Nitrogen Balance and Protein Intake at the RDA in Inactive Male Vegans

by

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ABSTRACT

Background: Vegan and vegetarian diets have gained in popularity in recent years. Stated reasons for this include some possible health benefits and concerns of animal welfare. Though considered to be nutritionally adequate, questions remain over whether current protein recommendations of 0.8 g/kg/d are sufficient to maintain body processes and growth. Protein is unique in that it is the only macronutrient that contains nitrogen. Its status can be determined through nitrogen balance analysis of the urine if protein content of the diet is known. Nitrogen balance is considered the gold standard for determining protein intake requirements. A negative balance indicates a catabolic state, whereas a positive nitrogen balance is seen during anabolism. In healthy people, nitrogen equilibrium is desired under normal circumstances. This equilibrium reflects the net synthesis and breakdown of proteins. While nitrogen balance techniques have been used for decades, currently, there are no known studies measuring nitrogen balance and protein intake in strict vegans.

Methods: Twenty vegan, inactive, male participants were recruited and received a 5-day eucaloric diet with a known protein content held constant at 0.8 g/kg/d. On day five, 24-hour urine was collected by participants and aliquoted for future analysis. Nitrogen content of the urine was determined through photometric assay and compared to the known nitrogen content of the diet to calculate nitrogen balance status.

Results: Mean absolute nitrogen balance (-1.38 ± 1.22 g/d, effect size = -1.13) was significantly lower than zero (equilibrium) ($p < .001$). Mean relative nitrogen balance (-18.60 ± 16.96 mg/kg/d, effect size = -1.10) was significantly lower than zero ($p < .001$).

There were no correlations seen between nitrogen balance and age, years as vegan, or fat-free mass.

Conclusion: Consuming 0.8 g/kg/d of protein is insufficient to produce nitrogen balance in long-term vegans.

DEDICATION

This project is dedicated to the 1,758 animals killed for food *every second* of every day in the United States alone (Animal Clock, n.d). May the information herein help show that their deaths were unnecessary and help provide better guidance for people to eat and live healthy lives free of animal suffering.

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CHAPTER 1

INTRODUCTION

In recent years vegetarian diets have gained in popularity with 5% of United States (U.S.) adults aged 18-34 years old identifying as vegetarian, half of those being vegan (Stahler, 2020). This amounts to 3.8 million vegetarians, nearly two million of which are vegan. The popularity of vegetarian diets in the U.S. has been linked to ethical and environmental concerns, but the desire to have a healthier lifestyle was the most prominent reason for adopting vegetarianism (Hopwood et al., 2020).

Following a vegetarian diet has shown mixed results in terms of various health benefits and risk factors for disease including cardiovascular disease (CVD) (Key et al., 1999; Kahleova et al., 2018; Kwok et al., 2014; Tonstad et al., 2009; Yokoyama et al., 2014; Tonstad et al., 2013; Kahleova et al., 2011; Barnard et al., 2006; Barnard et al., 1994; Barnard et al., 2009; Jenkins et al., 2003; Crane et al., 1994; Appleby & Key, 2016), cancer (Tantamango-Bartley et al., 2013; Key et al., 2009; Orlich et al., 2013; Key et al., 2014), metabolic syndrome (Yokoyama et al., 2014; Fraser, 2009; De Biase et al., 2007; Toohey et al., 1998; Berkow & Barnard, 2006; Jian et al., 2015; Wang et al., 2015; Yokoyama et al., 2017; Harland & Garton, 2016; Chuang et al., 2016; Appleby & Key, 2016; Orlich et al., 2013), and all-cause mortality (Appleby & Key, 2016). Given the totality of the evidence, it seems that many claims of health benefits of vegetarian diets may be overstated, though some benefits do exist.

The Academy of Nutrition and Dietetics has stated that "…appropriately planned vegetarian, including vegan, diets are healthful, nutritionally adequate, and…These diets are appropriate for all stages of the life cycle, including pregnancy, lactation, infancy, childhood, adolescence, older adulthood, and for athletes." (Melina et al., 2016). Despite this, concern remains over potential inadequacies of certain nutrients including protein (Kniskern & Johnston, 2011). Proteins are made up of long chains of amino acids in various sequences to form macromolecules with specific functions (Otten et al., 2006). Nine essential amino acids must be taken in through the diet, as the body cannot synthesize them. Animal-based sources such as meats, dairy, and eggs are considered complete protein sources since they contain adequate amounts of these essential amino acids. Plant-based, or vegan sources of protein typically lack one or more essential amino acids in adequate amounts and therefore are considered incomplete. Moreover, the fiber and matrix of plant foods lower the bioavailability of amino acids as well as vitamins and minerals (Gardner et al., 2019).

Protein is unique in that its amino acids contain a nitrogen atom that comprises 16% of the weight of proteins, whereas the other macronutrients, fat and carbohydrate, do not contain nitrogen (Otten et al., 2006). The recommended dietary allowance (RDA) for protein is 0.80 g/kg/d. This is based on the results of numerous nitrogen balance studies, in which nitrogen equilibrium is the criteria for determining protein requirements. These RDAs are estimated for those following an omnivore diet (Otten et al., 2006). Moreover, in their meta-analysis, Rand et al. (2003) reported that there is no significant difference in dietary protein source (e.g., animal vs. plant sources) in regards to protein needs (Rand et al., 2003). However, the 'plant-based diet' referenced in the meta-analysis included at least 10% of intake from animal foods of some sort, so therefore, does not truly represent the vegan population (Rand et al., 2003).

Proteins serve many roles in the body including enzymes, antibodies, messengers, transport and storage, and lastly structural functions (National Library of Medicine, 2020; Otten et al., 2006). Protein taken in through the diet is eventually hydrolyzed and broken down into amino acids. When amino acids are metabolized, they are broken down into creatinine, urea, ammonia, and uric acid, which are excreted in the urine and feces. Because of this, proteins are constantly being broken down and resynthesized, termed protein turnover. In cases where there is no excess growth or breakdown, protein is at a steady state, or net balance. When an individual takes in inadequate amounts of protein or essential amino acids, a net negative balance occurs which can cause many complications, affecting various organs and bodily systems (Otten et al., 2006). Nitrogen balance reflects net protein metabolism, or the difference between protein intake and excretion. It does not provide information regarding protein stores or nutritional status, but rather a global assessment of protein balance (Moizé et al., 2017; Gibson, 1990). This balance reflects the net synthesis and breakdown of proteins (Liu & Barrett, 2002), and can be estimated using the classic equation below where urinary urea nitrogen (UUN) is collected over a period of 24 hours (Dickerson, 2005b).

$$\text{Nitrogen Balance (g/d)} = (\text{PRO intake (g/d)}/6.25) - \text{UUN (g/d)} - 4$$

Percentage of nitrogen from proteins in foods ranges from 15-24%, thus 16% is used in the calculation of nitrogen content, resulting in a conversion factor of 6.25 g of nitrogen per g of protein (Richter et al., 2019). A constant of 4 represents 2 g of urinary non-urea nitrogen excretion (e.g., ammonia, uric acid, creatinine, and amino acids) and 2 g of incidental nitrogen losses (e.g., gastrointestinal, integumentary [dermal], and sweat) (Danielis et al., 2017).

Nitrogen balance is currently considered the gold standard for determining protein intake requirements (Darmaun & Rozé, 2008) and these studies have been conducted for decades. Some studies exist examining nitrogen balance in vegetarians (who consume dairy and/or eggs) or those eating a primarily plant-based diet with some animal flesh, however no known studies exist determining nitrogen balance in strict vegan participants, therefore this gap in knowledge should be filled (Rand et al., 2003). The purpose of this study was to determine nitrogen balance in vegans ingesting the protein RDA, 0.8 g/kg/d. It was hypothesized that vegans would exhibit a negative nitrogen balance in response to this diet, thus necessitating an amended, increased RDA for this population.

Project aims:

1. To determine nitrogen balance in vegan individuals following short-term, structured, eucaloric vegan diets at the protein RDA, 0.8 g/kg/d.

   Hypothesis 1: It was hypothesized that following five days of a eucaloric diet with a protein intake of 0.8 g/kg/d, vegans would have a negative nitrogen balance, significantly lower than zero balance.

2. To explore relationships between nitrogen balance in vegans with factors such as age, time as vegan, and fat free mass (FFM).

Delimitations:

1. This study was delimited to a convenience sample of inactive males living in the greater Phoenix, Arizona area.
2. This study was delimited to one protein intake (0.8 g/kg/d) and a five-day adaptation period.

Limitations:

1. Data are cross sectional; therefore, causal inferences cannot be determined.
2. Fecal and skin losses are estimated and not measured directly.
3. Participant compliance with the protocol (e.g., diet adherence, urine collection) cannot be guaranteed.

CHAPTER 2

REVIEW OF LITERATURE

Background

Vegetarianism can be defined as abstention from beef, pork, poultry, and fish. Sub-classifications include ovo-vegetarians who also consume eggs, lacto-vegetarians who consume dairy, and ovo-lacto-vegetarians who consume both eggs and dairy. Lastly, veganism is the strictest form of vegetarianism where adherents consume no animal products whatsoever (Appleby & Key, 2016). The prevalence of vegetarianism continues to grow with 5% of U.S. adults aged 18-34 years old identifying as vegetarian, with half of those being vegan (Stahler, 2020). This amounts to 3.8 million vegetarians, nearly 2 million of which are vegan. In other countries, such as India as much as 30% of the population is considered to be vegetarian due to religious reasons (Agrawal et al., 2014; Shridhar et al., 2014a). While the popularity of vegetarian diets in the U.S. has been linked to ethical and environmental concerns, the desire to live a healthier lifestyle was the most prominent reason for adopting vegetarianism (Hopwood et al., 2020).

Vegetarian Health

Following a vegetarian diet has shown mixed results in terms of various health benefits and risk factors for disease including cancer (Tantamango-Bartley et al., 2013; Key et al., 2009; Orlich et al., 2013; Key et al., 2014), CVD (Key et al., 1999; Kahleova et al., 2018; Kwok et al., 2014; Tonstad et al., 2009; Yokoyama et al., 2014; Tonstad et al., 2013; Kahleova et al., 2011; Barnard et al., 2006; Barnard et al., 1994; Barnard et al., 2009; Jenkins et al., 2003; Crane et al., 1994; Appleby & Key, 2016), metabolic syndrome (Yokoyama et al., 2014; Fraser, 2009; De Biase et al., 2007; Toohey et al., 1998; Berkow & Barnard, 2006; Jian et al., 2015; Wang et al., 2015; Yokoyama et al., 2017; Harland & Garton, 2016; Chuang et al., 2016; Appleby & Key, 2016; Orlich et al., 2013), and all-cause mortality (Appleby & Key, 2016).

Cancer

Cancer is the second leading cause of death in the U.S. and the number one cause of death worldwide (Ferlay et al., 2010). The National Center for Health Statistics estimates that there will be 1,806,590 new cancer cases in 2020 in the U.S., with a projected 606,520 mortalities (Siegel et al., 2020). Diet is estimated to account for 30% of cancer cases in first-world countries and 20% in developing countries with half of cancer cases being considered preventable (American Cancer Society, 2020). Research however concerning vegetarian diets and their effect on cancer prevention is equivocal (Tantamango-Bartley et al., 2013).

The EPIC-Oxford study was a prospective study following 63,550 vegetarians and meat eaters throughout the United Kingdom (UK) in the 1990s. Standardized incidence ratios (SIRs) were provided for meat eaters and vegetarian groups for various cancers. SIRs for all malignant cancers were significantly lower than the national average in nonvegetarians, vegetarians, and overall. Vegetarians had overall lower incidence of malignant cancers than meat eaters, however colorectal cancer was higher among vegetarians, though not significantly different than the national average. These results may have occurred due to the overall low smoking rate of the cohort, relatively low meat intake, and higher intake of fruits and vegetables in both groups. With lower meat intake than expected, coupled with higher fruit and vegetable consumption, this may explain the lower than average cancer incidence in the nonvegetarian group (Key et al., 2009).

In an updated analysis of their EPIC-Oxford study pooled with data from the Oxford vegetarian study (Appleby et al., 1999), Key et al. (2014) reported lower relative risks for certain cancers in vegetarians versus meat eaters. This included cancer of the stomach, lymphatic and hemapoietic tissue, multiple, and all sites combined. Again, smoking rates were low overall, and it was suggested that some cancers may be higher in those who consume or are even exposed to meat or livestock perhaps due to viral or other factors (Key et al., 2014).

In their collaborative analysis of five prospective studies examining overall mortality in nonvegetarians and vegetarians, Key et al. (1999) combined data from five studies including 76,172 vegetarians and nonvegetarians. After a mean 10.6-year follow-up, 8,330 deaths were reported. No significant differences in combined death rate ratios were seen in vegetarians compared to nonvegetarians for stomach, colorectal, lung, breast, and prostate cancers when adjusted for age, sex, and smoking status. Thus, based on this analysis it can be concluded that a vegetarian diet is not associated with a decreased risk in cancer mortality (Key et al., 1999).

The Adventist Health Study 2 (AHS2) examined the association between vegetarian diets and mortality in 73,308 Seventh Day Adventists from the Loma Linda, CA area. Mean follow-up time was 5.8 years with a total of 2,570 deaths during that period. Overall mortality rate was 6.05, 95% CI (5.82 to 6.29) deaths per 1,000 person years. The overall adjusted hazard ratio (HR) in vegetarians compared with nonvegetarians was significant for all-cause mortality. However, there was no reduced risk of cancer mortality in the vegetarian group compared to nonvegetarians. These data may not have reached significance due to insufficient power to detect a weaker association during follow-up. It is also interesting to note that when vegetarians were subdivided into specific groups, only the pesco-vegetarians showed a decreased HR, thus true vegetarians did not have a lower risk for cancer or all-cause mortality (Orlich et al., 2013).

Overall, it appears that vegetarian diets have little benefit in the reduction of cancer risk. However, it is proposed that increased fruit and vegetable intake and certain nutrients such as fiber and various antioxidants and phytochemicals as found in greater amounts in vegetarian diets may have a protective effect and reduce cancer incidence (Lanou & Svenson, 2011). Additionally, studies have shown a strong link between heterocyclic amines produced during the cooking of red meat and cancer incidence (Zheng & Lee, 2009). By definition, vegetarian diets would protect against this potential harm.

Metabolic Syndrome

Metabolic syndrome is defined as several metabolic abnormalities including hypertension, insulin resistance, central adiposity, and dyslipidemia and is associated with development of diabetes and CVD. Approximately 25% of the U.S. population has metabolic syndrome (O'Neill & O'Driscoll, 2015), which is caused by both genetic and acquired factors and is related to obesity (Rochlani et al., 2017).

A study examining the AHS2 cohort showed significantly lower blood pressure, glucose, body mass index (BMI), and waist circumference ($p < .05$) in vegetarians versus nonvegetarians when adjusting for age, sex, smoking, alcohol intake, ethnicity, physical activity, and energy intake. The prevalence of metabolic syndrome was lower in vegetarians (25.2%) compared to nonvegetarians (39.7%). Odds ratios (OR) for metabolic syndrome were lower in vegetarians (0.44) compared to nonvegetarians (Rizzo et al., 2011).

Another cross-sectional study of a Chinese population showed significantly lower BMI, systolic blood pressure (SBP), diastolic blood pressure (DBP), and fasting blood glucose, among other measures in vegetarians versus meat-eaters. This was attributed mostly to dietary factors, such as high fruit and vegetable intake, as well as grains and soy foods. The authors distinguished this from a Western vegetarian diet which is typically higher in nuts, seeds, dairy, and eggs than that of the traditional Chinese diet (Yang et al., 2012).

A recent meta-analysis of 71 studies of various designs (randomized control trial [RCT], cross-sectional, and cohort) determined that RCTs and cohort studies showed no association between vegetarian diets and metabolic syndrome components. Cross sectional studies showed a decreased risk for SBP, DBP, fasting glucose, waist circumference, and high-density lipoprotein (HDL), however the authors noted that there was high risk of bias and high heterogeneity of effects across studies, therefore concluding that cross sectional studies also show an uncertain association with vegetarian diets and metabolic syndrome components or metabolic syndrome overall (Picasso et al., 2019).

Overall, metabolic syndrome is suggested to occur in people with high BMI and low fruit and vegetable intake, the latter indicating low fiber and antioxidant intake, which contributes to reduced protection against systemic inflammation (Turner-McGrievy & Harris, 2014). Research suggests that systemic inflammatory markers should be included in defining metabolic syndrome (Marsland et al., 2010).

Cardiovascular Disease

CVD is the leading cause of death in the U.S. with approximately 647,000 deaths reported each year (Benjamin et al., 2019). CVD is characterized as coronary artery disease, stroke, peripheral artery disease, and aortic atherosclerosis (Olvera-Lopez et al., 2020). Risk factors for CVD include high plasma cholesterol and hypertension (Appleby & Key, 2016). Much research investigating the relationship between vegetarian diets and cardiometabolic disease has been conducted (Thorogood et al., 1987; Bradbury et al., 2014; Pettersen et al., 2012; Shridhar et al., 2014b).

An early study investigating cholesterol levels in vegetarians and meat eaters participating in a long-term prospective study found that vegans had significantly lower total cholesterol and low-density lipoprotein (LDL) cholesterol than both vegetarians and meat eaters with no difference in HDL cholesterol between diet groups when adjusted for age, sex, and LDL:HDL concentration (Thorogood et al., 1987). Controlling for LDL:HDL is important as this is a greater predictive risk indicator than HDL or LDL levels alone (Millán et al., 2009). The authors concluded from this study that both vegans and vegetarians have a lower incidence of CVD versus meat eaters (-57% and -24% respectively) (Thorogood et al., 1987). This is likely due to the lower intake of saturated fat, and higher mono- and polyunsaturated fatty acids, fiber, and complex carbohydrate content of vegetarian diet, which can lead to lower LDL levels.

A more recent cross-sectional study of the EPIC-Oxford cohort subsample of 3,358 participants examined lipid concentrations between vegetarians and meat eaters. Results showed significantly lower LDL in both vegans and vegetarians compared to meat eaters ($p < .001$) as well as lower non-HDL ($p < .001$). The authors attribute this to very low reported saturated fat and high polyunsaturated fat and fiber intakes in vegans. They concluded that vegans would have a much lower risk for ischemic heart disease (IHD) mortality compared to meat eaters (Bradbury et al., 2014).

A cross-sectional examination of blood pressure in 500 participants from the AHS2 cohort showed significantly lower SBP in vegans ($p < .05$) and vegetarians ($p < .001$) compared to meat eaters. DBP results were similar with vegans ($p < .001$) and vegetarians ($p < .001$) being lower than meat eaters. After adjustment for BMI, results were similar, however slightly attenuated. When examining ORs, vegetarians had lower risk than meat eaters. Thus, hypertension risk is reduced in vegans and vegetarians (Pettersen et al., 2012).

A study conducted in India paired vegetarian participants with a sibling. Researchers found significantly lower levels of SBP ($p = .07$) and DBP ($p = .02$) in vegetarians compared to their meat-eating siblings. While statistically significant, these differences are small (SBP -0.9 mmHg; DBP -0.7 mmHg) and unlikely to be clinically significant (Shridhar et al., 2014b).

One proposed mechanism that might explain some benefit of vegetarian diets is increased dietary nitric oxide. Normally produced in the cardiovascular system of mammals, nitric oxide helps regulate blood pressure, inflammation, and oxidative stress. Low nitric oxide levels have been shown to cause CVD. The functional form of nitric oxide is found naturally in vegetables such as leafy greens and beets, which tend to be higher in a healthful vegetarian diet, therefore increasing nitrate levels and potentially decreasing the risk for CVD (Bryan, 2018; Lanou & Svenson, 2011).

All-cause Mortality

All-cause mortality was shown to be decreased in people following a healthy plant-based diet in a sample of 11,879 where participants were categorized as following a healthy or unhealthy plant-based diet. They were then further categorized into quintiles based upon quality of diet. The healthy plant-based participants scoring above the median showed a non-linear association with lower risk of all-cause mortality. This study concluded that people with higher plant food intake had less all-cause mortality over a nine-year follow-up. The authors of this study however do not appear to distinguish between plant-based and vegetarian diets. It seems as though their definition of plant-based was somewhat blurred and may have included individuals who eat small amounts of meat or animal foods (Kim et al., 2018).

A meta-analysis by Kwok et al. (2014) found reduced risk of all cause mortality in vegetarian Seventh Day Adventists in a pooled analysis of seven different cohorts. Interestingly, they did not see any reduction in all-cause mortality in non-Seventh Day Adventist vegetarians. This suggests that other possible lifestyle factors exhibited by Seventh Day Adventists such as possible increased physical activity, diet quality, and low smoking and drinking rates may also play a role in this reduction, thus studies using this population should control for these and other possible factors (Kwok et al., 2014).

Contrary to the Kim at al. (2018) study, evidence from the 45 And Up study examining a cohort of 267,180 Australian participants found no significant differences in all-cause mortality after extensive adjustment for confounding factors. The authors were critical of other studies, including those using Seventh Day Adventists citing the potential for selection bias in groups with a high prevalence of vegetarianism (Mihrshahi et al., 2017).

Given the previous evidence, it seems that many claims of health benefits of vegetarian diets may be overstated, though some benefits do exist. Regardless, with a growing population of vegetarians and vegans, it is important to investigate nutritional considerations to provide information for adequate intake for this population.

Vegetarian Nutrition

Background

In their 2016 position stand, the Academy of Nutrition and Dietetics stated that "…appropriately planned vegetarian, including vegan, diets are healthful, nutritionally adequate, and may provide health benefits in the prevention and treatment of certain diseases. These diets are appropriate for all stages of the life cycle, including pregnancy, lactation, infancy, childhood, adolescence, older adulthood, and for athletes." (Melina et al., 2016). Despite this, concern remains over potential inadequacies of certain nutrients including protein (Kniskern & Johnston, 2011), vitamins B_{12} and D, calcium, iron, and zinc, among others (Melina et al., 2016). This section will address these key nutrients.

Dietary Reference Intakes

Dietary reference intakes (DRI) are comprised of four categories: estimated average requirement (EAR), RDA, adequate intake (AI), and tolerable upper intake level (UL). The DRIs are a relatively new set of standards, being developed in 1994 and replacing the exclusive use of RDAs, which had dated back to the 1940s (Trumbo et al., 2002; Otten et al., 2006).

EARs are defined as the daily intake of a nutrient that meets the requirement of half the individuals in an age or sex group. It serves three purposes, a) as the basis for the RDA; b) to assess adequacy of nutrient intakes in different groups; and c) planning for the intake of groups. RDAs estimate the minimum daily intake level that meets requirements for nearly all (97-98%) healthy people in an age or sex group. This intake is said to have a high probability of meeting nutrient requirements in any chosen individual. AI is used when sufficient data are lacking to determine an EAR, and therefore an RDA, thus there is much less certainty when using an AI versus RDA. It is however expected that the AI will meet or exceed nutrient requirements in all members of a healthy population. The UL is the highest intake level that is likely to pose no risk of ill effects in most members of an age or sex group (Trumbo et al., 2002; MacFarlane et al., 2019).

DRIs are all determined by life stage and clustered into groups based on age; infancy, toddlers, early childhood, puberty/adolescence, young adulthood, middle aged adults, adulthood, and older adults. There are also recommendations for pregnancy and lactation. DRIs are developed using evidence from animal models, human feeding studies, observational studies, and randomized clinical trials. There are various methods for determining requirements for all age groups, but for the purpose of this review of literature, the focus will be on determining requirements for adults (Atkinson, 2011; Institute of Medicine, 2000).

When determining DRIs, first the EAR must be determined. EARs are estimated using available evidence from the aforementioned study designs. Once the EAR has been determined, this value can be used to calculate the RDA. When data for distribution of a requirement among individuals in a group is normally distributed, the following computation can be used:

$$\text{RDA} = \text{EAR} + 2 \times \text{SD}_{\text{requirement}}$$

If standard deviation (SD) cannot be determined, yet data are still normally distributed, the coefficient of variation (CV) of 10% can be used:

$$\text{RDA} = \text{EAR} + 2\ (0.1 \times \text{EAR}) = 1.2 \times \text{EAR}$$

This 10% CV is assumed based on variation in basal metabolic rate. When adequate data are available, the RDA is the preferred method of estimating sufficient nutrient intake. AI is often determined from data on a single study, estimated dietary intakes in a population, or on a review of data. When analyzed alone, these studies do not supply sufficient evidence to provide an EAR, thus RDA cannot be determined. AIs may be different than RDAs for a nutrient; oftentimes higher, so special care must be given when using AIs rather than RDAs. The UL is based on risk assessment data for various nutrients and provides guidance for supplementation and/or fortification of nutrients in foods. Intakes above the UL may be toxic and potentially cause harm; however, not all nutrients have a UL indicating that risk associated with high intakes is not evident (Trumbo et al., 2002).

The acceptable macronutrient distribution range (AMDR) is the percentage of each energy constituent, or macronutrient, recommended for adequate energy intake and a healthful diet. Though AMDRs do not fall under the DRI grouping, they are considered important for dietary planning (Trumbo et al., 2002; Lee et al., 2015). For protein, the AMDR is 10-35% of total energy intake, for carbohydrate 45-65%, and fat 20-35% (Wolfe et al., 2017; Julibert et al., 2019; Otten et al., 2006). The primary interest of this literature review and research will be on protein consumption, so the main scope of this review will focus upon this in regard to macronutrients.

DRI - Protein

Proteins are made up of 20 amino acids, nine of which are considered essential, since the body cannot synthesize them, with the rest being nonessential. Essential amino acids must be taken in through the diet. Animal-based foods such as meats, dairy, and eggs are considered complete protein sources since they contain adequate amounts of the nine essential amino acids. Plant-based or vegan sources of protein such as legumes, grains, nuts, and seeds typically lack one or more essential amino acid in adequate amounts and therefore are considered incomplete (Gardner et al., 2019). Protein is unique in that its amino acids contain a nitrogen molecule, whereas the other macronutrients, fat and carbohydrate do not contain nitrogen (Otten et al., 2006). Amino acid levels are determined using isotopic tracer methods where amino acids are tagged using radioactive and stable isotopes that are detectable as they pass through or are distributed throughout the body (Wolfe, 2011). The EAR for protein for non-exercising adults aged 19 years and older is 0.66 g/kg/d, while the RDA is 0.80 g/kg/d. These recommendations are based on the results of numerous nitrogen balance studies, in which nitrogen equilibrium is the criteria for determining protein requirements (Otten et al., 2006). This will be discussed in detail later in this review of literature. This RDA however is purported to extend to vegetarians and vegans, although there is the assumption that at least 10% of intake includes animal foods of some sort. Furthermore, the RDA does not consider bioavailability or protein quality and therefore does not truly represent this population, especially those who follow a vegan diet (Rand et al., 2003). As of 2006, it was reported that no evidence exists to support a separate protein requirement for vegetarians and vegans, even though it is mentioned that these diets are low in lysine, threonine, and the sulfur amino acids (Otten et al., 2006). Further, it has been suggested that protein digestibility scores may be as much as 30% lower for plant-based foods due to inadequate amounts of essential amino acids in most plant based sources. (Kniskern & Johnston, 2011; Trumbo et al., 2002).

DRI - Vitamins – A, B_{12}, D

Several vitamins are of special concern to vegetarians and especially vegans. For example, vitamin A is a fat-soluble vitamin and preformed vitamin A is found primarily in animal foods such as liver, dairy, and fish. Provitamin A carotenoids which can be converted to vitamin A are found in plant-based sources such as broccoli and spinach as well as orange-colored foods like carrots and squash. Vitamin A plays a key role in vision, gene expression, and immune function, among others. Some functions include helping to produce neural signals for sight and coding for structural proteins and enzymes. It is absorbed via the small intestine and stored primarily in the liver. Absorption from preformed vitamin A is much higher (70-90%) than from provitamin A carotenoids (9-22%). DRIs for vitamin A are measured in retinol activity equivalents (RAE), which are complicated due to the various forms of vitamin A available in the diet. The RDA is 700 μg RAE/d for females and 900 μg RAE/d for males. Deficiency can cause xerophthalmia, or dryness of the eyes. Special care should also be taken as absorption of vitamin A competes with absorption of iron, zinc, and alcohol (Otten et al., 2006; Trumbo et al., 2002). Research suggests that plant-based diets are linked to lower intakes of vitamin A (Cifelli et al., 2016). For vegetarians and vegans, though no special recommendations exist, it is advised that people consume adequate amounts of deeply colored cooked fruits and vegetables as cooking enhances bioavailability. Lastly, fortified foods such as plant-based beverages, cereals, and margarines often contain vitamin A (Otten et al., 2006; Trumbo et al., 2002; Ross, n.d.).

Vitamin B_{12} is found primarily in animal foods such as liver and fish. Plant-based foods contain no naturally occurring B_{12}, thus vegetarians and vegans must take care to eat fortified foods or take a supplement to ensure adequacy. Significant reductions in vitamin B_{12} status occur in as little as four weeks of adherence to a vegan diet (Lederer et al., 2019), and B_{12} deficiency is reported among vegan populations that do not supplement (Selinger et al., 2019). B_{12} plays a key role in blood formation and neural function (Kennedy, 2016). Its main function is as a coenzyme that converts homocysteine to methionine in the metabolism of fats and amino acids. It is absorbed via an active process, which requires intrinsic factor from the stomach. Once combined with intrinsic factor, it is absorbed in the stomach and small intestine and stored primarily in the liver. DRIs for vitamin B_{12} are in the form of RDAs at 2.4 µg/d. Deficiency can cause neurological complications (Langan & Goodbred, 2017) and is linked to the potentially fatal condition, pernicious anemia. Special care should also be taken as absorption of vitamin B_{12} may be affected by folate, however evidence for this is insufficient. For vegetarians and vegans, though no special recommendations exist, it is advised that fortified foods are eaten, with possible use of supplemental B_{12} (Otten et al., 2006; Trumbo et al., 2002).

Vitamin D is found primarily in fatty fish, some eggs, and fortified foods. It is also synthesized by the body through exposure to sunlight (Jungert et al., 2014). It plays a key role in bone health where its main function is to aid in the absorption of calcium and phosphorus. It is absorbed via the small intestine and eventually lymphatic system and stored in the liver before entering the circulation (Nair & Maseeh, 2012; Maurya & Aggarwal, 2017). DRIs for vitamin D are in the form of AIs and 5 μg/d for adults less than 50 years old is recommended. Deficiency can cause demineralization of the skeleton or decreased bone accretion in order to keep circulating calcium levels high for other needed functions. Although no special recommendations exist and serum vitamin D concentrations are not associated with vegetarian status (Chan et al., 2009), it is advised that vegetarians and vegans have adequate exposure to sunlight and consume foods fortified with vitamin D (Otten et al., 2006; Trumbo et al., 2002).

DRI - Minerals Ca, Fe, Zn

Like vitamins, several minerals are also of concern to vegetarians and vegans. Calcium is found primarily in dairy foods, plant foods such as kale and broccoli, and fortified foods and plays a key role in bone health (Cormick & Belizán, 2019). It is also important for neural, vascular, and glandular functions. It is absorbed via active transport and passive diffusion in the small intestine and stored almost exclusively in the skeleton and teeth (Power et al., 1999). DRIs for calcium are in the form of an AI, since adequate evidence for an EAR is not available. Therefore, the AI for calcium is 1000 mg/d for adults < 50 years old. Interestingly, as calcium intake increases, absorption decreases, and vice versa (Cormick & Belizán, 2019). Deficiency can cause resorption of skeletal calcium, eventually leading to osteopenia and osteoporosis (Beto, 2015). Special care should also be taken as calcium absorption may be inhibited by caffeine, magnesium, phosphorus, protein, sodium, and oxalic and phytic acid. For vegetarians and vegans, though no special recommendations exist, it is advised to be mindful of intake, as many plant foods contain oxalic and phytic acid, which can severely inhibit its absorption

(Otten et al., 2006; Trumbo et al., 2002). Evidence suggests that both vegans and vegetarians may be at risk for lower bone mineral density and fracture, likely related to low intakes of vitamin D and calcium and higher circulating markers of bone turnover (Tucker, 2014; Hansen et al., 2018).

Iron is found in two forms, heme, and non-heme iron. Heme iron is better absorbed by the body, whereas non-heme iron tends to be poorly absorbed, thus requiring greater intakes in those who do not consume animal flesh. Heme iron is found exclusively in animal flesh foods such as meat, poultry, and fish. Non-heme iron is found in dairy, eggs, and plant foods such as whole grains and cereals and various fruits and vegetables (Abbaspour et al., 2014). It plays a key role as a component in hemoglobin, myoglobin, cytochromes, and enzymes, where its main function is oxygen transport. It is absorbed via the small intestine and two-thirds are incorporated in the hemoglobin of red blood cells. It is also stored in the liver, spleen, and bone marrow (Geissler & Singh, 2011). RDAs for iron are 8 mg and 18 mg/d for males and females, respectively for adults < 50 years old. Deficiency can cause iron deficiency anemia. Special care should also be taken as phytates, polyphenols, calcium, and vegetable proteins inhibit absorption of iron. Vitamin C increases the absorption of iron. About 25% of heme iron taken in is absorbed whereas only 10% of non-heme iron is absorbed; a concern in vegetarians and vegans. For vegetarians and vegans, special recommendations exist; it is advised that they consume 1.8 times the RDA to account for lower bioavailability (Otten et al., 2006; Trumbo et al., 2002). In one trial, low iron stores were more prevalent among vegetarian women in comparison to omnivore women (29% vs. 17%), but rates of iron-deficiency anemia were similar for these populations (≈ 3%) (Donovan & Gibson, 1995).

Zinc is found primarily in red meat, fish, whole grains, and fortified foods (Rangan & Samman, 2012). It plays a key role in enzymatic function where it is important in metabolism of protein, carbohydrate, and fat. It is absorbed via the small intestine and stored in the skeletal muscle and bone (Roohani et al., 2013; Gammoh & Rink, 2017). RDAs for zinc are 11 mg and 8 mg/d for adult males and females, respectively (Saper & Rash, 2009). Although zinc deficiency is relatively rare, data do suggest that vegetarians have lower serum zinc concentrations than their omnivore counterparts (Foster & Samman, 2015). Special care should be taken as absorption of zinc competes with absorption of iron, calcium, phosphorus, protein, phytic acid, and fiber (Rangan & Samman, 2012). For vegetarians and vegans, though no special recommendations exist, it is advised that vegetarians consume up to 50% more dietary zinc, as bioavailability is poor and dietary sources often contain high levels of phytates, which inhibit absorption (Otten et al., 2006; Trumbo et al., 2002).

Protein

Background

Proteins are made up of long chains of amino acids in various sequences to form macromolecules with specific functions. As mentioned previously, there are 20 different amino acids in the body, nine of which are considered essential because the body cannot synthesize them and they must be obtained through the diet. These are histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, and valine (Hou & Wu, 2018). Some plant-based sources of these amino acids are peas, soy, legumes, rice, quinoa, nuts, and seeds, among others (Mariotti & Gardner, 2019; Berrazaga et al., 2019). Of the non-essential amino acids, six of these are considered conditionally essential, meaning that in healthy people the body can synthesize them, however in certain pathological conditions they must be obtained through diet as well. The conditionally essential amino acids are arginine, cysteine, glutamine, glycine, proline, and tyrosine (Dioguardi, 2011; Otten et al., 2006). The remaining non-essential amino acids are alanine, aspartic acid, asparagine, glutamic acid, and serine (Reeds, 2000). Nitrogen is a defining constituent of amino acids not present in fats or carbohydrates, and comprises 16% of the weight of proteins (Otten et al., 2006; Richter et al., 2019; Watford & Wu, 2018).

Examples of various functions of proteins include enzymes, antibodies, messengers, transport and storage, and lastly structural functions (National Library of Medicine, 2020; Otten et al., 2006). Enzymes catalyze chemical reactions in the body and also bind to receptors signaling the building or breakdown of various other molecules. An example of this would be salivary amylase and its function of breaking down starch into simple sugars during digestion. However, much of the amylase required by the body is produced in the pancreas. (Peyrot des Cachons & Breslin, 2016). Antibodies such as immunoglobulins A, D, E, G, and M have differing functions (Chiu et al., 2019). For instance, IgEs help protect the body by binding to viruses and bacteria and facilitating their removal (Kelly & Grayson, 2016). Messenger proteins often come in the form of hormones, such as growth hormone which when bound to its specific receptor, regulates cell growth in the body (Campbell, 1997). Transport proteins like hemoglobin move other substances such as oxygen around the body. Structural proteins are a major component of every cell in the body. Actin provides structure to muscle cells and also participates in muscle activity, such as lengthening and shortening of the sarcomere. Another important structural protein is collagen, which makes up 25-30% of the protein in the body (Kirmse et al., 2019). It provides strength and cushioning to body tissues, and is found in ligaments and tendons, cartilage, and organs, among other places, where proline and glycine are some of the main amino acids found in its structure (Shoulders & Raines, 2009; National Library of Medicine, 2020).

Protein Intake

Protein taken in through the diet is eventually hydrolyzed and broken down to its amino acid constituents (Carbone & Pasiakos, 2019). In the stomach, proteins are denatured and cleaved into smaller peptides by the enzyme pepsin. In the small intestine these peptides, some of which are biologically active, are broken down into amino acids (Jahan-Mihan et al., 2011). During digestion, proteins and peptides may have a variety of functions in the gastrointestinal tract, including metabolic signaling, enzyme regulation, and controlling absorption of nutrients (Jahan-Mihan et al., 2011). Amino acids are then primarily passed into the blood where they eventually end up in the liver and are used or sent out to other peripheral tissues. Skeletal muscle makes up 43% of the proteins in the body, while skin and blood make up another 15%. Most of the body's protein is in the form of actin, myosin, collagen, and hemoglobin. Waste products of nitrogen metabolism are creatinine, urea, ammonia, and uric acid, which are excreted in the urine and feces (Wrong, 1978; Wright, 1995; Otten et al., 2006). Other losses are through sweat, hair, and fingernails. Because of this, proteins are constantly being broken down and resynthesized, a process termed protein turnover. Total body protein turnover is about 5.7 g/kg/day (Poortmans et al., 2012), and most released amino acids are recycled for protein synthesis. In cases where there is no excess growth or breakdown, protein is at a steady state, or net balance (Vogel & Marcotte, 2012). When an individual takes in inadequate amounts of protein or essential amino acids, a net negative balance occurs. Most protein turnover or loss occurs outside of the skeletal muscle, with only 25% contributed by the muscle tissue (Otten et al., 2006).

The caloric value of one gram of protein is approximately four Calories or kilocalories (kcal) (National Research Council (US) Committee on Diet and Health, 1989). Current RDAs for protein are 0.8 g/kg/d, which are based on analysis of nitrogen balance studies (Rand et al., 2003; Carbone & Pasiakos, 2019; Otten et al., 2006). While excess dietary protein has been shown to have low risk of adverse effects in healthy people (Phillips et al., 2016), low dietary protein can lead to many complications due to reductions in protein synthesis, which affects all organs and bodily systems. Furthermore, dietary proteins are not equal in providing for protein synthesis due to differing amino acid profiles and bioavailability. Generally, animal-based proteins possess more favorable amino acid profiles and bioavailability than plant-based proteins, and thus stimulate a higher degree of protein synthesis in vivo. One study from the lab of Stu Phillips showed that soy protein was inferior to whey for inducing muscle protein synthesis at rest as well as following resistance exercise in young, healthy men (Tang et al., 2009). Further work from the lab of Luc van Loon showed that casein protein produced greater myofibrillar protein synthetic response versus the same amount of wheat protein in healthy older adults and greater amounts of wheat protein were required to increase synthetic rates (Gorissen et al., 2016).

Protein energy malnutrition (PEM) contributes to the deaths of over 6 million people worldwide each year, mostly children in developing countries. In industrialized countries, PEM is mostly seen in clinical patients or the elderly. Deficiency of protein affects all of the body's organs and many systems. This includes the brain, immune system, digestive, and kidney function. Physical manifestations of PEM include failure to thrive in young children, weakness, edema, and fragile hair. Biochemically this is best determined through nitrogen balance testing. Formerly, examination of serum, pre-albumin and/or albumin were used, however, these are no longer used as measures of whole body protein status, as the consensus is now that they are markers of inflammation, rather than PEM and nutrition status. (Otten et al., 2006; Evans et al., 2021).

Animal and Plant Protein

Plant proteins, particularly cereal grains are the primary source of amino acids in many parts of the world. In developed countries meat and legumes are also widely consumed (Cervantes-Pahm et al., 2014). As mentioned previously, animal sources of protein such as meat, fish, dairy, and eggs are considered complete, in that they contain all nine essential amino acids. Plant proteins however, are often lacking or low in one or more essential amino acids, such as lysine, threonine, methionine, and the conditionally essential cysteine (Otten et al., 2006; Brosnan & Brosnan, 2006). Examples of plant protein foods include soy foods, legumes, and grains. The soybean is quite versatile and can be eaten whole as edamame and is often used in milk and meat analogues as well. Of the plant proteins, soy is the only source that is considered a high-quality protein, based on nitrogen balance studies in humans (Istfan et al., 1983; Young et al. 1984). Young et al. (1984) compared two normally omnivorous groups; both receiving 0.8 g/kg/d protein, one exclusively from beef, the other exclusively from soy protein isolate. Both groups remained in net nitrogen balance over the course of the 84-day study (Young et al., 1984). This study, and others using primarily plant proteins did not use vegan participants; however, at least one group in each study received all of their amino acids from plant-based sources for the duration of these short-term studies (Istfan et al., 1983; Nicol & Phillips, 1976; Atinmo et al., 1988).

Other plant sources of protein generally lack or contain insufficient amounts of a particular amino acid, such as the case of lysine and threonine in rice, which is considered a low-quality protein (Jiang et al., 2016). Also importantly, plant foods are known to contain anti-nutrients such as tannins and phytates. Tannins can inhibit digestive enzymes, while phytates can bind to proteins in the gut, thus reducing protein digestibility (Cirkovic Velickovic & Stanic-Vucinic, 2018; Kumar et al., 2021). Because of this, people who rely entirely on plant-based protein sources may require a greater total protein intake to compensate for the inferior digestibility of these proteins due to these anti-nutrients. Luckily, some preparation methods such as milling, cooking, soaking, sprouting, and fermentation can reduce these anti-nutritive properties (Hotz & Gibson, 2007).

It was long thought that vegetarians and vegans must combine different complementary protein sources in the same meal (Lappé, 1981; Young & Pellet, 1994); however, more recently experts do not believe this is necessarily the case. In individuals who eat a varied diet throughout the day, and caloric requirements are met, the proteins and amino acids taken in through the diet contribute to the intra- and extracellular amino acid pools. This pool of necessary amino acids can be drawn from, when needed across the day (Melina et al., 2016; Liu & Barrett, 2002).

Endogenous protein formation and breakdown occurs constantly with daily amino acid turnover amounting to ≈ 300 g (Tomé & Miller, 2000). Percentage of nitrogen from proteins in foods ranges from 15-24%, thus 16% is used in the calculation of nitrogen content, resulting in a conversion factor of 6.25 g of nitrogen per g of protein (Richter et al., 2019). Unlike carbohydrate and fat, the storage pool for protein (i.e., muscle tissue) is not oxidized as readily. In a healthy person, protein contributes only 15-20% towards fuel oxidation, primarily as amino acids through gluconeogenesis. Hence, fat and carbohydrate provide the majority of the daily energy needs while amino acids are used to build structural, enzymatic, and signaling proteins. Amino acids are acquired through dietary intake, or recycling of catabolized proteins, maintaining protein homeostasis such that the nitrogen ingested is equal to the nitrogen excreted (e.g., 'nitrogen balance'). However, during periods of growth or anabolism, amino acid requirements are elevated above maintenance levels creating a positive nitrogen balance in the body. Eventually, excess amino acids are converted to urea and ammonia in the liver, and excreted, primarily in the urine (Liu & Barrett, 2002). Nitrogen balance, the net synthesis and breakdown of proteins, can be estimated using the equation below where UUN is measured in urine collected over a period of 24 hours (Dickerson, 2005b).

$$\text{Nitrogen Balance (g/d)} = (\text{PRO intake (g/d)}/6.25) - \text{UUN (g/d)} - 4$$

A constant of 4 represents 2 g of urinary non-urea nitrogen excretion (e.g., ammonia, uric acid, creatinine, and amino acids) and 2 g of incidental nitrogen losses (e.g., gastrointestinal, integumentary [dermal], and sweat) (Danielis et al., 2017). Obligatory nitrogen losses are estimated at 54 mg/kg body weight for adults, with 34 mg/kg coming from urinary losses, which in a 77 kg person, accounts for approximately 4g (Munro, n.d.). To convert back to protein content of the urine, the following equation is used:

$$\text{Urinary PRO} = \text{Nitrogen Balance (g)} \times 6.25$$

This can then be compared to protein intake. Urea is the primary product of nitrogen metabolism and the main form of urinary nitrogen (Matthews & Downey, 1984; Dahms et al., 1989).

Protein Measurement Methods

Several methods of measuring protein exist including protein digestibility corrected amino acid scores (PDCAAS), digestible indispensible amino acid scores (DIAAS), and nitrogen balance. Both PDCAAS and DIAAS take into account digestibility and essential amino acid content, whereas nitrogen balance measures and compares protein intake and protein turnover in the body (Tomé & Miller, 2000; Mathai et al., 2017). The PDCAAS method was adopted by the World Health Organization (WHO), United States Department of Agriculture (USDA), and the Food and Agriculture Organization (FAO) in 1991 and was the standard used for the current protein and amino acid recommendations, however it has now been replaced by the DIAAS method. PDCAAS involves measurement of total gastrointestinal tract digestibility of crude protein in rats – something that has been scrutinized as this assumes all amino acids have the same digestibility. Other criticisms of this method include the fact that fecal samples are used taking into account total tract digestibility when it is known that amino acid absorption occurs at the ileum of the small intestine. Another limitation is that PDCAAS values are truncated at 1.00 so as to avoid values greater than 1.00, or 100% for high quality proteins egg, milk, and sometimes soy (Mathai et al., 2017; Rizzo & Baroni, 2018). This limits identification of higher quality proteins that may have values greater than 100%. Lastly, it was recently shown the PDCAAS tend to overestimate the value of low-quality proteins as well as underestimate the value of high quality proteins (Rutherford et al., 2015). PDCAAS values are determined by examining the relative nitrogen levels in the feces compared to those ingested, corrected for endogenous losses. Foods with lower coefficients of nitrogen digestibility determined in the feces and/or lower levels of essential amino acids will yield lower PDCAAS values and are considered of lower quality protein if the amino acid profile of the foods ingested is known (Marinangeli & House, 2017).

The DIAAS method was adopted in 2013 by the FAO and is now recognized as a better model for determining amino acid requirements for humans. This method determines the amino acid digestibility at the ileum in pigs, a better model for translation to humans than rats, as used in PDCAAS determination (Mathai et al., 2017). DIAAS are calculated by determining the disappearance of amino acids at the end of the ileum as compared to the amino acids ingested. These values are termed standard ileal digestibility and are used to calculate DIAAS in proteins (Cervantes-Pahm et al., 2014; Mathai et al., 2017). Like PDCAAS, this requires knowledge of the protein content and essential amino acids of the ingested food. When a mixed meal with multiple protein containing components is ingested, the sum of amino acids digested from each ingredient can be determined. Unlike PDCAAS, DIAAS values are not truncated and can exceed 100. This value indicates the DIAAS of a food as a percentage (Marinangeli & House, 2017; Munro, n.d.). DIAAS values ≥ 75 are considered good protein scores, while scores ≥ 100 are considered excellent or high-quality protein. For example, a score of 115% meets the daily requirement for the most limiting amino acid in a protein. A lower score of 60% means that 60% of the daily requirement is met with the ingestion of 0.66 g/kg/d of the test protein (Wolfe et al., 2016). DIAAS scores for whole wheat and brown rice are 20% and 42% respectively (Han et al., 2019), whereas scores for milk protein concentrate and soy protein isolate are 141% and 98%, respectively (Mathai et al., 2017).

Nitrogen balance reflects net protein metabolism, or the difference between protein intake and excretion. It does not provide information regarding protein stores or nutritional status, but rather a global assessment of protein balance (Moizé et al., 2017; Gibson, 1990). A negative nitrogen balance (e.g., nitrogen excreted > nitrogen ingested) may be brought on by inadequate protein or energy intake and/or poor amino acid quality of ingested foods. Malnourished individuals such as those with PEM or those experiencing chronic starvation will always have a negative nitrogen balance, as amino acids are being oxidized for fuel, thus more nitrogen is excreted as urea (Moizé et al., 2017). For example, chronic kidney disease patients often experience a negative energy balance, leading to protein catabolism, which leads to a negative nitrogen balance and muscle wasting (Zha & Qian, 2017). This may also affect skeletal muscle mass via decreased protein synthesis (Moizé et al., 2017). Nitrogen losses may also occur due to metabolic products of stress such as cortisol and epinephrine and their catabolic effects. Breakdown of skeletal muscle provides amino acids for gluconeogenesis as mentioned previously and the nitrogen moiety is excreted as urea. During periods of growth, the body will be in a positive nitrogen balance to support the synthesis of new protein tissues. A normal healthy adult consuming adequate protein and energy will be in net nitrogen balance, or equilibrium. The nitrogen balance method is based on feeding participants a series of diets containing different levels of nitrogen (as protein) and measuring the excretion of nitrogen in the urine (Bender, 2003). This is currently considered the gold standard for determining protein intake requirements (Darmaun & Rozé, 2008) and these studies have been conducted for decades. Despite this, no known studies exist determining nitrogen balance in strict vegan participants. Only a few studies exist examining nitrogen balance in vegetarians or those eating a primarily plant-based diet with some animal flesh, therefore this gap in knowledge should be filled (Rand et al., 2003).

Nitrogen Balance Methods

Methods of Assessing Nitrogen

Because UUN does not achieve a steady state immediately following changes in nitrogen intake, a lead in or adaptation period is needed prior to assessment in order to allow adjustment of UUN to the new, consistent protein intake. This consists of four days to three weeks of a known diet and protein intake, followed by a 24-hour complete urine collection (Bender, 2003; Margolis et al., 2016; Rand et al., 2003). Urine can then be analyzed via photometric assay to determine nitrogen content while the protein composition of the diet can be determined and nitrogen calculated as above. Nitrogen balance status can then be determined.

Though these methods are the currently accepted standard for measurement of nitrogen excretion and balance, they are not without shortcomings. Foremost is that non-UUN losses such as ammonia, creatinine, and fecal losses are estimated using a coefficient of 4 g/d. This can sometimes lead to an underestimate of UUN. While it is possible to measure fecal losses, this is often unnecessarily burdensome on the participant; therefore, most studies estimate these losses (Dickerson et al., 2005a). Additionally, due to the strict dietary control needed to measure nitrogen balance, a lack of long-term studies exist making it difficult to determine a true protein requirement (Bender, 2003; Rand et al., 2003). These studies can be expensive to perform, as all food must be provided to participants for the duration of the study. Additionally, food may be lost to cooking and utensils, leading to an overestimate of intake (Kopple, 1987). Because a complete 24-hour urine collection is needed, some participants in outpatient settings may find this burdensome so participant attrition or compliance may pose retention problems. Similarly, outpatient participants may find adherence to prescribed diets difficult and deviate from this. Furthermore, a negative nitrogen balance provides no information regarding the mechanism involved in a negative balance. It may be due to decreased protein synthesis, increased breakdown, or a combination thereof. Therefore, studies using stable isotope tracers or DIAAS studies would provide further information (Darmaun & Rozé, 2008).

Nitrogen Balance to Determine RDA

In 1985, the FAO/WHO/United Nations University (UNU) Expert Consultation on Energy and Protein Requirements was published. This report used data from nitrogen balance studies of both short and long-term durations to calculate a mean protein requirement for adults. They determined an EAR of 0.6 g/kg/d for consumption of high quality proteins – animal proteins such as meat, egg, and dairy (Rand et al., 2003). In their 2007 update, the FAO/WHO/UNU provided a RDA of 0.83 g/kg/d for protein intake of proteins with a PDCAAS value of 1.0. An equation for correction of protein quality was also given:

Total available protein in the diet = Total protein x PDCAAS of protein sources

which will change and augment the RDAs, depending on the protein makeup of a given meal especially in the case of plant-based protein intake (Joint FAO/WHO/UNU Expert Consultation on Protein and Amino Acid Requirements in Human Nutrition (2002 : Geneva, Switzerland) Food and Agriculture Organization of the United Nations, World Health Organization & United Nations University, 2007).

One nitrogen balance study examined eight young (25.5 years old) males given three different levels of protein (0.4, 0.8, and 1.6 g/kg/d) on a eucaloric diet for 11 days. The sole source of protein was a wheat-soy-milk shake mixture given four times daily (specifically, 25% of the shake was dry nonfat milk solids). Twenty-four hour urine was collected to determine nitrogen balance. At the low protein intake (0.4 g/kg/d), all participants exhibited a negative nitrogen balance (-1.56 ± 0.27 g/d). At the RDA of 0.8 g/kg/g, a nitrogen balance of -0.55 ± 0.34 g/d was determined. It should be noted that of the eight participants, three were in positive nitrogen balance while the remaining five were in negative balance under this feeding condition. Lastly, under the final protein feeding condition (1.6 g/kg/d) all participants remained in positive nitrogen balance (2.98 ± 0.27 g/d). This study showed that the RDA of 0.8 g/kg/d was not adequate for over half the sample studied at maintaining nitrogen equilibrium, however statistical analysis to see if the reported mean was different from zero was not completed by the authors (Cheng et al., 1978).

Another study examined 15 omnivore Nigerian medical students (age 19-21 years) of normal weight and body composition. They were provided with four different levels of protein (0.3, 0.45, 0.6, and 0.75 g/kg/d) from foods normally eaten by the participants on a eucaloric diet. They consumed each diet three times daily for 10 days with a 3-day washout period in which they returned to their normal eating habits. After a 5-day adaptation period to each diet, daily 24-hour urine and complete fecal samples were collected and analyzed for nitrogen determination. Apparent nitrogen balances actually measured for 0.3, 0.45, 0.6, and 0.75 g/kg/d were -11.02 ± 8.07 mg/kg/d, -9.90 ± 6.64 mg/kg/d, 1.97 ± 4.15 mg/kg/d, 5.13 ± 4.62 mg/kg/d, respectively. However, apparent balance does not take into account other losses such as through sweat. When corrected for total sweat losses, estimated total nitrogen balance was then -18.48 ± 8.07 mg/kg/d, -17.36 ± 6.67 mg/kg/d, -5.49 ± 4.15 mg/kg/d, -2.33 ± 4.62 mg/kg/d, respectively, thus creating a negative nitrogen balance under each condition which suggests that even a 0.75 g/kg/d protein intake is not high enough to achieve nitrogen equilibrium (Atinmo et al., 1988).

Istfan et al. (1983) studied six healthy males aged 18-26 years old and assessed nitrogen balance weekly over 82 days following a nine-day adaptation period. Participants received 0.8 g/kg/d of soy protein as the sole source of dietary protein with vitamins and minerals supplemented to achieve individual needs. No other dietary information was provided in terms of macronutrient intake. Throughout the study, daily 24-hour urine output and fecal samples were collected and pooled for weekly determination of total nitrogen excretion. Over the course of the study, individual nitrogen balances varied from slightly negative to positive, with mean values all in positive balance. The authors concluded that 0.8 g/kg/d of soy protein was adequate for maintaining nitrogen balance and protein nutritional status. However, though soy protein has been shown to be of similar high quality to animal sources of protein, it is unrealistic to assume that an individual would consume a diet of exclusively soy protein outside of a research setting, but rather instead a mixed diet with various sources of protein. Additionally, though this study used exclusively soy protein, the study was not conducted in vegans and the initial lead in diet consisted of nine days of exclusive egg protein consumption (Istfan et al., 1983).

Using the previously reviewed studies, among others, Rand et al. (2003) performed a meta-analysis of 19 separate nitrogen balance studies and determined an EAR of 0.65 g/kg/d and calculated RDA of 0.83 g/kg/d of good quality protein to achieve nitrogen equilibrium during energy balance in healthy people of normal body composition. No further subgroup recommendations are given for age, sex, or diet groups, however at this time there was no analysis specifically for those following a mixed protein vegan diet (Rand et al., 2003). Interestingly, in 2013 Pencharz contended that based on available nitrogen balance data in omnivores, the Food and Drug Administration (FDA) and WHO recommendations for protein are too low. It is then suggested that reanalysis of data show a mean requirement of 0.91 g/kg/d and population estimate of 0.99 g/kg/d protein in adults (Pencharz, 2013). Later in 2016, the same group suggested that protein intake recommendations should be 1.5 to 2.2 g/kg/d based on novel indicator amino acid oxidation (IAAO) analysis, which has been validated compared to nitrogen balance (Pencharz et al., 2016). This could have additional implications for those adhering to a strict vegan diet, increasing the RDA even further, by up to 30% (Kniskern & Johnston, 2011; Trumbo et al., 2002).

Nitrogen balance and metabolism are sensitive to changes in energy intake, therefore it is important to maintain a eucaloric diet (e.g., dietary energy is equal to maintenance energy need) when assessing for nitrogen balance (Rand et al., 2003). It has been shown that as much as one-third of variation in nitrogen balance can be attributed to variation in energy intake compared to actual energy requirement (Pellett & Young, 1992).

Nitrogen balance and metabolism can also be affected by physical activity level (Todd et al., 1984; Butterfield & Calloway, 1984; Wolfe, 2000). For active people, protein needs increase depending on the modality of exercise. Endurance training such as running, cycling, and swimming increases protein needs to 1.2-1.4 g/kg/d whereas people who engage in resistance training require 1.6-1.7 g/kg/d (Fielding & Parkington, 2002). This equates to a 1.5 to just over two-fold increase from baseline RDAs. In their position stand paper on nutrition and athletic performance, The Academy of Nutrition and Dietetics, Dietitians of Canada, and American College of Sports Medicine contend that protein intake for athletes should range from 1.2 to as high as 2.0 g/kg/d (Thomas et al., 2016). This is not only to prevent a protein deficiency, but also to allow for muscle protein accretion and recovery following an exercise stimulus (Phillips & van Loon, 2011; van Loon, 2014). It has also been shown that protein intakes up to 3.1 g/kg/d are effective at maintaining muscle mass during severe caloric deficits due to dieting and/or exercise (Jäger et al., 2017). Thus, study participants should be selected for physical activity level or this should be controlled for statistically.

CHAPTER 3

METHODS

Study Design

Following approval by the Institutional Review Board at Arizona State University, participants were recruited from the greater Phoenix, AZ area from October 2020 through October 2021 via fliers, word of mouth, email listservs, and local social media groups. Men, 22-45 years old who had followed a vegan diet for at least one year, were invited to enroll in the trial. This study examined exclusively male participants to control for hormonal differences, as it has been demonstrated that the stage of the menstrual cycle can impact nitrogen balance in females (Calloway & Kurzer, 1982; Draper et al., 2018). Participants were healthy by self-report and inactive (< 150 min/wk moderate to vigorous exercise). Potential participants were excluded if they partook in regular, structured exercise such as resistance training, running, etc. regardless of total weekly duration. Further exclusion criteria included anyone taking prescription medications, muscle-building supplements such as protein or creatine powders, those with food allergies or unwilling to consume the trial foods, and/or those training for, or who had participated in recent (< 1 year) competitive sports.

Following an initial screening via internet questionnaire, potential participants took part in a separate, follow-up phone screening to explain to them the procedures and requirements of the study and ask any follow-up screening questions that may need clarification by the investigator. Upon agreement, qualifying participants were then scheduled to visit the lab where written informed consent was obtained. At the visit, anthropometric data (height, weight, fat free mass [FFM], and waist circumference) were collected. FFM was assessed via bioelectrical impedance analysis following a 12-hour fasting period in which no food or beverages were consumed (Walter-Kroker et al., 2011). Participants received a full 5-day menu of food calculated to provide adequate energy (± 100 kcal/d) and protein based on their bodyweight and reported activity level using the Harris-Benedict equation. Diet consisted of mostly frozen meals, meal replacement shakes, and dried fruits with a protein intake from mixed, plant-based sources held constant at 0.8 g/kg/d (See Table 1). Participants were also allowed to eat select foods from a list of fruits and vegetables, and logged all foods eaten on a daily food log (see Table 2).

Table 1. Example Meal Plans for Vegan Diets*

VEGAN EXAMPLE MENU			
		Protein (g)	**Energy (kcal)**
Breakfast	Orgain Protein Shake	20	150
	Cinnamon Raisin Bagel	9	280
	Peanut Butter (2 T)	8	180
	Apple	0	65
Lunch	Amy's Indian Vegetable Korma	9	330
	Dried Pineapple (2 servings)	0	280
Dinner	Sweet Earth General Tso's Tofu	10	330
	Trader Joes Dried Mango (1/2 package)	0	280
	Peanut Butter (2 T)	8	180
	Fruit Snacks (2 packages)	0	160
Total		64	2235

*Example meal plan is for 80 kg male using the Harris-Benedict equation and a physical activity level of 1.3 (seated work, no exercise). Meal plans adjusted for protein (0.8 g/kg/d) and energy calculated. Frozen entrees from Amy's Kitchen and Sweet Earth.

Table 2. List of Permitted Foods to Supplement Diet Plan (up to 3 servings/d)

2 large celery stalks
2 cups shredded romaine lettuce
½ cucumber
1 medium tomato
½ cup sugar snap peas
1 carrot
1 cup jicama sticks
1 peach
½ grapefruit
1 cup winter mix vegetables
1 cup Tuscan-style vegetables
1 cup mixed broccoli, cauliflower, and carrots
¾ cup whole green beans

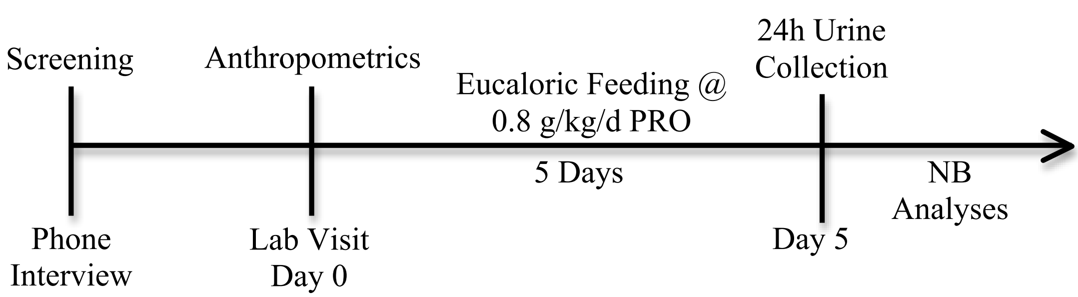
At the initial laboratory visit, participants were provided a container in which to collect a 24-hour urine sample on the final day of feeding, and were scheduled to begin the 5-day feeding period. During this dietary adaptation period, participants were asked to refrain from any moderate-vigorous physical activity and limit all other activities in general. Participants tracked activity daily using the validated Godin leisure time physical activity questionnaire. Participants were instructed to record any uneaten food portions or additional foods eaten. On day five of the adaptation diet, participants collected 24-hour urine specimens in the provided container. The first morning void was discarded, and all urine was collected throughout the day and overnight, including the first morning sample on day six. No urine preservative was necessary and participants were asked to refrigerate the sample. The 24-hour urine sample was delivered to the lab on the morning of day six (See Figure 1). Samples were thoroughly mixed, total volume determined, and aliquots frozen at -80º C for later analysis via photometric assay to determine nitrogen content by Sonora Quest Laboratories.

Nitrogen balance was determined using the known protein content of the diet on the fifth day of consumption and the nitrogen content of the urine (as UUN) using the following equation:

$$\text{Nitrogen Balance (g/d)} = (\text{PRO intake (g/d)}/6.25) - \text{UUN (g/d)} - 4$$

where the coefficient of 6.25 is derived from the fact that protein is made up of 16% nitrogen, thus there are 6.25 g of nitrogen per g of protein (Richter et al., 2019). A constant of 4 is used to account for 2 g of urinary non-urea nitrogen excretion (eg, ammonia, uric acid, creatinine, and amino acids) and 2 g for gastrointestinal, integumentary (dermal), and sweat losses (Dickerson, 2005b).

Figure 1: Study Protocol



Analysis

<u>Research Question #1</u>

Determine nitrogen balance in vegans following a eucaloric five-day diet with dietary protein held constant at 0.8 g/kg/d.

<u>Research Question # 2</u>

To explore predictor variables for nitrogen balance such as age, time as vegan, and fat free mass in vegans.

Statistical Analysis

Data for this cross-sectional study is reported as the mean ± SD, and an a priori α of .05 used. All outcome data were tested for normality and nonparametric statistics used as needed. Statistical analyses were performed using SPSS version 27 (IBM, Armonk, NY, USA). To determine if calculated nitrogen balance values were different from zero (nitrogen equilibrium), a one-sample t-test was used to compare mean difference. The Cohen's *d* statistic was used to compute effect size (ES). A left-sided α of .05 was used to determine statistical significance. Spearman rank-ordered correlations were used to investigate the associations of nitrogen balance with age, years vegan, and FFM. Spearman partial correlations were also used to examine these associations after adjustment for the other two covariates, respectively. The sample size was determined to detect a 5 mg/kg/d difference in nitrogen (SD = 6.4 mg/kg/d) over the five-day adaptation feeding period, based on Rand et al., 2003. With the effect size of 0.78, the power analysis showed that the recruitment of 14 participants was needed to have 90% statistical power at a one-sided .05 α level of significance. Allowing for a 30% attrition rate, 20 participants were recruited for this study.

CHAPTER 4

RESULTS

Participant Characteristics

One hundred twenty people responded to the online screening questionnaire to determine eligibility to participate in the study. Of these, 35 qualified based on their answers to the survey. Twelve of these qualifiers did not respond to emails requesting a brief phone interview and lab scheduling. Further, three declined to participate after a phone interview and two withdrew from the study prior to any participation. Thus, 18 participants were enrolled and completed the study. Mean participant characteristics are presented in Table 3.

Prior to analyses, nitrogen balance values were assessed for normality and potential outliers. A box plot analysis determined one outlier (Figure 2), which was confirmed by Shapiro-Wilk normality testing, $W(18) = 0.89$, $p < .047$. Once removed, Shapiro-Wilk testing confirmed normality of remaining data points, $W(17) = 0.93$, $p = .236$. Thus, this outlier was removed and all future analyses were performed using data from 17 participants.

Table 3: Mean Participant Characteristics.

	$N = 17$
Age (yrs)	31.6 ± 6.2
Years Vegan (yrs)	7.1 ± 6.5
Height (cm)	176.6 ± 8.1
Weight (kg)	75.5 ± 13.7
Waist Circumference (cm)	85.9 ± 10.5
Hip Circumference (cm)	101.3 ± 8.3
W:H	0.85 ± 0.06
Fat Free Mass (kg)	60.0 ± 7.5
Fat Mass (kg)	15.1 ± 8.2
Body Fat (%)	19.2 ± 7.2
BMI (kg/m^2)	24.2 ± 3.8
Kcal Needs (kcal/d)	2377.4 ± 361.7
Protein Needs (g/d)	60.9 ± 10.5
Nitrogen Balance (g/d)	-1.38 ± 1.22*
Nitrogen Balance (mg/kg/d)	-18.60 ± 16.96*

*Significantly different than nitrogen equilibrium (Nitrogen Balance = 0) ($p < .05$)

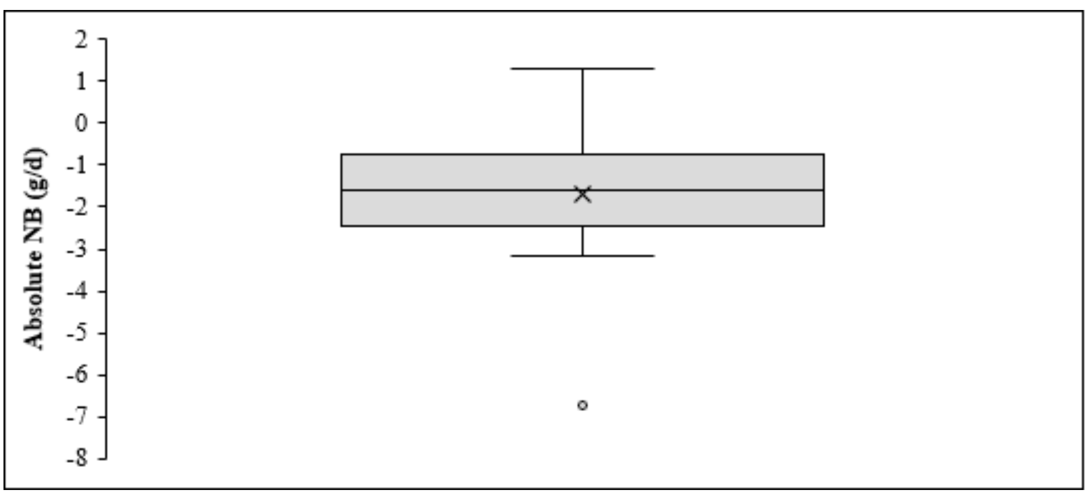


Figure 2: Nitrogen Balance (NB) (g/d) Box Plot Analysis

Nitrogen Balance

A one-sample t-test was performed to determine whether nitrogen balance values following the 5-day dietary protocol were statistically different than nitrogen equilibrium (a nitrogen balance value of zero). Nitrogen balance was analyzed in two ways – as absolute nitrogen balance in grams per day (g/d) and relative to body weight as relative nitrogen balance in milligrams per kilogram per day (mg/kg/d).

Mean absolute nitrogen balance score (-1.38 ± 1.22 g/d) was lower than the nitrogen equilibrium score of zero, a statistically significant difference of -1.38 (95% CI, -2.00 to -0.75), (ES = -1.13), $t(16) = -4.643$, $p < .001$. Mean relative nitrogen balance score (-18.60 ± 16.96 mg/kg/d) was lower than the nitrogen equilibrium score of zero, a statistically significant difference of -18.60 (95% CI, -27.32 to -9.88), (ES = -1.10), $t(16) = -4.522$, $p < .001$ (Table 3, Figures 3a and 3b). Individual participants' nitrogen balance values can be seen in Table 4 and Figures 4 and 5.

Table 4: Individual Participant Nitrogen Balance Values.

	Absolute NB (g/d)	Relative NB (mg/kg/d)
VNB S001	-0.59	-6.90
VNB S002	-3.17	-46.38
VNB S003	-1.58	-23.24
VNB S004†	-6.73†	-86.70†
VNB S005	0.83	8.32
VNB S006	0.02	0.31
VNB S007	-2.40	-30.81
VNB S008	-1.49	-23.00
VNB S009	-0.78	-11.42
VNB S010	-1.82	-28.00
VNB S011	1.31	21.49
VNB S012	-2.46	-36.09
VNB S013	-2.68	-34.65
VNB S014	-1.42	-24.66
VNB S015	-2.53	-24.02
VNB S016	-1.32	-14.42
VNB S017	-1.60	-19.00
VNB S018	-1.72	-23.69

Values represent Mean ± SD
†Outlier value (removed from analyses)

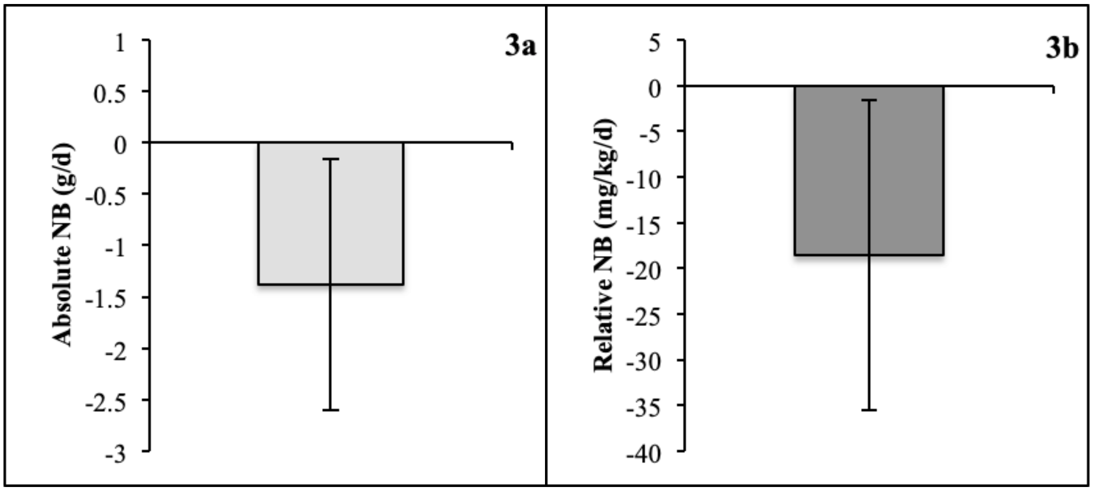


Figure 3: Mean Nitrogen Balance

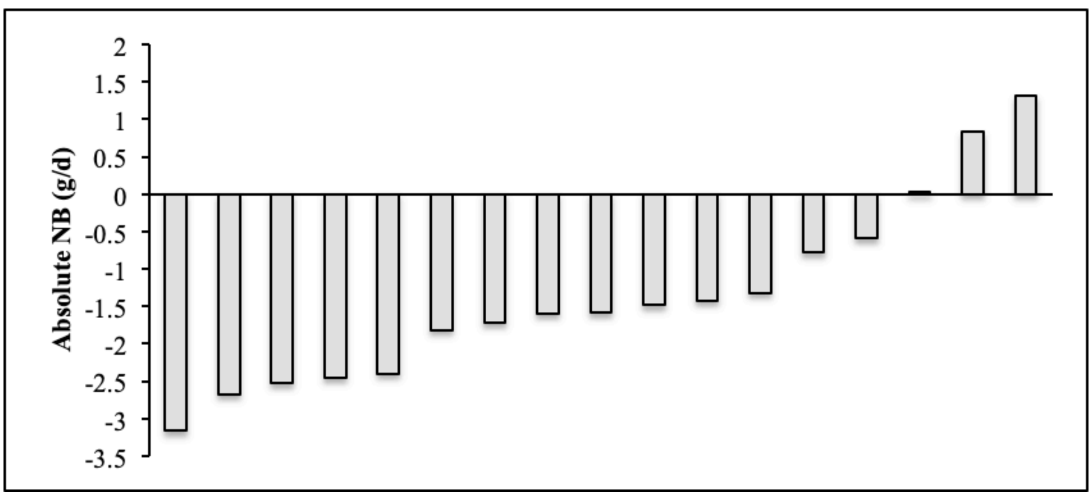


Figure 4: Individual Absolute Nitrogen Balance (g/d)

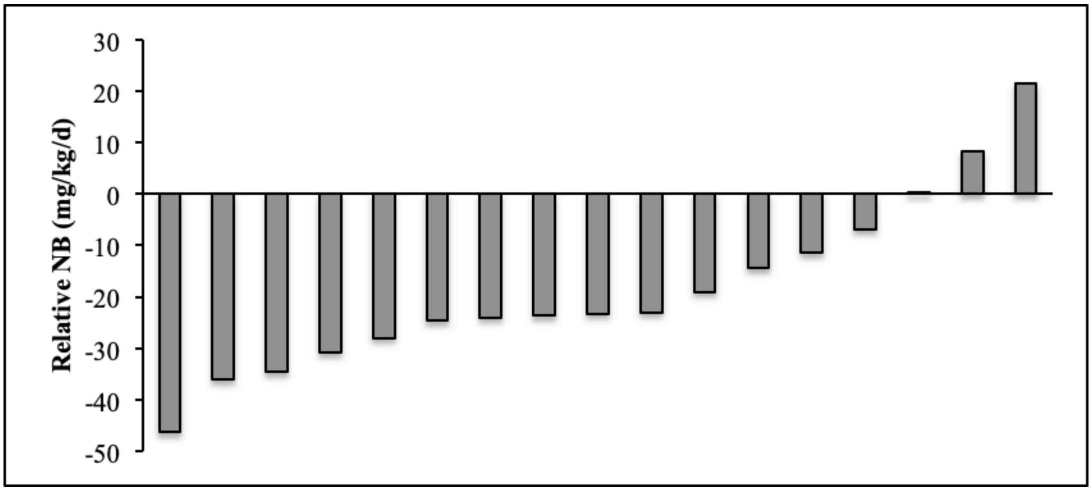


Figure 5: Individual Relative Nitrogen Balance (mg/kg/d)

Nitrogen Balance Predictor Variables

Prior to testing, data for age, years vegan, and FFM were checked for normality using the Shapiro-Wilk test of normality. FFM appeared to be normally distributed ($p < .455$), however age ($p < .015$) and years vegan ($p < .001$) were not. Thus, Spearman rank ordered correlation was used to determine if any correlation existed between the proposed predictor variables and nitrogen balance (Table 5). Spearman partial correlation was further used to examine correlations while controlling for other potential predictor variables (Table 6).

Table 5: Correlation and Significance of Proposed NB Predictor Variables

		Age	Years Vegan	FFM
NB (g/d)	Correlation Coefficient	-0.409	-0.182	-0.159
NB (g/d)	P-value	.103	.484	.541

Table 6: Partial Correlation and Significance of Proposed NB Predictor Variables

Control Variables			Age
Years Vegan & FFM	NB (g/d)	Correlation	-0.296
		P-Value	.285
			Years Vegan
Age & FFM	NB (g/d)	Correlation	-0.236
		P-Value	.397
			FFM
Age & Years Vegan	NB (g/d)	Correlation	-0.305
		P-Value	.269

CHAPTER 5

DISCUSSION

The purpose of the present study was to determine nitrogen balance in inactive, vegan males while following a eucaloric diet at the protein RDA of 0.8 g/kg/d. When performed correctly, nitrogen balance studies are still considered the gold standard for determination of dietary protein adequacy. In order to yield accurate data, there must be tight control over protein intake, adequate energy provided, physical activity standardized, and possible confounders limited and controlled for. There must also be an adequate lead-in, or adaptation period to the diet to ensure artifacts of prior dietary intake are minimized (Wernerman et al., 2017). The literature shows that four days to three weeks is adequate to allow UUN adjustment to changes in nitrogen intake (Bender, 2003; Margolis et al., 2016; Rand et al., 2003). Data from known dietary intake is then compared to UUN analyzed from the urine and nitrogen balance can be determined. It was herein demonstrated that there was a mean negative nitrogen balance in the sample studied in absolute terms (NB = -1.38 ± 1.22 g/d, ES = -1.13) as well as when adjusted for bodyweight (relative NB = -18.60 ± 16.96 mg/kg/d, ES = -1.10). Both means are significantly lower than the value of zero, which was used to indicate nitrogen equilibrium. In both cases, 14 of the 17 participants (82%) exhibited a net negative balance following the five day standardized adaptation diet. These findings are important because this is the first known study to examine nitrogen balance in strict, long-term vegans.

The RDA is informed from a series of nitrogen balance studies meta-analyzed by Rand et al. (2003). This meta-analysis primarily utilized studies feeding animal foods as the main source of protein, and the studies that fed plant-based protein sources either contained a certain portion of animal-based foods in the diet or studied participants who had only omitted animal foods for the purpose of these short-term studies. Thus, it can be argued that those results, and therefore the RDA, are only translatable to omnivores and are not truly applicable to a vegan population (Rand et al., 2003; Otten, et al., 2006). The present results, when examined in the context of the data used in Rand et al. (2003) show a markedly decreased relative nitrogen balance (-18.60 ± 16.96 mg/kg/d) compared to much of those analyzed for RDA determination (nitrogen balance range: -13.5 ± 21.2 to 46.3 ± 19.3 mg/kg/d). Also importantly, the RDA does not take into consideration protein quality and bioavailability, of which it has been suggested could be up to 30% lower in plant-based foods – further complicating this issue for vegans (Kniskern & Johnston, 2011; Trumbo et al., 2002).

Exploratory analyses of possible predictors of nitrogen balance in the present study such as age, years as vegan, or FFM yielded no significant correlations showing that these variables had no significant effect on the primary outcome of a eucaloric diet at the protein RDA on nitrogen balance. This is encouraging, as it demonstrates, at least in the male participants studied, that minimum protein requirements should in theory be relatively straight forward and use body weight as the predictor without having to account for other factors in otherwise healthy people, assuming overall energy intake is adequate and physical activity is minimal.

Negative nitrogen balance indicates a net loss of protein in the body, or protein catabolism. Nitrogen balance is a global assessment of protein balance in the body and does not measure protein quality (i.e. amino acid composition), however its outcome can be affected by it. In a healthy, inactive person consuming a eucaloric diet at the RDA of 0.8 g/kg/d, a negative nitrogen balance would suggest inferior protein quality/lack of essential amino acids, rather than quantity as the cause (Moizé et al., 2017). A consistent negative nitrogen balance will have a negative effect on the synthesis of new proteins, which, over time will decrease the body's protein stores, including skeletal muscle (Moizé et al., 2017). As discussed in chapter 3, protein is comprised of 16% nitrogen, thus 6.25 g of lean tissue contains approximately one gram of nitrogen (Richter et al., 2019). Putting this into perspective given the results of this study – a mean nitrogen balance of -1.38 g/d signifies a total loss of 8.63 g/d total body protein, or a 3.1 kg loss per year. Mason (2021) proposes that 6.25 g/d total protein loss is the equivalent of 30 g/d loss of hydrated lean tissue. So over time, this can accumulate to cause a substantial loss. This is important in clinical cases such as in people with PEM and older adults where sarcopenia becomes common, as well as people trying to gain skeletal muscle mass, and evidently, vegans following the RDA.

PEM results from inadequate protein and overall caloric intake. It is prevalent in developing countries, but also of concern in older populations worldwide (Satapathy et al., 2021; Mathewson et al., 2021). Sarcopenia, or age-related muscle loss begins typically near age 40 and has been documented to show losses of as much as 50% of muscle mass by age 80 (Mathewson et al., 2021; Walston, 2012; Metter et al., 1997; Mitchell et al., 2012). In fact, one study in the U.S. showed prevalence of sarcopenia in 18 to 29 year olds (males 12%, females 14%) and 30 to 39 year olds (males 20%, females 24%) to be of concern as well, thus making the importance of dietary protein evident in all diet populations, not just vegans (Janssen et al., 2002). Fortunately, sarcopenia is reversible and not inevitable with proper precautions taken. Combatting PEM and sarcopenia is best started before their onset through muscle strengthening activity and increased dietary protein consumption (Mathewson et al., 2021). It is recommended that individuals consume 25-30 g of high-quality protein per meal, with possible inclusion of supplemental leucine to enhance the protein synthetic response in those susceptible to sarcopenia (Paddon-Jones & Rasmussen, 2009). Adults over 65 years of age should take care to consume 1.0-1.2 g/kg/d of protein per day, at minimum to help prevent sarcopenia (Bauer et al., 2013). Unfortunately, no known data regarding the prevalence of sarcopenia in aging vegans exists in the literature, though concern has been raised that vegan diets in those over 65 years old may not be adequate to combat sarcopenia (Domić et al. 2022). This area should be examined in future research. Dietary protein adequacy and preservation of muscle mass becomes even more important in vegans, as Vanacore et al. (2018) showed a nearly 5 kg difference in muscle mass between omnivore (32.1 ± 0.81 kg) and long-term vegan (27.3 ± 1.2 kg) cohorts, with no difference between vegetarians (32.8 ± 1.4 kg) and omnivores. The study sample had a mean age of 29 ± 5 years and participants were purported to have been age-matched, however mean ages of individual diet cohorts were not provided. Another study examined the relationship between protein intake from animal-based sources and muscle mass in healthy women. They found that the vegetarian group (n = 19, with only one strict vegan, mean age = 48 ± 12 years, mean years on diet = 12) had significantly lower muscle mass compared to the omnivore control group (18.2 ± 3.9 kg vs. 22.6 ± 5.0 kg). There was also a significantly lower muscle mass index in vegetarians (6.7 ±1.2 kg/m^2) compared to omnivores (8.3 ± 1.5 kg/m^2). Interestingly, they also found that animal protein intake was an independent predictor of muscle mass index (adjusted $r^2 = 0.42$) (Aubertin-Leheudre & Adlercreutz, 2009). The data from these studies suggest that vegans typically start out with significantly lower muscle mass than omnivores leading into the years in which sarcopenia is known to become apparent, likely caused by years of negative nitrogen balance status. In their 2019 paper, Carbone and Pasiakos state strongly "The current protein RDA, however, is often incorrectly applied when used as the definition of recommended intake, rather than its true designation as the required minimum intake. This misapplication is problematic for healthy populations and aging adults, and disadvantageous for those with pathophysiological conditions that would necessitate higher-protein needs." (Carbone & Pasiakos, 2019). This statement is especially true in light of the results of the present study, where it is shown that even the RDA is not an adequate minimum for vegans.

The population examined in this study included males, aged 22-45 years who had followed a long-term vegan diet (≥ 1 year). All were healthy by self-report, with no prescription medications taken. All participants reported to be inactive (< 150 min/wk of moderate-vigorous physical activity) and not competitive athletes. This homogenous population was chosen for several reasons. First, to control for hormonal differences between sexes, as menstrual cycle stage in females can affect nitrogen balance (Calloway & Kurzer, 1982; Draper et al., 2018). The age range of participant recruitment was initially 25-40 years old, but eventually expanded due to recruitment difficulties. This window was chosen to minimize the possibilities of continued growth at the younger end and onset of sarcopenia at the higher end of the age spectrum. Participants with chronic diseases or taking prescription medications did not qualify due to metabolic changes that these variables might have caused on protein and nitrogen metabolism. Lastly, as explained previously, physical activity can have a strong effect on dietary protein utilization in the body, as well as breakdown of existing proteins, including skeletal muscle in the body. Participants were mostly inactive prior to the study and were asked to remain sedentary during the study protocol to standardize physical activity of the group. Lastly, Table 3 shows mean participant characteristics such as waist to hip ratio (0.85 ± 0.06), body fat percentage (19.2 ± 7.2), and BMI (24.2 ± 3.8) to all be within normal range (American College of Sports Medicine et al., 2018).

The current protein RDA for the general population is at minimum 0.8 g/kg/d of high-quality protein for sedentary individuals (Otten et al., 2006). This recommendation was based on analyses of available nitrogen balance studies at the time. However, Elango at al. (2010) conducted alternative statistical analyses using a two-phase linear regression analysis rather than single linear regression analysis as Rand et al. (2003) had done. The authors suggest this is more appropriate and realistic for protein requirement determination. This alternative analysis suggested that the recommendation should be closer to 1.0 g/kg/d, and again this was only considering omnivores and some vegetarians. As mentioned previously, it needs to be stressed that this is the absolute minimum level of protein needed per day (Carbone & Pasiakos, 2019). Most people are not completely sedentary or inactive, thus these recommendations would be inappropriate to consider adequate. Interestingly, the RDA is unchanged (0.8 g/kg/d) in adults aged 50 years and older, despite arguments from experts for a higher protein RDA for adults 65 years and older (Rafii et al., 2015a, Rafii et al., 2015b). The only differences occur in infants (1.2 g/kg/d), children (0.95 – 1.05 g/kg/d), and teens (0.85 g/kg/d) (Otten et al., 2006). The Academy of Nutrition and Dietetics recommends 1.2 – 2.0 g/kg/d of protein for athletes, depending on sport and activity level (Thomas et al., 2016). As mentioned in Chapter 2, even higher protein intakes (3.1 g/kg/d) have been shown to help maintain muscle mass during hypocaloric periods and are also sometimes used to increase satiety during these times (Jäger et al., 2017). Benefits of higher protein diets include aiding in the accretion of muscle mass, as well as an aid for weight loss through elevated diet-induced thermogenesis and increased satiety caused by higher protein intakes (Moon & Koh, 2020, Paddon-Jones et al., 2008).

A vegan diet *can* in fact be healthy and adequate, provided attention is paid to certain nutrients, most importantly for the discussion and purposes of the present study, protein. The RDA suggests a *minimum* of 60 g of protein per day for a non-exercising person weighing 75 kg. Given the results provided herein, this will produce a negative nitrogen balance, and is thus inadequate. Previous research has suggested up to 30% greater nitrogen requirement for vegetarians compared to omnivores, therefore increasing the RDA to greater than 1.0 g/kg/d or in this case, a minimum intake of 75 g of protein per day for the same individual (Kniskern & Johnston, 2011). Although empirical information regarding adequate protein intake in vegans is scant, given the totality of the available evidence, including data from the present study, a speculative recommendation for a sedentary male vegan would be to consume three to four meals daily containing at minimum 20 – 25 g of high-quality protein per meal for whole-body protein maintenance. This recommendation of course would increase or decrease depending on a person's bodyweight. Suggestions for high quality sources to incorporate should include an emphasis on soy (tofu, tempeh, edamame, etc.), with other mixed sources as well (legumes, grains, nuts, seeds).

It should be noted that additional protein intake within the AMDR range (10-35% of total daily energy intake) likely has no ceiling effect in terms of absorption and that this extra protein will not be wasted or merely excreted, but rather be beneficial to healthy individuals (Deutz & Wolfe, 2013, Schoenfeld & Aragon, 2018). As shown in the results of this study, there can be substantial interindividual variation regarding nitrogen balance, which will change markedly depending on lifestyle and activity levels, thus recommendations for active people and exercisers would of course increase as well.

Strengths of this study include utilizing a mixed diet with protein from various plant-based sources to best mimic participants' normal diets, thus enhancing ecological validity. This protocol provided proteins with complementary amino acid profiles, instead of limiting intake to a single protein source. Importantly, this study was limited to long-term (> 1 year), strict vegans, for which data in the literature was previously lacking. The present RDA was determined using nitrogen balance studies in omnivores and few vegetarians – all of whom consume animal products, at least in part. The results of this study will serve as a starting point to better inform protein recommendations for those following a vegan lifestyle.

This study had several limitations. First, this was a short-term study, with an adaptation feeding period of five days. This is at the short end of what is considered adequate for nitrogen balance determination with standard protocol being between four days to several weeks adaptation to the experimental diet before measurement of nitrogen balance (Bender, 2003; Margolis et al., 2016; Rand et al., 2003). Next, this was not an inpatient study, thus participants were free to live their daily lives during the study protocol. Because of this, dietary menu adherence was based on participants' trustworthiness in tracking their intake. Although all food was provided to participants at no cost, it is possible they may not have consumed all meals, added prohibited foods, or measured certain foods incorrectly, thus providing inaccurate information on total energy and protein intake. Like dietary intake, physical activity was based on participants' honesty of tracking outside of the laboratory as well. While participants were asked to keep all physical activity to a minimum, this definition may vary between individuals and can affect energy needs, thus nitrogen balance. Future studies of similar design should better assess physical activity using body worn accelerometers to gather objective data. This study utilized a single protein intake for all individuals. While our data show that the RDA was inadequate in achieving nitrogen balance, it is unknown at what intake this equilibrium could be achieved. Future studies could employ a crossover design, with participants consuming other protein amounts such as 1.0 g/kg/d, 1.2 g/kg/d, and so on in a randomized order to better determine protein adequacy. Lastly, this study used only a small sample of inactive males, thus these results are not generalizable to females or those engaging in physical activity or exercise. Further work is needed in these areas.

CHAPTER 6

CONCLUSION

Results of this study suggest that the current RDA for protein, of 0.8 g/kg/d, is not adequate to produce nitrogen equilibrium in inactive, vegan males. This is important, given the increasing numbers of people who follow a vegan lifestyle, and the fact that high quality and quantity protein foods are less common in a vegan diet compared to an omnivore diet. Due to inferior protein quality of most plant-based foods compared to animal-based foods, it is likely that the RDA should be amended with special recommendations for vegans, or at the very least highlight the importance of a diet with higher protein intake to better guide and assure nutrient adequacy in vegans.

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APPENDIX A

INFORMED CONSENT FORM, IRB APPROVAL

Informed Consent

Nitrogen Balance in Adult Vegan Males

INTRODUCTION

The purposes of this form are (1) to provide you with information that may affect your decision as to whether or not to participate in this research study, and (2) to record your consent if you choose to be involved in this study.

RESEARCHERS

Dr. Carol Johnston (ASU Nutrition professor) and Eric Bartholomae (Doctoral Candidate) have requested your participation in a research study.

STUDY PURPOSE

The purpose of this study is to investigate nitrogen balance in relation to protein intake at the RDA (0.8g/kg/d) in healthy male vegans.

DESCRIPTION OF RESEARCH STUDY

You have indicated to us that you are male, vegan, a non-smoker, 22-45 years of age, and healthy. You are not a competitive athlete, and you have not recently trained for an athletic event, Also, you are not currently ill or taking prescription medications for a medical condition, and you do not take any muscle building supplements such as creatine or protein powder. You will be assigned a calorie-adequate vegan diet consisting of frozen, microwaveable meals and liquid protein drinks for 5 days. No other foods are to be consumed during this time. Additionally, you will be asked to not exercise or perform any moderate- to-vigorous physical activity during this 5-day feeding protocol. On the fifth day of the feeding period, you will collect a complete, 24-h urine sample in a provided container. This sample will be refrigerated overnight and delivered to the laboratory the morning of day 6.

This research entails two in-person lab visits with Eric at the ASU downtown Phoenix campus. You will be asked to complete diet recalls and health questionnaires. You will receive a $50 e-gift card to Amazon.com once you have completed your final laboratory visit.

RISKS

You may feel hungry during the feeding protocol, though all meals provided will be calorie-adequate for weight maintenance; you are allowed to consume unlimited water and various fruits and vegetables (you will be provided with a list of these foods) throughout each day.

BENEFITS

You may not benefit from this study, but once the study is complete you will be provided with your data, if desired. You will need to complete a study release form to receive your test results.

NEW INFORMATION

If the researchers find new information during the study that would reasonably change your decision about participating, then they will provide this information to you.

CONFIDENTIALITY

All information obtained in this study is strictly confidential unless disclosure is required by law. The results of this research study may be used in reports, presentations, and publications, but your name or identity will not be revealed. In order to maintain confidentiality of your records, Dr. Johnston will use subject codes on all data collected, maintain a master list separate and secure from all data collected, and limit access to all confidential information to the study investigators.

WITHDRAWAL PRIVILEGE

You may withdraw from the study at any time for any reason without penalty or prejudice toward you. Your decision will not affect you any manner. We ask that you notify us in a timely manner if you decide to withdraw from the study, and we will ask you to complete the exit survey at that time.

COSTS AND PAYMENTS

You will receive a $50 Amazon e-gift card upon your completion of this trial. There are no payments required for this study.

COMPENSATION FOR ILLNESS AND INJURY

If you agree to participate in the study, then your consent does not waive any of your legal rights. However, in the event of harm, injury, or illness arising from this study, neither Arizona State University nor the researchers are able to give you any money, insurance coverage, free medical care, or any compensation for such injury. Major injury is not likely but if necessary, a call to 911 will be placed.

VOLUNTARY CONSENT

Any questions you have concerning the research study or your participation in the study, before or after your consent, will be answered by Dr. Carol Johnston, [redacted]

If you have questions about your rights as a subject/participant in this research, or if you feel you have been placed at risk, you can contact the Chair of the Human Subjects Institutional Review Board, through the ASU Research Compliance Office, at 480-965 6788.

This form explains the nature, demands, benefits and any risk of the project. By signing this form you agree knowingly to assume any risks involved. Remember, your participation is voluntary. You may choose not to participate or to withdraw your consent and discontinue participation at any time without penalty or loss of benefit. In signing this consent form, you are not waiving any legal claims, rights, or remedies. A copy of this consent form will be given to you.

Your signature below indicates that you consent to participate in the above study.

__________________ ______________________ ____________________

Subject's Signature   Printed Name   Date

_______________________________ _______________________________

Contact phone number   Email

INVESTIGATOR'S STATEMENT

"I certify that I have explained to the above individual the nature and purpose, the potential benefits, and possible risks associated with participation in this research study, have answered any questions that have been raised, and have witnessed the above signature. These elements of Informed Consent conform to the Assurance given by Arizona State University to the Office for Human Research Protections to protect the rights of human subjects. I have provided the subject/participant a copy of this signed consent document."

Signature of Investigator____________________ Date____________

ASU IRB IRB # STUDY00012662 | Approval Period 10/2/2020 – 10/1/2021

ASU Knowledge Enterprise Development

APPROVAL: EXPEDITED REVIEW

Carol Johnston
CHS: Health Solutions, College of
602/496-2539
CAROL.JOHNSTON@asu.edu

Dear Carol Johnston:

On 10/2/2020 the ASU IRB reviewed the following protocol:

Type of Review:	Initial Study
Title:	Nitrogen Balance and Protein Intake at the RDA in Underactive Male Vegans
Investigator:	Carol Johnston
IRB ID:	STUDY00012662
Category of review:	
Funding:	Name: Graduate College (GRAD)
Grant Title:	
Grant ID:	
Documents Reviewed:	• calendar, Category: Participant materials (specific directions for them); • consent, Category: Consent Form; • health history questionnaire, Category: Measures (Survey questions/Interview questions /interview guides/focus group questions); • KE approval for research to proceed, Category: Technical materials/diagrams; • protocol, Category: IRB Protocol; • release form, Category: Technical materials/diagrams; • screener, Category: Screening forms; • verbal script and ad, Category: Recruitment Materials;

The IRB approved the protocol from 10/2/2020 to 10/1/2021 inclusive. Three weeks before 10/1/2021 you are to submit a completed Continuing Review application and required attachments to request continuing approval or closure.

If continuing review approval is not granted before the expiration date of 10/1/2021 approval of this protocol expires on that date. When consent is appropriate, you must use final, watermarked versions available under the “Documents” tab in ERA-IRB.

In conducting this protocol you are required to follow the requirements listed in the INVESTIGATOR MANUAL (HRP-103).

Sincerely,

IRB Administrator

cc:
Hannah Rater
Eric Bartholomae

APPENDIX B

RECRUITMENT FLIER, ONLINE SCREENING SURVEY, SUBJECT QUESTIONNAIRES

ASU STUDY SEEKS PARTICIPANTS:
NITROGEN BALANCE IN VEGAN MALES

THE ASU NUTRITION PROGRAM IS RECRUITING
UNDERACTIVE (<150 min/wk) VEGAN MALES AGE 22-45
TO EXAMINE THE EFFECTS OF PROTEIN INTAKE ON NITROGEN BALANCE AT THE PROTEIN RDA (0.8g/kg/d)

Participation is voluntary and includes:

- Two in-person laboratory visits to ASU's downtown Phoenix campus.
- Completion of diet recalls and health questionnaires.
- You will eat a predetermined and provided diet consisting of frozen, microwaveable vegan meals for 5 days and collect a 24-hour urine sample on the final feeding day.

*You will receive a **$50** Amazon card for your participation*

INTERESTED?? Please visit our recruitment site:
https://asuhealthpromotion.co1.qualtrics.com/jfe/form/SV_bEJ1WiRV2wJ0boF

Researchers in the nutrition program at Arizona State University (ASU) are conducting a research study to determine protein adequacy in male vegans, a test called: Nitrogen Balance. We are inviting your participation in the screening process, which will consist of answering questions regarding health history, demographics, and scheduling availability. You have the right not to answer any question, and to stop participation at any time. We are recruiting healthy male vegans 22 to 45 years of age. Your participation in this survey and study is voluntary. If you choose not to participate or to withdraw from the study at any time, there will be no penalty. Your responses to this survey will not be linked to your name but may be used in aggregate form for publications and presentations. If you meet the criteria for this study, you will be contacted to schedule an in-person appointment at the downtown campus of ASU. If you have any questions concerning the research study, please contact Eric Bartholomae, a nutrition doctoral student, at ebartho2@asu.edu. You may also contact Dr. Carol Johnston, ASU Nutrition Professor who is leading this study at carol.johnston@asu.edu. If you have any questions about your rights as a subject/participant in this research, or if you feel you have been placed at risk, you can contact the Chair of the Human Subjects Institutional Review Board, through the ASU Office of Research Integrity and Assurance, at (480) 965-6788.

Completion of this survey will indicate your consent to participate in this screening. You will be contacted by investigators (via e-mail) to schedule an appointment, should you qualify.

ASU Vegan Protein Study

Start of Block: Default Question Block

Please provide your email address

____

How old are you in years?

____

Please select your sex.

○ Male (1)

○ Female (2)

What is your height in inches? (5 feet = 60 inches) (6 feet = 72 inches)

____

What is your weight in pounds?

____

Do you smoke or vape?

○ Yes (1)

○ No (2)

Which diet best describes your day to day eating?

○ Vegan (1)

○ Vegetarian (2)

○ Omnivore (3)

How long have you followed this diet?

____

Do you consider yourself healthy?

____

Are you currently taking any prescription medications?

○ Yes (1)

○ No (2)

If yes, please list which medications.

____

Do you have any food allergies?

○ Yes (1)

○ No (2)

Page 3 of 5

If yes, what food allergies do you have?

____

Do you consume any dietary supplements? (vitamins, minerals, protein powders, etc)

◯ Yes (1)

◯ No (2)

If selected for this study, are you willing to exclusively consume frozen meals (vegan) provided by the investigators for 5 days?

◯ Yes (1)

◯ No (2)

On average, how many minutes of moderate to vigorous physical activity do you perform weekly? (e.g., exercise that breaks a sweat)

____

Have you competed in any athletic event in the past year?

◯ Yes (1)

◯ No (2)

If selected for this study, are you willing to refrain from exercise or strenuous physical activity for 5 days?

- ◯ Yes (1)
- ◯ No (2)

Are you able to commute to the downtown Phoenix campus of Arizona State University (north of Van Buren and 5th Streets in Phoenix, AZ) for a total of 2 laboratory visits, 6 days apart (depending on your eligibility for this study)?

- ◯ Yes (1)
- ◯ No (2)

End of Block: Default Question Block

HEALTH HISTORY QUESTIONNAIRE ID#___________________

1. Gender: M F

2. Age: __________

3. Have you lost or gained more than 10 lbs in the last 12 months? Yes No
   If yes, how much lost or gained? _________ How long ago? ___________

4. Ethnicity: (please circle one) American Indian/Alaska Native Black or African-American White or Caucasian Native Hawaiian or Other Pacific Islander Hispanic or Latino Asian

5. Education (please circle) High school diploma AA/vocational degree College degree MS degree PhD degree

6. Do you smoke? No, never _________
   Yes ________ # Cigarettes per day = _________
   I used to, but I quit ________ months/years (circle) ago

7. Do you take any medications regularly? Yes No *If yes, list type and frequency:*

Medication Dosage Frequency

____

____

____

8. Do you currently take supplements (vitamins, minerals, protein powders, etc.) ? Yes No
If yes, list type and frequency:

Supplement Dosage Frequency

____

____

____

9. Please check (YES/NO) if you currently have or if you have ever been clinically diagnosed with any of the following diseases or symptoms:

	YES	NO		YES	NO
Coronary Heart Disease			Chest Pain		
High Blood Pressure			Shortness of Breath		
Heart Murmur			Heart Palpitations		
Rheumatic Fever			Any Heart Problems		
Irregular Heart Beat			Coughing of Blood		
Varicose Veins			Feeling Faint or Dizzy		
Stroke			Lung Disease		
Diabetes			Liver Disease		
Low Blood Sugar			Kidney Disease		
Bronchial Asthma			Thyroid Disease		
Hay Fever			Anemia		
Leg or Ankle Swelling			Hormone Imbalances		
Eating Disorder			Depression		

10. Please circle the **number of times** you did the following kinds of exercises **for more than 15 minutes** last week.

Mild exercise (minimal effort):
Easy walking, golf, gardening, bowling, yoga, fishing, horseshoes, archery, etc.

Times per week: 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14+

Moderate exercise (not exhausting):
Fast walking, easy bicycling, tennis, easy swimming, badminton, dancing, volleyball, baseball, etc.

Times per week: 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14+

Strenuous exercise activities (heart beats rapidly):
Running, jogging, hockey, football, soccer, squash, basketball, cross country skiing, judo, roller skating, vigorous swimming, vigorous long distance bicycling, etc.

Times per week: 0 1 2 3 4 5 6 7 8 9 10 11 12 13 14+

11. Are you healthy and fit right now? Yes No

Comments: ______________________________

12. How much alcohol do you drink? (average #drinks per week) ____________

13. Do you have any food allergies? Yes No

If yes, explain:

____

14. Do you follow a special diet? Yes No

If yes, explain: ______________________________

15. Are you willing to refrain from exercise and physical activity for 5 days? Yes No

16. Are you willing to consume 3 frozen meals (vegan) and liquid protein supplements exclusively daily for 5 days? Yes No

17. Are you willing to provide a 24-hour complete urine sample on day 5 of the study? Yes No

Godin Leisure-Time Exercise *Questionnaire*

During a typical **7-Day period** (a week), how many times on the average do you do the following kinds of exercise for **more than 15 minutes** during your free time (write on each line the appropriate number).

Weekly leisure activity score = (9 × Strenuous) + (5 × Moderate) + (3 × Light)

	Times per week		Totals
a) **STRENUOUS EXERCISE (HEART BEATS RAPIDLY)** (e.g., running, jogging, hockey, football, soccer, squash, basketball, cross country skiing, judo, roller skating, vigorous swimming, vigorous long distance bicycling)		**X9**	
b) **MODERATE EXERCISE (NOT EXHAUSTING)** (e.g., fast walking, baseball, tennis, easy bicycling, volleyball, badminton, easy swimming, alpine skiing, popular and folk dancing)		**X5**	
c) **MILD/LIGHT EXERCISE (MINIMAL EFFORT)** (e.g., yoga, archery, fishing from river bank, bowling, horseshoes, golf, snow-mobiling, easy walking)		**X3**	
WEEKLY LEISURE-TIME ACTIVITY SCORE			

EXAMPLE
Strenuous = **3** times/wk
Moderate = **6** times/wk
Light = **14** times/wk
Total leisure activity score = (9 × **3**) + (5 × **6**) + (3 × **14**) = 27 + 30 + 42 = 99

Godin Scale Score	**Interpretation**
24 units or more	Active
14 – 23 units	Moderately Active
Less than 14 units	Insufficiently Active/Sedentary

Adapted from: Godin, G. (2011). The Godin-Shephard leisure-time physical activity questionnaire. Health & Fitness Journal of Canada, 4(1), 18-22.

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ID# _______________

Exit Survey – *Vegan Nitrogen Balance questionnaire*

1. Did you have problems collecting the 24h urine sample? Yes No

2. Did you have problems consuming your prescribed diet daily during the study?
   Circle the most appropriate answer:

   Never Occasionally Daily

3. What were your favorite foods during the study?

____

4. What were your least favorite foods during the study?

____

5. Would you consider eating a similar diet regularly after the study is over? Yes No

6. Did you experience any negative symptoms during the study? Yes No
If yes, please explain:____

7. Did you experience any positive symptoms during the study? Yes No
If yes, please explain: ____

8. If you have any other comments please write below: