value at the two higher intensities when compared to whole-body washdown (Lemon, Yarasheski et al. 1986). Urea-nitrogen excretion measured at the thigh most accurately predicted the washdown value. The researchers concluded that the use of several body sites significantly improved the estimate of whole-body sweat-urea loss when compared to a single site, however, the intra and inter-individual variability of nitrogen content collected from respective body sites compromises the validity of the method (Lemon, Yarasheski et al. 1986). Despite these findings, the regional collection method is widely used to quantify the excretion of compounds from the sweat (Patterson, Galloway et al. 2000).

2. 3. 5. Fecal nitrogen loss

Although fecal nitrogen loss does not vary greatly in absolute magnitude ($\sim 1\text{-}3.5\text{ gN}\cdot\text{d}^{-1}$), it is an appreciable percentage of total nitrogen loss, and must be accounted for when calculating nitrogen balance. Fecal nitrogen content is determined by a number of factors, and may explain the individual variation seen in actual measurements. While undigested protein contributes to fecal nitrogen content, there is net endogenous movement of nitrogenous compounds into the gut lumen from digestive and mucosal secretion, urea, and cellular turnover (Fuller and Reeds 1998). Furthermore, the gut microflora utilises exogenous and endogenous nitrogen sources, adding to the complexity of nitrogen cycling by the gastrointestinal tract. An early investigation of fecal nitrogen loss during prolonged constant nitrogen intake ($14.42\text{ gN}\cdot\text{d}^{-1}$) over 99 different periods indicated large variation both between individuals and repeated measures in the same individual (6-16% of intake), while the peak distribution of fecal loss was between 8 and 9% of an intake (Toscani and Whedon 1951). Fecal nitrogen loss appears to be loosely correlated to body size and dietary protein intake, although it is most closely related to fecal weight ($r = 0.95$) (Bingham and Cummings 1985), which possibly implicates food quantity or caloric intake as a contributing factor. In an assessment total nitrogen loss in healthy men eating a 3000 Kcal diet comprising of 75-g of protein, fecal nitrogen loss was reported to be 12.7% of total nitrogen loss or an absolute loss of $1.5\text{ gN}\cdot\text{d}^{-1}$ (Calloway, Odell et al. 1970). Fecal nitrogen loss during a modest dietary protein intake ($<1.0\text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) was similar between genders (females: $1.46\text{ gN}\cdot\text{d}^{-1}$, males: $1.53\text{ gN}\cdot\text{d}^{-1}$) (Phillips, Atkinson et al. 1993), and appeared to increase in proportion to dietary protein intake (Tarnopolsky, MacDougall et al. 1988).

2. 3. 6. Limitations of methodology

The nitrogen balance method is a relatively simple technique used to determine protein balance and calculate dietary protein requirement. However the method has a number of limitations and assumptions. Firstly, there is no indication of the precise adaptation of the complex metabolic processes in which specific amino acids are involved (Tarnopolsky 2004). Therefore, it is assumed that a mixed protein diet achieving nitrogen balance may not satisfy the specific amino acid requirement. Researchers typically set a range of intake to balance methodological limitations. Secondly, although an adaptation period of at least 10 days is recommended and commonly employed for a new equilibrium of protein turnover to be reached, equilibrium is difficult to determine (Oddoye and Margen 1979). It is conceptualised that there are broad physiological adaptations to chronic states of dietary protein deficiency and excess. During nutrient deficit, these involve the loss of critically less essential body protein stores (muscle mass), and the down regulation of physiologically advantageous processes (intermediates of oxidative energy pathways). In times of chronic excess and adaptation, optimal utilisation of protein results in the remodelling of enzyme concentration and muscle ultra structure (training-induced adaptations) and leads to increased oxidation of amino acids (Young, Bier et al. 1989; Tarnopolsky 2004). The specific fate and requirement of individual amino acids, however, cannot be elucidated by nitrogen balance. A study by Oddoye and Margen (1979) outlined limitations of the nitrogen balance method to accurately measure balance at high protein intakes. These limitations included the inability to determine the adaptation time to a change in protein intake and errors in the calculation of nitrogen intake and output (Oddoye and Margen 1979).

Protein balance is a dynamic interaction between dietary intake and exercise-stimulated protein turnover. There is a cycling between states of protein breakdown and synthesis in which exercise and diet combine to equilibrate. Nitrogen balance is insensitive to the acute changes in exercise-induced protein turnover; therefore, no insight can be given into the required level, composition, and timing of dietary nutrient intake around an exercise bout to optimise recovery.

2. 4. Protein in the diet

2. 4. 1. The need for adequate dietary protein

Dietary protein intake serves to satisfy two biological requirements. Firstly, there is a critical need for the consumption of essential amino acids as they cannot be biologically derived. Secondly, there is the requirement of an adequate nitrogen pool for the synthesis of non-essential amino acids and proteins (Young and Pellet 1994). From a dietary standpoint, amino acids are categorised as essential or non-essential depending upon whether the de novo synthesis of the amino acid is possible within the body. Non-essential amino acids are able to be formed through transamination reactions based on their requirement. Essential amino acids cannot be synthesised by the body and therefore must be supplied by the diet. The need for the exogenous supply of essential amino acids creates the basis for the dietary requirement for protein. The oxidative loss of essential amino acids during exercise may further increase the dietary requirement for protein. Figure 3 illustrates the interactions between fasting losses, feeding gains, and the metabolic demand for protein.

change is imposed (Tarnopolsky, MacDougall et al. 1988; Phillips, Atkinson et al. 1993).

Adaptation to a high level of dietary protein ($>2.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) consistently yields positive nitrogen balance values in the order of $1.6 - 3.0 \text{ gN}\cdot\text{d}^{-1}$ (Oddoye and Margen 1979; Price, Halliday et al. 1994; Forslund, El Khoury et al. 1999). A positive balance of $3 \text{ gN}\cdot\text{d}^{-1}$ corresponds to approximately 18-g of protein per day. Adaptation to a given dietary protein intake represents a new equilibrium between the obligatory and adaptive metabolic demand for protein (Millward 2003). The obligatory metabolic demand is dependent upon need for indispensable amino acids for net protein synthesis, production of non-protein compounds, and nitrogen loss from the intestine (Millward 2003). An insufficient level of habitual protein intake largely determines whether net body-protein degradation is needed to satisfy the obligatory metabolic demand. Figure 4 illustrates the relationship between protein turnover and the regulatory and obligatory amino acid losses.

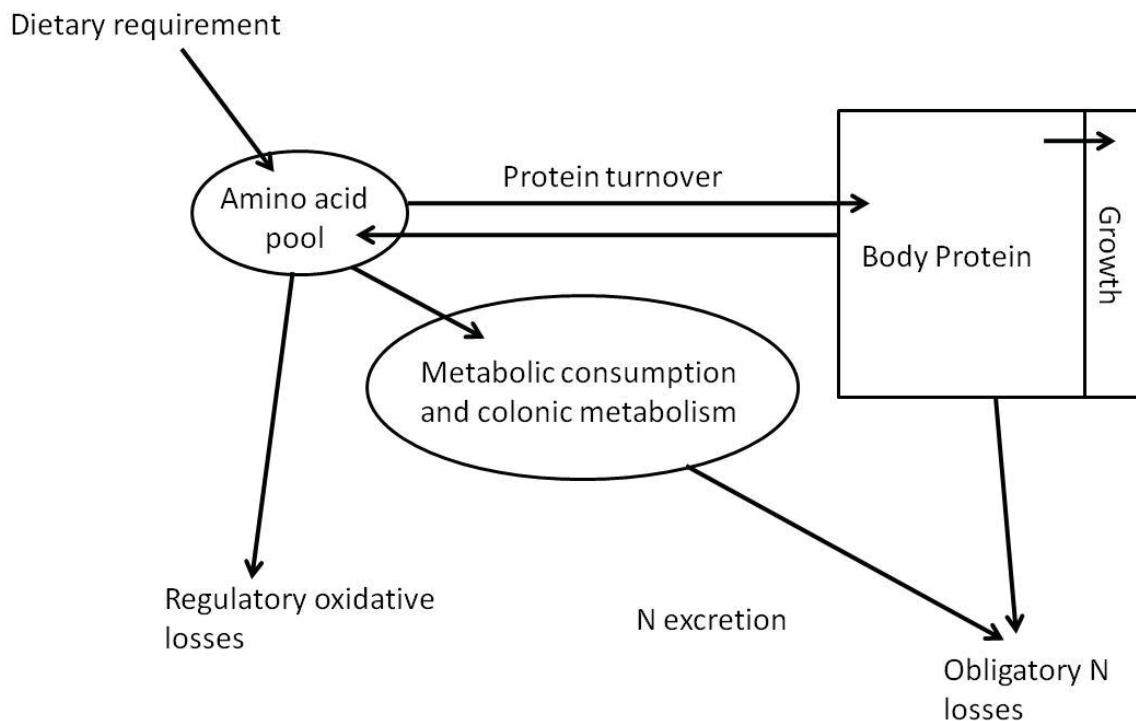


Figure 4. Regulation of dietary protein requirement as a balance between intake, obligatory losses, protein turnover and regulatory oxidative losses. Adapted from Millward (1994)

The adaptive metabolic demand for protein is the rate of amino acid oxidation serving to dispose of unnecessarily high concentrations in the amino acid pools to maintain tissue concentration of amino acids required to meet the obligatory metabolic demand (Millward, Bowtell et al. 1994). Changes in the adaptive metabolic demand are insensitive to acute feeding and change slowly with a change in protein intake, hence, there is a need for an adaptation period to a change in protein intake in balance studies (Millward 2003). As a result, adaptation to a given dietary protein intake represents the fulfillment of the obligatory metabolic demand, either via dietary adequacy and a measured increase in amino acid oxidation resulting in positive nitrogen balance, or in the case of dietary insufficiency, the loss of body protein to satisfy the need for indispensable amino acids involved in processes that make up the obligatory metabolic demand for protein.

2. 4. 3. Analysis of protein content in foods

Nitrogen balance methodology requires an accurate account of dietary nitrogen intake. Therefore, it is necessary to either estimate nitrogen intake from known protein content in the foods provided, or to analyse the diet for nitrogen content and convert it to protein intake. The measurement of total nitrogen is a relatively simple and cost effective method of analysis; however, there are several limitations. Firstly, the assumption is made that all of the nitrogen comes from amino acids and not other nitrogen containing compounds known to be found in food such as creatine, choline and nucleotides. Secondly, food items contain proteins made up of specific amino acid concentrations, some of which contain more nitrogen than others; therefore a nitrogen factor known as a Jones factor is applied to the food item based on its amino acid make up.

2. 4. 4. Jones' factors

The original method of converting a known nitrogen content of food to protein content was to apply a blanket factor of 6.25 on the basis that proteins contain roughly 16% nitrogen (Jones 1941). However, the analysis of proteins for nitrogen content have shown a range of between 13 and 19%, and is largely due to non-protein nitrogen content and the specific amino acid makeup of proteins within foods. Based on this, D. B. Jones (1941) proposed that food items were given their own factor for the calculation of protein content from a nitrogen value. Table 1 provides an example of some common food items and their corresponding Jones factor.

Table 1. Jones' factors for common food items (Merrill and Watt 1973).

Food	Factor
Animal Origin	
Eggs	6.25
Meat	6.25
Milk	6.38
Vegetable Origin	
Corn	6.25
Oats	5.83
Rice	5.95
Wheat (Whole Kernel)	5.83
Peanuts	5.46

In a practical sense, it is known that those food items with a low factor also have low protein content. Therefore, applying a lower factor to these foods does not result in a markedly different protein content for a mixed diet than the blanket factor of 6.25 (Merrill and Watt 1973).

An alternative method of protein analysis of foods is to measure the individual amino acid content by breaking down proteins using high-performance liquid chromatography (King-Brink and Sebranek 1993). However, this method is more costly than measuring total nitrogen and requires specific equipment, making nitrogen analysis a more attractive option in cases of budget constraint.

2. 5. Practical application of dietary knowledge

2. 5. 1. Recommended dietary protein intake for athletes

Several studies have been conducted that indicate an increased protein requirement (Tarnopolsky, MacDougall et al. 1988; Friedman and Lemon 1989; Phillips, Atkinson
A measure of dietary protein requirement in endurance trained women

et al. 1993) and determine a recommended range of dietary protein intake (Tarnopolsky, MacDougall et al. 1988) for endurance trained males undergoing heavy training. The first definitive evidence was published in the late 1980s. Tarnopolsky and colleagues investigated nitrogen balance in six male sedentary individuals, endurance athletes, and body builders (Tarnopolsky, MacDougall et al. 1988). Participants were habituated for 10 days to two isocaloric diets containing normal and altered dietary protein intake. The protein content in diet A was a representation of each group's normal protein intake. In diet B, a protein intake differential was created by increasing dietary protein for the sedentary and endurance-trained groups but reducing intake for the resistance trained group. The recall diaries from the resistance trained group showed a high habitual protein intake relative to energy content, therefore, in diet B, protein intake was reduced to provide a differential to their normal intake.

A 72-h nitrogen balance period was completed for each diet. To calculate nitrogen balance, complete urine, fecal, and sweat (exercise and rest) collections were analysed for total nitrogen loss and subtracted from dietary nitrogen intake. A correlation of the two nitrogen balance values with protein intake was made. The regression line of the mean balance values was extrapolated to estimate the daily protein intake to achieve zero nitrogen balance for each group. Values were stated as a range of protein intake to achieve zero or mildly positive nitrogen balance. The estimated dietary protein requirement to achieve nitrogen balance was greater for male endurance athletes ($1.3\text{-}1.7\text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) than body builders ($1\text{-}1.2\text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) and sedentary individuals ($0.8\text{-}1\text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) (Tarnopolsky, MacDougall et al. 1988). This data is supported by subsequent work that indicates that for male endurance athletes

involved in heavy training, the range to achieve zero or mildly positive nitrogen balance is 1.4-1.7 g·kg⁻¹·d⁻¹ (Tarnopolsky, MacDougall et al. 1988; Brouns, Saris et al. 1989; Friedman and Lemon 1989).

Subsequently, nitrogen balance in male and females endurance athletes stated an increase in protein requirement in trained females, but to a lesser extent than males (Phillips, Atkinson et al. 1993). In this study, 72-h nitrogen balance was measured in participants who were habituated for 10 days to a level close to the Canadian recommended dietary intake for protein (0.94 g·kg⁻¹·d⁻¹ for males, 0.80 g·kg⁻¹·d⁻¹ for females). The diet represented a reduction in protein intake from the participants' normal diet as indicated by four day food records. The males were in more negative nitrogen balance than the females, despite a higher relative protein intake (men: -26.3 mg·kg⁻¹·d⁻¹, women: -15.9 mg·kg⁻¹·d⁻¹).

2. 5. 2. Protein requirement for female endurance athletes: The current standpoint

There is currently no data on the habitual protein requirement for female endurance athletes. In a comprehensive review by Tarnopolsky, M., (2004) used inference from dietary surveys, measurements of amino acid turnover and gender comparison of nitrogen balance to conclude that the female protein requirement is 15-20% below that for equally trained males (Tarnopolsky 2004). A recommended range for female endurance athletes of 1.2-1.4 g·kg⁻¹·d⁻¹ has been suggested based on reviews of protein requirement (Tarnopolsky 2004; Phillips, Moore et al. 2007) and measured requirement in males (Tarnopolsky, MacDougall et al. 1988; Brouns, Saris et al. 1989; Rowlands and Wadsworth 2010).

2. 5. 3. Dietary practices of female endurance athletes

Evidence from a collection of dietary surveys of female endurance athletes states an average habitual intake of $1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ (range: $0.7\text{-}1.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) (Tarnopolsky 2004). In a study of dietary practices of elite female cyclists, calculated habitual protein intake was $2.7 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ during training and $2.3 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ during stage races (Martin, Martin et al. 2002). When comparing protein requirement with intake, the heavy energy demand of high-volume and high-intensity training and racing is likely to raise protein intake greatly above recommended intake levels. The data mentioned above, along with similar data in male cyclists that state a range of intake of $1.4\text{-}2.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ (Burke 2001) provide strong evidence that athletes achieve protein requirement through maintaining energy balance.

2. 6. Gaps in knowledge

There is a body of evidence that suggests the required dietary protein intake for female athletes undergoing heavy endurance training is less than that for fitness matched males (Tarnopolsky, MacDougall et al. 1990; Phillips, Atkinson et al. 1993; McKenzie, Phillips et al. 2000; Rowlands and Wadsworth 2010). However, nitrogen balance in habituated female endurance athletes is yet to be conducted. To date, metabolic research conducted on females has focused intently on the effect of ovarian hormones and corresponding menstrual cycle phases on substrate utilisation and exercise performance (Oosthuyse and Bosch 2010). Furthermore, there has been considerable focus on gender differences in exercise capacity and performance with regard to substrate utilisation and physiological response to exercise. Work is required to calculate and set a recommended protein requirement

for female athletes. Furthermore, it would be useful for highly-trained females to understand the cyclical impact of the menstrual cycle and the effect of amenorrhea on protein requirement.

2. 7. Summary

The required daily protein intake for male athletes is well documented (Tarnopolsky, MacDougall et al. 1988; Tarnopolsky 2004), and data exists indicating a reduced requirement for female athletes (Rowlands and Wadsworth 2010). However, the habitual protein requirement for female endurance athlete is yet to be measured. Differences between the sexes in fat and carbohydrate utilisation provide a possible explanation for the variance seen in nitrogen balance in gender comparisons studies (Lamont, Patel et al. 1990; Phillips, Atkinson et al. 1993). The effects of estradiol and progesterone on substrate utilisation provide a mechanistic rationale for these variances. However, the antagonistic nature of their effects adds complexity to the hormonal mediation of metabolism in females. As a result, the menstrual cycle is tightly controlled during research to mitigate their influence.

Nitrogen balance is a proven technique to determine a required protein intake. The factors involved in the calculation of nitrogen balance (dietary intake, nitrogen output, intensity and duration of exercise) must be tightly controlled and accurately measured. Although such methodology cannot provide information regarding changes in specific functions in which amino acids are involved, it does provide practical information for populations who seek to optimise training and performance through an optimal dietary approach.

3. Objective

The primary objective of the investigation was to calculate the mean relative dietary protein requirement for well-trained female endurance athletes undergoing daily moderate intensity and duration endurance training.

4. Materials and methods

4. 1. Study design

Each participant took part in two 72-h nitrogen balance periods, one while consuming a diet containing the equivalent of their normal habitual (NH) dietary protein and energy intake, and the other while consuming an isocaloric high-protein diet (HP) containing approximately twice the dietary protein intake. For a new equilibrium in protein turnover to be reached, participants were prescribed the isocaloric high-protein diet for 10 days preceding the HP block. No adaptation period was used proceeding the NH block as it was assumed that participants were already adapted to their normal protein intake. Each experiment was conducted in the mid-follicular phase of the menstrual cycle (days 4-11) to standardise for the effect of hormonal profile on metabolism. Figure 5 below graphically illustrates the experimental design.

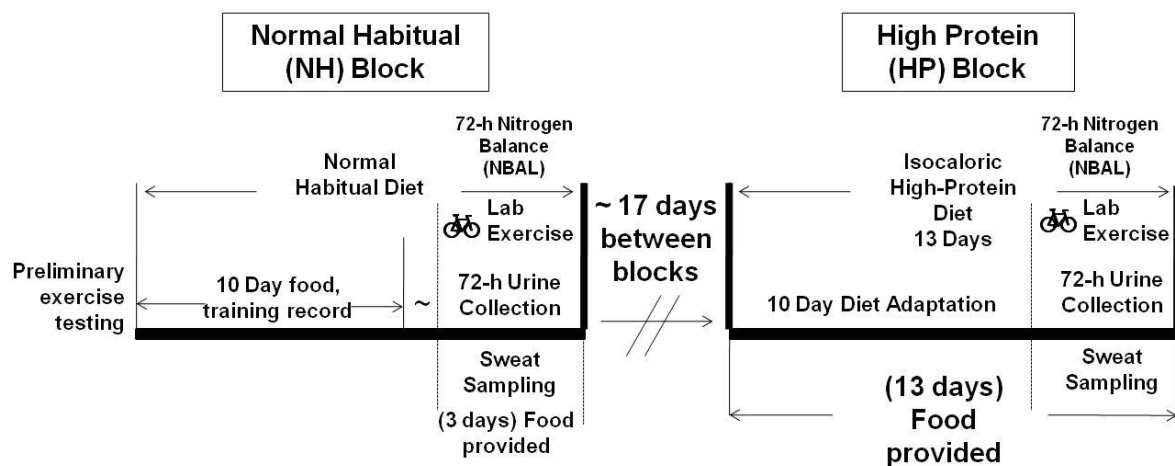


Figure 5. Illustration of experimental design. Within each 72-h collection period, cyclists completed three 90-min moderate and variable intensity training rides. Training rides are described in section 3.5.1.

4. 2. Participants

Ten well-trained female cyclists and triathletes completed the study. Average (SD) maximal oxygen uptake ($\text{VO}_{2\text{max}}$) and peak power outputs were 55.1 (5.74 $\text{ml}\cdot\text{kg}^{-1}\cdot\text{min}^{-1}$) and 246 (16.22 W) respectively. Participants were aged 30 (4.3 y), standing 166 (4 cm) tall, with a body mass of 61.3 (6.2 kg). Individual characteristic data is summarised in Appendix 1. All participants received verbal and written descriptions from the researchers detailing the purpose of the study, rights, requirements, and associated risks; participants were screened for precluding health conditions and gave written consent to participate. The study was approved by the Massey University Human Ethics Committee (Southern A, Application 10/76).

4. 3. Preliminary testing

4. 3. 1. Maximal aerobic power

Maximal aerobic power output (W_{max}) and $\text{VO}_{2\text{max}}$ were estimated using a 2.5-min stage, 25-W incremental test on an electromagnetically-braked cycle ergometer (Velotron, Version 1.5 Software, Racermate Inc., Seattle, U.S.A.). Participants cycled at 100 W for 5-min immediately prior. The incremental test began at 130 W and was completed when the cadence dropped below 70 rpm for the third time or for a duration longer than three seconds. W_{max} was defined as the last completed work rate plus the fraction of time spent in the non-completed work rate multiplied by the 25 W work rate increment. Expired gas fractions were collected by douglas bag and analysed using a gas analyser (Servomex 1440, Applied Instruments Auckland, New Zealand) calibrated with a blank (99.9% N_2), calibration gas (16% O_2 and 4% CO_2), and ambient air prior to each test. Ambient bag volume was measured using a dry

gas meter (Harvard, Biolab, New Zealand). All cycle ergometer testing was performed under standardised conditions (20°C and 45-65% humidity) and subjects were cooled with fans to minimise thermal distress.

4. 3. 2. Body composition

Body fat percentage was estimated using the method adapted for females by Jackson et al. (1980). Body density was calculated using the sum of 7 sites (pectoral, axilla, abdominal, suprailium, subscapular, tricep, mid-thigh) and was used to calculate body fat percentage. Measured body-fat percentage is summarised in Appendix 1.

4. 4. Nutritional procedures

4. 4. 1. Determination of habitual dietary intake

Preceding the NH block, each participant completed a 10-d diet record. Participants were asked to accurately describe the ingredients, quantity, and time of consumption of all food and beverages on a dietary recall spreadsheet. The diet was analysed using Foodworks diet analysis software (Xyris software Pty Ltd, Australia. Version 6.0.2562). Then, calculated habitual energy intake and macronutrient composition were used to prescribe the diet for each experiment. A summary of the calculated intakes can be found in Appendix 2.1.

4. 4. 2. Diet

The prescribed diets were made up of commercially available food items. To account for individual tastes, approximately 80% of the food items provided were consumed by every participant. The remaining 20% of food items were prescribed according to each participant's preference. Fruit was consumed ad libitum and added to the

A measure of dietary protein requirement in endurance trained women

prescribed diet. To create the isocaloric high-protein condition, food items were removed to reduce calories from fat and carbohydrate in relative equal proportion and two commercially available protein supplements were added. The protein supplements were a whey protein isolate beverage (Raiseys Original, Napier, New Zealand) delivering $170 \text{ g}\cdot\text{L}^{-1}$ of protein and a bar (Balance Sports Nutrition. Vitaco Health Ltd, Auckland, New Zealand) containing 35 g of protein. The beverage was portioned relative to the individual increase protein intake required. The diets from the respective conditions are summarised in Appendix 2. 2. and 2. 3.

4. 5. Experimental protocol

4. 5. 1. Exercise protocol

On days -2, -1, and 0 of each block, participants attended the lab to perform 90-min of submaximal cycling. The same exercise protocol was used for each day and consisted of a 20-min warmup at 50% W_{max} followed by five blocks of 5-min intervals at 70% W_{max} interspersed between 5-min blocks at 50% W_{max} and concluded with a 25-min warmdown at 50% PPO. Participants were cooled using a fan. On days -2 and -1, water was supplied and consumed ad libitum.

4. 5. 2. Urine collection

The 72-hour urine collection began with the second void on day -2 of the NBAL period and ended with the first void upon waking and prior to breakfast on the morning after day 0. Urine was collected in daily quantities using 5 L containers and delivered to the lab where it was stored at 4°C. Each container was supplied containing 15 ml of 6 M hydrochloric acid to inhibit bacterial utilisation of nitrogen. Total 72-h urine volume was then measured, pooled and mixed thoroughly to ensure

homogenous pH and analyte concentration. Samples (5 x 12 ml) were collected and stored at -80°C to await biochemical analysis for nitrogen concentration.

4. 5. 3. Exercise sweat collection

Regional exercise sweat collections were made according to the method of Colombani et al. (1997) for quantification of sweat urea concentration (Colombani, Späti et al. 1997). Gauze pads (Propax 75 mm x 75 mm) and waterproof adhesive patches (Opsite) were placed on the right side of the body at the chest and abdomen during the training ride on day 0. Participants were weighed prior to and immediately following exercise and were given 500 ml of water that was to be fully ingested during the ride. The gauze sweat pads were weighed, diluted approximately x 2 using distilled water, and centrifuged at 3000 rpm for 15-min at 4°C. Duplicate samples were then taken and stored at -80°C to await biochemical analysis.

4. 5. 4. Resting sweat collection

Regional 24-h resting sweat collections were taken on the right side of the body at the chest and abdomen between post exercise on day -1 and prior to exercise on day 0. The gauze sweat pads were weighed, diluted approximately x 2 using distilled water, and centrifuged at 3000 rpm for 15-min at 4°C. Duplicate samples were then taken and stored at -80°C to await biochemical analysis.

4. 5. 5. Calculation of total sweat urea nitrogen excretion

To estimate exercise sweat loss, participants were weighed prior to and immediately post-exercise on day 0 and given 500 ml of water to be fully consumed during the training ride. Exercise sweat loss was calculated as the difference between pre and post-ride body weight minus the water consumed during the ride.

To estimate 24-h resting sweat loss, participants were asked to record all fluid consumed between the training ride on day -1 and day 0. Participants were weighed immediately prior and post the 24-h period. Total urine volume for the 24-h period was recorded. Resting 24-h sweat loss was calculated by subtracting the volume of urine from the estimated and measured fluid volume consumed from food and drink. The net body weight difference was then added.

To calculate sweat urea nitrogen excretion, the respective sweat volumes were multiplied by the average of the measured sweat urea nitrogen concentrations from the regional collections.

4. 5. 6. Estimation of fecal nitrogen excretion

To account for nitrogen loss in feces, we applied the mean fecal loss of $1.48 \text{ g}\cdot\text{d}^{-1}$ previously measured in females (Phillips, Atkinson et al. 1993) to the normal habitual condition. The differential across the conditions was estimated based on the magnitude increase (~20%) in mean fecal loss from nitrogen balance measured during two habituated protein intakes in males endurance athletes (Tarnopolsky, MacDougall et al. 1988). Estimated mean fecal nitrogen loss for the high-protein condition was $1.78 \text{ g}\cdot\text{d}^{-1}$.

4. 6. Biochemical analysis

4. 6. 1. Urinary nitrogen concentration and total quantity

To determine 72-h urinary nitrogen concentration, duplicate samples were analysed using the Micro-Kjeldahl technique. Samples were accurately weighed combined with two kjeltabs (each containing 3.5 g potassium sulphate and 0.0035 g selenium)

and 15 ml of concentrated sulfuric acid. The digest was then undertaken at 420°C for 45 to 60-min and continued for an additional 10-min after the sample became clear. Once cooled approximately 70 ml of hot distilled water was added. The sample then underwent distillation with 40% sodium hydroxide and 25 ml of 4% boric acid. The resultant solution was then titrated with 0.1 M hydrochloric acid (HCL) to a grey/mauve end point.

The percentage nitrogen of the sample was calculated using the following equation:

$$\text{Percentage nitrogen} = \frac{(\text{milliliters of HCL} \times \text{molarity of HCL}) \times 14 \times 100}{1000 \times \text{weight of urine sample in grams}}$$

To account for the dilution of the urine sample by the 6 M HCL added to the collection containers described in section 4. 5. 2., the calculated fraction of HCL in urine was subtracted from the sample weight in the above equation.

The density of the urine samples was measured using a specific gravity refractometer (Atago Co. Ltd, Tokyo, Japan). Total 72-h urine weight was calculated by multiplying the density by the 72-h urine volume. Total quantity of nitrogen in 72-h urine collections was calculated by multiplying the percentage nitrogen by the weight of the 72-h collection.

4. 6. 2. Sweat urea concentration

Urinary and sweat urea concentration were measured using an Infinity Urea liquid stable reagent (Thermo Fisher Scientific Inc., Middletown, Virginia, USA).

4. 6. 3. Analysis of dietary nitrogen intake

To determine total 72-h dietary nitrogen intake, a representative portion of each food item was prepared as an edible sample and analysed for percentage nitrogen

A measure of dietary protein requirement in endurance trained women

content using the total combustion method (Leco, AOAC 968.06). Total consumed nitrogen was then calculated by applying the individual food nitrogen percentage to the portion weight of each food item in the respective diets. A summary of the nitrogen content of individual food items can be found in Appendix 2.4.

4. 6. 4. Estimation of protein intake from food nitrogen content

To determine total protein ingestion, the nitrogen content of each food items was multiplied by a converting factor from a table produced by McCance and Widdowson (2002).

4. 7. Statistical analysis

To determine nitrogen balance in the NH and HP conditions, measured and estimated daily nitrogen excretion data (urine, sweat, feces, misc) were pooled, converted to a daily total and subtracted from total daily nitrogen intake. Total intake and total excretion were expressed in milligrams of nitrogen per kilogram of fat free mass ($\text{mgN.kgFFM}^{-1}.\text{d}^{-1}$). Mean nitrogen intake to achieve nitrogen balance was calculated from a linear regression of two estimates of mean nitrogen balance versus nitrogen intake relative to fat free mass. To estimate the equivalent mean protein requirement relative to body weight ($\text{g.kg}^{-1}.\text{d}^{-1}$), nitrogen factors of respective food items were used to convert nitrogen intake to an equivalent relative protein intake. Mean protein requirement was determined as the equivalent relative protein requirement at the intercept of the regression line at zero nitrogen balance.

4. 7. 1. Sample size

The sample size required to generate an acceptable degree of certainty for the estimate of mean dietary protein requirement was determined from the assumed magnitude of the correlation ($r=0.87$) reported in the earlier study by Tarnopolsky (1988). At $r=0.87$, $n=8$ is required with a >95% likelihood of a minimum threshold for r of 0.5, which is the cutoff for a large correlation (Hopkins 2007). It was decided that $n=10$ would be employed to cater for possible dropouts.

5. Results

All participants successfully completed the NH and HP blocks between days 4-11 post the first day of menses (mid-follicular).

5. 1. Dietary intake

The macronutrient breakdown and energy content of the diets for each condition are summarised on Table 2. Individual dietary compositions can be found in Appendix 2.

Table 2. Summary of habitual and high-protein diets

Parameter		Condition	
		Normal Habitual Diet mean (SD)	High-Protein Diet mean (SD)
Energy	$\text{kJ}\cdot\text{d}^{-1}$	9078 (1492)	8909 (1411)
CHO	$\text{g}\cdot\text{d}^{-1}$	286 (52)	236 (44)
	$\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$	4.68	3.86
	% energy	54	45
Fat	$\text{g}\cdot\text{d}^{-1}$	75 (17)	55 (13)
	$\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$	1.23	0.9
	% energy	30	23
Protein	$\text{g}\cdot\text{d}^{-1}$	85 (11)	166 (19)
	$\text{g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$	1.39	2.72
	% energy	16	32

5. 2. Nitrogen turnover

Total mean daily nitrogen intake and measures nitrogen excretion are summarised in Table 3. Mean nitrogen intake was 1.85-fold (range: 1.72-2.14) higher during the HP versus the NH diet.

Table 3. Summary of dietary nitrogen intake and excretion pathways

	N Intake, g·d ⁻¹ mean (SD)	Nitrogen Excretion, g·d ⁻¹ mean (SD)						Balance, g·d ⁻¹ mean (SD)
		Urine	Daily Sweat		Feces	Miscellaneous	Total	
			Exercise	Rest				
Normal Habitual Diet	14.5	13.19	0.11	0.22	1.48	0.1	15.09	-0.59
	(2.3)	(2.39)	(0.05)	(0.11)			(2.35)	(1.64)
High Protein Diet	26.8	21.53	0.19	0.35	1.78	0.05	23.89	2.69
	(3.6)	(3.94)	(0.09)	(0.17)			(3.92)	(3.09)

The relationship between nitrogen intake and total excretion across the whole study is shown in Figure 3. There was a very large correlation between total nitrogen excretion and dietary nitrogen intake ($R^2 = 0.83$).

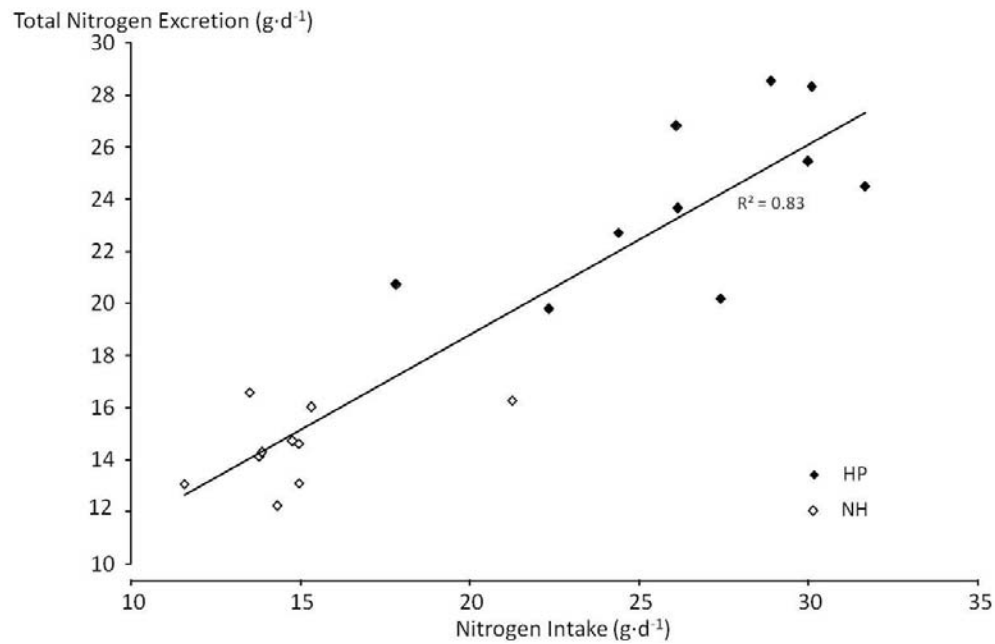


Figure 6. Relationship between nitrogen intake to total nitrogen excretion

5. 2. 1. Sweat urea nitrogen excretion

Figure 6 below shows the proportion of resting and exercise sweat to total sweat urea nitrogen loss. Total sweat urea nitrogen excretion was 1.6-fold higher during the HP condition compared to the NH condition (NH: 0.33 g·d⁻¹, HP: 0.53 g·d⁻¹, 95% confidence limits: 0.06-0.87).

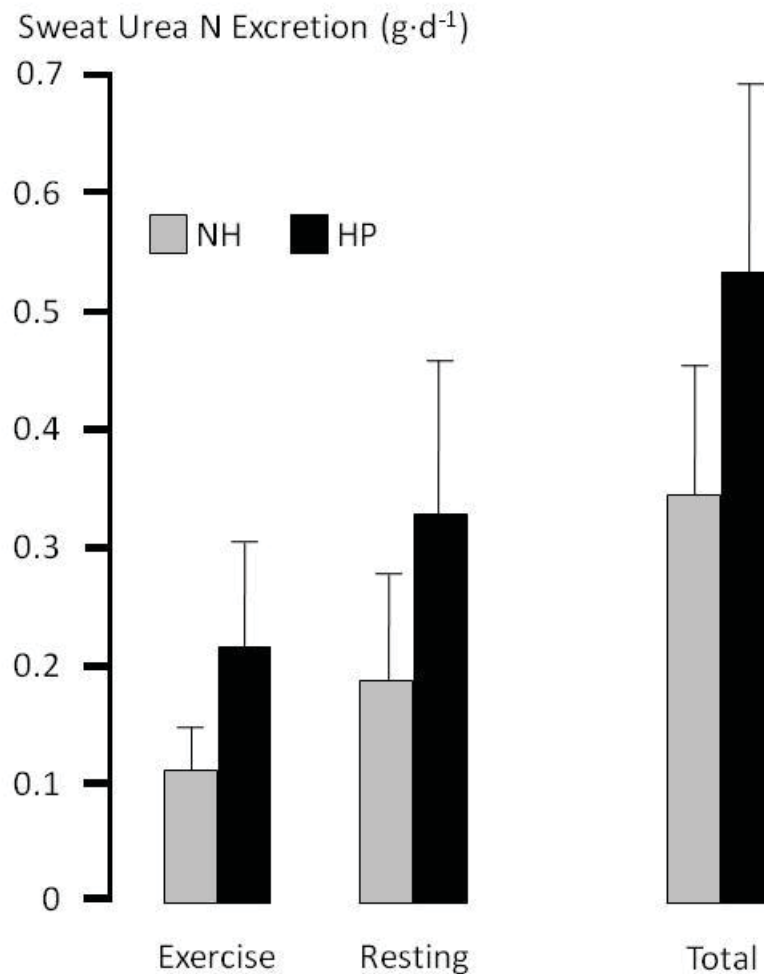


Figure 7. Sweat urea nitrogen excretion

5. 3. Nitrogen balance

Individual nitrogen balance is presented in Figure 8. All participants were in more positive nitrogen balance during the HP compared to the NH condition (mean balance: NH: -11 mgN·kgFFM⁻¹·d⁻¹, HP: 60 mgN·kgFFM⁻¹·d⁻¹). Across the experiment, the greatest net difference in nitrogen balance within participants was 203 mN·kgFFM⁻¹·d⁻¹, and the smallest was 5.93 mN·kgFFM⁻¹·d⁻¹. The net mean difference between conditions was 71 mgN·kgFFM⁻¹·day⁻¹.

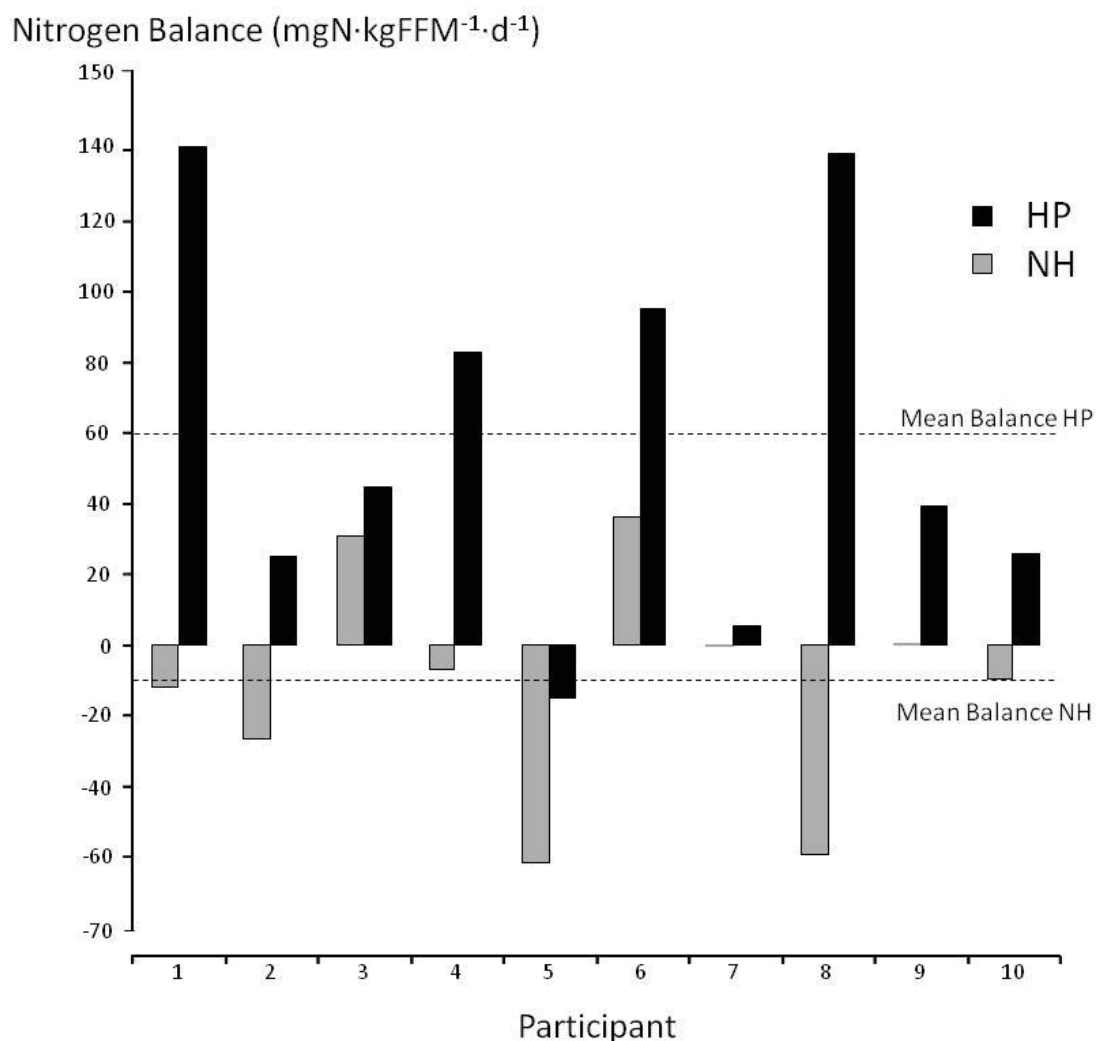


Figure 8. Individual nitrogen balance during the habitual and high-protein dietary conditions

It was observed that 7 of the 10 participants in negative nitrogen balance consuming a representation of their normal habitual diet (mean balance: $-11 \text{ mgN} \cdot \text{kgFFM}^{-1} \cdot \text{d}^{-1}$, mean intake: $1.42 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), while a further participant was very close to balance ($0.19 \text{ mgN} \cdot \text{kgFFM}^{-1} \cdot \text{d}^{-1}$). One participant was in negative balance during the NH and HP conditions (HB: $-63 \text{ mgN} \cdot \text{kgFFM}^{-1} \cdot \text{d}^{-1}$, HP: $-15 \text{ mgN} \cdot \text{kgFFM}^{-1} \cdot \text{d}^{-1}$). Nitrogen balance in this participant was the most negative of all participants during both conditions, and the extrapolated protein requirement to estimate balance was $2.83 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$. During the NH condition, a nitrogen deficit of $-61.3 \text{ mgN} \cdot \text{kgFFM}^{-1} \cdot \text{d}^{-1}$ occurred in the participant consuming the highest relative protein intake ($1.8 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$). For this participant, nitrogen balance in the NH condition equated to an insufficiency of $\sim 0.3 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ of protein.

5. 4. Estimated dietary protein requirement

The correlation between nitrogen balance and nitrogen intake is presented in Figure 9. The interpolation of the regression of the two mean nitrogen balance values estimated an average daily protein requirement of 1.63 g·kg⁻¹·d⁻¹ (95% confidence interval = 1.14-3.77 g·kg⁻¹·d⁻¹).

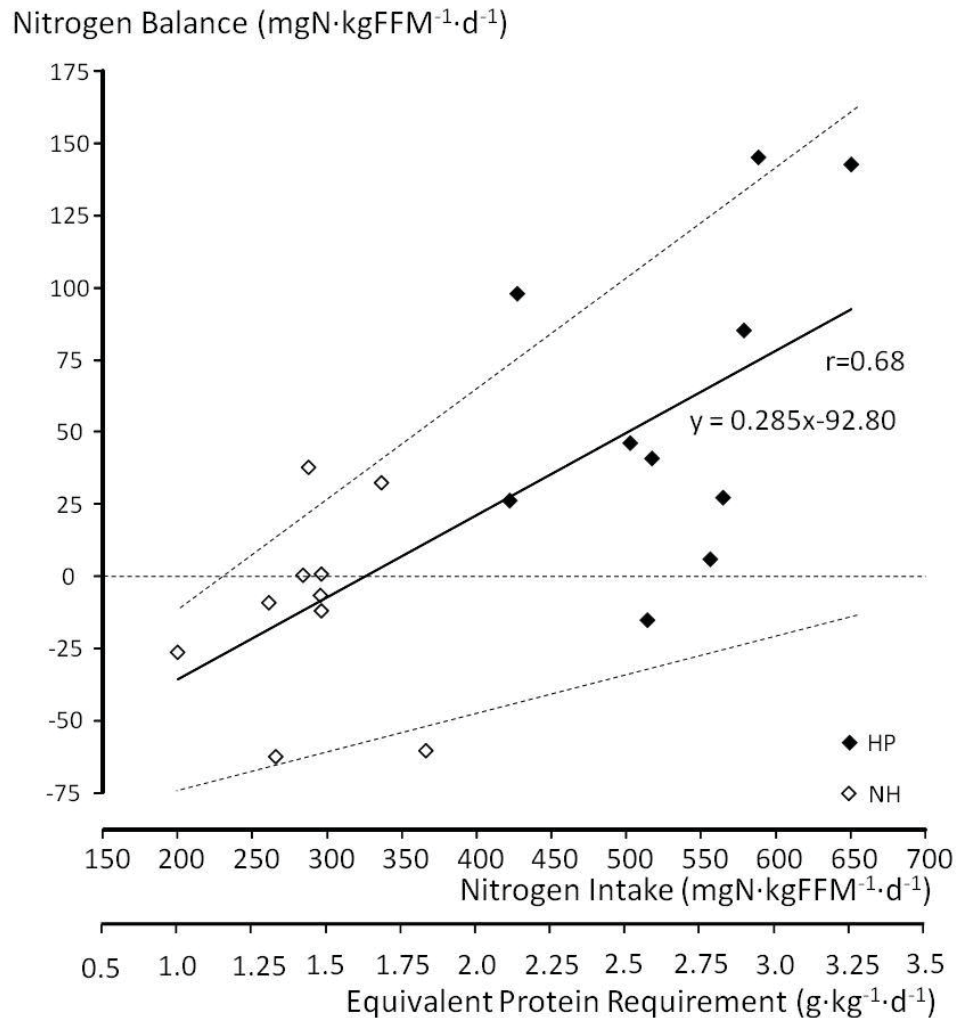


Figure 9. Estimated dietary protein requirement. The fill line represents the mean regression; dashed line the 95% confidence limits.

6. Discussion

The primary finding was an estimated mean dietary protein requirement of $1.63 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ (95% confidence interval = $1.14\text{-}3.77 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) for well-trained young-adult female endurance athletes in the mid-follicular phase of the menstrual cycle undergoing daily training. It was also observed that a diet representing normal individual protein intake was inadequate to achieve NBAL across the cohort, while in one participant, habituation to the high-protein diet was not sufficient to reach balance. The data shows that daily moderate-intensity ($1.5 \text{ h}\cdot\text{d}^{-1}$) endurance training raises dietary protein requirement in female athletes to within the range of literature estimates previously reported for endurance-training males.

To our knowledge, the current data provides the first empirical measurement of habituated protein requirement in female endurance athletes. The study was modeled on the classic study by Tarnopolsky et al. (1988). Their linear regression of two 72-h nitrogen balance calculations produced an estimated safe level of intake of $1.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ for endurance trained men (Tarnopolsky, MacDougall et al. 1988). Since then, two further nitrogen balance studies in male endurance athletes suggests an intake of between $1.8\text{-}2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ may be required to maintain nitrogen balance during intensive training (Brouns, Saris et al. 1989; Rowlands, Rossler et al. 2008; Rowlands and Wadsworth 2010). In review, Mark Tarnopolsky (2004) presented a rationale that females require on average approximately 15-20% less dietary protein than equally trained males. His estimation was inferred from: a) lower habitual protein intakes observed from dietary surveys, b) less exercise-induced amino acid turnover (Phillips, Atkinson et al. 1993; McKenzie, Phillips et al. 2000), and c) gender comparisons of nitrogen balance indicating 25-35% lower estimated

protein requirement for females compared to males (Lamont, Patel et al. 1990; Phillips, Atkinson et al. 1993). In the first estimate of female protein requirement during a 4-d block of high-intensity training, Rowland and Wadsworth (2010) reported a mean protein requirement of $1.28 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, which was 0.65 times that of a fitness matched male cohort (Rowlands and Wadsworth 2010). The findings in the current study provide empirical evidence that the female requirement is within the range of the measured requirement for males. Furthermore, a novel point was that individual variability in nitrogen balance suggests that the earlier literature estimate of $1.2\text{-}1.4 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ for females may be too conservative for some athletes, leaving them in negative nitrogen balance.

The estimation of $1.28 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ in the earlier study by Rowlands and Wadsworth (2010) fell within the literature range for female protein requirement. Their estimate was calculated from two nitrogen balance measurements during acute protein feeding. The current study was designed to ensure each balance calculation represented protein turnover at equilibrium, an approach consistently adopted in measurements of protein requirement (Oddoye and Margen 1979; Tarnopolsky, MacDougall et al. 1988; Phillips, Atkinson et al. 1993; Millward 2003). Although it was not possible to define whether an equilibrium of protein turnover was reached, particularly prior to the high-protein balance period, the adopted 10-d dietary adaptation window adopted is consistent with literature recommendations for adaptation to a change in dietary protein (Hegsted 1975). Therefore, it is very likely that equilibrium in protein turnover is accurately represented in the respective conditions.

The current study is an estimation of protein requirement in the mid-follicular phase of the menstrual cycle. Inference from measures of amino acid catabolism (Lariviere, Moussalli et al. 1994; Kriengsinyos, Wykes et al. 2004; Toth, Sites et al. 2006) and nitrogen balance (Lamont, Lemon et al. 1987) suggest greater protein utilisation during the luteal phase. Additionally, estradiol administration in men has been shown to reduce leucine oxidation by 16% at rest and during exercise (Tarnopolsky 2008). In the current study, the effect of the hormonal profile on protein turnover and metabolism was controlled by testing within a given menstrual phase (days 4-11 post the first day of menses). Day 11 after the first day of menses was a suitable cutoff to confidently avoid the normal pre-ovulatory surge in estradiol secretion (Oosthuyse and Bosch 2010). Nevertheless, in view of the increased amino acid turnover in the luteal phase, nitrogen balance and other studies are required to quantify the effect of menstrual phases on protein requirement to appreciate the cyclical picture of female protein metabolism and dietary requirement.

The exercise volume during the balance periods in the present study was greater than in previous measures of male and female protein turnover, and may in part explain why calculated protein requirement was greater than other estimates for well-trained females. In the present study, participants performed 90 minutes of moderate variable intensity cycling on each day of the 72-h nitrogen balance period, whereas Phillips et al. (1993) measured 72-h nitrogen balance in well-trained females who exercised for 90 minutes on days 1 and 3 of the nitrogen-balance sampling period. Protein requirement was estimated at $0.96 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ (Phillips, Atkinson et al. 1993). In the study in men by Tarnopolsky and MacDougall et al. (1988), exercise was also performed on days 1 and 3 of the sampling period. Estimated mean protein

requirement was $1.37 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ (Tarnopolsky, MacDougall et al. 1988). Lamont and Patel et al. (1990) measured nitrogen balance in well-trained females during a period of no exercise, and reported mildly negative nitrogen balance $-4.3 \text{ mgN}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ during a diet containing $1.0 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ of protein. Calculated mean protein requirement was $1.03 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ (Lamont, Patel et al. 1990). In the present study, the mean balance calculation of $-11 \text{ mgN}\cdot\text{kgFFM}^{-1}\cdot\text{d}^{-1}$ during a mean habitual protein intake of $1.42 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$, and the overall balance calculation of $1.63 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ suggests that a 90-min moderate intensity training bout performed on consecutive days increased protein turnover and raised the daily protein requirement.

The mean weekly training volume, energy intake and resultant protein turnover of the cohort in the present study more closely resembled that of well-trained top-sport endurance athletes than the recreational values of the participants in previous studies of nitrogen balance (Phillips, Atkinson et al. 1993) and protein turnover (Lamont, McCullough et al. 1999). In the present study, the mean habitual training load of the cohort estimated from 10-d training diaries was $10.8 \pm 2.8 \text{ h}\cdot\text{wk}^{-1}$, whereas in the study by Phillips et al. (1993), mean weekly training load was $3.4 \pm 0.1 \text{ h}\cdot\text{wk}^{-1}$. Although no comparison of daily energy intake and expenditure can be made between the two studies because of the lack of detail in the earlier works, it is fair to speculate that the observed nitrogen balance in the present study was a function of the level of protein turnover resulting from higher levels of energy intake, expenditure, and relative protein intake compared to the study by Phillips et al. (1993). Despite not having a comparative male cohort, it is plausible to assume that a mean estimated requirement for females of $1.63 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ is within the estimation of 15-20% lower than males based on the calculated male requirement of $1.98 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$.

$1 \cdot d^{-1}$ by Rowlands and Rossler et al. (2008) and $1.8-2.0 \text{ g} \cdot \text{kg}^{-1} \cdot d^{-1}$ by Brouns and Saris et al. (1989).

The timing relative to exercise of the ingestion of supplementary protein may also have contributed to the calculated requirements in the high-protein recovery feeding study of Rowlands and Wadsworth (2010) compared with the present habitual-diet intervention study. In contrast with the post-exercise feeding intervention study, in the current study, the high-protein condition was achieved by replacing normal food items from the normal habitual condition with high-protein supplements consumed ad libitum per 24-h period. Any high-protein supplements consumed immediately post-exercise may have resulted in lower urea production than if consumed at other times because urea production has been shown not to increase after the provision of post-exercise amino acids (Tipton, Ferrando et al. 1999; Rasmussen, Tipton et al. 2000; Tipton, Rasmussen et al. 2001; Miller, Tipton et al. 2003; Tipton, Elliott et al. 2004). Ingestion of protein in high quantity may have exceeded the immediate demand for amino acids and led to acute nitrogen disposal via urea production. Therefore, it is reasonable to conclude that tighter control of timing and quantity of high-protein feeding may have produced a truer reflection of 72-h nitrogen balance in the present study.

Energy requirement and dietary macronutrient composition were estimated from dietary recall diaries, which means that it is not certain whether participants were in energy balance, and it is not possible to discount increased protein utilisation due to dietary insufficiency. Amino acid catabolism is known to increase during energy deficit (Pikosky, Smith et al. 2008), and may have been greater in some participants due to a modest energy and carbohydrate intake. In one participant, nitrogen

balance during the normal habitual condition was substantially negative despite the highest relative protein intake (NBAL: $-61 \text{ mgN}\cdot\text{kgFFM}^{-1}\cdot\text{d}^{-1}$, protein intake: $\sim 1.8 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$). Carbohydrate intake for this participant during the normal habitual condition was the lowest across the cohort ($184 \text{ g}\cdot\text{d}^{-1}$), and represented only 43.8% of energy. According to diet records, carbohydrate intake was habitually low ($<200 \text{ g}\cdot\text{d}^{-1}$). Although being less pronounced in females, the relationship between a low carbohydrate diet and increased protein utilisation (Lemon and Mullin 1980) may explain the high negative balance observed in this participant. Across conditions, the magnitude of increase of nitrogen balance between the normal habitual ($-61.3 \text{ mgN}\cdot\text{kgFFM}^{-1}\cdot\text{d}^{-1}$) and high-protein diet ($142.2 \text{ mgN}\cdot\text{kgFFM}^{-1}\cdot\text{d}^{-1}$) was the greatest of any participant. Moreover, the positive balance attained by this participant was the greatest despite further reduction in carbohydrate intake. Despite being unable to account for the influence of energy balance on protein utilisation, the dietary protocol was designed to self-regulate energy need by providing additional food items for the regulation of hunger. No participants complained of hunger during either of the balance periods. Therefore, it can be assumed that self-regulation of energy need was sufficient to ensure energy balance within the margin where nitrogen balance was not greatly influenced.

It is of concern that the slope and position of the regression line in Figure 9 is not representative of some individual responses. When single participants are removed from the data set, the shift in the regression line is dependent on which participant is removed. There is a maximum shift of $100 \text{ mgN}\cdot\text{kgFFM}^{-1}\cdot\text{d}^{-1}$ when a single participant is omitted, effectively reducing the estimated mean requirement by $0.3 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ of protein. No reason could be found to omit any data points from the

calculations, and therefore it is accepted that individual variability is the primary contributor to the range of balance values observed across the cohort.

Although the high-protein condition improved nitrogen balance in every participant, individual responses were greatly variable. For example, in one participant, nitrogen balance was negative in both the normal habitual ($-63.4 \text{ mgN} \cdot \text{kgFFM}^{-1} \cdot \text{d}^{-1}$) and high-protein conditions ($-15.6 \text{ mgN} \cdot \text{kgFFM}^{-1} \cdot \text{d}^{-1}$), with a calculated individual protein requirement of $2.83 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$, while another was positive in both conditions (NH: $36.9 \text{ mgN} \cdot \text{kgFFM}^{-1} \cdot \text{d}^{-1}$, HP: $97.4 \text{ mgN} \cdot \text{kgFFM}^{-1} \cdot \text{d}^{-1}$, individual requirement: $1.02 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$). No explanation could be determined for the observed variability. Despite the inherent variability and tendency for overestimation of nitrogen balance at high protein intakes, the mean requirement of $1.63 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$ is a fair representation of the dietary protein required when the balance data for normal habitual condition is viewed in isolation. In determining the breadth of individual requirement, the 95% confidence interval ($1.14\text{-}3.77 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), and individual regressions indicate that in some cases protein requirement is modest ($\sim 1.2 \text{ g} \cdot \text{kg}^{-1} \cdot \text{d}^{-1}$), while in others it greatly exceeds the mean requirement.

The size of the correlation in Figure 9 raises the uncertainty of the inference made to population recommendations. From a sample size of $n=10$, a large correlation ($r > 0.5$) was required to be certain that the estimated mean requirement is accurately representative of the true requirement. In the study by Tarnopolsky et al. (1988) in men, the correlation was $r=0.87$ from $n=6$. In the present study, the correlation of $r=0.68$ (Figure 9) implies that an estimated mean requirement is unsuitable as a population recommendation and that a recommended range may be more appropriate. To settle individual variation in minimum protein requirement, a safe

range of dietary protein intake was calculated using the same approach as Tarnopolsky et al. (1988), who added 1 standard deviation to the mean requirement. The calculated range for the present study was 1.63-1.87 g·kg⁻¹·d⁻¹.

It is interesting to speculate whether a 10-d adapted dietary protein intake standardised to body weight or fat-free mass would have produced a more reliable estimate of mean protein requirement. In designing the dietary protocol, habitual protein intakes were measured from diet diaries, and the magnitude increase in protein intake from the normal habitual to high-protein conditions was not consistent between participants (range: 1.72-2.14 fold, mean: 1.8 fold). It is possible that noise created by the diet variability may have been reduced if protein intake was standardised and the magnitude increase in intake was more tightly controlled. Whether by adopting a standardised dietary protocol, the estimation of mean protein requirement would be a truer reflection is debatable because of the inherent individual variability in nitrogen balance during a clamped diet (Tarnopolsky, MacDougall et al. 1988; Rowlands and Wadsworth 2010).

There are noteworthy limitations in the nitrogen balance calculations and estimation of protein requirement in the current study. Imposing fecal collections on the cohort would have been impractical and likely to compromise participation. Therefore, fecal nitrogen loss for the normal habitual condition was estimated according to previous measures in females (Phillips, Atkinson et al. 1993), and adjusted for the high-protein condition according to the magnitude of increase observed in males relative to the normal protein intake (Tarnopolsky, MacDougall et al. 1988). In adopting an estimation, it is difficult to determine whether fecal nitrogen loss is accurately represented. Measured fecal nitrogen loss in females consuming a low protein intake

($0.86 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) was $1.39 \text{ gN}\cdot\text{d}^{-1}$ (Phillips, Atkinson et al. 1993). Fecal loss in males during their habitual intake ($1.7 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$) was appreciably greater ($2.68 \text{ gN}\cdot\text{d}^{-1}$). The estimates in the current study (NH: $1.48 \text{ gN}\cdot\text{d}^{-1}$, HP: $1.78 \text{ gN}\cdot\text{d}^{-1}$) reflect these measures, but they do not reflect the variability of measured fecal loss in previous balance studies (Tarnopolsky, MacDougall et al. 1988; Phillips, Atkinson et al. 1993), and it is possible they underestimate actual fecal loss in some cases. Despite this, the conservative estimations may offset the inherent methodological tendency for overestimation of balance, especially at high protein intakes (Tarnopolsky, MacDougall et al. 1988). Therefore, the true protein requirement may still be marginally greater than the calculation in the current study.

Despite the outlined limitations, the current study provides the first empirical estimate of habituated dietary protein requirement for female endurance athletes performing daily training. The empirical data further strengthen the notion that endurance athletes require appreciably more dietary protein than sedentary persons. The calculated safe range of minimum requirement of $1.63\text{-}1.87 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ can be adequately achieved by consuming a eucaloric diet containing ~15% of energy from protein. However, substantial individual variability suggests that some females require only very modest daily protein intakes ($\sim 1.0\text{-}1.2 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$), while some may require in excess of $2.0 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$. No direct cause could be found for the variation, but it is fair to speculate that macronutrient breakdown, energy intake, hormonal difference and meal timing may have been contributing factors in this experiment and are candidate modifying covariates worthy of future investigation.

7. Future directions

The current study was the first to measure nitrogen balance in habituated female endurance athletes. Measures were taken in the mid-follicular phase (between days 4-11) of the menstrual cycle. Dietary protein requirement for females may be higher in the luteal phase compared to the mid-follicular phase due to higher protein turnover in the luteal phase associated with the metabolic effects of ovarian hormones (Oosthuyse and Bosch 2010), and consequently, variation associated inter-individual difference in sex hormone concentration and metabolic responsiveness. In order to gain an overall picture of protein requirement in females, it may be important to directly compare estimates of protein requirement during the mid-follicular and luteal phases. For top-sport or elite female athletes, it will also be useful to understand the effect of amenorrhea on protein requirement.

8. Conclusions

According to the data in the current study, endurance trained females undergoing daily training may require a protein intake exceeding $1.6 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ to achieve nitrogen balance. In some cases, an intake above $2.0 \text{ g}\cdot\text{kg}^{-1}\cdot\text{d}^{-1}$ may be required. These findings provide evidence that some female endurance athletes require a similar relative protein intake to equally trained males, and that the current literature estimates may be inadequate as a guideline for females involved in daily moderate intensity and duration training.

9. References

- Aftiring, R. P., K. P. Block, et al. (1986). "Leucine and isoleucine activate skeletal muscle branched-chain alpha-keto acid dehydrogenase in vivo." Am J Physiol Endocrinol Metab **250**(5): E599-604.
- Ashley, C. D., M. L. Kramer, et al. (2000). "Estrogen and Substrate Metabolism: A Review of Contradictory Research." Sports Medicine **29**: 221-227.
- Ashworth, A. and D. B. Harrower (1967). "Protein requirements in tropical countries: nitrogen losses in sweat and their relation to nitrogen balance." British Journal Of Nutrition **21**(04): 833-843.
- Benoit, V., A. Valette, et al. (1982). "Potentiation of epinephrine-induced lipolysis in fat cells from estrogen-treated rats." Biochemical and Biophysical Research Communications **109**(4): 1186-1191.
- Bingham, S. (1994). "The use of 24-h urine samples and energy expenditure to validate dietary assessments." Am J Clin Nutr **59**(1): 227S-231.
- Bingham, S. and J. Cummings (1985). "Urine nitrogen as an independent validatory measure of dietary intake: a study of nitrogen balance in individuals consuming their normal diet." Am J Clin Nutr **42**(6): 1276-1289.
- Bingham, S. A. (2003). "Urine Nitrogen as a Biomarker for the Validation of Dietary Protein Intake." J. Nutr. **133**(3): 921S-924.
- Bolster, D. R., M. A. Pikosky, et al. (2005). "Dietary protein intake impacts human skeletal muscle protein fractional synthetic rates after endurance exercise." Am J Physiol Endocrinol Metabol **289**(4): E678-683.
- Boyer, B. and R. Odessey (1991). "Kinetic characterization of branched chain ketoacid dehydrogenase." Archives of Biochemistry and Biophysics **285**(1): 1-7.
- Brooks, G. A., T. D. Fahey, et al. (2005). Exercise Physiology: Human Bioenergetics and its Application. New York, McGraw-Hill Companies Inc.
- Brouns, F., W. H. Saris, et al. (1989). "Eating, drinking, and cycling. A controlled Tour de France simulation study, Part I." International Journal of Sports Medicine **10 Suppl 1**: S32-40.
- Brouns, F., W. H. Saris, et al. (1989). "Eating, drinking, and cycling. A controlled Tour de France simulation study, Part II. Effect of diet manipulation." International Journal of Sports Medicine **10 Suppl 1**: S41-48.
- Burke, L. M. (2001). "Nutritional practices of male and female endurance cyclists. [Review] [56 refs]." Sports Medicine **31**(7): 521-532.
- Calloway, d. H., A. C. F. Odell, et al. (1970). "Sweat and miscellaneous nitrogen losses in human balance studies." J Nutr **101**: 775-786.
- Campbell, S. E., D. J. Angus, et al. (2001). "Glucose kinetics and exercise performance during phases of the menstrual cycle: effect of glucose ingestion." American Journal of Physiology-Endocrinology and Metabolism **281**(4): E817-E825.
- Carter, S., S. McKenzie, et al. (2001). "Short-term 17beta-estradiol decreases glucose R(a) but not whole body metabolism during endurance exercise." Journal of Applied Physiology **90**(1): 139-146.
- Colombani, P., S. Späti, et al. (1997). "Exercise-induced sweat nitrogen excretion: evaluation of a regional collection method using gauze pads." Zeitschrift für Ernährungswissenschaft **36**(3): 237-243.
- Cooper, B. C., C. K. Sites, et al. (2007). "Ovarian suppression with a gonadotropin-releasing hormone agonist does not alter insulin-stimulated glucose disposal." Fertil Steril **87**(5): 1131-1138.
- D'Eon, T. M., C. Sharoff, et al. (2002). "Regulation of exercise carbohydrate metabolism by estrogen and progesterone in women." American Journal of Physiology - Endocrinology And Metabolism **283**(5): E1046-E1055.

- Devries, M. C., M. J. Hamadeh, et al. (2006). "Menstrual cycle phase and sex influence muscle glycogen utilization and glucose turnover during moderate intensity endurance exercise. ." Am J Physiol Regul Integr Comp Physiol **291**: R1120–R1128.
- Dolny, D. G. and P. W. Lemon (1988). "Effect of ambient temperature on protein breakdown during prolonged exercise." J Appl Physiol **64**(2): 550-555.
- Faria, A. C. S., L. W. Bekenstein, et al. (1992). "Pulsatile growth hormone release in normal women during the menstrual cycle." Clinical Endocrinology **36**(6): 591-596.
- Felig, P. and J. Wahren (1971). "Amino acid metabolism in exercising man." J Clin Invest **50**(12): 2703-2714.
- Forslund, A., A. El Khoury, et al. (1999). "Effect of protein intake and physical activity on 24-h pattern and rate of macronutrient utilization." Am J Physiol **276**: E964 - E976.
- Friedman, J. E. and P. W. R. Lemon (1989). "Effect of Chronic Endurance Exercise on Retention of Dietary Protein." Int J Sports Med **10**(02): 118,123.
- Fuller, M. F. and P. J. Reeds (1998). "Nitrogen cycling in the gut." Nutrition **18**: 385-411.
- Hackney, A. (1999). "Influence of oestrogen on muscle glycogen utilization during exercise." Acta Physiologica Scandinavica **167**: 273-274.
- Hackney, A. C., M. A. McCracken-Compton, et al. (1994). "Substrate responses to submaximal exercise in the midfollicular and midluteal phases of the menstrual cycle." Int J Sports Nutr. Sep(4(3)): 299-308.
- Hamadeh, M. J., M. C. Devries, et al. (2005). "Estrogen supplementation reduces whole body leucine and carbohydrate oxidation and increases lipid oxidation in men during endurance exercise." Journal of Clinical Endocrinology & Metabolism **90**(6): 3592-3599.
- Hargreaves, M. and M. Thompson (1999). Biochemistry of Exercise. Champaign, Human Kinetics.
- Hegsted, D. M. (1975). "Balance Studies." The Journal of Nutrition **36**: 110-117.
- Hopkins, W. G. (2007) "Typical Error of Measurement."
<http://www.sportsci.org/resource/stats/precision.html#loa>.
- Horton, T. J., E. K. Miller, et al. (2002). "No effect of menstrual cycle phase on glucose kinetics and fuel oxidation during moderate-intensity exercise." American Journal of Physiology-Endocrinology and Metabolism **282**(4): E752-E762.
- Jackson, A. S., M. L. Pollock, et al. (1980). "Generalized equations for predicting body density of women." Medicine & Science in Sports & Exercise **12**(3): 175-182.
- Jones, D. B. (1941). Factors for converting percentages of nitrogen in foods and feeds into percentages of proteins U. S. D. o. Agriculture. Washington D.C., Bureau of Agricultural Chemistry and Engineering. **183**: 1-16.
- King-Brink, M. and J. G. Sebranek (1993). "Combustion Method for Determination of Crude Protein in Meat and Meat Products:A Collaborative Study." J AOAC Int. **76**(4): 787-793.
- Kriengsinyos, W., L. J. Wykes, et al. (2004). "Phase of menstrual cycle affects lysine requirement in healthy women." Am J Physiol Endocrinol Metab **287**(3): E489-496.
- Lamont, L. S., P. W. Lemon, et al. (1987). "Menstrual cycle and exercise effects on protein catabolism." Medicine & Science in Sports & Exercise **19**(2): 106-110.
- Lamont, L. S., A. J. McCullough, et al. (1999). Comparison of leucine kinetics in endurance-trained and sedentary humans. **86**: 320-325.
- Lamont, L. S., D. G. Patel, et al. (1990). "Leucine kinetics in endurance-trained humans." J Appl Physiol **69**(1): 1-6.
- Lariviere, F., R. Moussalli, et al. (1994). "Increased leucine flux and leucine oxidation during the luteal phase of the menstrual cycle in women." Am J Physiol Endocrinol Metab **267**(3): E422-428.
- Layman, D. K. (2002). "Role of Leucine in protein metabolism during exercise and recovery." Applied Physiology, Nutrition, and Metabolism **27**(6): 646-662.
- Lemon, P. W. and J. P. Mullin (1980). "Effect of initial muscle glycogen levels on protein catabolism during exercise." Journal of Applied Physiology **48**(4): 624-629.

- Lemon, P. W., K. E. Yarasheski, et al. (1986). "Validity/reliability of sweat analysis by whole-body washdown vs. regional collections." J Appl Physiol **61**(5): 1967-1971.
- Lemon, P. W. R. and K. E. Yarasheski (1985). "Feasibility of Sweat Collection by Whole Body Washdown in Moderate to High Humidity Environments." Int J Sports Med **06**(01): 41,43.
- Liappis, N. and H. Hungerland (1972). "Quantitative study of free amino acids in human eccrine sweat during normal conditions and exercise." Am J Clin Nutr **25**(7): 661-663.
- Martin, M., D. Martin, et al. (2002). "Voluntary food intake by elite female cyclists during training and racing: influence of daily energy expenditure on bdy composition." Int J Sports Nutr Exerc Metab **12**: 249-267.
- McCance, R. A. and E. M. Widdowson (2002). The Composition of Foods, Poyal Society of Chemsitry.
- McCracken, M., B. Ainsworth, et al. (1994). "Effects of the menstrual cycle phase on the blood lactate responses to exercise." European Journal of Applied Physiology and Occupational Physiology **69**(2): 174-175.
- McKenzie, S., S. M. Phillips, et al. (2000). "Endurance exercise training attenuates leucine oxidation and BCOAD activation during exercise in humans." American Journal of Physiology Endocrinology & Metabolism **278**(4).
- McLay, R. T., C. D. Thomson, et al. (2007). "Carbohydrate loading and female endurance athletes: effect of menstrual cycle phase." International Journal of Sport Nutrition and Exercise Metabolism **17**(2): 189-205.
- Merrill, A. L. and B. K. Watt (1973). Energy value of foods-Basis and derivation. D. o. Agriculture. Washington D.C., Agriculture research service. **Agriculture Handbook**.
- Miller, S. L., K. D. Tipton, et al. (2003). "Independent and combined effects of amino acids and glucose after resistance exercise." Medicine & Science in Sports & Exercise **35**(3): 449-455.
- Millward, D. J. (2003). "An adaptive metabolic demand model for protein and amino acid requirements." British Journal of Nutrition **90**: pp 249-260.
- Millward, D. J., J. L. Bowtell, et al. (1994). "Physical activity, protein metabolism and protein requirements. [Review] [62 refs]." Proceedings of the Nutrition Society **53**(1): 223-240.
- Nicklas, B. J., A. C. Hackney, et al. (1989). "The Menstrual Cycle and Exercise: Performance, Muscle Glycogen, and Substrate Responses." Int J Sports Med **10**(04): 264,269.
- Oddoye, E. A. and S. Margen (1979). "Nitrogen Balance Studies in Humans: Long-Term Effect of High Nitrogen Intake on Nitrogen Accretion." The Journal of Nutrition **109**(3): 363-377.
- Oosthuysen, T. and A. N. Bosch (2010). "The effect of the menstrual cycle on exercise metabolism: Implications for exercise performance in eumenorrhoeic women." Sports Medicine **40**(3): 207-227.
- Patterson, M. J., S. D. R. Galloway, et al. (2000). "Variations in Regional Sweat Composition in Normal Human Males." Experimental Physiology **85**(6): 869-875.
- Phillips, S. M., S. A. Atkinson, et al. (1993). "Gender differences in leucine kinetics and nitrogen balance in endurance athletes." Journal of Applied Physiology **75**(5): 2134-2141.
- Phillips, S. M., D. R. Moore, et al. (2007). "A critical examination of dietary protein requirements, benefits, and excesses in athletes." Int J Sport Nutr Exer Metab **17**: S58-S76.
- Phillips, S. M. and S. M. Phillips (2006). "Dietary protein for athletes: from requirements to metabolic advantage." Applied Physiology, Nutrition, & Metabolism = Physiologie Appliquee, Nutrition et Metabolisme **31**(6): 647-654.
- Pikosky, M. A., T. J. Smith, et al. (2008). "Increased Protein Maintains Nitrogen Balance during Exercise-Induced Energy Deficit." Medicine & Science in Sports & Exercise **40**(3): 505-512.
- Price, G. M., D. Halliday, et al. (1994). "Nitrogen homeostasis in man: influence of protein intake on the amplitude of diurnal cycling of body nitrogen." Clin. Sci. **86**(1): 91-102.
- Rasmussen, B. B., K. D. Tipton, et al. (2000). "An oral essential amino acid-carbohydrate supplement enhances muscle protein anabolism after resistance exercise." J Appl Physiol **88**(2): 386-392.

- Rodriguez, N. R., L. M. Vislocky, et al. (2007). "Dietary protein, endurance exercise, and human skeletal-muscle protein turnover." Current Opinion in Clinical Nutrition & Metabolic Care **10**(1): 40-45 10.1097/MCO.1090b1013e3280115e3280113b.
- Rowlands, D. S., K. Rossler, et al. (2008). "Effect of dietary protein content during recovery from high-intensity cycling on subsequent performance and markers of stress, inflammation, and muscle damage in well-trained men." Appl Physiol Nutr Metabol **33**: 39-51.
- Rowlands, D. S. and D. P. Wadsworth (2010). "Effect of high-protein feeding on performance and nitrogen balance in female cyclists." Medicine & Science in Sports & Exercise.
- Ruby, B. C., R. A. Robergs, et al. (1997). "Effects of estradiol on substrate turnover during exercise in amenorrheic females." Medicine & Science in Sports & Exercise **29**(9): 1160-1169.
- Smith, K. and M. J. Rennie (1996). "The measurement of tissue protein turnover." Baillière's Clinical Endocrinology and Metabolism **10**(4): 469-495.
- Tarnopolsky, L. J., J. D. MacDougall, et al. (1990). "Gender differences in substrate for endurance exercise." Journal of Applied Physiology **68**(1): 302-308.
- Tarnopolsky, M. (2004). "Protein Requirements for Endurance Athletes." European Journal of Sport Science **4**(1): 1 - 15.
- Tarnopolsky, M. A. (2000). "Gender differences in substrate metabolism during endurance exercise." Canadian Journal of Applied Physiology **25**(4): 312-327.
- Tarnopolsky, M. A. (2008). "Sex Differences in Exercise Metabolism and the Role of 17-Beta Estradiol." Medicine & Science in Sports & Exercise **40**(4): 648-654
610.1249/MSS.1240b1013e31816212ff.
- Tarnopolsky, M. A., S. A. Atkinson, et al. (1995). "Carbohydrate loading and metabolism during exercise in men and women." Journal of Applied Physiology **78**(4): 1360-1368.
- Tarnopolsky, M. A., J. D. MacDougall, et al. (1988). "Influence of protein intake and training status on nitrogen balance and lean body mass." J Appl Physiol **64**(1): 187-193.
- Tipton, K. D., T. A. Elliott, et al. (2004). "Ingestion of Casein and Whey Proteins Result in Muscle Anabolism after Resistance Exercise." Medicine & Science in Sports & Exercise **36**(12): 2073-2081.
- Tipton, K. D., A. A. Ferrando, et al. (1999). "Postexercise net protein synthesis in human muscle from orally administered amino acids." Am J Physiol **276**(4 Pt 1): E628-E634.
- Tipton, K. D., B. B. Rasmussen, et al. (2001). "Timing of amino acid-carbohydrate ingestion alters anabolic response of muscle to resistance exercise." Am J Physiol Endocrinol Metabol, **281**(2): E197-206.
- Tome, D. and C. Bos (2000). "Dietary Protein and Nitrogen Utilization." The Journal of Nutrition **130**(7): 1868S-1873S.
- Toscani, V. and G. D. Whedon (1951). "Nitrogen loss in the feces: the variability of excretion in normal subjects on constant dietary intakes." The Journal of Nutrition **45**(1): 119-130.
- Toth, M. J., C. K. Sites, et al. (2006). "Ovarian suppression with gonadotropin-releasing hormone agonist reduces whole body protein turnover in women." American Journal of Physiology - Endocrinology And Metabolism **291**(3): E483-E490.
- van Hall, G., G. J. van der Vusse, et al. (1995). "Deamination of amino acids as a source for ammonia production in human skeletal muscle during prolonged exercise." The Journal of Physiology **489**(Pt 1): 251-261.
- van Pelt, R. E., W. S. Gozansky, et al. (2003). "Intravenous estrogens increase insulin clearance and action in postmenopausal women." American Journal of Physiology - Endocrinology And Metabolism **285**(2): E311-E317.
- Wagenmakers, A. J., E. J. Beckers, et al. (1991). "Carbohydrate supplementation, glycogen depletion, and amino acid metabolism during exercise." American Journal of Physiology **260**(6 Pt 1): E883-890.

- Young, V., D. Bier, et al. (1989). "A theoretical basis for increasing current estimates of the amino acid requirements in adult man, with experimental support." The American Journal of Clinical Nutrition **50**(1): 80-92.
- Young, V. and P. Pellet (1994). "Plant proteins in relation to human protein and amino acid nutrition." American Journal of Clinical Nutrition **59**: 12035-12125.
- Zderic, T. W., A. R. Coggan, et al. (2001). "Glucose kinetics and substrate oxidation during exercise in the follicular and luteal phases." Journal of Applied Physiology **90**(2): 447-453.

10. Appendix 1: Characteristic data

Participant	Age	Weight (kg)	Height (cm)	% Body Fat	VO _{2max} (ml.kg ⁻¹ .min ⁻¹)	Peak Aerobic Power (w)
1	26	54.3	166	14	62.3	243
2	33	69.6	165	17.7	58	254
3	34	52.4	159	15	52.5	218
4	30	61.75	168	16.4	54.7	261
5	37	62.8	169	19.1	58.8	255
6	30	64.85	171	23.3	49.3	258
7	27	68.1	169	23.7	44.4	238
8	34	56.6	160	14	62	260
9	24	61.4	164	17.6	59.5	280
10	33	60.9	171	12.6	57.7	263
Mean	30.8	61	166	17.3	55.9	252
SD	4.13	5.4	4	3.6	5.7	16

11. Appendix 2: Nutritional data

A2. 1. Habitual intake from diet diaries

Participant	Energy Intake	CHO (g)	Fat (g)	Protein (g)	% CHO to Energy	% Fat to Energy	% Protein to Energy
1	9902	286	92	92	49.1	34.3	15.8
2	7171	195	63	74	46.2	32.8	17.5
3	9700	291	83	78	51	31.7	13.7
4	8649	248	78	91	49	33	18
5	6836	249	60	69	61.9	32.5	17.2
6	7603	225	77	56	50.3	37.5	12.5
7	9307	233	58	81	53.5	29	18.6
8	7195	178	75	82	42	38.5	19
9	9307	310	73	81	56.6	29	14.8
10	13068	290	166	119	37.7	47	15.5
Mean	8955	266	83	82	51.4	33.6	15.7
SD	1863	44	31	16	6.9	5.4	2.1

A2. 2. Normal habitual diet

Participant	Energy Intake (kJ)	CHO (g)	Fat (g)	Protein (g)	% CHO to Energy	% Fat to Energy	% Protein to Energy
1	9632	308	77	91	54.4	29.6	16.1
2	6997	217	60	64	52.7	31.7	15.5
3	9635	314	78	83	55.4	30	14.6
4	8699	257	72	98	50.2	30.6	19.2
5	8380	285	62	73	57.8	27.4	14.8
6	11691	356	106	101	51.8	33.5	14.7
7	8393	297	55	77	60.2	24.2	15.6
8	7143	184	69	86	43.8	35.7	20.5
9	9296	317	67	84	58	26.7	15.4
10	10917	326	103	92	50.8	34.9	14.3
Mean	9078	286	75	85	53.5	30.4	16.1
SD	1493	53	17	11	4.8	3.7	2.1

A2. 3. High-protein diet

Participant	Energy Intake	CHO	Fat	Protein	% CHO to Energy	% Fat to Energy	% Protein to Energy
1	9517	271	50	180	48.4	19.4	32.2
2	7181	194	42	137	45.9	21.6	32.4
3	7336	208	37	143	48.2	18.7	33.1
4	8539	229	47	171	45.6	20.4	34
5	8600	228	56	156	45.1	24.1	30.8
6	11460	300	80	200	44.5	25.8	29.7
7	8722	228	57	161	44.4	24.2	21.4
8	7730	163	55	172	35.8	26.3	37.8
9	9133	248	58	163	46.2	23.5	30.3
10	10878	297	73	184	46.4	24.8	28.8
Mean	8909.6	236.6	56	167	45.1	22.9	31.1
SD	1411	65	13	19	3.5	2.7	4.3

A2. 4. Nitrogen content in food items

Sample Name	Nitrogen %
Almonds	3.96
Apple	0.05
Bacon	3.71
Banana	0.22
Bread	2.19
Cheese	3.14
Chicken	4.49
Cocoa Powder	0.93
Crackers	2.02
Crumbed Fish	1.83
Egg	2.48
Ham	3.14
Milk	0.65
Muesli	1.78
Muesli Bar	1.15
Orange	0.20
Pasta (Supermarket)	0.74
Pasta Continental	0.79
Plain Pasta	0.91
Plain Rice	0.46
Porridge	0.37
Protein Bar	6.11
Protein Shake	13.54
Salmon	3.39
Soy Protein Isolate	13.45
Steak	4.16
Stir Fry Veg	0.31
Sushi	0.75
Udon Noodle	0.60
Uncle Bens Rice	0.65
Vege Pattie	2.83
Wheetbix	2.06
Yoghurt	0.69

12. Appendix 3: Participant information and screening

A3. 1. Participant information sheet



Massey University

Institute of
Food Nutrition & Human Health



Te Kunenga
ki Pūrehuroa

What is the dietary protein requirement for female endurance athletes?

INFORMATION SHEET

The School of Sport and Exercise

The School of Sport and Exercise is part of Massey University. It includes a large team of scientists that are interested in human nutrition, exercise science, physiology, and health. The project coordinator, Mr. Stuart Houltham (Masters student), supervised by Dr. David Rowlands, Senior Lecturer in Sport and Exercise Science hereby invite potential participants to take part in this study.

Why Are We Doing This Study?

The recommended dietary protein intake for active individuals is a controversial topic. Much of the research is focused on males undergoing resistance training programs and more recently, endurance athletes. There is evidence which suggests an increased dietary protein requirement for these exercising populations. However, little research has been conducted to investigate the protein requirements of females and whether they differ from males. During exercise, females use less carbohydrate and more fat as a fuel source than do males, indicating a likely a difference in protein requirement between males and females. Furthermore, females turnover less protein during exercise than males. There is evidence that the adapted habitual dietary protein intake for female athletes is 25-35% lower than in males, however this is yet to be scientifically verified.

In Scientific Terms

The purpose of this study is to determine the dietary protein requirement of female endurance athletes.

Participant Recruitment

We would like to recruit eight females who are:

A measure of dietary protein requirement in endurance trained women

- Aged 18 to 40 years
- *Not pregnant*
- Cyclists, mountain bikers, du- or tri-athletes or runners
- *In regular training – 8 or more hours of cycling and/or running per week including commuting for the last 12 months or greater.*
- $\text{VO}_{2\text{max}}$ of at least $55 \text{ ml} \cdot (\text{kg min})^{-1}$
- *Free from food allergies*

NOTE

Potential participants must not have participated in a similar trial within the last 2 months prior to starting this trial – discuss with us if you have questions regarding suitable start dates.

What is involved

If you decide to participate in our study, we will invite you to make one visit to the laboratory several weeks before the experiment to discuss any questions you may have with the researchers, complete consent forms and a health screening questionnaire, and to perform a $\text{VO}_{2\text{max}}$ test.

This will be followed by two experimental periods (blocks), each 13 days in duration.

Block One

During the first 10 days you will be asked to accurately record your food intake for 6 days and your training for 10 days. Easy to understand forms will be provided for you.

You will then be asked to collect all of your urine passed in a 72 hour period.

Commencing the first day of the urine collection we will provide you with all of your food until the end of urine collection (3 days). The diet in this time will be a representation of your normal diet prepared from your food diary.

We will ask you to come into the lab during each day of the urine collection period to complete moderate intensity cycling or running sessions. During the first session, patches will be placed on your back, thigh, chest and upper arm for a 24 hour period to measure your sweat rate.

Each 13 day block will begin on the same day of your menstrual cycle. This caters for the effect the changing hormone environment may have on metabolism of nutrients. For this reason there is around 17 days between each block.

Block Two

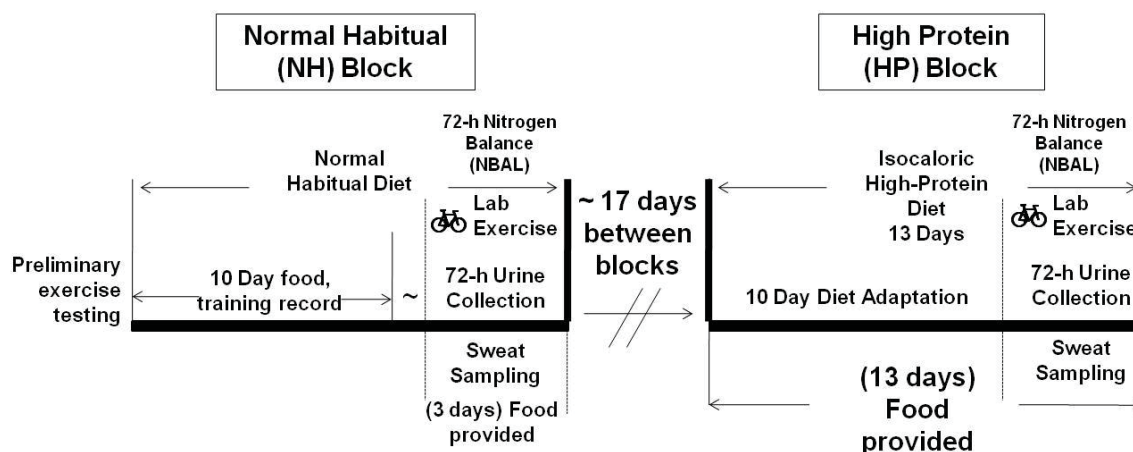
During the second block we ask that your training be replicated from the training diary recorded in the first block.

Most (90%) of your food will be provided for you in this block (13 days). The diet will be altered based on your normal diet but to increase protein intake. The energy content will be the same as your normal diet. You will be given a list of food items which you can choose food to make up the remainder 10% of the diet to provide you with some freedom of food choice.

It is important that you adhere strictly to the diet provided, as the food will be analysed for protein content to provide an accurate account of the protein that is ingested.

The three day urine and sweat collection will then be replicated as in the first block.

The below diagram provides a graphical illustration of the experiment.



Standardisation of Training

During each 10 day period, we require that your training is standardised. In the initial 10 day period we ask that a training diary be recorded, and that this training is replicated on the subsequent 10 day period. Training sessions will be of your own preference to be discussed with the researchers. This provides us with some control over the impact of exercise on metabolism and will enable us to more accurately compare the two diets.

Standardisation of Diet

During the 2nd block, we will provide you with all of your food for the 13 days. The energy content of the diet will remain the same, but protein will be altered.

Urine Collection

The measurement of Nitrogen in the urine is an accurate indicator of protein turnover in the body. It is therefore possible to measure protein loss or gain in the body as a result of diet and exercise, through the nitrogen content in urine. Containers will be provided for you.

Are any of the Procedures Harmful or Painful?

Exercise

There is often some physical and psychological discomfort associated with heavy exercise. Recent evidence has indicated that even among healthy populations of athletes who exercise strenuously and regularly, there is some risk of sudden death due to heart failure. Though rare, such cases can occur in people who may have an undiagnosed condition. If you have any reason to suspect that you may have a cardiovascular problem, we suggest that you see your physician and get an ECG before you agree to participate.

If you have any additional medical concerns associated with this project, please contact your GP, or discuss with the researcher

Time Commitment

Experiment Component	Time Commitment (h)
Screening, consent, VO ₂ max test & resting and exercise sweat collection	3 h
Daily recording of food for 13 days	10-15 min per day (2-3 hours total)
Exercise in Lab	3-4 h
Total	~7-10 h

Note. The majority of the time will be spent recording a food diary

Benefits

You will learn your VO₂max and peak power output. You will partake in some landmark interesting research. You will receive a summary of the results once the final results are available, which will contain a summary of the effectiveness of the recovery formulation. \$50 MTA vouchers for reimbursement of your time contribution.

What if I Suffer a Personal Injury?

If physical injury results from your participation in this study, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic and your claim will be assessed by ACC in accordance with the Injury Prevention, Rehabilitation and Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlements, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim.

Participant's Rights

You are under no obligation to accept this invitation. If you decide to participate, you have the right to:

- decline to answer any particular question
- withdraw from the study

A measure of dietary protein requirement in endurance trained women

- ask any questions about the study at any time during participation
- provide information on the understanding that your name will not be used unless you give permission to the researcher;
- be given access to a summary of the project findings when it is concluded.

If you are Interested in Taking Part

CONTACT:

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"This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 10/76. If you have any concerns about the conduct of this research, please contact Professor Julie Boddy, Chair, Massey University Human Ethics Committee: Southern A telephone 06 350 5799 x 2541, email humanethicsoutha@massey.ac.nz."

A3. 2. Consent form



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What is the dietary protein requirement for female endurance athletes?

PARTICIPANT CONSENT FORM

This consent form will be held for a period of five (5) years

I have read the Information Sheet and have had the details of the study explained to me. My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.

I agree to participate in this study under the conditions set out in the Information Sheet

Signature:

Date:

Full Name - printed

"This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 10/76. If you have any concerns about the conduct of this research, please contact Professor Julie Boddy, Chair, Massey University Human Ethics Committee: Southern A telephone 06 350 5799 x 2541, email humanethicsoutha@massey.ac.nz."

A3. 3. Health screening questionnaire

Health Screening Questionnaire

Massey University

Sport and Exercise Science

General Health Questionnaire

Name:

Address:

.....

Email:

Phone:

Name of the investigator responsible for the study:

Stuart Houltham

Please answer the following questions. This questionnaire has been designed to identify the small number of persons (15-69 years of age) for whom physical activity might be inappropriate and to provide the researchers with descriptive information about the participant. If you have any doubts or difficulty with the questions, please ask the investigator for guidance. These questions are to determine whether the proposed exercise is appropriate for you. Your answers will be kept strictly confidential.

1.	What is your exact date of birth? Day..... Month.....Year..19..... So your age is..... Years		
2.	Are you currently taking any medication?	YES	NO
3.	When was your last menstrual cycle (first day of menses)? Date: Day.....Month.....		

4.	Are you currently taking an oral contraceptive pill?	YES	NO
5.	Has your GP ever advised you not to take vigorous exercise?	YES	NO
6.	Has your GP ever said you have "heart trouble"?	YES	NO
7.	Has your GP ever said you have high blood pressure?	YES	NO
8.	Have you ever taken medication for blood pressure or your heart?	YES	NO
9.	Do you feel pain in your chest when you undertake physical activity?	YES	NO
10.	In the last month have you had pains in your chest when not doing any physical activity?	YES	NO
11.	Has your GP (or anyone else) said that you have raised blood cholesterol?	YES	NO
12.	Have you had a cold or feverish illness in the last month?	YES	NO
13.	Do you ever lose balance because of dizziness, or do you	YES	NO

	ever lose consciousness?		
14.	a) Do you suffer from back pain	YES	NO
	b) If yes, does it ever prevent you from exercising?	YES	NO
15.	Do you have moderate-severe liver or kidney disease?	YES	NO
16.	Do you suffer from asthma?	YES	NO
	If yes, do you control it with medication?	YES	NO
17.	Do you have any joint or bone problems which may be made worse by exercise?	YES	NO
18.	a) Has your doctor ever said you have diabetes?	YES	NO
	b) Do you think you have diabetes?	YES	NO
19.	Have you ever had a blood-borne viral disease?	YES	NO
20.	a) Do you have any allergies?	YES	NO
	b) If yes, which ones?		
21.	Are you accustomed to vigorous exercise (~8h / week)?	YES	NO

Diet Related Questions:

22.	a) Do you have any food allergies? b) If yes, which ones?	YES	NO
23.	Are you Vegetarian?	YES	NO
24.	a) Do you eat milk, milk products and eggs? b) If no, what exactly do you not eat?	YES	NO
25.	Are you lactose-intolerant?	YES	NO
26.	Are there any fruits or vegetables you can't eat? If yes, which ones?	YES	NO
27.	Do you suffer from celiac disease?	YES	NO
28.	Is there any food, which is not mentioned yet, you can't eat at all? If yes, which?	YES	NO

I have completed the questionnaire to the best of my knowledge and any questions I had have been answered to my full satisfaction.

Signed:

Date:

13. Appendix 4: Letter notifying ethics approval



MASSEY UNIVERSITY

2 December 2010

Stuart Houltham
144 Parkvale Road
Karori
WELLINGTON 6012

Dear Stuart

Re: HEC: Southern A Application – 10/76
What is the dietary protein requirement for female endurance athletes?

Thank you for your letter dated 2 December 2010.

On behalf of the Massey University Human Ethics Committee: Southern A I am pleased to advise you that the ethics of your application are now approved. Approval is for three years. If this project has not been completed within three years from the date of this letter, reapproval must be requested.

If the nature, content, location, procedures or personnel of your approved application change, please advise the Secretary of the Committee.

Yours sincerely

Prof Julie Boddy, Chair
Massey University Human Ethics Committee: Southern A

cc Dr David Rowlands
IFNHH
WELLINGTON

Prof Richard Archer, HoI
IFNHH
PN452

Te Kunenga
ki Pūrehuroa

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