THE EFFECTS OF GREATER DIETARY PROTEIN SPREAD AND QUALITY ON MUSCLE HEALTH IN HEALTHY ADULTS

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Title

THE EFFECTS OF GREATER DIETARY PROTEIN SPREAD AND QUALITY ON MUSCLE HEALTH IN MIDDLE-AGED ADULTS

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The Supervisory Committee certifies that this ***disquisition*** complies with North Dakota State University's regulations and meets the accepted standards for the degree of

DOCTOR OF PHILOSOPHY

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ABSTRACT

Skeletal muscle is critically important, but is often overlooked, not getting the respect or attention the tissue demands. Muscle is responsible for locomotion and physical performance, uses large amount of energy preventing gains in fat mass, and acts as an amino acid reservoir during trauma. Nonetheless, as individuals age, they lose muscle and to a greater extent strength. Maintaining muscle mass and strength is paramount for preventing disability and mortality. There are many aspects of diet that affect muscle tissue, but dietary protein directly activates muscle protein synthesis, so is important to consider as part of a balanced diet. Research regarding dietary protein intake has focused on the amount of protein consumed, but the quality and distribution of dietary protein also determines the body's anabolic response. Two different cross-sectional studies were completed to determine the associations between dietary protein intake and muscular performance. Dietary intake was measured using three-day food diaries. Isokinetic dynamometry determined lower-body strength and endurance. Handgrip strength measured upper-body strength. Dual x-ray absorptiometry evaluated lean body mass. Thirty-second chair stand and six-meter gait speed tests determined functional ability. Self-reported age and moderate-to-vigorous physical activty, assessed via accelerometry, were included in all models as covariates. Increased intake of higher quality proteins from animal sources was positively associated with lower-body strength ($\beta \pm$ S.E.; 65.874±19.855, $p = 0.001$), lower-body endurance (549.944±232.478, $p = 0.020$), and handgrip strength (0.349±0.171, $p = 0.045$) in the cross-sectional sample of 91 middle-aged men (n=41) and women (n=50) when controlling for relative energy intake and percent energy from the macronutrients. Using another sample of 192 women 18 to 79 years, achieving intakes of at 25 grams per meal was positively associated with lean mass (1.067±0.273 kg, $p<0.001$) and upper-body (3.274±0.737 kg, $p<0.001$) and lower-

body strength (22.858±7.918 Nm, p=0.004) controlling for relative energy intake and percent of energy from protein. In a subgroup of this sample aged 61-79, animal-based protein intake was related to increased lower-body strength (14.834±7.287 Nm, p=0.049) and faster gait speed (-0.177±0.087 s, p=0.049). To benefit muscle and performance, people should strive to consume enough high-quality protein at each meal.

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DEDICATION

This work is dedicated to the many older people in my life who have cared for and mentored me, my wife for loving me, and the many animals who bless our lives. Lastly, I would like to dedicate the work to the late Dr. Stephanie P. Waletzko whose unending kindness, witty sense of humor, and passion touched so many hearts, including mine and my wife's. We miss you Steph.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CFT .....Controlled feeding trial.

CI.....Confidence interval.

CSA.....Cross sectional area.

CT .....Computed tomography.

CV.....Coefficient of variance.

FFQ .....Food Frequency Questionnaire.

DIAAS .....Digestible indispensable amino acid score.

DXA.....Dual x-ray absorptiometry.

HR.....Hazard ratio.

IU .....International unit.

MRI.....Magnetic resonance imaging.

OR.....Odds ratio.

PDCAAS.....Protein digestibility corrected amino acid score.

RCT.....Randomized control trial.

RM .....Repetition maximum.

SD .....Standard deviation.

SE.....Standard error.

SPPB .....Short physical performance battery.

TUG .....Timed up and go.

USDA.....United States Department of Agriculture.

LIST OF DEFINITIONS

Appendicular Skeletal Muscle Mass	The skeletal muscle mass of the appendages (i.e., the arms and legs) assessed using dual-x-ray absorptiometry[1] or bioelectrical impedance.[2]
Dynapenia	A condition characterized by age-related losses in muscle strength, a term coined by Clark and Manini .[3]
Echogenicity	A measure of muscle quality involving computer gray-scale analysis of the intensity of light reflection from an image from magnetic resonance imaging[4,5] or ultrasonography[6] scored in arbitrary units from 0 (i.e., black) to 255 (i.e., white), where higher scores indicate greater intramuscular fat content[7] and poorer muscle quality.
Frailty	A condition characterized by an increased susceptibility to negative stimuli leading to increased mortality and morbidity.[8]
Hounsfield Units	A measure of muscle quality involving computer-aided gray-scale analysis of the intensity of light reflection from an image from computed tomography, scored in arbitrary units from -1,000 (i.e., black) corresponding to air, to 1,000 (i.e., white) corresponding to bone or teeth in the human body; water has value of 0.0.[9] Muscle tissue is considered to have values between 0-100 with higher values indicating lower intramuscular fat content[10] and better muscle quality.
Muscle Endurance	"The ability of muscles to exert force against resistance over a sustained period of time."[11]
Muscle Power	"The ability to exert a maximal force in as short a time as possible, as in accelerating, jumping and throwing implements."[11]
Muscle Strength	"The amount of force a muscle can produce with a single maximal effort."[11] Tools to measure muscle strength include handgrip strength,[12] 1-repetition maximums,[13–18] and isometric[18–27] and isokinetic[7,13,14,17,18,20,23,28–32] dynamometry.

Physical Performance	"An objectively measured whole body function related with mobility."[11] Tools to measure physical performance include the Short Physical Performance Battery,[17,33] the Timed Up and Go test,[34] a 400 m walk test,[35] and gait speed.[36]
Sarcopenia	A condition characterized by age-related losses in muscle strength, quantity, and quality and physical performance, as assessed according to European Working Group's revised (2019) criteria.[37]
Specific Force	A measure of muscle quality, defined as the amount of force or torque a muscle produces relative to its size or mass. Greater specific force values indicate better muscle quality.[38]

1. INTRODUCTION

Skeletal muscle mass comprises 40 to 50% of bodyweight[39] and is, of course, essential for locomotion and muscle strength. Skeletal muscle, though, is more than the tissue that moves us and the objects around us. It contains approximately 45% of the human body's total protein content[40] and acts as an "amino acid reservoir"[41,42] catabolizing itself to provide amino acids or energy to other tissues after traumatic injuries or infections[43] or during periods of negative energy balance.[44] Naturally then, sarcopenia, a condition characterized by reduced muscle mass and strength, is related to both an increased risk of all-cause mortality (odds ratio (OR) [95% confidence interval (CI)]: 3.596 [2.96, 4.37]) and disability (OR [95% CI]: 3.03 [1.80, 5.12].[45] Increasing or maintaining muscle mass and strength is important throughout the lifespan, as is indicated by both experts[37] and the United States Department of Agriculture (USDA),[46] yet muscle mass and strength decline as individuals age.[7]

1.1. Statement of the Problem

1.1.1. Echogenicity and Specific Force

There is no agreed upon definition of muscle quality, yet the measurement of muscle quality by researchers investigating sarcopenia is advocated for by experts.[37] Echogenicity[21] and specific force[47] are both considered measures of muscle quality, yet they have been infrequently compared to one another.[26,48,49] The association between these two distinct methodologies to determine muscle quality warrants further investigation and may help establish a definition of muscle quality.

1.1.2. Protein Distribution and Muscle Strength, Quantity, and Quality

The link between the evenness of protein intake distribution and muscle mass is well established, but the relationship between the evenness of protein intake distribution and strength

and physical performance is more tenuous.[50] Achieving at least 0.24 g of protein per kg per meal or at least 25 grams of protein at each meal should be related to muscle strength and physical performance when controlling for other dietary variables like relative energy intake and the intakes of the macronutrients in addition to other covariates such as age, sex, and physical activity in adults.

1.1.3. Protein Quality and Muscle Strength, Quantity, and Quality

Proteins from animal sources (i.e., animal-based proteins) have better protein quality[51,52] and are thought to stimulate muscle protein synthesis to a greater extent than lower quality plant-based proteins.[53,54] Therefore, animal-based protein intake should contribute to muscle quantity, strength, and performance to greater extent than total protein intake.

1.2. Purpose of the Literature Review

The objectives of this review were threefold: one, to determine the effects of aging on muscle, strength, and the development of sarcopenia, two, to describe the methods used to diagnose sarcopenia, and three, to investigate the role of dietary intake on muscle health. More specifically, this work focuses on dietary protein intake and its effects on muscle.

2. LITERATURE REVIEW

2.1. Literature Search Methods

The Pub-Med, Web of Science, and Science Direct Databases were searched using the terms "sarcopenia," "dynapenia," "specific force," "muscle mass," "muscle cross-sectional area," "muscle quality," "strength," "physical performance," "performance," AND "diet," "nutrition," or "protein." Search results were limited to studies performed in humans and published after 2009. The references of search articles were reviewed to add additional, relevant, older works.

2.2. Muscle Strength and Aging

It is well accepted that decreases in muscle mass are the result of age-related processes. E Excluding animal models, the best evidence to support age-related decline in muscle mass comes from longitudinal studies, as these studies are free from some of the confounding variables that affect cross-sectional research. In the "Health Aging and Body Composition Study," a longitudinal sample of adults aged 70 to 79 at baseline, men (n = 813) lost (Mean ± standard deviation [SD]) 6.8 ± 10.0 cm^2 (4.9 ± 7.4%; $p < 0.001$), whereas women lost 3.2 ± 7.6 cm^2 (n = 865; 3.2 ± 7.9%; $p < 0.001$) of thigh muscle cross-sectional area (CSA) assessed via computed tomography (CT) across a five year period.[7] Another group of researchers utilizing the same dataset reported that men lost a mean of 0.145 kg or 0.8% of lean leg mass per year measured using dual x-ray absorptiometry (DXA); women, on the other hand, lost a mean of 0.088 kg, 0.7%, of lean leg mass per year during the same now eight-year period.[30] In support of these findings, "The Baltimore Longitudinal Study of Aging," which followed 412 men for (Mean ± SD) 15.4 ± 3.9 years, reported declines in both muscle CSA determined using arm circumference with skinfold measurements and muscle mass assessed using deuterated creatine/creatinine excretion analysis.[25]

Longitudinal declines in muscle mass reported in the "Health Aging and Body Composition Study"[7,30] and other longitudinal studies[25] are supported by data from a host of cross-sectional studies.[25,55–57] Some of earliest cross-sectional studies of muscle and aging utilized cadavers.[55,56] Perhaps more important than linking muscle mass to age, these early studies on cadavers established that the loss of muscle mass associated with aging does not affect all muscles or fiber types equally. Lindboe and Torvik (1982)[55] reported a respective 9 and 14% difference in Type II muscle fiber areas of the *biceps brachii* and *tibialis anterior* among 23 healthy men who were either 60 years or older (n = 10) or under 60 years (n = 13) when they died suddenly; however, there was not a significant difference in Type I fibers. Several years later in another examination of cadavers, Oertel (1986)[56] not only found that Type II fibers atrophy with age to a greater extent than Type I fibers, but also that these changes begin in young adults and occur more predominantly in the *vastus lateralis* compared to the *deltoid*. Not only do declines in muscle health start at earlier than age than older adulthood, but the differential loss of Type II fibers between the two muscles is concerning because the *vastus lateralis* is more directly involved with locomotion and balance than the *deltoid*. Thus, losses of Type II fibers and therefore strength in the *vastus lateralis* are more likely to lead to loss of function and disability than losses from the *deltoid.*

In fact, although many physiological factors contribute to age related losses in muscle mass and strength,[58] the loss of Type II fibers is critical because some older adults use close to their maximal muscular power to stand from a chair.[59] Thus, any change that adversely affects muscular power can dramatically affect older adults' quality of life, particularly if these changes occur in the lower body, as described by Oertel.[56] Type II, or fast-twitch muscle fibers, adhere to Henneman's Size Principle[60,61] and are recruited after slower-twitch Type I fibers, when the body

needs to generate maximum force or power.[62] Therefore, the loss of Type II fibers has a greater effect on physical performance than a loss of an equivalent amount of Type I and II fibers combined. In other words, due to the loss of Type II fibers, it is expected that muscle strength and power decline more rapidly than what would be expected from corresponding losses in muscle mass.

Even though losses of muscle strength have been included in the definition of sarcopenia since at least 2001,[63] the differential impact of age related processes on performance and muscle mass were largely overlooked until a special "Green Banana" article by Clark and Manini (2008)[3] in which they coined the term "dynapenia," the age related loss of strength. In support of their arguments in the special article, Clark and Manini also reference the "Health Aging and Body Composition Study," where men (n = 814) and women (n = 865) 70 to 79 years of age respectively lost (Mean ± SD) 4.9 ± 7.4% and 3.2 ± 7.9% of thigh muscle cross sectional area, whereas they lost a 16.1 ± 20.6% and 13.4 ± 23.0% of muscle strength over a five-year period.[7] Since then, others have reported losses in strength and physical performance that are greater than those observed in muscle mass or area. Using a longitudinal sample of habitually active men (n = 59; Mean ± SD: 58.6 ± 7.3 years) and women (n = 35; Mean ± SD: 56.9 ± 8.2 years) with an average follow-up period of 4.8 years, Marcell, Hawkins, and Wiswell (2014) reported isometric knee extension strength losses of about 5% per year even though lean body mass measured using hydrodensitometry did not change.[23] Others using less advanced techniques of anthropometry, namely leg circumference 10 cm above the knee, found no difference in several measures of leg circumference between individuals aged under 40 (n = 14) and those over 40 (n = 12), but saw significant differences in isometric strength at 60° flexion between the groups (Mean ± SD: <40: right leg = 716.96 ± 291.88 N, >40: right leg = 423.84 ± 179.45 N, p = 0.00448; <40: left leg

757.55 ± 291.08 N, >40 left leg 520.97 ± 220.25 N, p = 0.0234).[64] Nonetheless, the current consensus is to use the term sarcopenia to describe both losses of muscle strength and muscle quantity,[37,65] so sarcopenia in this work will also refer to age related losses in both muscle quantity and strength.

2.3. Diagnosing Sarcopenia

In 2010, the European Working Group on Sarcopenia in Older People released the first widely known algorithm for diagnosing sarcopenia.[65] This algorithm had three criteria. The first criterion, low muscle mass, had to be met before the other two criteria, low muscle strength and low physical performance, could be used to confirm the condition. More specifically, a person with low muscle mass and either of the other two criteria was considered to have sarcopenia, whereas a person meeting all three criteria was considered to have "severe sarcopenia."[65] Following the publication of this algorithm, the Asian Working Group for Sarcopenia released their criteria for diagnosing the condition, which were similar to that of the European Working Group; sarcopenia was defined as "age-related loss of muscle mass, plus low muscle strength, and/or low physical performance."[66] Likely as direct result of these works, sarcopenia eventually a earned an International Classification of Disease code.[67]

Both the European[37] and Asian Working Groups[68] have since updated their criteria for diagnosing sarcopenia to include more relevant metrics and more fine-tuned cut-points for muscle quantity, muscle strength, and physical performance measures. More specifically, the updated cut-points reflect the different populations evaluated by the two different working groups. The European working group considered cut-points for people of European ancestry,[37] whereas the Asian working group considered cut-points for those of Asian ancestry.[68] Because the population of the United States (72.0%) and that of Fargo in particular (84.6%) is mostly

people of European descent,[69] this work uses the European Working Group's criteria.[37] Notably, the European Working Group's criteria changed from 2010;[65] the first criterion was changed from low muscle mass to low muscle strength, which was followed by low muscle quantity or quality as the second criterion, with low physical performance only as a third criterion for "severe sarcopenia."[37]

2.3.1. Assessing Muscle Strength

As indicated, the first criterion of sarcopenia according to the European Working Group's revised consensus is low muscle strength.[37] Fortunately, measuring muscle strength is relatively straightforward and inexpensive and can be completed using a variety of laboratory, clinical, or field tests. The European Working Group[37] specifically recommends using two clinical or field tests to appraise muscle strength. The working group's preference is handgrip strength[12] or if handgrip strength cannot be performed, a 5-repetition chair stand test,[70] a derivative of the 30-second chair stand test,[71] to evaluate muscle strength because of their low cost, accessibility, and relationship to critical health outcomes. For instance, a Jamar Handheld Dynamometer (Bolingbrook, IL), the most widely cited tool to measure grip strength,[12] costs less than $300.00 and can be brought directly to participants, even those unable to get out of bed, for strength testing. Beyond this, decreases in or low handgrip strength have been related to important health outcomes such as mortality, disability, and cognitive impairment.[72]

Outside of the European Working Group's recommendations,[37] others have used isometric,[18–27] isokinetic,[7,13,14,17,18,20,23,28–32] and 1-repetition maximum (RM)[13–18] tests to measure muscle strength. Isometric and isokinetic tests are laboratory measures of strength, although portable isometric dynamometers that allow for clinical or field testing are sometimes used.[22] In fact, isokinetic dynamometry has been hailed as the "Gold Standard" of muscle strength

measures.[73] While isokinetic dynamometry may be considered the best measure of muscle strength, it is not portable, nor does every organization, researcher, or clinician have access to an isokinetic dynamometer, limiting its use in clinical and field settings. When selecting measures of muscle strength for research, one must obviously consider their feasibility; isokinetic measures of strength may not always be possible because of their limited portability and accessibility.

The 1-RM, on the other hand, is a field test; equipment for 1-RMs is more accessible to researchers and readily available in most gyms, but it is still not portable like isokinetic and most forms of isometric dynamometry, limiting the use of 1-RMs in clinical settings. Surprisingly, 1-RMs have not only been found to be both safe in older adults[74] and those in cardiopulmonary rehabilitation programs,[75,76] but have also been shown to be reliable in untrained men and women (i.e., in men and women not exercising).[15] Nonetheless, of these two techniques the 1-RM is least optimal as the technique seems to be biased, overestimating strength gains compared to isokinetic measures of strength.[13,14,16]

Handgrip strength, in addition to being associated with various important health outcomes,[72] is related to isokinetic strength.[73] Thus, handgrip strength can be considered a stand-in for isokinetic measures in clinical populations, which is another reason why the European Working Group advocates for using handgrip strength in their revised consensus.[37] Ideally, laboratory research investigating sarcopenia will have participants perform isokinetic dynamometry for its ability to measure muscle endurance (i.e., work), and muscle power in addition to muscle strength[77] and other measures of muscle strength such as handgrip strength or a chair stand test that can easily be compared to epidemiological[71,78–80] or clinical data.[48,81]

2.3.1. Assessing Physical Performance

Poor physical performance is the third and final criterion of the European Working Group's revised consensus, distinguishing "confirmed sarcopenia" from "severe sarcopenia."[37] The European Working Group[37] offers several different methods to assess physical performance including the Short Physical Performance Battery (SPPB),[17,33] the Timed Up and Go (TUG) test,[34] a 400 m walk test,[35] and gait speed.[36] These assessments, like some techniques to measure muscle strength, require little equipment and can be completed in a variety of settings. In fact, walking is a critical element of all of these tests.

Nevertheless, the SPPB is the most complex of these methods, consisting of three different assessments: an eight-foot gait speed test, tandem, semi-tandem, and side by side standing 10 s balance tests, and a 5-repetition chair stand test.[82] Each one of these three types of assessment is scored as 0-4; thus, the maximum score on the SPPB is 12, indicating the highest level of physical performance.[82] The TUG is the next most sophisticated assessment of physical performance, demanding participants stand from sitting, walk 3 m, and return to sitting.[83,84] The 400 m walk test[35] and assessments of gait speed[36] are least complicated, only requiring participants to walk. Of these two types of assessment, measurements of gait speed are easier for participants to complete as gait speed only asks participants to walk up to 6 m[36] as opposed to 400 m.[35] In fact, not being able to complete the 400 m walk test is a determinant of "severe sarcopenia" according to the European Working Group.[37] Because walking is an element of all four of these measures of physical performance, researchers investigating sarcopenia should at the minimum include a measure of gait speed in their work.

However, measures of physical performance in the context of the European Working Group's revised consensus[37] are intended to differentiate between those with sarcopenia and

those with "severe sarcopenia." In other words, in studies utilizing non-sarcopenic populations it may not be useful to assess physical performance. In support of this, Buchner and colleagues,[28] using a cross-sectional sample of 409 adults aged 60 to 96 years, reported no relationship between isokinetic leg strength and gait speed in stronger older adults, whereas leg strength was related to gait speed in weaker older adults when using a quadratic regression model. Thus, depending on the population, researchers examining sarcopenia or investigating the associations between lifestyle factors and the condition, may choose to omit measures of physical performance from their research.

Although sarcopenia can be prevented and treated through drugs such as myostatin inhibitors, testosterone and its derivatives, and selective androgen receptor modulators, these pharmacological interventions can cause negative side effects and can also be expensive.[85,86] Diet,[87–92] including heavy or binge drinking[93] but not moderate alcohol consumption,[94] physical activity or exercise,[17,87,95–98] sleep,[99,100] and cigarette smoking[101,102] are aspects of people's lifestyles that affect muscle strength, quantity or quality and represent low cost and well tolerated ways to mitigate sarcopenia. In fact, resistance exercise and dietary interventions are the most common non-pharmacological methods to address sarcopenia.[86] This work focuses on lifestyle changes to address sarcopenia, and more specifically, on dietary interventions that included other factors such as physical activity/exercise, sleep, and smoking status as covariates.

2.3.2. Assessing Muscle Quantity

According to the European Working Group's revised criteria, low muscle quantity or quality needs to be established after low muscle strength for a person to be considered to have sarcopenia.[37] There are many ways to assess muscle quantity, including CT,[7,22,57,103–108] DXA,[17,18,27,57,95,102,109–111] hydrostatic densitometry or weighing,[23,112] air displacement

plethysmography,[113] magnetic resonance imaging (MRI),[17,109,114–118] bioelectrical impedance,[119–121] ultrasonography,[6,21,26,29,108,115,122] and deuterated-creatine/urinary creatinine analysis.[25,109,123,124] It must be noted that some of these methods, namely DXA, hydrostatic weighing, air displacement plethysmography, and bioelectrical impedance technically do not quantify muscle but rather lean tissue, which includes muscle in addition to connective tissue and organs.[125] Deuterated-creatine/urinary creatinine analysis, on the other hand, is thought to measure muscle mass alone.[109] Nonetheless, these methods estimate whole body lean tissue or muscle mass, whereas CT, MRI, and ultrasonography produce images of individual muscles or muscle groups.

Of these measures of muscle quantity, only CT, MRI, and ultrasonography also measure muscle quality when performed alone.[126] Echogenicity, echo intensity, or computer-aided gray-scale analysis, is a measure of the intensity of light reflection from an image. Brighter images indicate greater amounts of intramuscular adipose and fibrous tissue which affect muscle quality,[7] and these images can be captured from MRI[4,5] or ultrasonography.[6] In older Japanese women, the echogenicity of images taken from ultrasonography were related to both age ($r = 0.34$; $p < 0.01$) and isometric strength ($r = -0.40$; $p < 0.01$).[6] Another way to evaluate muscle quality without a performance measure is to use Hounsfield Units. Hounsfield Units are the intensity units derived from CT,[10] and the technique is similar to echogenicity. A couple of studies have found positive associations between Hounsfield Units and mortality,[106,107] and one of these works reported a decrease in muscle quality with aging when using Hounsfield Units,[106] supporting their use for assessing sarcopenia. However, Hounsfield Units did not change in the "Health Aging and Body Composition" study, a longitudinal study with the goal of determining the effects of aging on muscle quality.[7,105] Although using either echogenicity or Hounsfield

Units to evaluate muscle quality does not require a measure of performance, neither is perfect. Echogenicity, at least from images captured using ultrasonography, is highly dependent on the sonographer's skill, and measurements of Hounsfield Units require exposure to x-rays. Perhaps more important, images from CT, MRI, and ultrasonography require analysis by a trained researcher or clinician, using a program such as ImageJ.[127,128]

When coupled with a measure of muscle strength, any method of quantifying muscle or lean tissue can produce a measure of muscle quality in the form of specific force, the amount of force produced divided by the quantity of muscle. In fact, specific force is the most common method of assessing or defining muscle quality. When investigating specific force, lean body mass has been determined using DXA in many studies,[30,47,95,98,105,110,129,130] whereas measures of muscle CSA are less common and have been measured using CT,[7] MRI,[117,118] and ultrasonography.[29] Performance has been assessed using a multitude of test and protocols including handgrip strength,[47,48,95] knee extensor strength, assessed using either 1-RMs[47,117] or strain gauges,[95,98] maximal knee flexor strength,[98] maximal leg press strength,[98] isokinetic knee extensor torque or strength,[7,30,105,129,130] knee extensor power,[110] isometric dorsiflexor strength,[118] and isokinetic dorsi/plantarflexion.[29,131]

Defining muscle quality as a performance measure relative to a measure of muscle mass or size has perhaps been most useful for describing age-related losses in muscle quality and their effects on health.[7,30,105,129] One group reported that for each SD increase in muscle quality mortality risk decreased by 11%.[106] Similarly, in patients receiving hemodialysis, greater muscle quality was related to lower 10-year mortality rates.[132] Lynch and colleagues[129] (1998) observed losses in muscle quality, as defined as concentric peak torque divided by muscle mass, across the lifespan using a cross-sectional design. Although statistically significant, r-squared values of

regression models where age was used to predict muscle quality were low (Men: Arm $R^2 = 0.15$; Leg $R^2 = 0.26$; Women: Arm $R^2 = 0.07$; Leg $R^2 = 0.27$) indicating that age only marginally contributes to muscle quality at least using this methodology. Others[7,30,105] did not report r-values making it difficult to evaluate the effects of aging on muscle quality when it is defined in this manner.

The low explained variance (i.e., r-squared values) reported by Lynch and colleagues[129] may be the result of a fundamental flaw of using specific force, that is this performance divided by muscle mass or area methodology, to define muscle quality. It seems that slower twitch muscle fibers are more affected by disuse than faster twitch fibers.[133] Thus, disuse may lead to increases in specific force values, and therefore populations who are less active may show greater specific forces (i.e., better muscle quality) than active populations. Indeed, in one study, there was a positive correlation between time spent in sedentary behavior and muscle quality when it was defined as knee extensor power divided by lower limb lean body mass in 16 older men ($r = 0.607$; $p < 0.001$),[110] despite the fact that faster twitch Type II muscle fibers are lost to greater extent during aging.[55,56] Another work found that appendicular muscle mass and upper ($r = -0.53$; $p < 0.001$) and lower-body ($r = -0.23$; $p < 0.001$) specific forces, determined using handgrip strength and knee extensor strength respectively divided by upper and lower body muscle mass determined with DXA, were inversely related.[47] Including intramuscular fibrous and adipose tissue in measures of muscle mass or area helps remedy this issue and has been performed in at least two other works.[106,118] A one SD increase in specific force relative to lean muscle area was associated with an 11% (95% CI of hazard ratio (HR): [0.83, 0.95]) decrease in risk of mortality,[106] and there was a difference in lean area but not total muscle CSA between healthy participants in the control group and those undergoing dialysis.[118]

Even if intramuscular fibrous and adipose tissue are included when measuring specific force, measures of muscle performance including strength testing are dependent on participant skill, motivation, and anthropometry, which may make measures of muscle quality that are dependent on performance measures spurious. Moreover, these confounders are not easily controlled for. One group of researchers sought to overcome these limitations by electrically stimulating participants' muscles during strength testing.[118] This technique reduces participants' motivation and skill as confounders, but it still does not alleviate concerns regarding anthropometry. More specifically, a person with a muscle whose insertion is farther away from the fulcrum of the joint will produce more force than the same person would if the insertion of the muscle is closer to fulcrum, potentially limiting the usefulness of specific force as an indicator of muscle quality. Yet, as there is no consensus about the definition of muscle quality,[37] researchers should strive to include both definitions of muscle quality, that is specific force and echogenicity or Hounsfield Units, in their work evaluating sarcopenia.

Despite the fact that specific force[47] and echogenicity[21] are considered measures of muscle quality and the fact that the echogenicity of the *rectus femoris* determined using ultrasound is related to muscle quality assessed using CT (i.e., Hounsfield units),[108] echogenicity of a muscle has not been directly related to the specific force of that muscle. In support of this disparity between these two broad methods of assessing muscle quality (i.e., specific force vs. echogenicity), Strasser and colleagues (2013) reported no relationship between the echogenicity of the four quadriceps muscles from ultrasound and maximal isometric knee extensor strength in a cross-sectional sample of 26 older (60-80 years) patients at an Austrian hospital.[26] In contrast, echogenicity has been inversely related to the specific force of individual muscle fibers ($r = -0.62$; $p = 0.02$) in 12 obese, older adults (68 ± 3 years),[49] and Ismail and colleagues (2015)

reported a significant relationship between the echogenicity of *rectus femoris* and handgrip strength relative to bodyweight, a very crude measure of specific force, in a sample of 20 middle-aged women (43.4 ± 3.4 years).[48] Again, as there is no consensus regarding muscle quality[37] and because both specific force[47] and echogenicity[21] are considered measures of muscle quality, researchers investigating muscle quality should be not only be performing both types of measures of muscle quality, but should also be comparing the two types of measures.

2.4. Dietary Intake

Dietary intake is comprised of energy, macronutrient, micronutrient, phytonutrient, water, and alcohol intakes. Beyond the detrimental effects of aging on muscle strength, quantity, and quality, people's ability to taste decreases with aging[134] as does their oral health[135] and ability to masticate.[136] As the result of these change among other factors, dietary intake decreases by about 25% from age 40 to 70, predisposing middle-aged and older adults to malnutrition which can hasten the development of sarcopenia.[137] Aging not only results in loses of lean tissue and strength,[7,25,30] but also due to the fact that aging reduces energy expenditure,[138] older adults can gain fat tissue despite reduced dietary intake; this can result in sarcopenic obesity,[139] which can further increase one's risk of death[140] or disability.[141] Thus, older adults should choose nutrient dense foods, as is recommended by the USDA[142,143] and other experts.[144,145]

2.4.1. Assessing or Manipulating Dietary Intake

Assessing or manipulating dietary intake is fundamental to nutritional research which informs recommendations, such as those of the USDA.[142,143] In experimental designs, such as randomized control trials (RCTs) and controlled feeding trials (CFTs) dietary intake is both manipulated and assessed. Observational designs, such as cross-sectional and longitudinal studies, on the other hand, only utilize assessments of dietary intake. Therefore, dietary

assessment is essential and ubiquitous to nutritional research, and within human nutritional research, several broad categories of assessment are used: biomarkers, food-frequency questionnaires (FFQs), recalls, and food diaries or logs. Study design often dictates which type or types of assessment are used.

Of all nutritional study designs, CFTs, experiments where participants are given all of their dietary intake by researchers, are considered the Gold Standard,[111,146] as the methodology minimizes confounding variables such as participants' ability to accurately recall, estimate, or record their intakes. As the nutrient content of all food provided in a CFT is known, researchers have an objective measure of dietary intake by determining or dictating the amount of food eaten.[147] Other study designs utilize correlations between biomarkers and dietary intakes or use self-reported dietary assessments, such as 24-hour or multiple day recalls, dietary food logs or journals, or FFQs. Self-reported methods can be biased by factors such as memory,[148,149] the desirability of responses[148,150] or participants' ability to estimate portion size.[151] Biomarkers, although more objective, do not capture all aspects of dietary intake measured by self-reported measures.[152] Additionally, biomarkers vary across individuals[153,154] and therefore are more useful to measure changes in intake within participants than to determine differences in intake between participants. For instance, serum albumin, a biomarker of protein intake, was only weakly related to nutritional status ($r = -0.13$; $p = 0.003$) assessed by the Short Global Assessment[155] and moderately associated with protein intake ($r = 0.42$; $p < 0.001$) measured via another biomarker, normalized protein equivalent of nitrogen appearance (i.e., the amount of urinary nitrogen relative to serum albumin),[156] in respectively 383 or 104 patients with end stage renal disease. Thus, instead of being used to compare between participants, changes in biomarkers within

participants are often used as outcome or dependent variables, or to validate the results of self-reported measures.[153,157,158]

In addition to providing all food, a "pure" or "true" CFT is not free-living; participants are locked down in a facility or they are monitored by clinicians or researchers for the entirety of the study, and all dietary intake is provided, meticulously measured, and recorded.[147,148,159] The advantage is that researchers are more confident of participants' dietary intakes, as participants only have access to foods the researchers provide for them and do not have access to other foods.[147] This is in contrast to other experiments, including some CFTs, where dietary intake is manipulated under free-living conditions, although some studies utilize both free-living and controlled-feeding methods.[111,160] Even if ample food is provided to participants under free-living conditions, there is risk of participants "going off menu" and consuming food not planned to be available to them, confounding the results. Thus, "absolute" CFTs where participants remain in a facility or are followed by researchers for the duration of the study are best.

The minimization of bias in CFTs performed entirely in a laboratory or other facility comes at a cost to the feasibility of the design. Because these studies are done entirely in a controlled setting, their generalizability to free-living settings may be somewhat limited as is their feasibility. Participants are monitored closely during CFTs, and observation can affect eating behaviors, potentially decreasing intake.[161] Thus, findings from CFTs may not be generalizable to free living settings. In addition, free-living study designs are not only more generalizable but also more feasible. Researchers do not need to find and pay for, one, a lockable housing and research facility, two, staff to administer the controlled feedings, monitor participants, and maintain the facility, and three, participants who are willing to stay "locked-in"

at the research facility or be observed the entirety of the study.[147] For these reasons, free-living experiments are performed much more often than CFTs.

Despite being more feasible, free-living experiments in the context of aging, nutrition, and sarcopenia are also somewhat limited in their feasibility. The detrimental effects of aging alone on muscle strength,[23] quantity,[7,25,30] and quality[105] are noticeable over longer periods of time (i.e., years) that are often greater than what is practical to investigate in many CFTs or RCTs (i.e., weeks). Thus, some of the strongest evidence linking diet, aging, and sarcopenia comes from observational studies, particularly longitudinal works, such as the "Health Aging and Body Composition Study,"[7,30,105] the "Baltimore Study of aging,"[25] and "The InCHIANTI Study."[162]

Regardless of the strengths and drawbacks of different study designs, assessment of dietary intake is *ipso facto* an aspect of nutritional research. The advantages of CFTs and biomarkers is that the methods are objective. However, true CFTs are relatively infeasible,[147] and biomarkers do not fully capture or estimate dietary intake.[152] Thus, many works utilize self-reported and therefore subjective dietary assessments like recalls, FFQs, and food diaries, which as previously indicated, are biased.[148–151] In recalls, researchers interview participants asking about their dietary intake usually over the last 24 hours,[148,151,163] although some recalls ask participants to remember intake from as long as a week ago.[149,164] Food frequency questionnaires, on the other hand, are surveys containing questions that have a discrete number of answers intended to assess dietary intake. This in contrast to recalls where participants are asked open ended questions (e.g., What did you eat for breakfast yesterday?). FFQs can be guided with an interview or self-adminstered,[148] and usually ask participants about intake over a longer of period of time such as several months,[163] as opposed to the last 24 hours[148,151,163] or

week.[149,164] A critical limitation of FFQs is that they contain a limited number of foods and options for frequencies of consumption. Food choices and motivations vary due to social, economic, cultural, biological, and environmental factors.[165,166] Thus, FFQs must be specific to a context,[27,167] limiting their generalizability, or they are nonspecific to their context, decreasing their validity.

Food diaries, unlike recalls and FFQs, demand that participants record their intake in real-time as they eat, and therefore, do not rely on memory, an advantage of the method.[152] In fact, food diaries, collected for a period of four days, explained a larger proportion of energy intake (partial adjusted $R^2 = 13.3$) and protein intake (partial adjusted $R^2 = 44.2$) measured using two biomarkers, doubly-labeled water and urinary nitrogen, than a 24 hour recall (energy partial adjusted $R^2 = 4.8$; protein partial adjusted $R^2 = 31.7$) or a FFQ (energy partial adjusted $R^2 = 6.5$; protein partial adjusted $R^2 = 16.4$) in a sample of 450 older women.[157] Another group of researchers found that nutrient intakes from 3-day food diaries (mean $r = 0.29$) were more closely related to nutrient intakes from 9-day food diaries than intakes from a population specific FFQ (mean $r = 0.21$).[167,168] Moreover, some works have used food diaries to validate other self-report methods.[167–170] In addition, food diaries can be used across contexts, so long as their instructions are clearly translated and participants can write. Nonetheless, food diaries are reactionary and thus are still biased;[152,158] participants are recording foods as they consume them, and therefore are more aware of their food choices, which affects which foods participants eat and how much they eat. Beyond this limitation, all self-report measures rely on nutrient databases that may have their own errors or omissions.[152]

In sum, if the goal of research is to make high-quality inferences about human nutrition, then researchers ideally should perform CFTs because of their objectivity, but due to practical

considerations, much nutritional research has been done using subjective, self-reported assessments. Although all of these self-reported assessments are biased to some extent,[148–151,158] food diaries seem to be the best as they limit errors due to recall.[157,158,168] In fact, food diaries have even been added to the ASA24, which was originally a 24-hour food recall developed by the NIH.[171] Biomarkers are useful, objective measures of nutrition, but no group of biomarkers can fully describe dietary intake.[152] Instead, biomarkers are often used with other forms of nutritional assessment or as a dependent variable. When choosing methods to assess or manipulate dietary intake, researchers need to consider both the feasibility and limitations of their methods.

2.4.2. Protein as a Nutrient to Address Sarcopenia

Several nutrients are particularly important for preserving muscle strength, quantity, and quality including protein, fatty acids, vitamin D, antioxidants, and minerals such as iron, magnesium, calcium, selenium, and zinc.[91,172] For example, using a longitudinal sample of 884 participants followed for 3 years from "The InCHIANTI Study," Abbatecola and colleagues (2009)[162] reported an inverse relationship between n-3 polyunsaturated fatty acid (i.e., α-linolenic, eicosapentanoic, and docosahexaenoic acids) intake and a decrease in SPPB score to 9 or less, an indication of poor physical performance (95% CI of OR: [0.081-0.530]). In a RCT, 1,000 international units of supplemental vitamin D3 significantly improved TUG performance ($p < 0.001$), but not muscle strength in vitamin D deficient middle aged women.[121] Another group reported positive correlations between iron (partial $r = 0.08$; $p = 0.02$), zinc (partial $r = 0.07$; $p = 0.02$), and magnesium (partial $r = 0.07$; $p = 0.02$) determined using an FFQ specific to the setting, Australia,[169] with lean body mass assessed via DXA.[27] Protein, though, is of

particular interest because of the nutrient's ability to directly affect muscle protein synthesis and breakdown.[159,173–176]

Proteins, of course, are long chains of amino acids. Beyond being the literal "building-blocks" of proteins, amino acids contribute to muscle protein synthesis by activating the mammalian target of rapamycin complex 1 (mTOR1).[174,177] This cytosolic protein complex controls translation, the process of producing proteins from messenger ribonucleic acids (i.e., mRNAs),[178,179] thereby regulating muscle protein synthesis.[174] Moreover, mTORC1 is at the interconnection of several different signaling pathways including ones related to energy balance (i.e., low adenosine triphosphate), hypoxia, hormones or growth factors,[180,181] and mechanotransduction.[182–185] Although aging alone seems to have a negligible effect on muscle protein synthesis and mTORC1 activity,[186] Fry and colleagues (2011) found that phosphoproteomic markers of mTORC1 activity (e.g., ribosomal protein S6 kinase beta-1 p-Thr 389) and muscle protein synthesis were lower in 16 older adults (70 ± 2 years) compared to 16 younger adults (27 ± 2) following resistance exercise (i.e., 8 sets of 10 repetitions of leg extension at 70% of 1-RM), suggesting that aging blunts mTORC1's response to mechanical stimuli and thus muscle protein synthesis.[187] However, others reported that a protein rich meal (i.e., 660 kcal, 90 grams of protein, 33 grams of fat) can help mitigate these losses in muscle protein synthesis,[188] supporting the role of protein in preventing sarcopenia.

Several aspects of mTORC1 signaling are sensitive to amino acids.[189] In the "classical" pathway of mTORC1 activation by amino acids,[189] amino acids are sensed in the lumen of lysosomes by vacuolar adenosine triphopastase[190] which through another protein, the "Ragulator," recruits mTORC1 to the surface of the lysosome,[191] where the complex can be activated by a lysosomal membrane-tethered guanosine triphosphatase, Ras homolog enriched in

brain.[192,193] Ras homolog enriched in brain binds to and activates mTORC1, and like most guanosine triphosphatases, Ras homolog enriched in brain is active when bound to guanosine triphosphate (GTP);[192,193] thus, proteins that enhance the catalytic activity of Ras homolog enriched in brain, facilitating the hydrolysis of GTP to guanosine diphosphate (GDP), inhibit mTORC1.[194] Four other guanosine triphosphatases, the "Rags" (i.e., Rag A, B, C, and D) also play critical roles in mTORC1 signaling related to amino acids. The Rag proteins function as heterodimers of Rag A or B and Rag C or D and are involved with the Ragulator to help relocate mTORC1 to the lysosomal membrane when amino acid concentrations are high.[190,191,195] Rag A/B is active when bound to GTP, a function of the guanine exchange activity of the Ragulator,[195] whereas Rag C is counterintuitively active when bound to GDP.[196,197] In other words, when Rag A or B is GTP bound and Rag C or D is GDP bound, the Rag/Ragulator complex is active, translocating mTORC1 to the lysosome. Although the guanosine loading of the Rag proteins are not directly affected by amino acids,[198] upstream regulator proteins capable of sensing amino acids (i.e., GATOR 1/2 or Folliculin) affect the rate of hydrolysis of GTP bound to the Rag proteins.[197,199,200] Thus, mTORC1 is capable of sensing amino acid inputs from both the lysosome and the cytosol.

Although all 20 naturally occurring amino acids likely impact mTROC1 signaling pathways to some extent, both leucine and arginine are needed to activate mTORC1,[201] and each has its own cytosolic receptor in mTORC1 signaling.[202,203] Of these two amino acids, leucine likely has greater importance as a nutrient not only because it is an essential amino acid, incapable of being synthesized by the body, but also because leucine is considered by some as "the strongest determinant of the capacity of a protein to affect [muscle protein synthesis] and likely hypertrophy."[204] In support of this notion, 4 g of supplemental leucine given at each meal

(i.e., three times daily) was found to increase both muscle protein synthesis and phosphoproteomic markers of mTORC1 activity in eight older adults (Mean ± Standard Error [SE]: 68 ± 2 years).[205]

Despite the effectiveness of leucine at stimulating muscle protein synthesis, it is incapable of fully activating mTORC1 without some degree of mechanical stimulation. Rats fed 1.35 g of leucine per kg bodyweight and with one hindlimb immobilized showed decreased ribosomal protein S6 kinase beta-1 phosphorylation, an indicator or mTORC1 activity, in their immobilized limbs but not in their free hindlimbs.[206] This finding was later supported by the identification of two mechanically controlled phosphorylation sites on an upstream regulator of Ras homolog enriched in brain,[185] which is a distinct mTORC1 signaling mechanism from those associated with amino acids.[180,181] Beyond affecting the phosphorylation of this upstream protein complex, mechanical stimulation, such as exercise, increases the amino acid transporter for leucine, leading to increased leucine uptake and mTORC1 activity,[207] as amino acid transporters are also directly involved in amino acid signaling.[189] In addition, essential amino acids also increase the amino acid transporter for leucine,[208] potentially leading to a multiplicative effect of exercise and protein intake on muscle protein synthesis. Thus, studies of mTORC1 signaling indicate that some level of mechanical stimulation (i.e., physical activity or exercise) is needed for participants to benefit from increased protein intake. In a nine year longitudinal analysis of nutrition and physical activity data from the "Framingham Offspring Study," high levels of physical activity were needed for participants' legumes, soy, nuts, and seeds intake to be related to increased muscle mass, supporting the need for some "mechanical stimulation," yet other proteins, namely animal-based proteins, were related to increased muscle mass even in those with lower physical activity levels, reiterating the importance of protein to muscle quantity.[96]

Nonetheless, within all groups, those who were more active had greater muscle mass.[96] Physical activity and exercise then, although a critical element of benefiting from increased protein intake, must be monitored and controlled for in studies examining nutrition and muscle health.

2.4.2.1. Physical activity and Exercise: Important covariates in Nutrition Research

Beyond being necessary to fully activate mTORC1,[185,206] the beneficial role of mechanical stimuli such as physical activity[96] and exercise, particularly resistance exercise,[32,209] on physical performance, and muscle quantity, quality, and strength is well documented.[18,210–212] By increasing muscle mass and strength through independently activating mTORC1 from dietary intake,[182–185,206] physical activity and exercise are confounders in nutritional research that must be controlled for. Although both direct or indirect calorimetry and observation are the most valid methods of measuring physical activity, these methods have a high burden for researchers and participants[213,214] and do not represent physical activity under non-experimental conditions. Unlike free-living measures of dietary intake, which as previously indicated are all subjective and self-reported,[148–151,158] free-living physical activity can be objectively measured using methods such as accelerometry, pedometry, heart rate monitoring, and doubly labeled water.

Although subjective, self-reported tools to measure physical activity, akin to those used in nutritional research (e.g., questionnaires, recalls, diaries), are sometimes used to estimate physical activity, they are not as valid as objective methods.[215] In a systematic review of physical activity questionnaires, the most valid questionnaires were only moderately related to objective measures of physical activity;[216] the strongest of these associations only explained 55% of total energy expenditure estimated using accelerometry.[217] Moreover, unlike performing CFTs, the only truly objective method of human nutritional research, it is feasible to perform objective measures of physical activity on a large scale. For example, the "National Health and Nutrition

Examination Survey" (NHANES) has included accelerometry in each of its waves since 2003-2004, in which over 10,000 participants wore a device.[218] Objective methods of measuring free-living physical activity are therefore not only more valid than subjective measures, but are also feasible to perform which is in contrast to performing objective measures of dietary intake.

Nonetheless, each of these objective methods to measure free-living physical activity is limited in some way. Doubly labeled water uses heavy isotopes of hydrogen and oxygen (i.e., deuterium and Oxygen-18) to determine carbon dioxide production; deuterium is lost only in urine as water, whereas heavy oxygen is lost in both urine and as carbon dioxide.[219] The rate of hydrogen elimination subtracted from oxygen elimination yields carbon dioxide. Therefore, the doubly labeled water method is actually a type of indirect calorimetry like peak oxygen uptake (i.e., $_{peak}VO_2$); though doubly labeled water can be used under free-living conditions.[213] Although the doubly labeled water method is considered the "Gold Standard" to capture free-living energy expenditure,[213,220] the method does not capture the duration, intensity, type, or timing of physical activity,[215] and is expensive to perform,[214] as heavy isotopes are used.

Although a poorer measure of energy expenditure than doubly labeled water,[214] accelerometry can measure the duration, intensity, and timing of physical activity, a crucial advanatage.[215] Accelerometry, as the name indicates, measures acceleration through the compression of piezoelectric crystals either in one (uniaxial),[221] two (biaxial) or three (triaxial) planes, and through complex data processing the acceleration recorded is converted to time spent in different intensities of physical activity.[222–224] Heart rate monitoring is also capable of measuring the duration, intensity, or timing of physical activity but is only useful during moderate to vigorous physical activity when heart rate is elevated.[215] Accelerometry, on the other hand, is a reasonably valid measure of total energy expenditure and not just physical activity

energy expenditure, explaining as much as 83% of the variance in total energy expenditure assessed using doubly labeled water when using only one device at a single location (i.e., non-dominant wrist).[225] Others in an effort to improve free-living physical activity measurements have used multi-sensing devices that record both acceleration and other biometrics like heart rate or body temperature. It is unclear how much more valid these multi-sensing devices are relative to accelerometry. For instance, a multi-sensing armband capable of measuring acceleration, skin temperature and Galvanic response[226] yielded significantly lower physical activity energy expenditure than doubly labeled water, whereas results from uniaxial accelerometry were not different than those from doubly labeled water.[221]

Pedometry is the least specific method of measuring free-living physical activity, only recording the number of steps participants take and not the intensity, duration, or frequency of physical activity.[215] Although accelerometry, heart rate monitoring, and pedometry all represent similar levels of burden for participants, that is in all cases participants must wear a device, using accelerometry places higher burden on researchers due to quantity of data that is collected and the complexity of processing of this glut of data.[214] Commonly used accelerometers are set to collect data at a minimum of 30 and up to 100 Hz,[227] producing as many 2,592,000 to 8,640,000 data points per participant per axis per day. Thus, a typical "uniaxial" day collected at 30 Hz produces a raw ".csv" accelerometry file that is more than one megabyte in size, and demands several levels of data processing.[224] In essence, researchers must include some measure of physical activity to control for its effects on muscle and physical performance, preferably an objective measure of physical activity. Accelerometry, although burdensome for researchers, advantageously records the duration, intensity, and timing of physical activity and is appropriate for large samples, making the method an ideal component of free-living nutritional research.

2.4.2.2. The Quantity of Protein Intake

Currently, the Institute of Medicine and the National Institutes of Health recommend a relative protein intake of 0.8 g per kg of bodyweight per day throughout adulthood for both men and women to prevent losses of lean tissue.[228] Experts in aging and muscle health, on the other hand, recommend greater amounts. For example, The European Society for Clinical Nutrition and Metabolism, The Society for Sarcopenia, Cachexia, and Wasting Disease, and those associated with the "PROT-AGE" study recommend at least 1.0 g per kg per day for healthy older adults and more for those with chronic diseases.[175,229,230] Others recommend at least 25 to 30 g of protein at each meal,[176] which, assuming three meals are eaten daily, is still greater than current recommendations in the 2020-2025 Dietary Guidelines for Americans.[143] Moreover, the per meal recommendations illustrate another important dimension of protein intake outside of quantity: distribution.

The Institute of Medicine, National Institutes of Health, and the USDA's justification for lower protein intakes comes largely from nitrogen balance studies,[175] and in particular a 2003 meta-analysis of nitrogen balance studies that indicated 0.8 g per kg was sufficient to prevent losses of lean tissue.[231] To clarify, the nitrogen balance method involves tracking both nitrogen intake and elimination. Ideally, nitrogen intake is recorded using a CFT methodology where all food is given, and urine and fecal samples are collected to determine nitrogen elimination, as is the case of 27 works included in the aforementioned meta-analysis.[231] In lieu of performing a CFT, nitrogen balance can be estimated using food diaries and urinary nitrogen analysis.[232,233] However, this method makes assumptions about the nitrogen content of proteins, that is proteins are 16% nitrogen by weight, and about other losses of nitrogen outside of urine.[232,233] Regardless

of the exact methodology, nitrogen balance and other protein balance studies are often flawed as they fail to consider all elements of muscle protein turnover.

The nitrogen balance method and other methods of protein balance postulate that positive net nitrogen or protein balances result in muscle gains, whereas negative net balances result in losses. Yet, some researchers using isotopic amino acid tracers to track muscle protein synthesis and breakdown have reported negative net protein balances following resistance exercise, despite increases in muscle protein synthesis.[234,235] One of these two groups even cleverly showed that changes in muscle protein turnover were positively associated with *vastus lateralis* thickness ($r = 0.555$; $p = 0.0027$) following 12 weeks of resistance training.[235] In other words, the crux of the issue when using a "net balance" method is that it assumes all protein synthesis is beneficial, and all autophagy is in fact harmful, a serious error.

Although protein synthesis is generally beneficial for the body, particularly in the context of muscle protein synthesis, it also is an essential element of cancer cell proliferation,[236] viral replication,[237] and Alzheimer's Disease progression.[238] Autophagy, though, is more even more mischaracterized, as the process helps protect against disease by degrading and recycling damaged cell components like proteins, and impaired autophagy is related to aging and disease progression.[239,240] In fact, muscle protein synthesis and muscle protein breakdown before and after resistance exercise are strongly correlated ($r = 0.84$; $p < 0.001$),[241] highlighting the importance of autophagy in muscle and strength gains and difficulty of using nitrogen or protein balance to determine optimal protein intake.

The recommendations of experts in muscle health and aging,[175,229,230] that protein intakes greater than 0.8 g per kg per day are needed to mitigate the determinantal effects of aging on muscle are naturally informed by studies that evaluated the effects of increased protein intake on

muscle health. Perhaps the most compelling evidence regarding increased protein intake comes from studies investigating the combined effects of protein supplements and resistance exercise. A meta-analysis and regression of 15 to 28 different works that investigated the effects of various protein supplements and resistance exercise programs on muscle mass and strength found that supplemental protein increased participants' 1-RM by 9% ($p = 0.01$), lean mass by 27% ($p = 0.007$), and muscle fiber CSA by 38% ($p = 0.02$), despite participants' high mean relative protein intakes before (Mean ± SD: Supplement: 1.4 ± 0.4; Control 1.4 ± 0.3) and after the interventions (Mean ± SD: Supplement: 1.8 ± 0.7; Control 1.3 ± 0.4 g/kg/day).[209] In a unique piece of analysis, this same group of authors showed that an even higher dose of supplemental protein with resistance exercise was beneficial for protein intakes up to 1.62 g per kg per day, although this analysis only approached significance ($p = 0.079$).[209] Nonetheless, it is clear that protein intakes greater than 0.8 g per kg per day benefit those performing resistance exercise. As the Second Edition of the Physical Activity Guidelines for Americans advocate for adults to perform muscle strengthening activities (e.g., resistance exercise) at least twice a week,[46] there is a clear impetus to increase the dietary reference intake for protein.

Aside from the effects of resistance exercise, others also using meta-analytical techniques reported that participants with greater protein intakes were less likely to be frail (OR [95% CI]: 0.67 [0.56, 0.82]) across four cross-sectional studies.[242] Using three years of longitudinal data from the "Health Aging and Body Composition Study" totaling 2,732 older adults (i.e., greater than 70 years at baseline), one group found that protein intake as percentage of total energy was positively associated with lean body mass when controlling for a variety of other covariates in a regression analysis (β ± SE: 8.76 ± 3.00; $p = 0.004$).[243] Another group analyzed cross-sectional data from 2,675 participants in the "Framingham Offspring Study" and reported that protein

intake was related to increased muscle mass in both men ($p = 0.005$) and women ($p = 0.003$).[244] On the other hand, one group found no difference between those consuming ≥ 1.1 g per kg per day and those eating < 0.83 g per kg per day in measures of muscular performance, such as handgrip and knee extensor strength, and 30-second chair stand test performance, in a cross-sectional sample of 184 older (Mean ± SD: 70.2 ± 3.9 years) Danish adults.[245] However, another one of the principle findings from this same work was that relative protein and relative energy intakes are highly correlated (Women: $r = 0.69$; $p < 0.0001$; Men: $r = 0.70$; $p < 0.0001$), and these authors did not control for relative energy intake, among other dietary intake variables, when investigating protein intake.[245]

Analyzing nutritional data is complex as there are many nutritional variables (e.g., macro and micronutrient intakes alone total over 30 different variables) and a high degree of collinearity between nutritional variables. Multicollinearity is problematic, biasing estimates in multivariate analyses,[246] and not only are the intakes of the macronutrients related to one another, but together with alcohol intake they equal energy intake. Thus, outside of the issues associated with collinearity, one cannot enter both total or relative energy intake and the total or relative intakes of all the macronutrients into the same statistical model as the intakes of the macronutrients explain all of the variance in energy intake. To overcome this particular issue and to include all dietary variables in analyses, researchers can utilize the "density method"[247,248] where macronutrients are expressed as percentages of energy intake and micronutrient intakes are expressed per 1,000 kcal.

2.4.2.3. Protein Intake Distribution

As previously indicated, some experts in aging and muscle health recommend greater amounts of protein on a relative basis (i.e., 1.0 > g/kg/day),[175,229,230] whereas other advocate for a

certain amount of protein to be consumed at each meal.[176] These recommendations for protein intake per meal[176] demonstrate not just the importance of the quantity of protein consumed, but also the distribution of protein intake. In fact, the same nitrogen balance studies that informed the National Institutes of Health 0.8 g per kg per day recommendation only included works where all participants ate at least three meals,[231] guaranteeing some level of protein spread. Moreover, the authors of a recent (i.e., January 2021) systematic review of 15 studies investigating protein intake distribution concluded that evenness (i.e., increased spread) of protein intake distribution was related to increased muscle mass but not increased strength or muscle protein turnover.[50]

More than a useful overview of other works evaluating protein distribution and muscle health, this review highlights the inadequacies of using the coefficient of variation (CV) to determine protein intake distribution, which unfortunately is the most common method of assessment,[50] having been performed in four works[249–252] included in the review[50] and in another even more recent work not included.[245] The CV is equal to the standard deviation of participants' protein intake across meals or time-periods divided by participants' mean intake, sometimes multiplied by 100 to yield a percentage. Although the CV does give some indication of protein intake distribution, it does not consider the quantity of protein consumed, a critical flaw. For example, a person eating one g of protein at each meal would have a CV of 0.00 (0%) indicating perfectly even distribution, whereas a person eating 15 g for breakfast, 30 g for lunch, and 45 g for dinner would have a CV of 0.50 (50%). Thus, researchers must control for total intake when using the CV to investigate the effects of protein intake distribution, yet three of these aforementioned works failed to do this.[245,249,250] Two of these three reported no association between protein intake distribution and muscle strength,[245,249] illustrating the potential impact of controlling for protein intake when using the CV to investigate protein intake distribution.

The CV not only fails to take into account the total amount of protein eaten, but also ignores the notion of the "anabolic threshold,"[175] that is the fact that it takes 25 to 30 grams of protein to maximally stimulate muscle protein synthesis.[173,176,253] Participants achieving at least 30 grams of protein at each of their three meals, but distributed as 30, 40, and 50 g would have a CV of 0.25 (25%); this is problematically equal to the CV of participants only meeting the anabolic threshold at one meal, but with a distribution of 15, 20, and 25 g of protein at each meal. In fact, the recommendations of experts, that people should strive for at least 25 to 30 g of protein at each meal,[175,176] are based the "anabolic threshold."

The best support for the anabolic threshold and of spreading protein intake distribution is from a seven-day crossover CFT with a 30-day washout period that investigated even protein intake distribution (i.e., 30 grams at each meal) versus a skewed distribution (10, 15, and 65 g).[253] These investigators found that eating 30 grams of protein at each meal led to higher rates of muscle protein synthesis in healthy adults (N = 8; Mean ± SE: 36.9 ± 3.1 years; Even: 0.077 ± 0.006; Skewed: 0.056 ± 0.006 % / hour; p = 0.001).[253] More recently though, another group also using a CFT methodology reported no differences in muscle mass, strength, or protein synthesis between those eating an even pattern (1.1 g/kg/day; 33%, 33%, 33%; n = 7; 58.1 ± 2.4 years) and those eating a skewed pattern (1.1 g/kg/day; 15%, 20%, 65%; n = 7; 60.3 ± 2.4 years) for eight weeks in a sample of 14 older adults.[254] However, the differences between these two groups' findings may be attributable to several factors.

Most notably, the former work utilized a crossover design, increasing the power of their analyses.[253] Next, although the latter group included measures of muscle mass and strength in their work,[254] eight weeks is likely not long enough to detect changes in these measures as the result of increasing the evenness or spread of protein intake. For example, the same meta-

analysis and regression that investigated the effects of various protein supplements and resistance exercise programs concluded across studies with a minimum intervention of period 6 weeks (Mean ± SD: 13 ± 8 weeks) that supplemental protein had significant but much smaller effects than resistance exercise on muscle strength (Mean change in 1-RM: Resistance Exercise = 27 kg, Protein = 2.49 kg) and muscle mass (Mean change in lean mass: Resistance Exercise = 1.1 kg, Protein = 0.3 kg).[209] Additionally, the authors of the latter work[254] excluded those participating in resistance exercise, and mTORC1 signaling studies[185,206] suggest that resistance exercise is necessary to benefit from increased protein intake. Moreover, these authors used the simplest method of statistical analysis for their design: a one-factor analysis of variance;[254] they did not control for confounding variables outside of baseline values for each measure. The other group of authors used a mixed effects linear regression model allowing for group by time interaction, which is a more robust form of analysis.[253] Thus, of these two CFTs, the former is a better indicator of the effects of spreading protein intake on muscle protein synthesis.

Outside of these CFTs and other works that have used the CV to determine protein intake distribution, studies that considered protein intake distribution as the number of meals (i.e., zero to three meals) meeting a specific quantity of protein intake (e.g., 25 or 30 g per meal) all produced significant results,[255–257] further supporting the idea of the anabolic threshold and of increasing the evenness or spread of protein intake. Two of these works used nationally representative data from NHANES;[255,257] one reported significant results when controlling for a variety of covariates,[255] whereas the other found significant results only in an unadjusted model.[257] Similar to the CV, this number of meals at a specific protein intake per meal methodology can be biased as those achieving more meals at a specific protein level are more likely to eat more protein, another problem highlighted by the authors of the recent systematic

review.[50] In other words, researchers must control for total or relative protein intake when using either the CV or when using this number of meals with a specific intake per meal method. Of the two studies that used NHANES data but produced conflicting results in fully adjusted statistical models,[255,257] only one research group controlled for protein intake in their fully adjusted models, and it was this work that reported significant differences between those achieving one meal of at least 30 g protein and those eating no meals of at least 30 g protein in isokinetic strength (β [95% CI]: 23.6 N [9.5, 37.7]) and lean mass (β [95% CI]: 1.160 kg [0.678, 1.643]).[255] Assuming one is unable perform a CFT, this number of meals at specific intake method is a more appropriate measure of protein intake distribution than the CV as it as it considers the anabolic threshold, although both techniques demand that protein intake is controlled for.

Ideally though, protein intake recommendations and analytical techniques are made on a g per kg of bodyweight basis (e.g., 1.2 g per kg per day),[175] including those for protein intake distibution.[50] More specifically, the authors of the recent systematic review advocate for cut-offs of 0.24 g per kg per meal for younger adults and 0.40 g per kg per meal for older adults,[50] as these cut-offs were informed by a breakpoint analysis of muscle protein synthesis data between healthy younger (n = 44; Mean [95% CI]: 22 [18, 26]) and older men (n = 43; Mean [95% CI]: 71 [70, 72]).[258] These cut-offs were separately evaluated in two different works. Younger Japanese adults (N = 266; Mean ± SD: 21.4 ± 2.4 years) achieving at least 0.24 g per kg per meal at all three meals for a period of three days had better body composition (n = 83; Mean ± SD: 77.0 ± 0.5% lean mass) than those not meeting that goal at one or more meals (n = 153; Mean ± SD: 75.2 ± 0.4% lean mass; p = 0.008).[259] A higher-cut-off of 0.4 g per kg per meal was investigated in a sample of 97 healthy German adults 75 to 85 years of age; however, these authors did not find an association between increased protein intake spread, assessed using both

the CV and relative intake per meal, and muscle mass, strength, or physical pefromance.[260] Although 0.4 g per kg per meal is the amount of protein indicated from a muscle protein synthesis study,[258] it may too high a cut-off to practically use in cross-sectional research as only 4.1% of men and none of the women ate 0.4 g per kg per meal for an entire week in this work.[260] Thus, few older adults truly met the 0.4 g per kg per meal goal, so the authors examined those eating at least two meals a day at 0.4 g per kg per meal as opposed to those eating all three.[260] In addition, these authors did not control for any other variables that may confound their results such as physical activity and total or relative protein intake.[260]

Beyond discrepancies in studies investigating cut-off points for younger and older people, using the relative intakes per meal may increase multicollinearity, as relative protein and energy intakes are related.[245] In other words, as relative protein intake increases so does energy intake. This is problematic because as previously indicated multicollinearity can bias estimates in multivariate analyses.[246] Assuming one wants to control for energy intake which is critical for nutritional research,[248] then even this more robust relative intakes per meal (e.g., 0.24 g per kg per meal) method is still somewhat limited. The number of meals at a specific protein intake per meal method is arguably as robust so long as one controls for protein intake.

2.4.2.4. Protein Quality

Not only does the amount of protein eaten[175,209,229,230] and its distribution[50,249,250] affect muscle health, but does so the quality of protein.[175,204,261] Historically, protein quality has been determined using the Protein Digestibility Corrected Amino Acid Score (PDCAAS), which has been used extensively since its adoption by the World Health Organization in 1989 as the organization's preferred method to calculate protein quality.[262,263] In 2011, another method to calculate protein quality, the Digestible Indispensable Amino Acid Score (DIAAS), was

proposed by the Food and Agriculture Organization of the United Nations to replace PDCAAS,[264,265] as there were concerns about the PDCAAS overestimating the amount of amino acids absorbed by the body,[266] thus decreasing differences in protein quality between high and low quality proteins. Later, another group confirmed this discrepancy. These authors reported significantly higher protein quality for pea protein concentrate, soya isolate, soya flour, and wheat when using various forms of the PDCAAS compared to DIAAS.[52] Although there are other differences in methodology, higher scores indicate better protein quality.

The greatest differences between the PDCAAS and the DIAAS are: one, the PDCAAS considers the digestion and absorption of crude protein, whereas the DIAAS considers individual amino acids, two, the PDCAAS determined digestibility at the end of rats' digestive tracts, whereas DIAAS measures digestibility at the end of pigs' ilea (i.e., the end of the small intestine), a better model for the human digestive system, and, three, the methods use different amino acid scores.[264,265] The amino acid score in PDCAAS is determined as the amount of limiting amino acid (i.e., the amino acid with the lowest quantity relative to a reference protein) in one g of protein divided by the amount of the same amino acid in one g of the reference protein.[51] To determine the full PDCAAS, these amino acid scores are multiplied by values determined from fecal digestibility studies.[51] Similarly, in DIAAS, the amino acid score is also determined as the lowest amount of an amino acid relative to a reference protein, but in this case the values for amino acids in the reference protein differ from those used in PDCAAS.[264,265] Regardless of how the amino acid score is determined, both methods demand that researchers determine the amino acid content of a protein to evaluate its quality, and this determination is both complex and expensive.

The first step in evaluating the amino acid content of a protein involves the complete hydrolysis of the protein down to the individual amino acid. Proteases, enzymes that catalyze the hydrolysis of peptide bonds, cannot be used in this process because proteases cleave peptide bonds after specific residues (e.g., trypsin prefers the positive amino acids arginine or lysine)[267] often generating small peptides as opposed to amino acids. Additionally, if one were to add a variety of proteases in an effort to produce single amino acids, there is a possibility that the various proteases may degrade one another, confounding the results. Moreover, if a protease were used to break down a protein, it would likely need to be removed from the sample before the sample's amino acid content could be analyzed. Thus, instead of using proteases to break down proteins into amino acids, which could be done under mild experimental conditions, researchers often use acid hydrolysis which involves heating proteins in strong acids within vacuum or hermetically sealed containers typically for periods of around one day.[268] One acid hydrolysis method, for instance, involves treating proteins with 4 M methanesulfonic acid at 110° C for 24 hours.[269] In addition to this process of breaking down proteins, hydrolyzed, individual amino acids must be separated from one another, and this is often achieved through high-performance liquid cation exchange chromatography,[270,271] before individual amino acids can be quantified by a variety of methods such as ninhydrin derivatization.[272] In addition to the amino acid content of a protein, its digestibility needs to be determined as well, and this is done using animal studies.[264,265] In sum, determining the amino acid content of a protein is not easy nor inexpensive, yet this is an essential element of determining protein quality when using the PDCAAS or DIAAS. For these reasons, many foods do not have a reported PDCAAS or DIAAS.

In an effort to work around this dearth of information, researchers have used a crude method of estimating protein quality: splitting proteins into "high" and "low quality" according

to their origin. Even though the PDCAAS overestimates the quality of low quality proteins,[266] animal-based proteins (i.e., proteins from animals) such as egg (PDCAAS = 118), cow's milk (PDCAAS = 121), and beef (PDCAAS = 92) have greater protein quality than plant-based proteins such soy (PDCAAS = 91) and wheat (PDCAAS = 42).[51] These differences in protein quality between animal and plant-based foods are further magnified when the more appropriate DIAAS is used to measure protein quality. For example, the DIAAS of soy protein isolate was 84 and its PDCAAS was equal to 93, whereas whey protein isolate had a PDCAAS of 99 and a DIAAS of 100, resulting in a difference of 6 when using the PDCAAS and of 16 when using the DIAAS.[52] Thus, animal-based proteins tend to have better protein quality than plant-based proteins spurring some researchers to investigate the effects of protein quality using the source (i.e., animal or plant-based) of the food as a rough gauge of protein quality.

Works that investigated the effects of animal or plant-based protein generally report that higher quality animal-based proteins are more related to better muscle health than lower quality plant-based proteins.[53,54] Using isotopic amino acid tracers, one group reported significantly greater increases in net protein balance following an egg breakfast compared to a cereal breakfast in a crossover sample of 12 older adults aged 57 to 74 years.[159] Another group of authors utilizing nine years of longitudinal nutrition and physical activity data from the "Framingham Offspring Study," reported that animal-based proteins were related to increased muscle mass even in those with lower physical activity levels, whereas greater physical activity levels were needed for participants' plant-based protein intake to be related to increased muscle mass.[96] In a cross-sectional sample of 1,853 Italian adults, those in highest tertile of animal-based protein intake had greater arm (Mean ± SE: low intake 23.3 ± 0.1; high intake 24.0 ± 0.1 cm) and calf (Mean ± SE: low intake 35.5 ± 0.09; high intake 36.1 ± 0.09 cm) circumferences and handgrip

strength (Mean ± SE: low intake 32.6 ± 0.4; high intake 34.5 ± 0.4 kg) compared to those in the lowest tertile when controlling for variety of covariates.[273] Thus, although animal-based protein intake is only a crude estimate of protein quality, examining animal-based protein intake is more feasible than using either the PDCAAS or the DIAAS as these values are not available for many proteins. Examining animal-based protein intake therefore offers an opportunity to investigate protein quality in free-living nutritional research.

2.5. Conclusions

Changing individuals' protein intake represents a relatively well-tolerated and modifiable lifestyle factor that can help increase or maintain muscle mass and strength throughout aging. Although the amount of protein consumed has been investigated frequently, other dimensions of protein intake, namely its distribution and quality, have received less attention by researchers. In addition to this, a definition of muscle quality has not been set. Research investigating the effects of protein distribution and quality on muscle mass and strength should also strive to investigate measures of muscle quality in an effort to reach a consensus.

2.6. Research Questions

2.6.1. Echogenicity and Specific Force

Echogenicity and specific force are thought to both assess the same factor: muscle quality. However, there are methodological differences between the two measures; most notably, echogenicity is not dependent on human performance, whereas specific force is. As both these measures are intended to determine the same variable, they should be highly correlated.

2.6.2. Protein Intake Distribution

A recent systematic review[50] indicated that more even distribution of protein is related to greater muscle mass but not strength. However, there was a wide degree of heterogeneity in the

methods used to assess protein intake distribution. Beyond these differences, several studies failed to control for total or relative protein intake,[245,249,250,257,260] a critical limitation. More complete statistical models that control for total or relative protein intake in addition to other confounders such as age, MVPA, and sex should better reflect the effects of protein intake distribution on muscle mass and performance.

2.6.3. Protein Quality

The quality of protein consumed affects muscle health.[175,204,261] Protein quality has been determined using two methods the PDCAAS[262,263] and DIASS,[264,265] but PDCAAS and DIAAS values are not available for all foods. Regardless of which method is used, dietary proteins from animals tend to have better protein quality.[52,266] Thus, those with greater intakes of animal-based protein should have more muscle mass and perform better than those who eat less. However, protein quality is not the same as dietary quality, and some foods, whole milk for instance, despite having high quality protein are also sources of saturated fat and sugars that limit their nutritional quality.

3. METHODS

Data for this project are from three cross-sectional studies, two separate studies performed using middle-aged men and later women (i.e., “Beef protein intake, physical activity, and muscle quality in middle-aged men” and “Beef protein intake, physical activity, and muscle quality in middle-aged women”) and the third performed with women aged 18 to 80 (i.e., "The influence of animal-based protein and beef consumption on ability to perform functional activities, muscle quality and bone mineral density among adolescent to older females"). Although the goals and methodologies of the three studies are similar, there are differences other than the populations examined within the three studies. The methods of the first two studies performed in middle-aged men and women are nearly identical and will be analyzed together. The third project examining women aged 18 to 80 will be analyzed separately.

3.1. Beef Protein Intake, Physical Activity, and Muscle Quality in Middle-aged Men and Women

These two studies were conducted in the North Dakota State University Healthy Aging Lab from October 2016 to December 2018. A total of 50 women and 41 men from the local community were recruited using e-mail, flyers, and word-of-mouth to visit the research lab for two sessions. During the first session, anthropometric, ultrasonographic, and performance variables were measured, and accelerometers and three-day food diaries were provided. Within 7 to 14 days later, participants returned their accelerometers and their completed food diaries to the lab. Participants were between 40 and 67 years of age, not currently using any nicotine product, free of any untreated or nonresponsive diseases or conditions including neuromuscular disease or conditions such as diabetes that might undermine muscle health, ambulatory without any assistance, and had to include both animal-based and plant-based foods in their diets. Participants

were screened using, a diabetes risk screener, the 2011 Physical Activity Readiness Questionnaire,[274] a more detailed health history questionnaire, and an orthostatic hypotension test. Participants were also instructed to refrain from exercise and strenuous physical activity at least 48 hours prior to the first session. The study was approved by the North Dakota State University Institutional Review Board (IRB) (#HE26929, Appendix A; #HE26153 Appendix B) and complied with the Helsinki Declaration of 2013. Written informed consent was obtained from all participants in this study.

3.1.1. Participant Health Screening and Anthropometric Measures

To screen participants for orthostatic hypotension, related to regulatory and safety concerns set forth by the IRB, resting blood pressure and standing blood pressure were measured manually with a stethoscope and Diagnostix 703 sphygmomanometer (American Diagnostic Corporation, Hauppauge, NY). Those whose blood pressure dropped by more than 10 mm Hg, either systolic or diastolic, from resting to standing during the orthostatic hypotension test were excluded (n = 0). Following the orthostatic hypotension test, anthropometric variables were measured. Age (years) was self-reported. Height , to the nearest 0.1 cm, was measured using a stadiometer (Seca 213, Chino, CA) and body mass. to the nearest 0.1 kg, was recorded using a digital balance (Denver Instrument DA-150, Arvada, CO). Waist and hip circumferences were completed using a Gulick (Fitness Mart Division of Country Technology Inc., Gays Mills, WI) spring-loaded measuring tape to the nearest mm.

3.1.2. Ultrasonography

Images of the right *rectus femoris* muscle were captured using a Philips ultrasound system (model HD11 XE; Bothell, WA) with a L12-5 50 mm linear array probe by three trained research assistants. Images were taken while participants were standing at marked sites 50% and

75% of the measured distance from the superior iliac spine of the hip to the lateral condyle of the knee. Participants were instructed to use their left leg as a base of support, while relaxing their right, resulting in a slight bend in the right knee. Previous works have shown high test–retest reliability of ultrasound measures of muscle thickness of healthy adults taken in the standing position.[275,276] A more recent study found the intraclass correlation coefficient (ICC) for standing measures of the anterior thigh muscles was 0.89, while the ICC for the same measures taken while participants were recumbent was 0.90.[277] Following generous application of ultrasonic gel, the probe was placed on the skin perpendicular to the leg and light, consistent pressure was applied to avoid excessive depression of the dermal surface until a full, clear image was obtained. The probe was removed from participants' skin between each image acquisition, and markings were used to ensure the same area was measured. Because participants were younger and likely have greater muscle size, the panoramic feature was used at the 50% site to record the entire transverse *rectus femoris*.[278] For panoramic ultrasonography, the lateral side of the right *rectus femoris* was identified, and the probe was moved medially until the entire transverse *rectus femoris* was recorded. B-mode image captures were taken at the 75% site where transverse sections of the *rectus femoris* are smaller. Three images were captured at each site using a frequency of 37 Hz with a standardized depth of 7 cm and gain of 100%.

After each image was captured, a 1 cm line was added to each image to act as a known distance during analysis. Images were transferred to personal computers, calibrated, and analyzed. ImageJ software (version 1.42) was used to analyze echogenicity, cross-sectional area (CSA), and muscle thickness.[128] Echogenicity was defined as the mean pixel intensity of the *rectus femoris* measured in arbitrary units (A.U.) ranging between 0 (i.e., black) and 255 (i.e., white). Anatomical muscle CSA was determined by tracing the inside of the epimysium of the

rectus femoris using the polygon tool. *Rectus femoris* thickness was assessed with a single measurement using the straight-line tool; using ImageJ, a line was made through the largest, middle portion of the muscle perpendicular to the skin. Intraclass correlation coefficients (ICC) were used to examine the reliability of these analyses. All three research assistants completed reliability training prior to being allowed to be an operator for the testing in the study. The test-retest reliability of three images obtained by the research assistants using intraclass correlation coefficients (ICCs) and 95% confidence intervals were as follows: panoramic muscle thickness = 0.98 [0.90, 0.95], B-mode muscle thickness = 0.98 [0.97, 0.99], panoramic muscle area = 0.95 [0.93, 0.96], B-mode muscle area =0.97 [0.97, 0.98], panoramic muscle echogenicity = 0.98 [0.97, 0.98], B-mode echogenicity = 0.81 [0.75, 0.87]. For consistency, these measurements were all analyzed by the same member of the research team. The mean of each participant's values across the three images at each site (i.e., 50% and 75%) will be used in analyses. Figure 1 displays an example of muscle thickness and CSA captured and analyzed at each site.

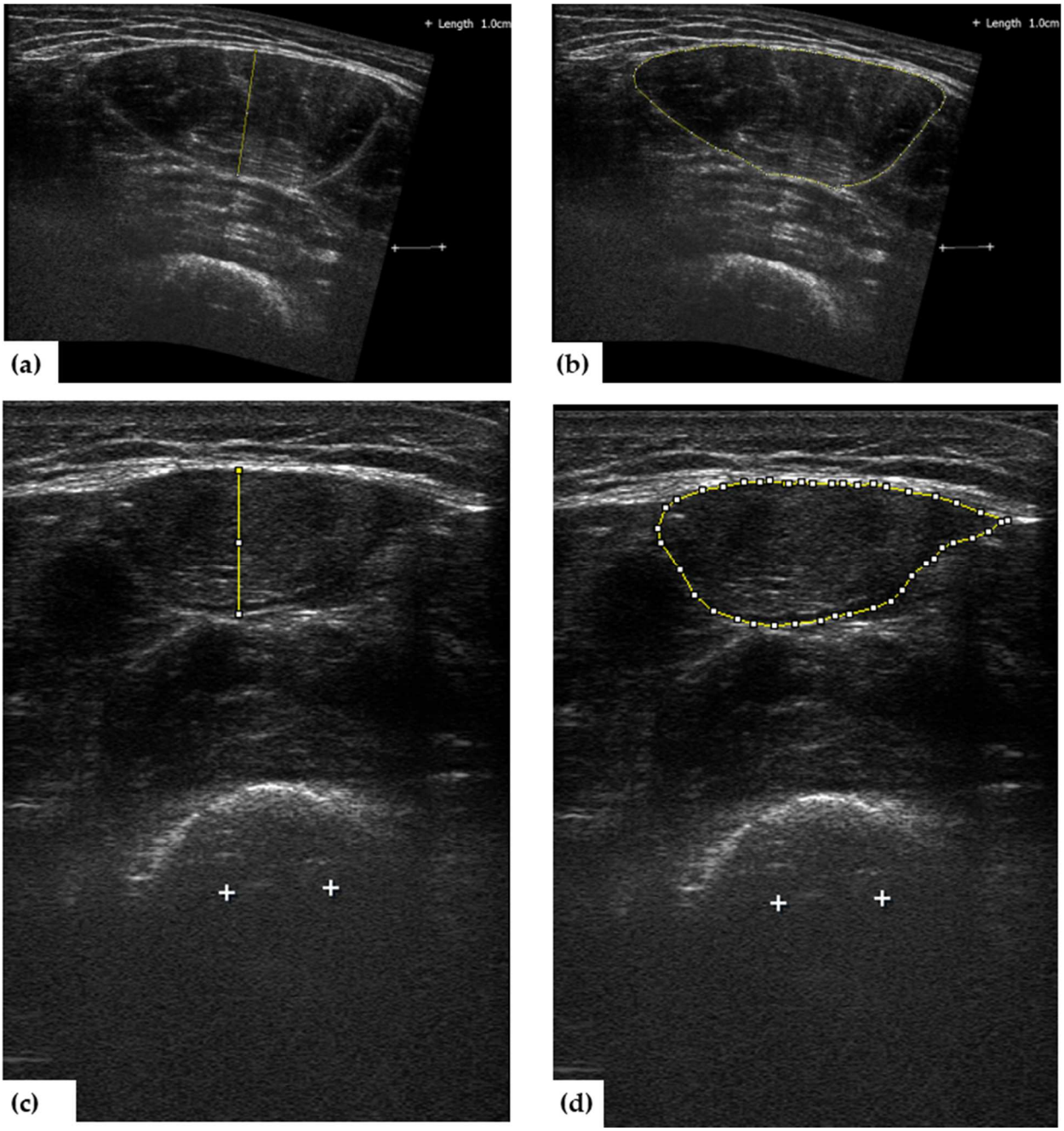

Figure 1. Examples of *rectus femoris* muscle thickness and CSA captured via ultrasonography for one participant. **(a)** *Rectus femoris* muscle thickness at 50% of leg length captured using the panoramic feature. **(b)** Same as A but showing muscle CSA. **(c)** *Rectus femoris* muscle thickness at 75% of leg length captured using a standardized B-mode image. **(d)** Same as C but showing muscle CSA.

3.1.3. Performance Measures

Participants performed a self-paced, low to moderate intensity warm-up for five minutes using a cycle ergometer. Muscle strength and endurance of the lower body were tested using isokinetic dynamometry on a Biodex Pro IV System (Biodex Medical Systems, Shirley, NY). Lower body muscular strength was assessed using peak torque performed during a three-repetition test at 60° per second for knee extension-flexion and a three-repetition test at 30° per second for plantar-dorsiflexion. Lower body muscular endurance was evaluated using the total amount of work performed during a 21-repetition test at 180° per second for knee extension-flexion and 60° per second for plantar-dorsiflexion.[279] Muscular strength and then endurance were first assessed in upper leg (i.e., knee extension-flexion) and then in the lower leg (i.e., plantar-dorsiflexion). A warm-up set was completed before each lower-body strength test (i.e., knee extension-flexion, and plantar-dorsiflexion); participants were instructed to perform three repetitions at <75% of their perceived maximal effort. Thirty seconds of rest was given between all extension-flexion tests. One minute of rest was provided between plantar-dorsiflexion tests. To optimize performance, participants were encouraged to employ "all-out effort" by research staff during all muscle function tests. To better capture muscular performance of the entire right leg, peak torques from the isokinetic strength test and total work from the isokinetic endurance test were added together to create summed peak torque and summed total work (i.e., knee extension + knee flexion + plantarflexion + dorsiflexion).

Maximal handgrip strength (kg) was assessed using an analog Jamar Handheld Dynamometer (Bolingbrook, IL). Participants were instructed to grasp the dynamometer in their dominant hand and to keep their elbow at their side with a 90° bend between the upper arm and forearm, while standing. Participants were told to squeeze the dynamometer as hard as possible

for two to three seconds. Each participant performed three maximal attempts; the highest grip strength was used.

Participants then performed a 30-second chair stand test on a chair with a 43cm floor-to-seat height. All trials were performed with participants' arms crossed and feet at a comfortable distance apart (i.e., about hip to shoulder width). With a straight back, participants were instructed to fully sit down and stand-up for each repetition, and practice repetitions were performed to ensure adequate performance during the test. The total number of repetitions completed in 30-second period was recorded, and the 30-second period began when participants started to rise.

3.1.4. Physical Activity Assessment

Following performance testing, participants were given accelerometers and three-day food diaries. Physical activity was recorded using Actigraph (Pensacola, FL) GT9X accelerometers. Participants were instructed to wear accelerometers on their right hip during all waking hours, excluding activities where the device may get wet (e.g., bathing or swimming), for a period of one week and to keep a sleep log to record the time that the accelerometer was removed at night and put back on in the morning. The raw acceleration data were collected at 80Hz, and processed in R software (http://cran.r-project.org) using the GGIR package (version 1.10-10).[224] Non-wear time was defined as intervals of at least 90 minutes of zero counts with allowance of two-minute interval of non-zero counts within a 30-minute window,[280] thus only valid time during waking hours of each day was included for statistical analyses. Although accelerometry captures many aspects of physical activity (e.g., sedentary time, light physical activity, etc.,), moderate-to-vigorous physical activity (MVPA) will be included in analyses because of its relationship with performance variables.[281,282]

3.1.5. Nutrition Analysis

During recruitment, participants received classroom food diary training that was provided by a registered, licensed dietitian. Then, after performance testing, participants were given three-day food diaries and the Arizona Food Frequency Questionnaire (FFQ) and reviewed the food diary training and associated portion and other guiding handouts with a member of the research team. Dietary intakes from three-day food diaries, including nutritional supplements, were entered into Food Processor Nutrition Analysis Software (ESHA Research, Salem, OR) which uses Food Data Central (i.e., the USDA Nutrient Database)[283] by trained research assistants. Data entry, including animal- and plant-based protein intakes, were then line-by-line verified by a registered dietitian. Food items that contained less than 1g of total protein were excluded from these calculations. Foods containing both animal- and plant-based protein were split according to their ingredients to distinguish protein sources. Animal-based protein sources included meat, fish and seafood, dairy, eggs, poultry, and wild game.

3.1.6. Statistical Analyses

Three male participants could not be included in analyses of ultrasonography because the ultrasound machine suffered a catastrophic failure near the very end of the data collection window, precluding ultrasonography for these male participants. Thus, all analyses related to ultrasonography have 88 as opposed to 91 participants. Separate multiple-linear regression models will be also used to evaluate the relationship between echogenicity and specific force of the *rectus femoris*, two measures of muscle quality. All aforementioned regression models will be adjusted for sex (i.e., 0 = women, 1 = men), age, and BMI, because these variables are routinely collected in both clinical and research settings.

All participants completed a three-day food diary, all performance measures (i.e., isokinetic dynamometry, handgrip strength, and 30-second chair stand test), and wore an accelerometer. For our analyses investigating nutritional variables, simple linear regression models will be used to verify that estimates of animal-based and plant-based protein intakes together agree with total protein intake. Animal-based and plant-based protein intakes, determined by line-by-line analysis of three-day food diaries by a registered dietitian and expressed either as relative intakes or percentages of energy intakes, will be entered as predictor variables and total protein, without partitioning into animal- or plant-based protein intakes, will be the outcome variable.

Analyses of nutritional data are complicated by the shared variance of many variables.[248] Energy intake and macronutrient intakes, which will be examined in this work, are directly related, that is, a person's macronutrient intake, plus alcohol intake, determines their energy intake (i.e., protein + carbohydrates + fat = energy). Therefore, when analyzing dietary variables, relative energy (kcals/kg/day) and the relative intakes of all the macronutrients (g/kg/day) cannot be entered simultaneously. Pearson Product-Moment Coefficients will be used to examine the collinearity of both relative macronutrient intakes and macronutrient intakes as percentages of energy intake with one another and with relative energy intake. Although there are other methodologies, the nutrient density approach[248] will be used where relative energy intake (kcal/kg/day) and the intake of the macronutrients as percentages of energy intake were included in our analyses. This method allows one to control for both relative energy intake and macronutrient intakes in statistical models.

Mixed linear models will be used to evaluate the impact of animal-based protein intake on muscular performance. The 41 men and 50 women will first be blocked according to self-

reported sex (0 = women, 1 = men). Then, each sex will be split at their median of energy intake from animal-based protein. More specifically, sex and animal-based protein intake (below median = 0, above median = 1) will be entered as fixed factors. Age, BMI, MVPA, relative energy intake, and percent energy from protein, fat, and carbohydrates will be entered as continuous covariates. Models will be evaluated for equality of error of variance using Levene's Test of Equality of Variance and for heteroscedasticity using White's Test of Heteroscedasticity; mixed models that are significantly unequal in their variances or heteroscedastic will be transformed using the square root function. Out of an abundance of caution, the HC3 method will be used to calculate the standard errors of variables as it is more robust to unequal variances, heteroscedasticity, and multicollinearity than the ordinary least squares method.[284] It was not hypothesized that there would be interaction between sex and animal-based protein intake, so only main effects will be examined in these mixed models. For those models in which animal-based protein intake is significant, effect sizes will be evaluated using partial η^2. To verify that animal-based protein intake and not total protein intake is important to performance the same aforementioned methods will be performed, but each sex will be split at the median of total protein intake as a percentage of energy intake and animal-based protein intake as a percentage of energy intake will be included as a continuous covariate.

Estimates of physical activity from accelerometry are considered valid when the devices are worn for 10 hours per day for at least four days,[282] and three participants failed to meet these criteria despite instruction to wear the devices during all waking hours for one week. Nonetheless, all other participants achieved at least four or more days including one weekend day with an average of 10 or more hours of time wearing the device. These three participants who failed to wear accelerometers as directed represents a small portion of the sample (3.3%),

and physical activity will be included in the mixed models as a covariate; physical activity is not the focus of this work, but it is essential to control for in our mixed models evaluating animal-based protein intake. For these reasons and due to small sample size, particularly when split into groups, these three participants will be included.

3.2. The Influence of Animal-based Protein and Beef Consumption on Ability to Perform Functional Activities, Muscle Quality and Bone Mineral Density Among Adolescent to Older Females

This project was also conducted in the North Dakota State University Healthy Aging Lab from October 2017 to December 2019. A total of 195 women from the local community were recruited using e-mail, flyers, and word-of-mouth to visit the research lab for two sessions. During the first session, anthropometric and performance variables were measured, and accelerometers, three-day food diaries, and Arizona FFQs were provided. Within 7-14 days later, participants returned to the lab to return their accelerometers and food diaries and have a fasting capillary blood sample collected. Participants were between 18 and 80 years of age, not currently using any nicotine product, free of any untreated or nonresponsive diseases or conditions, ambulatory without any assistance, and had to include both animal-based and plant-based foods in their diets. Participants were screened using the 2017 Physical Activity Readiness Questionnaire,[285] a more detailed health history questionnaire, a DXA screener, and an orthostatic hypotension test. The study was approved by the North Dakota State University Institutional Review Board (#HE18010; Appendix D) and complied with the Helsinki Declaration of 2013. Written informed consent was obtained from all participants in this study.

3.2.1. Participant Health Screening and Anthropometric Measures

Again, to screen participants for orthostatic hypotension, related to regulatory and safety concerns set forth by IRB, resting blood pressure and standing blood pressure were measured manually with a stethoscope and Diagnostix 703 sphygmomanometer (American Diagnostic Corporation, Hauppauge, NY). Those whose blood pressure dropped by more than 10 mm Hg, either systolic or diastolic, from resting to standing during the orthostatic hypotension test were excluded (n = 0). Following the orthostatic hypotension test, anthropometric variables were measured. Age (years) was self-reported. Height, to the nearest 0.1cm was measured using a stadiometer (Seca 213, Chino, CA) and body mass, to the nearest 0.1kg was recorded using a digital balance scale (Denver Instrument DA-150, Arvada, CO). Waist and hip circumferences were completed using a Gulick (Fitness Mart Division of Country Technology Inc., Gays Mills, WI) spring-loaded measuring tape to the nearest mm.

3.2.2. Performance Measures

Prior to performance testing, participants completed a light, self-paced, five-minute warm-up on a cycle ergometer. Handgrip strength (kg) was assessed first using an analog Jamar Handheld Dynamometer (Bolingbrook, IL). Participants were instructed to grasp the dynamometer in their dominant hands and to keep their elbows at their sides with a 90° bend between the upper arms and forearms in standing position. Participants were told to squeeze the dynamometer as hard as possible for two to three seconds. Each participant performed three maximal attempts; the highest grip strength was used. Gait speed was then measured using a Brower TCi system (Draper, UT). Participants were instructed to walk at their normal pace over a 10m distance. Timing gates were placed 6m apart. Gait speed was recorded three times, and mean time was used in analyses. Participants then performed a 30s chair-stand test on a 43cm

chair. All trials were performed with participants' arms crossed and feet at a comfortable distance apart (i.e., about hip to shoulder width). Participants were instructed to fully sit down and stand up for each repetition, and practice repetitions were performed to ensure adequate performance during the test. The total number of repetitions completed in 30s was recorded. Participants were seated, and the 30s period began when participants started to rise.

After these three assessments, muscle strength and endurance of the lower body were tested using isokinetic dynamometry on a Biodex Pro IV System (Biodex Medical Systems, Shirley, NY) in a manner identical to that of the previous studies. Lower body muscular strength was assessed using peak torque performed during a three-repetition test at 60° per second for knee extension-flexion and a three-repetition test at 30° per second for plantar-dorsiflexion. Lower body muscular endurance was evaluated using the total amount of work performed during a 21-repetition test at 180° per second for knee extension-flexion and 60° per second for plantar-dorsiflexion.[279] Muscular strength and then endurance were first assessed in upper leg (i.e., knee extension-flexion) and then in the lower leg (i.e., plantar-dorsiflexion). A warm-up set was completed before each lower-body strength test (i.e., knee extension-flexion, and plantar-dorsiflexion); participants were instructed to perform three repetitions at <75% of their perceived maximal effort. Thirty seconds of rest was given between all extension-flexion tests. One minute of rest was provided between plantar-dorsiflexion tests. To optimize performance, participants were encouraged to employ "all-out effort" by research staff during all muscle function tests. Again, to better capture muscular performance of the entire right leg, peak torques from the isokinetic strength test and total work from the isokinetic endurance test were added together to create summed peak torque and summed total work (i.e., knee extension + knee flexion + plantarflexion + dorsiflexion).

3.2.3. Physical Activity Assessment

Following performance testing, accelerometers, three-day food diaries, and ARIZONA FFQs were given to participants. Physical activity was recorded using Actigraph (Pensacola, FL) GT9X accelerometers worn on the non-dominant wrist, as opposed to the hip as in the case of the previous works, for seven consecutive days. Participants were instructed to wear the accelerometer during all waking hours except activities involving water (e.g., bathing or swimming). The raw acceleration data were collected at 80Hz, and processed in R software using the GGIR package (version 1.10-10).[224] A sleep log was provided to help delineate non-wear time from time spent sleeping. Non-wear time was defined as intervals of at least 90 minutes of zero counts with allowance of two-minute interval of non-zero counts within a 30 minute window,[280] thus only valid time during waking hours of each day was included for statistical analyses. The minimum number of wear days was four, including one weekend or one non-routine day, over the weeklong collection period, with a minimum wear time of 10h/day.

3.2.4. Nutrition Analysis

Similar to the previous two studies, participants were also given three-day food diaries and received training on how to record dietary intake by a member of the research team. Participants were also required to watch a prerecorded training video. Dietary intakes from three-day food diaries, including nutritional supplements, were entered into Food Processor Nutrition Analysis Software (ESHA Research, Salem, OR) which uses Food Data Central (i.e., the USDA Nutrient Data Base)[283] by trained research assistants. Data entry was then line-by-line verified by a registered dietitian. Animal- and plant-based protein intakes were estimated using a line-by-line examination of dietary intake by a registered dietitian. Food items that contained less than 1g of total protein were excluded from these calculations. Foods containing both animal- and plant-

based protein were split according to their ingredients to distinguish protein sources. Animal-based protein sources included meat, fish and seafood, dairy, eggs, poultry, and wild game.

Participants were also given the Arizona FFQ. The Arizona FFQ is a validated[286] 153 item questionnaire that can be scanned and read by a computer. For this project, participants were asked to recall their intakes over the last three months. As the three-day food diary asks participants to record their intakes in real-time and the Arizona FFQ ask participants about their intake over the last several months, the two methods do not assess exactly the same nutritional variables; the former represents immediate intake, whereas the latter represents some level of historical intake. Nonetheless, the Arizona FFQ was validated against data from three-day food diaries,[286] and three-day food diaries were more related to intake assessed across a year-long period than a FFQ.[168] As this project lacked a measure of criterion validity for dietary intake (i.e., an objective measure of dietary intake was not performed), the data from the ARIZONA FFQ will be used to verify estimates from the three-day food diaries.

3.2.5. Follow-up Visit

After 7 to 14 days, participants returned to the lab to turn in accelerometers, food diaries, and food frequency questionnaires, have their body composition measured, and give a blood sample. Body composition was measured using DXA on a Lunar Prodigy, model #8915 (GE Healthcare, Waukesha, WI), with enCORE software.

3.2.6. Statistical Analyses

A total of 192 women completed both a three-day food diary and the Arizona FFQ and wore an accelerometer for at least 10 hours a day for four or more days. Unlike the previous studies in middle-aged men and women, three participants will be excluded from analyses

because they failed to wear the accelerometer as directed. Thus, all analyses will have at most 192 participants.

First, total and relative intakes, including the percent of energy from each of the macronutrients, will be verified using paired t-tests between data from the three-day food diary and the Arizona FFQ. Next, similar to the analysis of the previous studies, simple linear regression models will be used to verify that estimates of animal-based and plant-based protein intakes together agreed with total protein intake. Then, animal-based and plant-based protein intakes, determined by line-by-line analysis of three-day food diaries by a registered dietitian and expressed either as relative intakes or percentages of energy intakes, will be entered as predictor variables and total protein, without partitioning into animal- or plant-based protein intakes, will be the outcome variable.

Then, to examine the effects of protein intake distribution, data collected from three-day food diaries were blocked into three periods: waking to 11:30, afternoon, 11:31 to 16:30 and evening after 16:30. Protein intakes of 0.24 g/kg or more per meal or of 25 grams or more per meal during one of these periods will be recorded as "1"s and will be summed to create two ordinal variables each with four levels, achieving greater than 0.24 g/kg per meal or 25 grams at 0, 1, 2, or 3 periods. These ordinal variables will be entered into multiple linear regression models controlling for age, BMI, MVPA, relative energy intake, and percent of energy from carbohydrates, fats, and proteins.

To investigate the role of animal-based protein dietary intake in muscle health, the sample will be subdivided into four cohorts: college-aged women (18 – 25), young women (26 – 45), middle-aged women (46 – 60 years), and older women (61 – 79). Multiple linear regression models will be used to investigate the effects of animal-based protein intake for each cohort and

in aggregate. Animal-based protein intake will be expressed as a continuous variable; more specifically, it will be represented as animal-based protein intake divided by total protein intake times 100 (i.e., the percentage of total protein from animal-based sources). This variable will be simultaneously entered into regression equations controlling for age, BMI, MVPA, relative energy intake, and percent of energy from carbohydrates, proteins, and fats.

Then, to verify these results, regression models where animal-based protein intake, expressed as the percentage of total protein intake, is the dependent variable and relative energy intake, and percent of energy from carbohydrates, proteins, and fats are the predictor variables will be run for each cohort; participants with residuals more than 0.5 standard deviations away from the regression line will be considered "Low" (Low = 0) or "High" (High = 1) consumers of animal-based protein. Then, mixed linear models, where measures of muscle health are the outcome variables, age group and animal-based protein intake (i.e., Low or High) are fixed factors, and age, BMI, MVPA, relative energy intake, and percent of energy from carbohydrates, proteins, and fats are entered as continuous covariates, will be used to determine the difference between those eating more or less animal-based protein.

4. MEASURES DERIVED FROM PANORAMIC ULTRASONOGRAPHY AND ANIMAL-BASED PROTEIN INTAKE ARE RELATED TO MUSCULAR PERFORMANCE IN MIDDLE-AGED ADULTS[287]*

To briefly recapitulate methods: although regression models were used to examine the relationship between echogenicity and specific force, mixed linear models were used to evaluate the effects of animal-based protein intake and muscle health in the same sample. For these mixed models, participants (N = 91) from "Beef protein intake, physical activity, and muscle quality in middle-aged men and women" were first separated according to self-reported sex (female n = 50; male n = 41) and then split at the median of animal-based protein intake as a percentage of total intake. Thus, participants' median self-reported age, measured height, weight, and calculated BMI are displayed in Table 1 according to these groups even though only gender (women = 0; men =1) and not animal-based protein intake (below median = 0; above median = 1) were included in regression models examining echogenicity and specific force. There were no statistically significant differences between those below or above the median of animal-based protein intake as a percentage of total energy within each sex when using the Brown- Forsythe method (i.e., assuming unequal variances).

4.1. Abstract

Ultrasonography advantageously measures skeletal muscle size and quality, but some muscles may be too large to capture with standardized brightness mode (B-mode) imaging. Panoramic ultrasonography can capture more complete images and may more accurately

* This chapter is a co-authored manuscript that can be reproduced in its entirety if clearly cited.[287] It is available in its final form at DOI: 10.3390/jcm10050988. In addition to collecting data, I Nathaniel R. Johnson, completed all statistical analyses, wrote the manuscript, and made all revisions.

measure muscle size. We investigated measurements made using panoramic compared to B-mode ultrasonography images of the *rectus femoris* with muscular performance. Concurrently, protein intake plays an important role in preventing sarcopenia; therefore, we also sought to investigate the association between animal-based protein intake and muscular performance. Ninety-one middle-aged adults were recruited. Muscle cross-sectional area (CSA) and thickness were obtained using B-mode and panoramic ultrasound and analyzed with Image J software. Muscular performance was assessed using isokinetic dynamometry, a 30-s chair test, and handgrip strength. Three-day food diaries estimated dietary intakes. Linear regression models determined relationships between measures from ultrasonography and muscular performance. Mixed linear models were used to evaluate the association between animal-based protein intake and muscular performance. Muscle CSA from panoramic ultrasonography and animal-based protein intake were positively associated with lower-body strength (β ± S.E.; CSA, 42.622 ± 20.024, p = 0.005; animal-based protein intake, 65.874 ± 19.855, p = 0.001), lower-body endurance (β ± S.E.; CSA, 595 ± 200.221, p = 0.001; animal-based protein intake, 549.944 ± 232.478, p = 0.020), and handgrip strength (β ± S.E.; CSA, 6.966 ± 3.328, p = 0.004; animal-based protein intake, 0.349 ± 0.171, p = 0.045). Panoramic ultrasound shows promise as a method for assessing sarcopenia. Animal-based protein intake is related to better muscular performance.

4.2. Introduction

Earlier and more frequent assessments of muscle strength, mass, size, and quality and physical performance could help prevent sarcopenia by indicating a need for treatment or other intervention. According to the European Working Group on Sarcopenia in Older People 2, low muscle strength is the first criteria of sarcopenia, and low muscle mass or quality is the second;

both must be assessed to determine sarcopenia.[37] Low physical performance in addition to low muscle strength and quantity is considered severe sarcopenia.[37] Measures of muscle strength, such as handgrip strength, and physical performance, (e.g. 30-second chair stand), however, can be performed with minimal equipment and are used across various settings.[37] Although several methods can be used to accurately assess muscle quantity and quality such as computed tomography (CT), magnetic resonance imaging (MRI), and dual x-ray absorptiometry, these techniques require expensive equipment and are not portable, limiting their utility. Ultrasonography is a portable and relatively low-cost method of assessing muscle size,[288] making it a potentially useful tool for evaluating sarcopenia for clinical or research purposes.[126,289] Beyond this, ultrasonography records a measure of muscle quality in the form of echogenicity or echo intensity,[6,21,48] making ultrasound a potentially more powerful tool than bioelectrical impedance for assessing sarcopenia or signs of pre-sarcopenia in middle age.

Others have used ultrasonography to successfully diagnose sarcopenia.[48,108,290] However, two of these studies were performed with either frail elderly patients or older adults diagnosed with chronic kidney disease.[108,290] Not only are the causes of sarcopenia thought to start earlier in life,[37] making middle-aged-adults a population of interest, but also older adults often have smaller muscles that can be captured using a traditional ultrasound image at 50% of leg length. Although Ismail and colleagues[48] were able to discriminate between those with sarcopenia and those without in a younger cohort, they did this by using longitudinal and not transverse images of the *rectus femoris*. The crux of the issue is that in populations that have greater muscle mass at the midpoint of the thigh, such as younger populations, the entire transverse *rectus femoris* may be too large to capture in one image.[278] Assuming the goal is to image the entire transverse *rectus femoris*, then there are two workarounds: one is to use a feature, like the panoramic feature, to

record the entire *rectus femoris* at the midpoint of the thigh, and the other is to move the imaging site distally down the leg where the *rectus femoris* has smaller transverse sections. Other researchers have validated panoramic ultrasound of the quadriceps with MRI[291] but to our knowledge, the relationship between ultrasonographic measures of the transverse *rectus femoris* captured using the panoramic feature and muscular performance, in particular that of the knee extensors, has not been investigated. Because muscle strength is more closely related to sarcopenia than muscle mass,[37,47] the association warranted investigation.

Beyond this, specific force, the amount of force produced per unit of muscle, like echogenicity,[21] is considered a measure of muscle quality.[47] Although echogenicity of the *rectus femoris* is related to muscle quality assessed using CT,[108] and to a lesser extent knee extensor strength,[26] the echogenicity of the *rectus femoris* has not been directly related to the specific force of the muscle. However, Ismail and colleagues[48] reported a significant relationship between echogenicity of *rectus femoris* and handgrip strength relative to bodyweight, a crude measure of specific force. If echogenicity and specific force reflect the muscle quality of the *rectus femoris*, then they should be closely related. We also sought to determine this relationship.

Outside of assessing the condition, nutrition is another important consideration for preventing and treating sarcopenia. Although there are many nutritional factors that can impact sarcopenia,[91] dietary protein is perhaps of greatest interest because of its ability to stimulate muscle protein synthesis.[173] Recently though, the role of protein intake in performance has come into question, with one group finding no relationship between protein intake and measures of muscular performance, such as handgrip strength, knee extensor strength, and 30-second chair stand test performance.[245] Foods from animal and plant sources, of course, differ in their digestibility and amino acid content,[292] and therefore in their ability to stimulate muscle protein

synthesis.[54] Due to the differential impact that animal-based protein has on muscle protein synthesis, we secondarily sought to determine the relationship between animal-based protein intake and lower-body strength and endurance, handgrip strength, and 30-second chair stand performance, measures of muscular performance.

4.3. Methods

This was a cross-sectional study conducted in the North Dakota State University Healthy Aging Lab from October 2016 to December 2018. A total of 50 women and 41 men from the local community were recruited using e-mail, flyers, and word-of-mouth to visit the research lab for two sessions. During the first session, anthropometric, ultrasonographic, and performance variables were measured, and accelerometers and three-day food diaries were provided. Within 7 to 14 days later, participants returned their accelerometers and their completed food diaries to the lab. Participants were between 40 and 67 years of age, not currently using any nicotine product, free of any untreated or nonresponsive diseases or conditions including neuromuscular disease or conditions such as diabetes that might undermine muscle health, ambulatory without any assistance, and had to include both animal-based and plant-based foods in their diets. Participants were screened using the 2011 Physical Activity Readiness Questionnaire,[274] a more detailed health history questionnaire, and an orthostatic hypotension test. Participants were also instructed to refrain from exercise and strenuous physical activity at least 48 hours prior to the first session. The study was approved by the North Dakota State University Institutional Review Board (#HE26929 & 26153) and complied with the Helsinki Declaration of 1983. Written informed consent was obtained from all participants in this study.

4.3.1. Participant Health Screening and Anthropometric Measures

To screen participants for orthostatic hypotension, related to regulatory and safety concerns, resting blood pressure and standing blood pressure were measured manually with a stethoscope and Diagnostix 703 sphygmomanometer (American Diagnostic Corporation, Hauppauge, NY). Those whose blood pressure dropped by more than 10 mm Hg, either systolic or diastolic, from resting to standing during the orthostatic hypotension test were excluded (n = 0). Following the orthostatic hypotension test, anthropometric variables were measured. Age (years) was self-reported. Height (cm) was measured using a stadiometer (Seca 213, Chino, CA) and body mass (kg) was recorded using a digital balance (Denver Instrument DA-150, Arvada, CO).

4.3.2. Ultrasonography

Images of the right *rectus femoris* muscle were captured using a Philips ultrasound system (model HD11 XE; Bothell, WA) with a L12-5 50 mm linear array probe used by three trained research assistants. Images were taken while participants were standing at marked sites 50% and 75% of the measured distance from the superior iliac spine of the hip to the lateral condyle of the knee. Participants were instructed to use their left leg as a base of support, while relaxing their right, resulting in a slight bend in the right knee. Previous works have shown high test–retest reliability of ultrasound measures of muscle thickness of healthy adults taken in the standing position.[275,276] A more recent study found the intraclass correlation coefficient (ICC) for standing measures of the anterior thigh muscles was 0.89, while the ICC for the same measures taken while participants were recumbent was 0.90.[277] Following generous application of ultrasonic gel, the probe was placed on the skin perpendicular to the leg and light, consistent pressure was applied to avoid excessive depression of the dermal surface until a full, clear image

was obtained. The probe was removed from participants' skin between each image acquisition, and markings were used to ensure the same area was measured. Because our participants were younger and likely have greater muscle size, the panoramic feature was used at the 50% site to record the entire transverse *rectus femoris*.[278] For panoramic ultrasonography, the lateral side of the right *rectus femoris* was identified, and the probe was moved medially until the entire transverse *rectus femoris* was recorded. B-mode image captures were taken at the 75% site where transverse sections of the *rectus femoris* are smaller. Three images were captured at each site using a frequency of 37 hz with a standardized depth of 7 cm and gain of 100%.

After each image was captured, a 1 cm line was added to each image to act as a known distance during analysis. Images were transferred to personal computers, calibrated, and analyzed. ImageJ software (version 1.42) was used to analyze echogenicity, cross-sectional area (CSA), and muscle thickness.[128] Echogenicity was defined as the mean pixel intensity of the *rectus femoris* measured in arbitrary units (A.U.) ranging between 0 (i.e., black) and 255 (i.e., white). Anatomical muscle CSA was determined by tracing the inside of the epimysium of the *rectus femoris* using the polygon tool. *Rectus femoris* thickness was assessed with a single measurement using the straight-line tool; using ImageJ, a line was made through the largest, middle portion of the muscle perpendicular to the skin. Intraclass correlation coefficients (ICC) were used to examine the reliability of these analyses. All three research assistants completed reliability training prior to being allowed to be an operator for the testing in the study. The test-retest reliability of three images obtained by the research assistants using intraclass correlation coefficients (ICCs) and 95% confidence intervals were as follows: panoramic muscle thickness = 0.98 [0.90, 0.95], B-mode muscle thickness = 0.98 [0.97, 0.99], panoramic muscle area = 0.95 [0.93, 0.96], B-mode muscle area =0.97 [0.97, 0.98], panoramic muscle echogenicity = 0.98

[0.97, 0.98], B-mode echogenicity = 0.81 [0.75, 0.87]. For consistency, these measurements were all analyzed by the same member of the research team. The mean of each participant's values across the three images at each site (i.e., 50% and 75%) were used in our analyses. Figure 2 displays an example of muscle thickness and CSA captured and analyzed at each site.

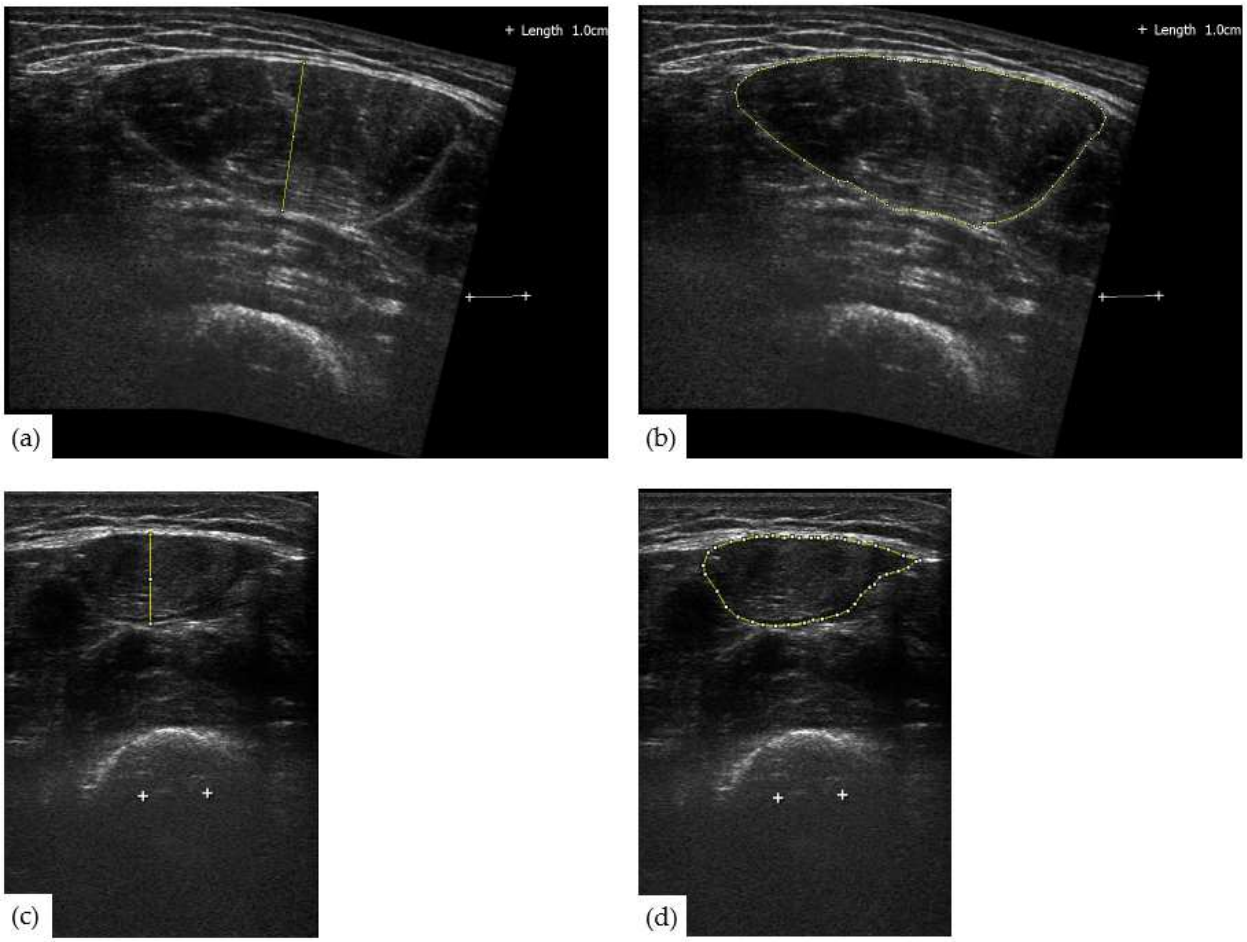

Figure 2. Examples of *rectus femoris* muscle thickness and CSA captured via ultrasonography for one participant. **(a)** *Rectus femoris* muscle thickness at 50% of leg length captured using the panoramic feature. **(b)** Same as A but showing muscle CSA. **(c)** *Rectus femoris* muscle thickness at 50% of leg length captured using a traditional image. **(d)** Same as C but showing muscle cross sectional area.

4.3.3. Performance Measures

Participants performed a self-paced, low to moderate intensity, warm-up for five minutes using a cycle ergometer. Muscle strength and endurance of the lower body were tested using

isokinetic dynamometry on a Biodex Pro IV System (Biodex Medical Systems, Shirley, NY). Lower body muscular strength was assessed using peak torque performed during a three-repetition test at 60° per second for knee extension-flexion and a three-repetition test at 30° per second for plantar-dorsiflexion. Lower body muscular endurance was evaluated using the total amount of work performed during a 21-repetition test at 180° per second for knee extension-flexion and 60° per second for plantar-dorsiflexion.[279] Muscular strength and then endurance were first assessed in upper leg (i.e., knee extension-flexion) and then in the lower leg (i.e., plantar-dorsiflexion). A warm-up set was completed before each lower-body strength test (i.e., knee extension-flexion, and plantar-dorsiflexion); participants were instructed to perform three repetitions at <75% of their perceived maximal effort. Thirty seconds of rest was given between all extension-flexion tests. One minute of rest was provided between plantar-dorsiflexion tests. To optimize performance, participants were encouraged to employ "all-out effort" by research staff during all muscle function tests. To better capture muscular performance of the entire right leg, peak torques from the isokinetic strength test and total work from the isokinetic endurance test were added together to create summed peak torque and summed total work (i.e., knee extension + knee flexion + plantarflexion + dorsiflexion).

Maximal handgrip strength (kg) was assessed using an analog Jamar Handheld Dynamometer (Bolingbrook, IL). Participants were instructed to grasp the dynamometer in their dominant hand and to keep their elbow at their side with a 90° bend between the upper arm and forearm, while standing. Participants were told to squeeze the dynamometer as hard as possible for two to three seconds. Each participant performed three maximal attempts; the highest grip strength was used.

Participants then performed a 30-second chair stand test on a chair with a 43cm floor-to-seat height. All trials were performed with participants' arms crossed and feet at a comfortable distance apart (i.e., about hip to shoulder width). With a straight back, participants were instructed to fully sit down and stand up for each repetition, and practice repetitions were performed to ensure adequate performance during the test. The total number of repetitions completed in 30-second period was recorded, and the 30-second period began when participants started to rise.

4.3.4. Physical Activity Assessment

Following performance testing, participants were given accelerometers and three-day food diaries. Physical activity was recorded using Actigraph (Pensacola, FL) GT9X accelerometers. Participants were instructed to wear accelerometers on their right hip during all waking hours, excluding activities where the device may get wet (e.g., bathing or swimming), for a period of one week and to keep a sleep log to record the time that the accelerometer was removed at night and put back on in the morning. The raw acceleration data were collected at 80Hz, and processed in R software using the GGIR package (version 1.10-10).[224] Non-wear time was defined as intervals of at least 90 minutes of zero counts with allowance of two-minute interval of non-zero counts within a 30-minute window,[280] thus only valid time during waking hours of each day was included for statistical analyses. Although accelerometry captures many aspects of physical activity (e.g., sedentary time, light physical activity, etc.,), we decided to use moderate-to-vigorous physical activity (MVPA) in our analyses because of its relationship with performance variables.[281,282]

4.3.5. Nutrition Analysis

After performance testing, participants were also given three-day food diaries, received training on how to record dietary intakes by a member of the research team, and were required to watch a prerecorded training video. Dietary intakes from three-day food diaries, including nutritional supplements, were entered into Food Processor Nutrition Analysis Software (ESHA Research, Salem, OR) which uses Food Data Central (USDA Nutrient Data Base) by trained research assistants. Data entry was then line-by-line verified by a registered dietitian. Animal- and plant-based protein intakes were estimated using a line-by-line examination of dietary intake by a registered dietitian. Food items that contained less than 1g of total protein were excluded from these calculations. Foods containing both animal- and plant-based protein were split according to their ingredients to distinguish protein sources. Animal-based protein sources included meat, fish and seafood, dairy, eggs, poultry, and wild game.

4.3.6. Statistical Analyses

Alpha was set at 0.05 and all statistics were performed in SPSS version 27 (IBM, Armonk, NY). Three male participants could not be included in analyses of ultrasonography because our ultrasound machine suffered a catastrophic failure near the very end of the data collection window, precluding ultrasonography for these male participants. Thus, all analyses related to ultrasonography have 88 as opposed to 91 participants. We used multiple-linear regression models to determine the relationships between variables derived from ultrasonography (i.e., *rectus femoris* muscle thickness, echogenicity, and CSA) using the two different methodologies (i.e., panoramic versus B-mode images) and sites (i.e., 50 and 75% of right leg length) with measures of muscular performance. Each of these variables from ultrasonography were assessed in separate multiple-linear regression models. Although we consider summed peak

torque and summed total work to be more representative of lower-body performance, we specifically included knee extensor peak torque and total work in these analyses because ultrasonography was used to measure the *rectus femoris*, one of the knee extensors. Separate multiple-linear regression models were also used to evaluate the relationship between echogenicity and specific force of the *rectus femoris,* two measures of muscle quality. All aforementioned regression models were adjusted for gender (i.e., 0 = women, 1 = men), age, and BMI, because these variables are routinely collected in both clinical and research settings.

All participants completed a three-day food diary, all performance measures (i.e., isokinetic dynamometry, handgrip strength, and 30-second chair stand test), and wore an accelerometer. For our analyses investigating nutritional variables, we first used simple linear regression models to verify that our estimates of animal-based and plant-based protein intakes together agreed with total protein intake. Animal-based and plant-based protein intakes, determined by line-by-line analysis of three-day food diaries by a registered dietitian and expressed either as relative intakes or percentages of energy intakes, were entered as predictor variables and total protein, without partitioning into animal- or plant-based protein intakes, was the outcome variable.

Analyses of nutritional data are complicated by the shared variance of many variables. Energy intake and macronutrient intakes, which we examined in this work, are directly related, that is, a person's macronutrient intake, plus alcohol intake, determines their energy intake (i.e., protein + carbohydrates + fat = energy). Therefore, when analyzing dietary variables, relative energy (kcals/kg/day) and the relative intakes of all the macronutrients (g/kg/day) cannot be entered simultaneously. We used Pearson Product-Moment Coefficients to examine the collinearity of both relative macronutrient intakes and macronutrient intakes as percentages of

energy intake with one another and with relative energy intake. Although there are other methodologies, we chose to include relative energy intake (kcal/kg/day) in our analyses and to express the intake of the macronutrients as percentages of energy intake. This method allowed us to control for both relative energy intake and macronutrient intakes in our statistical models.

Mixed linear models were used to evaluate the impact of animal-based protein intake on muscular performance. The 41 men and 50 women were first blocked according to self-reported gender (0 = women, 1 = men). Then, each gender was split at their median of energy intake from animal-based protein. More specifically, gender and animal-based protein intake (below median = 0, above median = 1) were entered as fixed factors. Age, BMI, MVPA, relative energy intake, and percent energy from protein, fat, and carbohydrates were entered as continuous covariates. Models were evaluated for equality of error of variance using Levene's Test of Equality of Variance and for heteroscedasticity using White's Test of Heteroscedasticity; mixed models that were significantly unequal in their variances or heteroscedastic were transformed using the square root function. Out of an abundance of caution, we chose to use the HC3 method to calculate the standard errors of our variables as it is more robust to unequal variances, heteroscedasticity, and multicollinearity than the ordinary least squares method.[284] We did not hypothesize that there would be interaction between gender and animal-based protein intake, so only main effects were examined in these mixed models. For those models in which animal-based protein intake is significant, we evaluated effect size using partial η^2. We also sought to verify that animal-based protein intake and not total protein intake is important to performance. We verified our results by performing the same aforementioned methods, but we split each gender at median of total protein intake as a percentage of energy intake and included animal-based protein intake as a percentage of energy intake as a continuous covariate.

Estimates of physical activity from accelerometry are considered valid when the devices are worn for 10 hours per day for at least four days,[282] and three participants failed to meet these criteria despite our instruction to wear the devices during all waking hours for one week. Nonetheless, all other participants achieved at least four or more days including one weekend day with an average of 10 or more hours of time wearing the device. These three participants who failed to wear accelerometers as directed represents a small portion of our sample (3.3%), and physical activity was included in our mixed models as a covariate; physical activity is not the focus of this work, but we feel it is essential to control for in our mixed models evaluating animal-based protein intake. For these reasons and due to small sample size, particularly when split into groups, we decided to include these three participants, using their limited physical activity data in our analyses.

For our descriptive statistics, we described the four groups from the secondary analyses in our all of our tables, even though we choose not to investigate the association between animal-based protein intake and measures from ultrasonography because the three of men who were precluded from ultrasonography were, coincidently, above the median for animal-based protein intake as a percentage of energy. Within these tables, we chose to use the Brown-Forsythe method for comparisons, because we did not assume equal variances. We compared those above the median of animal-based protein intake as a percentage of energy to those below the median within each gender, so we did not adjust for multiple comparisons.

4.4. Results

Table 1 describes participants self-reported age, measured height, weight, and calculated BMI. There were no statistically significant differences between those below or above the median of animal-based protein intake as a percentage of total energy within each gender.

Table 1. Participants' age, height, weight, and body mass index by group.

	Women			Men		
	Total (n =50)	Below Median (n =25)	Above Median (n =25)	Total (n =41)	Below Median (n =21)	Above Median (n =20)
Age (years)	54.00	55.00	54.00	51.00	55.00	50.00
Height (cm)	165.20	164.00	165.50	181.00	176.70	181.05
Weight (kg)	68.30	67.33	69.12	87.7	85.20	92.36
BMI	25.11	24.43	25.54	26.57	26.57	26.32

All values are medians. Comparisons within gender and between those below and above the median for animal-based protein intake as a percentage of energy intake were made using the Brown-Forsythe method.

Table 2 describes right *rectus femoris* muscle thickness, echogenicity, and CSA measured using the panoramic ultrasonography at 50% and B-mode images at 75% of the distance of the right leg. Within each gender, there were no statistically significant differences in these measures between those above the median of animal-based protein intake and those below.

Table 2. *Rectus femoris* muscle thickness, echogenicity, and cross-sectional area assessed via ultrasonography captured using the panoramic feature at 50% and with regular B-mode images at 75% of the right leg in 88 middle-aged men and women.

	Women			Men		
	Total (n =50)	Below Median (n =25)	Above Median (n =25)	Total (n =38)	Below Median (n =21)	Above Median (n =17)
Muscle Thickness at 50% (cm)	2.109	2.038	2.178	2.339	2.275	2.345
Muscle Thickness at 75% (cm)	0.707	0.710	0.706	0.994	0.918	1.070
Echogenicity at 50% (A.U.)	96.70	97.86	96.64	35.90	34.85	41.73
Echogenicity at 75% (A.U.)	91.99	93.34	90.63	81.99	74.56	84.54
Muscle CSA at 50% (cm^2)	7.384	6.569	7.861	10.593	10.470	10.963
Muscle CSA at 75% (cm^2)	0.957	0.790	1.055	1.934	1.660	2.088

All values are medians. CSA = Muscle Cross-Sectional Area. A.U. = Arbitrary Units. Comparisons within gender and between those below and above the median for animal-based protein intake as a percentage of energy intake were made using the Brown-Forsythe method.

Table 3 presents the results of the separate multiple linear regression models investigating the relationship between different measures derived from ultrasonography and muscular performance. Measures of *rectus femoris* size assessed using panoramic ultrasonography were

less related to knee extensor performance but more strongly related to overall muscular performance. More specifically, both muscle thickness ($p = 0.302$) and CSA ($p = 0.056$) assessed using the panoramic feature of the right leg were unrelated to knee extensor peak torque, whereas the same measures assessed using a B-mode image at of the right leg at 75% of leg length were related to knee extensor peak torque. Similarly, muscle thickness assessed using the panoramic feature was unrelated to knee extensor total work ($p = 0.197$). Although muscle CSA captured with the panoramic feature was related to knee extensor total work ($p = 0.049$), it was less closely related than muscle CSA ($p = 0.013$) or thickness ($p = 0.036$) assessed with a B-mode image at 75% of leg length. Conversely, measures of muscle thickness ($p = 0.001$) and CSA ($p = 0.004$) derived from panoramic ultrasound were significantly related to handgrip strength performance, whereas the same measures collected using B-mode were not. Muscle CSA from panoramic ultrasound was also most closely related to summed peaked torque ($p = 0.005$), a relationship that was only close to significance ($p = 0.051$) with a B-mode image. Both methodologies (i.e., panoramic and B-mode) produced measures of muscle thickness and CSA that were associated with summed total work.

Echogenicity of *rectus femoris* was unrelated to both knee extensor and summed peak torque but was significantly associated with knee extensor total work when captured using either panoramic ($p = 0.001$) or B-mode images ($p = 0.004$). Echogenicity of the *rectus femoris* from both panoramic ($p = 0.008$) and B-mode ($p = 0.007$) images was also associated with handgrip strength. Interestingly, although echogenicity was related to knee extensor total work, it was not related to summed total work when using either methodology. No ultrasonographic measure was associated with 30-second chair stand performance.

Table 4 describes our evaluation of echogenicity with specific force, two measures of muscle quality. Echogenicity was not related to specific force in any regression model nor was any model significant. We found measures from the 50% site, taken using the panoramic feature, created better fitting models. In fact, echogenicity assessed at 50% trended toward significance ($p = 0.077$).

Table 3. The associations between different ultrasonographic measures of the right *rectus femoris* using the panoramic feature (50% of leg upper length) and a B-mode image (75% of upper leg length) in a sample of 88 middle-aged men and women when controlling for age, gender, and BMI.

Variable Entered	Dependent Variable											
	Knee Extensor Peak Torque (Nm)		Summed Peak Torque (Nm)		Knee Extensor Total Work (J)		Summed Total Work (J)		30-Second Chair Stand Test (repetitions)		Handgrip Strength (kg)	
	R	B ± S.E.	R	B ± S.E.	R	B ± S.E.	R	B ± S.E.	R	B ± S.E.	R	B ± S.E.
Muscle Thickness at 50% (cm)	0.816 p <0.001	11.098 ± 10.286 p =0.302	0.861 p <0.001	42.622 ± 20.024 p =0.036	0.707 p <0.001	174.654 ± 134.410 p =0.197	0.850 p <0.001	595.980 ± 200.221 p =0.004	0.353 p =0.025	1.348 ± 1.415 p =0.334	0.900 p <0.001	6.966 ± 3.328 p =0.001
Muscle Thickness at 75% (cm)	0.826 p <0.001	23.166 ± 9.955 p =0.022	0.862 p <0.001	42.533 ± 19.076 p =0.025	0.719 p <0.001	269.252 ± 126.430 p =0.036	0.849 p <0.001	555.550 ± 191.981 p =0.005	0.347 p =0.029	0.963 ± 1.357 p =0.480	0.885 p <0.001	0.307 ± 2.131 p =0.886
Echogenicity at 50% (A.U.)	0.822 p <0.001	-0.271 ± 0.141 p =0.059	0.854 p <0.001	-0.237 ± 0.275 p =0.389	0.854 p <0.001	-5.809 ± 1.710 p =0.001	0.836 p <0.001	-3.622 ± 2.804 p =0.200	0.349 p =0.027	-0.016 ± 0.019 p =0.412	0.895 p <0.001	-0.078 ± 0.029 p =0.008
Echogenicity at 75% (A.U.)	0.817 p <0.001	-0.142 ± 0.129 p =0.274	0.853 p <0.001	-0.058 ± 0.248 p =0.815	0.853 p <0.001	-4.763 ± 1.550 p =0.003	0.834 p <0.001	-4.763 ± 1.550 p =0.370	0.376 p =0.012	-0.027 ± 0.017 p =0.113	0.895 p <0.001	-0.071 ± 0.026 p =0.007
Muscle CSA at 50% (cm^2)	0.823 p <0.001	3.406 ± 1.754 p =0.056	0.867 p <0.001	9.915 ± 3.271 p =0.005	0.717 p <0.001	44.281 ± 22.142 p =0.049	0.860 p <0.001	126.648 ± 32.205 p <0.001	0.349 p =0.028	0.193 ± 0.237 p =0.418	0.897 p <0.001	1.050 ± 0.354 p =0.004
Muscle CSA at 75% (cm^2)	0.828 p <0.001	8.120 ± 3.245 p =0.014	0.860 p <0.001	12.464 ± 6.294 p =0.051	0.726 p <0.001	104.435 ± 40.951 p =0.013	0.844 p <0.001	153.621 ± 63.783 p =0.018	0.341 p =0.034	0.165 ± 0.445 p =0.713	0.885 p <0.001	-0.154 ± 0.698 p =0.826

S.E. = standard error. Age: years. Gender: Women = 0, Men = 1; CSA = Muscle Cross-Sectional Area; BMI: kg/m^2. Summed peak torque was calculated by adding the peak torques recorded during the isokinetic strength test, 60° per second for knee extension-flexion and 30° per second for plantar-dorsiflexion. Summed isokinetic endurance was calculated by adding total work performed during a 21-repetition test at 180° per second for the knee extension-flexion and 60° per second for plantar-dorsiflexion. The height of the chair for the 30-second chair stand test was 43 cm.

Table 4. Association of echogenicity assessed via ultrasonography captured using the panoramic feature and B-mode images of the right leg with various assessments of knee extensor specific force in 88 middle-aged men and women.

Variable Entered	Specific Force Variable	R	$F_{4,83}$	Age (beta ± S.E.)	Gender (beta ± S.E.)	BMI (beta ± S.E.)	Entered Variable (beta ± S.E.)
Echogenicity at 50% (A.U.)	Peak KE Torque by Muscle Thickness at 50% (Nm/cm)	0.299	2.030 p =0.098	-0.799 ± 3.154 p =0.801	-106.185 ± 54.253 p =0.054	10.527 ± 4.306 p =0.017	-1.381 ± 0.770 p =0.077
	Peak KE Torque by Muscle CSA at 50% (Nm/cm^2)	0.311	2.226 p =0.073	-0.625 ± 2.187 p =0.776	-5.110 ± 37.627 p =0.892	6.163 ± 2.986 p =0.042	-0.831 ± 0.534 p =0.123
Echogenicity at 75% (A.U.)	Peak KE Torque by Muscle Thickness at 75% (Nm/cm)	0.239	1.253 p =0.295	-0.074 ± 3.181 p =0.982	-45.255 ± 41.943 p =0.284	9.403 ± 4.341 p =0.033	-0.370 ± 0.702 p =0.600
	Peak KE Torque by Muscle CSA at 75% (Nm/cm^2)	0.267	1.594 p =0.184	-0.161 ± 2.199 p =0.535	32.388 ± 28.991 p =0.267	5.416 ± 3.001 p =0.075	-0.131 ± 0.485 p =0.788

A.U. = Arbitrary Units. S.E. = Standard Error. Age: years. Gender: Women = 0; Men = 1. BMI: kg/m^2.

Table 5 describes the nutritional variables assessed from three-day food diaries for study participants. There were significant differences in macronutrient intake between those above the median for animal-based protein intake as a percentage of energy intake and those below within each gender; relative carbohydrate intake, carbohydrate intake as percentage of energy, protein intake as percentage of energy, relative animal-based protein intake, animal-based protein intake as a percentage of energy, and relative plant-based protein intake were all significantly different in both men and women. Those above the median consumed fewer carbohydrates, more protein, and more animal-based protein than those below. In women, there were also significant differences in relative fat and calcium intake with those above the median consuming less fat and more calcium. In men, on the other hand, there was a significant difference in relative energy intake with those below the median of animal-based protein intake consuming more energy.

Table 5. Dietary intakes accessed from three-day food diaries in 41 middle-aged men and 50 middle-aged women.

	Women			Men		
	Total (n =50)	Below Median (n =25)	Above Median (n =25)	Total (n =41)	Below Median (n =21)	Above Median (n =20)
Relative Energy (kcal/kg/day)	24.46	30.51	22.51	28.41	31.08*	26.73
Relative Fat (g/kg/day)	1.04	1.14*	0.90	1.15	1.20	0.99
Fat Percent Energy (%)	35.66	37.03	34.88	34.85	34.02	35.63
Relative Carbohydrate (g/kg/day)	2.85	3.22**	2.30	3.56	4.12**	2.81
Carbohydrate Percent Energy (%)	46.20	48.56*	44.36	46.86	48.82***	41.16
Relative Protein (g/kg/day)	1.19	1.15*	1.25	1.28	1.28	1.24
Protein Percent Energy (%)	17.99	14.40**	21.27	17.35	14.54***	18.65
Relative Animal Protein (g/kg/day)	0.77	0.61***	1.00	0.87	0.82*	0.96
Animal Protein Percent Energy (%)	11.99	8.59***	16.08	11.74	10.39***	15.16
Relative Plant Protein (g/kg/day)	0.31	0.37*	0.27	0.34	0.39**	0.29
Plant Protein Percent Energy (%)	4.92	5.23	4.81	4.56	4.77	4.26
Vitamin D (IU/day)	155.28	105.58	236.41	149.70	206.52	135.49
Calcium (mg/day)	849.06	743.91**	951.94	1166.69	1103.57	1212.28
Mg (mg/day)	202.96	196.17	210.15	315.96	254.04	332.94
Mn (mg/day)	1.67	1.50	1.98	2.03	2.31	1.89
Vitamin K (mcg/day)	72.01	88.31	59.97	70.72	52.02	77.98
Fe (mg/day)	12.49	12.51	12.03	16.10	18.43	14.80
Vitamin C (mg/day)	107.42	84.78	115.31	79.03	86.42	54.11
Vitamin E (mg/day)	7.716	7.00	13.06	7.71	5.37	8.10
P (mg/day)	772.54	809.96	765.45	1314.39	1265.21	1349.81
K (mg/day)	1693.39	1692.27	1754.97	2577.01	2577.01	2576.71

Table 6 lists physical activity variables recorded using accelerometry. Excluding wear days, which was greater in men below the median compared to men above the median, there were no significant differences between those above the median of animal-based protein as percentage of energy intake and those below.

Regression models examining estimates of animal-based and plant-based protein intakes with total protein intake showed good agreement between our estimates and total protein. Estimates of relative animal-based and relative plant-based protein intakes explained 98.4% of the variance in relative protein intake ($F_{2,88} = 2{,}788.702$, $p < 0.001$), and estimates of animal- and plant-based protein intakes as percentages of energy explained 94.0% of the variance in protein as a percentage of energy ($F_{2,88} = 683.550$, $p < 0.001$).

Table 7 shows Pearson Product-Moment Correlations between relative macronutrient intakes, macronutrient intakes as percentages of energy intake, and relative energy intake. Relative macronutrient intakes showed stronger relationships with relative energy intake than macronutrient intakes expressed as a percentage of energy intake. Outside of the association between percent of energy from fats and carbohydrates, macronutrient intakes expressed as percentages of energy were less strongly correlated amongst one another than relative macronutrient intakes. These results suggest macronutrient intakes should be expressed as percentages of energy intake in statistical models including relative energy intake to limit collinearity.

Table 6. Physical activity variables assessed using accelerometry in 41 middle-aged men and 50 middle-aged women.

	Women			Men		
	Total (n =50)	Below Median (n =25)	Above Median (n =25)	Total (n =41)	Below Median (n =21)	Above Median (n =20)
Wear Days (days)	7.00	6.00	7.007	7.00	7.00*	6.00
Wear Time (min/day)	867.04	869.50	864.57	895.33	895.71	891.87
Sedentary Time (min/day)	559.58	556.00	563.001	613.14	606.00	620.91
Light Physical Activity (min/day)	265.13	285.83	260.33	242.38	269.43	210.11
Moderate Physical Activity (min/day)	27.46	30.67	22.00	27.86	31.83	25.85
Vigorous Physical Activity (min/day)	0.15	0.14	0.29	0.33	2.00	0.00
Moderate to Vigorous Physical Activity (min/day)	31.05	31.20	27.14	33.25	33.83	27.00

All values are medians. Comparisons between those below and above the median for animal-based protein intake as a percentage of energy intake within gender were made using the Brown-Forsythe method. * $p < 0.05$; ** $p < 0.01$; *** $P < 0.001$.

Table 7. Pearson Product-Moment Correlations of macronutrient intakes, including animal-based protein, and relative energy intake in 41 middle-aged men and 50 middle-aged women.

Variable	Variable							
	Relative energy intake	Relative Fat (g/kg/day)	Fat Percent Energy (%)	Relative Carbohydrate (g/kg/day)	Carbohydrate Percent Energy (%)	Relative Protein (g/kg/day)	Protein Percent Energy (%)	Relative Animal Protein (g/kg/day)
Relative Fat (g/kg/day)	0.819 p <0.001	-	-	-	-	-	-	-
Fat Percent Energy (%)	-0.120 p =0.258	0.435 p <0.001	-	-	-	-	-	-
Relative Carbohydrate (g/kg/day)	0.911 p <0.001	0.534 p <0.001	-0.440 p <0.001	-	-	-	-	-
Carbohydrate Percent Energy (%)	0.315 p =0.002	-0.188 p =0.074	-0.845 p <0.001	0.648 p <0.001	-	-	-	-
Relative Protein (g/kg/day)	0.755 p <0.001	0.617 p < 0.001	-0.144 p = 0.174	0.570 p <0.001	-0.019 p = 0.858	-	-	-
Protein Percent Energy (%)	-0.353 p =0.001	-0.351 p =0.001	-0.114 p = 0.281	-0.438 p <0.001	-0.438 p <0.001	0.297 p =0.004	-	-
Relative Animal Protein (g/kg/day)	0.548 p <0.001	0.452 p <0.001	-0.122 p = 0.248	0.357 p =0.001	-0.138 p = 0.191	0.922 p <0.001	0.473 p <0.001	-
Animal Protein Percent Energy (%)	-0.350 p <0.001	-0.332 p =0.001	-0.082 p = 0.439	-0.440 p <0.001	-0.431 p <0.001	0.277 p =0.008	0.916 p <0.001	0.550 p <0.001

Table 8 and Figure 3 present the results of our investigation of the relationship between animal-based protein intake with performance measures. In order to create homoscedastic models with equal variances, data from the handgrip strength test (kg) and the 30-second chair stand test (repetitions) were transformed using the square root function. Using these transformed variables, all of these mixed models had equal variances according to Levene's Test and were homoscedastic according to White's test (i.e., $p > 0.05$).

Our mixed models explained 78.6% of the variance of summed peak torque performed during the isokinetic strength test, 75.7% of the variance of summed work performed during the isokinetic endurance test, and 83.3% of the variance in handgrip strength transformed using the square root function, indicating good model fit for these performance variables. However, our mixed model investigating the results of the 30-second chair stand test only explained 19.1% of the variance in this measure indicating relatively poor model fit. Nonetheless, all models were significant.

Animal-based protein intake was significant to mixed models evaluating lower-body muscular strength, lower-body muscular endurance, and handgrip strength. Those consuming above the median of animal-based protein as percentage of energy intake performed better on these tests of muscular strength and endurance than those below the median. The effect sizes assessed using partial $\eta 2$ of the animal-based protein intake median split were 0.120, 0.065, and 0.049 for summed lower-body peak torque, summed lower-body total work, and handgrip strength, respectively. Animal-based protein intake was not related to performance in the 30-second chair stand test.

Because animal-based protein intake was significant to lower-body muscular strength, lower-body muscular endurance, and handgrip strength, we wanted to verify that these findings

were due to animal-based protein intake and not to greater total protein intake. Although we did control for total protein intake as percentage of energy in our mixed models where participants were split at the median of animal-based protein intake, Table 9 shows our analyses where participants were split at the median of total protein intake as percentage of energy intake and animal-based protein intake as a percent of energy intake was entered as a continuous covariate. With the exception of square root transformed 30-second chair stand repetitions, all of these mixed models had equal variances according to Levene's Test and were homoscedastic according to White's test (i.e., $p > 0.05$). Square root transformed 30-second chair stand performance was homoscedastic but showed unequal variances between groups ($p = 0.024$) according to Levene's test. Because our earlier analysis of square root transformed 30-second chair stand performance (i.e., Table 8) showed equal variances between groups, was homoscedastic, and produced nonsignificant results regarding protein intake and animal-based protein intake, we did not transform 30-second chair stand performance using a different methodology (e.g., Log). In other words, square root transformed 30-second chair stand performance was included in Table 9 despite showing unequal variances between groups, although the HC3 method is considered to be more robust to violations of unequal variance.[284] Total protein intake split at the median of energy intake was not significant to any performance variable, whereas APBI split at the median was significant to lower-body muscular strength, lower-body muscular endurance, and handgrip strength, indicating that APBI is more closely related to muscular performance than total protein intake.

Table 8. Animal-based protein intake and muscular performance in middle-aged men and women.

Performance Variable	R	$F_{9,81}$	Age (beta ± S.E.)	Gender (beta ± S.E.)	BMI (beta ± S.E.)	MVPA (beta ± S.E.)	Relative Energy (beta ± S.E.)	Fat Percent Energy (beta ± S.E.)	Carbohydrate Percent Energy (beta ± S.E.)	Protein Percent Energy (beta ± S.E.)	Animal-Based Protein Intake Median Split (beta ± S.E.)
Summed Isokinetic Peak Torque (Nm)	0.887	33.111	-3.767 ± 1.138	190.543 ± 13.850	1.694 ± 1.874	0.287 ± 0.395	-0.829 ± 0.862	-3.754 ± 8.467	-3.889 ± 8.351	-5.769 ± 8.007	65.874 ± 19.855
		p <0.001	p =0.001	p <0.001	p =0.369	p =0.469	p =0.339	p =0.659	p =0.643	p =0.473	p =0.001
Summed Isokinetic Work (J)	0.870	28.032	-46.224 ± 11.546	1671.298 ± 126.695	29.436 ± 19.814	2.842 ± 4.617	16.825 ± 9.500	-100.977 ± 76.033	-95.794 ± 76.033	-92.620 ± 71.011	549.944 ± 232.478
		p <0.001	p <0.001	p <0.001	p =0.141	p =0.540	p =0.080	p =0.188	p =0.204	p =0.196	p =0.020
Transformed 30-Second Chair Stand (repetitions #)	0.437	2.128	0.004 ± 0.010	0.316 ± 0.128	-0.024 ± 0.013	0.000 ± 0.003	0.008 ± 0.009	-0.092 ± 0.077	-0.103 ± 0.076	-0.095 ± 0.076	0.086 ± 0.156
		p =0.036	p =0.700	p =0.016	p =0.081	p =0.940	p =0.859	p =0.237	p =0.182	p =0.214	p =0.584
Transformed Handgrip Strength (kg)	0.913	45.026	-0.029 ± 0.008	1.898 ± 0.105	0.001 ± 0.018	0.003 ± 0.003	-0.008 ± 0.008	-0.083 ± 0.042	-0.091 ± 0.041	-0.111 ± 0.040	0.349 ± 0.171
		p <0.001	p =0.001	p <0.001	p =0.956	p =0.295	p =0.323	p =0.052	p =0.027	p =0.007	p =0.045

S.E. = standard error. Age: years. Gender: Women = 0, Men = 1. BMI: kg/m^2. Relative energy intake: kcal/kg/day. Animal-based protein intake was split at the median of percent energy from animal-based protein within both men and women; below median = 0, above median = 1. Nutritional variables were assessed using three-day food diaries. Summed isokinetic peak torque was calculated by adding the peak torques recorded during the isokinetic strength test, 60° per second for knee extension-flexion and 30° per second for plantar-dorsiflexion. Summed isokinetic endurance was calculated by adding total work performed during a 21-repetition test at 180° per second for the knee extension-flexion and 60° per second for plantar-dorsiflexion. Total repetitions performed during the 30-second chair stand test and handgrip strength were transformed using the square root function. The height of the chair for the 30-second chair stand test was 43 cm.

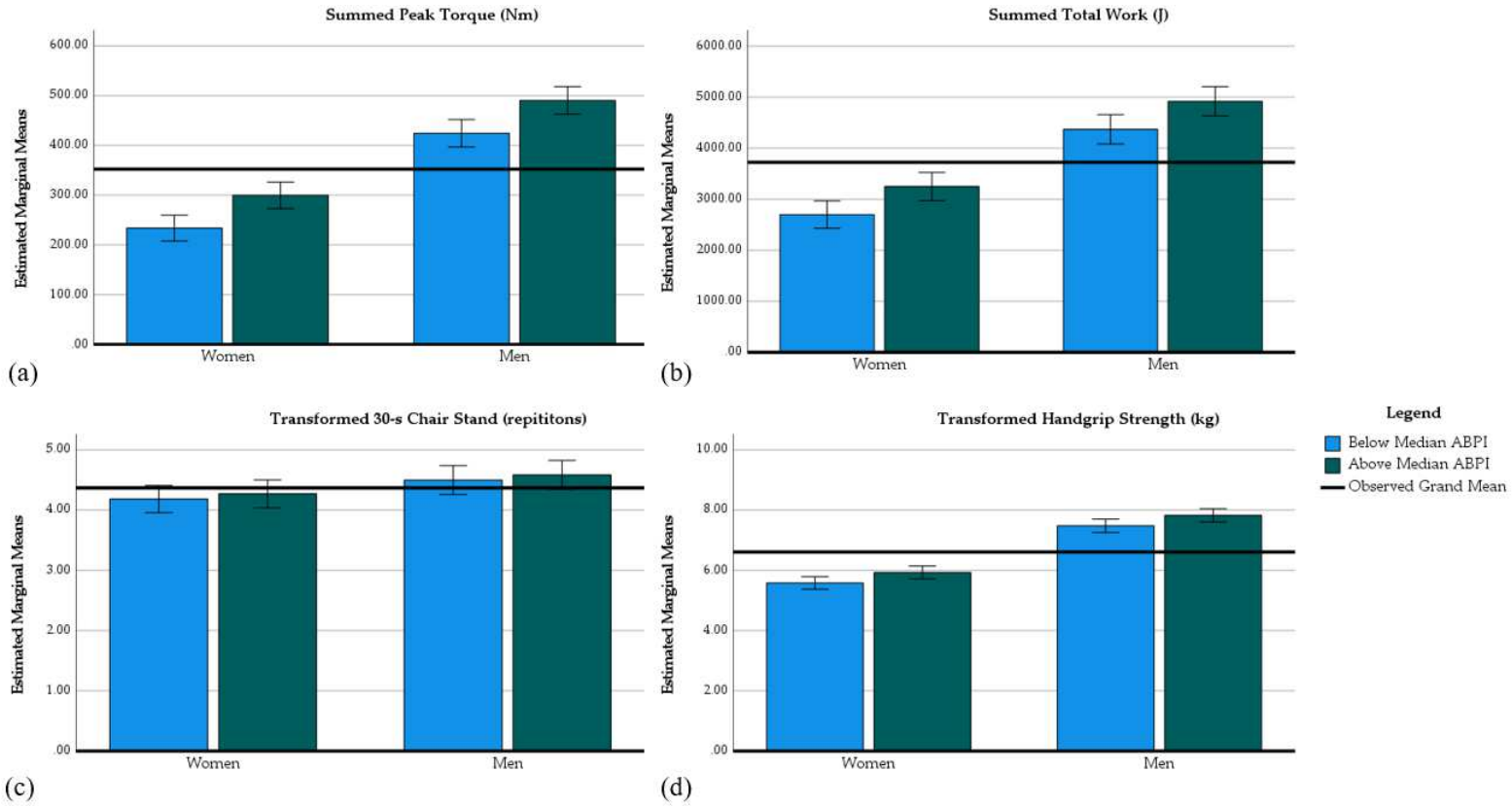

Figure 3. Animal-based protein intake and muscular performance. Animal-based protein intake (ABPI) was split at the median of percent energy from animal-based protein within both men and women; below median = 0, above median = 1. Covariates included age, gender, BMI, MVPA, relative energy intake, and percentages of energy intake from fat, carbohydrate, and protein. All bars are means, and error bars represent 95% confidence intervals. **(a)** Summed isokinetic peak torque by gender and animal-based protein intake. Summed isokinetic peak torque was calculated by adding the peak torques recorded during the isokinetic strength test, 60° per second for knee extension-flexion and 30° per second for plantar-dorsiflexion. **(b)** Summed isokinetic endurance by gender and animal-based protein intake. Summed isokinetic endurance was calculated by adding total work performed during a 21-repetition test at 180° per second for the knee extension-flexion and 60° per second for plantar-dorsiflexion. **(c)** Square root transformed 30-second chair stand test repetitions by gender and animal-based protein intake. The height of the chair for the 30-second chair stand test was 43 cm. **(d)** Square root transformed handgrip strength by gender and animal-based protein intake.

Table 9. Total protein intake and muscular performance in middle-aged men and women.

Performance Variable	R	$F_{9,81}$	Age (beta ± S.E.)	Gender (beta ± S.E.)	BMI (beta ± S.E.)	MVPA (beta ± S.E.)	Relative Energy (beta ± S.E.)	Fat Percent Energy (beta ± S.E.)	Carbohydrate Percent Energy (beta ± S.E.)	ABPI Energy (beta ± S.E.)	Total Protein Intake Median Split (beta ± S.E.)
Summed Isokinetic Peak Torque (Nm)	0.871	28.366	-4.013 ± 1.171	189.571 ± 14.575	2.003 ± 2.029	0.194 ± 0.427	-0.792 ± 0.962	-0.049 ± 4.836	-0.681 ± 4.576	1.754 ± 3.637	19.397 ± 23.176
		p <0.001	p =0.001	p <0.001	p =0.326	p =0.651	p =0.413	p =0.992	p =0.882	p =0.631	p =0.405
Summed Isokinetic Work (J)	0.856	24.638	-47.751 ± 12.387	1654.781 ± 134.463	32.111 ± 22.256	2.090 ± 5.296	16.687 ± 10.609	-24.735 ± 61.303	-24.971 ± 58.990	29.836 ± 43.397	-2.405± 258.849
		p <0.001	p <0.001	p <0.001	p =0.153	p =0.694	p =0.120	p =0.688	p =0.673	p =0.494	p =0.993
Transformed 30-Second Chair Stand (repetitions #)	0.409	1.806	0.004 ± 0.011	0.313 ± 0.130	-0.024 ± 0.013	0.000 ± 0.003	0.007 ± 0.009	-0.011 ± 0.043	-0.024 ± 0.040	0.003 ± 0.043	-0.112 ± 0.172
		p =0.080	p =0.728	p =0.018	p =0.084	p =0.958	p =0.466	p =0.803	p =0.549	p =0.939	p =0.519
Transformed Handgrip Strength (kg)	0.904	40.523	-0.030 ± 0.009	1.901 ± 0.121	0.004 ± 0.0.019	0.002 ± 0.003	-0.008 ± 0.008	-0.018 ± 0.043	-0.031 ± 0.042	0.000 ± 0.032	0.187 ± 0.197
		p <0.001	p =0.001	p <0.001	p =0.834	p =0.680	p =0.360	p =0.683	p =0.459	p =0.997	p =0.953

S.E. = standard error. ABPI = animal-based protein intake. Age: years. Gender: Women = 0, Men = 1. BMI: kg/m^2. Relative energy intake: kcal/kg/day. Total protein intake was split at the median of percent energy from protein within both men and women; below median = 0, above median = 1. Nutritional variables were assessed using three-day food diaries. Summed isokinetic peak torque was calculated by adding the peak torques recorded during the isokinetic strength test, 60° per second for knee extension-flexion and 30° per second for plantar-dorsiflexion. Summed isokinetic endurance was calculated by adding total work performed during a 21-repetition test at 180° per second for the knee extension-flexion and 60° per second for plantar-dorsiflexion. Total repetitions performed during the 30-second chair stand test and handgrip strength were transformed using the square root function. The height of the chair for the 30-second chair stand test was 43 cm.

4.5. Discussion

We found that measures of muscle size from standardized B-mode ultrasound images better captured the performance of the knee extensors, whereas measures of muscle size assessed from panoramic images were more closely related to overall muscular performance, producing significant associations between muscle size with summed peak torque and handgrip strength. However, our methodology differed from that of others who have utilized panoramic ultrasound. We took panoramic images of the *rectus femoris* at one location (i.e., 50% of leg length) as opposed to using a template to image the entire length of the quadriceps, although one research group advocated for an investigation of a single site at the mid-quadriceps.[291]

Nonetheless, the lack of a significant relationship between muscle thickness and CSA measured using the panoramic feature and knee extensor strength is surprising, considering these measures of muscle size were more closely related both to lower-body strength (i.e., summed peak torque) and upper-body strength. Low muscle strength is the first criterion of sarcopenia according to the European Working Group on Sarcopenia in Older People 2 and should be, albeit not necessarily linearly, related to muscle mass.[37] In other words, changes in muscle mass or size are not as meaningful as changes in muscle strength. Measures of muscle size or mass that are unrelated to muscle strength then may have limited utility in assessing or screening for sarcopenia. Despite the fact measures from panoramic ultrasonography lacked face validity in the form of a significant relationship with knee extensor peak torque, our findings suggest that the panoramic feature is a suitable method for assessing sarcopenia in those with greater muscle at the midpoint of thigh as it is related to both lower body and upper-body strength.

We also report that in our sample echogenicity was unrelated to both knee extensor, strength, overall lower-body strength, and *rectus femoris* specific force, another measure of

muscle quality. Although Strasser and colleagues[26] reported a significant correlation between echogenicity and knee extensor strength, the relationship was only found in younger and not older adults. In contrast, Akima and colleagues[293] found a significant relationship between echogenicity and sit-to-stand performance in older Japanese men and women. However, in a subsequent work, the same research group reported no relationship between echogenicity and knee extensor strength.[21] We also did not find a significant relationship between echogenicity and knee extensor strength, and we were the first, at least to our knowledge, to directly compare the echogenicity of the *rectus femoris* to the muscle's specific force. None of the relationships were significant. However, we did find an association between echogenicity with handgrip strength and knee extensor muscular endurance. Echogenicity has been related to both intramuscular fat[294] and fibrous tissue[295] content of muscle. In a large study of older Italian men and women, De Stefano and colleagues[79] reported a negative association between intramuscular fat and physical performance but found that those who were overweight or 'Class I' obese had greater knee extensor strength than those with a normal BMI, suggesting that intramuscular fat plays a greater role in physical performance than in maximal strength. Our findings regarding echogenicity support that view. Echogenicity, then, is not closely related to specific force as it is with other muscular qualities such as endurance, because specific force is dependent on maximal muscle strength.

Our secondary findings regarding dietary intake indicate a positive relationship between animal-based protein intake and muscle strength when controlling for gender, age, BMI, relative energy intake, and macronutrient composition. More specifically, those above the median of animal-based protein intake as percentage of energy intake showed greater lower body strength and endurance and greater handgrip strength than those below. Although greater protein intake is

thought to be protective from developing sarcopenia,[175,296,297] a recent cross-sectional study of older Danish adults utilizing methods similar to ours (e.g., three-day food diary and physical activity assessment) reported that protein intake was not related to knee extensor strength, handgrip strength, and 30-second chair stand test performance.[245] In contrast to their methodology where participants were divided into groups based on relative protein intake, we split ours according to animal-based protein intake as a percentage of energy intake. Although recommendations for protein intake are made on a g/kg basis,[175] one of advantages expressing intakes as percentages of energy intake is that one can control for relative energy intakes and for macronutrient composition in the same statistical model. There is a high degree of collinearity between relative intakes of macronutrients and relative energy intake. In fact, one of the main findings from Højfeldt and colleagues' study of older Danish adults was that relative protein intakes and relative energy intakes are related.[245] Collinearity can bias estimates of betas in multivariate analyses.[246] Although there is still a degree of collinearity between macronutrient intakes as percentages of energy and relative energy intakes, we addressed this issue by using the HC3 method of calculating standard errors which is more robust to collinearity and heteroscedasticity.[284] Outside of expressing intakes as percentages of energy, our methodology also differed because we evaluated animal-based protein intake. Plant-based proteins generally contain amino acids that are oxidized to be used as energy to a greater extent than higher quality animal-based proteins.[54] Thus, total protein intake is likely less strongly related to muscle mass and strength than protein intake from higher quality sources, and our findings particularly support this notion. When split at its median, total protein intake as a percentage of energy intake was not related to lower body strength, lower-body endurance, and handgrip strength, whereas

animal-based protein intake split at the median was positively associated with all of these measures.

There are some limitations to our investigations. We cannot determine from our primary results if the panoramic feature inaccurately quantified muscle size because our study lacked a measure of criterion validity in the form *rectus femoris* muscle thickness and cross-sectional area assessed using MRI or CT. Another caveat to our findings regarding ultrasonography is the skill of our sonographers. Although our sonographers were trained and showed good reliability and ICCs were greater than 0.95 for all measures other than B-mode echo intensity which was equal to 0.81, they were and are not professional sonographers. Panoramic ultrasound is a more difficult method to perform as the probe must be moved while keeping light, consistent pressure during imaging. Our results regarding panoramic ultrasonography and knee extensor performance may indicate, then, that the method should only be performed by those with highest levels of skill. Nonetheless, measures from panoramic ultrasonography were related to summed peak torque and handgrip strength, indicating these measures were related to overall performance. Another potential limitation was the assessment of anatomical as opposed to physiological CSA, as physiological CSA of pennate muscles, such as the *rectus femoris* , is thought to be more closely related to strength.[278]

Regarding the limitations of our secondary analysis, this was a cross-sectional study incapable of establishing causality, the self-reported nature of our food-diary recording limits their accuracy, and we included three participants' physical activity data despite the fact these participants did not have enough valid wear days. Our secondary investigation did have some strengths. We objectively measured and controlled for physical activity. We verified our partitioning of protein intake into animal- and plant-based sources using regression models. We

included relative energy and macronutrient intakes in our mixed models to control for differences in participants' diets outside of animal-based protein intake. Lastly, we confirmed the importance of animal-based protein intake to muscular performance by performing another set on analyses where participants were split at the median of percent energy from total protein.

We report that measures of muscle thickness and CSA derived from panoramic ultrasonography are more closely related to overall strength than the same measures derived from B-mode ultrasound images. Thus, panoramic images may be a suitable method to measure muscle size and estimate overall muscle mass when the entire transverse area of a muscle cannot be measured with a standardized B-mode image. However, measures of muscle size from B-mode images were more closely related to the performance of knee extensors alone, suggesting that B-mode images may be better measures of individual muscles or muscle groups. Echogenicity of the *rectus femoris* was unrelated to its specific force and to overall lower body strength. Instead, echogenicity was related to handgrip strength and knee extensor endurance. Finally, we found a positive relationship between animal-based protein intake and lower-body strength, lower-body endurance, and handgrip strength when controlling for physical activity and diet.

5. EVENNESS OF DIETARY PROTEIN INTAKE IS POSITIVELY ASSOCIATED WITH LEAN BODY MASS AND STRENGTH IN HEALTHY WOMEN[298]*

5.1. Abstract

Background: Evenness of protein intake is associated with increased lean mass, but its relationship with muscle strength and performance is uncertain. **Objectives:** We determined the association of evenness of protein intake with lean mass, muscle strength and endurance, and functional ability. **Design:** This was a cross-sectional study. **Setting:** Data were collected at a research university in the upper midwestern United States. **Participants:** 192 healthy women, aged 18-79 years, mean ± SEM 41.9 ± 1.3, completed the study. **Measurements**: Dietary intake was assessed using three-day food diaries verified with food frequency questionnaires. To assess evenness of protein intake, the day was divided into three periods: waking to 11:30, 11:31 to 16:30, and after 16:30. Lean mass was measured with dual energy x-ray absorptiometry. Lower-body muscle strength and endurance were determined using isokinetic dynamometry. Upper-body muscle strength was maximal handgrip strength. Functional ability was assessed using 6-meter gait speed and 30-second chair stand tests. Accelerometry measured physical activity. **Results:** Intakes of 25 g or more of protein at one or more of the three periods was positively associated with lean mass (β ± S.E; 1.067 ± 0.273 kg, $p<0.001$) and upper-body (3.274 ± 0.737 kg, $p<0.001$) and lower-body strength (22.858 ± 7.918 Nm, $p=0.004$) when controlling for age, body mass index, physical activity, and energy and protein intakes. Consuming at least 0.24 g/kg/period for those under 60 years and 0.4 g/kg/period for those 60 years and older was related

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* This chapter is a co-authored manuscript that can be reproduced in its original form, here, if clearly cited.[298] This article is currently under review for *Nutrition and Metabolic Insights,* but does not yet have a DOI. In addition to collecting data, I Nathaniel R. Johnson, completed all statistical analyses, wrote the manuscript, and made all revisions.

to lean mass (0.754 ± 0.244 kg, p=0.002), upper-body strength (2.451 ± 0.658 kg, p<0.001) and lower-body endurance (184.852 ± 77.185 J, p=0.018), controlling for the same variables.

Conclusions: Evenness of protein intake is related to lean mass, muscle strength, and muscle endurance in women. Spreading protein intake throughout the day maximizes the anabolic response to dietary protein, benefitting muscle mass and performance.

Keywords: Protein distribution, dietary protein, muscle endurance, and muscle strength

5.2. Introduction

Skeletal muscle mass comprises 40 to 50% of body mass[39] and contains approximately 45% of the human body's total protein content.[40] Muscle tissue acts as an "amino acid reservoir,"[41,42] catabolizing itself to provide amino acids or energy to other tissues after traumatic injuries or infections[43] or during periods of negative energy balance.[44] Naturally then, sarcopenia, a condition characterized by reduced muscle quantity and strength, is related to both an increased risk of disability and all-cause mortality.[45] Increasing or maintaining muscle quantity and strength is important throughout the lifespan, as is indicated by both experts[37,68] and the United States Department of Health and Human Services,[46] yet muscle mass and strength decline as individuals age.[7,25,30]

Having adequate dietary intake represents a relatively well-tolerated and low-cost method to mitigate losses of muscle quantity and strength associated with aging, bedrest, or trauma.[88,90,91,175] In addition to the detrimental effects of aging on muscle strength and quantity, an individual's ability to taste decreases with aging[134] as does one's oral health[135] and ability to masticate.[136] As the result of these changes, among other factors, dietary intake decreases by about 25% from age 40 to 70 and predisposes middle-aged and older adults to malnutrition which can hasten the development of sarcopenia.[137] Several nutrients are particularly important

for preserving muscle quantity and strength including protein, fatty acids, vitamin D, antioxidants, and minerals such as iron, magnesium, calcium, selenium, and zinc.[91,172]

Beyond being the "building-blocks" of proteins, dietary amino acids contribute to muscle protein synthesis by activating the mammalian target of rapamycin complex 1.[174,177] This makes dietary protein of particular interest because of the nutrient's ability to directly affect muscle protein synthesis and breakdown.[159,174–176] In fact, about 25 to 30 g of protein is the amount required for muscle protein synthesis,[176] and it is thought that by achieving intakes of this amount more frequently, such as at each meal, one would maximize muscle protein synthesis, benefitting muscle mass and strength.[50] In support of this notion, the primary estimation studies of nitrogen balance that informed the National Institutes of Health 0.8 g per kg body weight per day recommendation for dietary protein intake only included studies where all participants ate at least three meals,[231,299] guaranteeing some level of evenness in dietary protein spread.

A systematic review of 15 studies investigating the evenness of dietary protein intake concluded there was enough evidence to determine that evenness of protein intake distribution was related to increased muscle mass, but there was not enough evidence to determine its effects on muscle strength or protein turnover.[50] Considering this conclusion, we sought to determine the association of evenness of dietary protein intake with lean mass, muscle strength and endurance, and functional ability. Other investigators of dietary protein intake distribution have not controlled for energy intake,[245,249,250,260] which is critical to include in statistical models investigating nutritional variables.[50,248] Moreover, some of these groups,[245,250,260] when investigating dietary protein intake distribution, did not control for total, relative, or percent of energy from protein intake, which can also affect muscle mass and performance.[242,300]

Additionally, the authors of the systematic review advocate for cut-points of 0.24 g per kg body weight per meal for younger adults and 0.4 g per kg per meal for older adults,[50] as these cut-points were informed by a breakpoint analysis of muscle protein synthesis data between healthy younger and older men.[258] However, as there is lack of consensus regarding how to measure or define dietary protein intake distribution,[50] we sought to compare the previous recommendation of 25 to 30 g of protein per meal, the minimum amount thought to elicit a maximal anabolic response,[176] to these newer relative cut-points in a population of healthy women.

5.3. Methods

This project was conducted in the North Dakota State University Healthy Aging Research Lab from October 2017 to December 2019. A total of 195 women from the local community were recruited using e-mail, flyers, and word-of-mouth to visit the research lab for two sessions. During the first session, anthropometric and performance variables were measured, and accelerometers, three-day food diaries, and food frequency questionnaires (FFQ)[286] were provided. Within 7 to 14 days later, participants returned to the lab to return their accelerometers food diaries, and FFQs and have a full-body dual energy x-ray absorptiometry (DXA) scan performed. Participants were between 18 and 80 years of age, not currently using any nicotine products, free of any untreated or nonresponsive diseases or conditions, ambulatory without any assistance, and had to include both animal-based and plant-based foods in their diets. Those who reported working during the night were excluded. Participants were eligibility screened using the 2017 Physical Activity Readiness Questionnaire,[285] a more detailed health history questionnaire, and an orthostatic hypotension test. The study was approved by the North Dakota State University Institutional Review Board (#HE18010) and complied with the Helsinki Declaration of 2013. All Participants completed an informed consent.

5.3.1. Participant Health Screening and Anthropometric Measures

To screen participants for orthostatic hypotension, related to regulatory and safety concerns set forth by the Institutional Review Board, resting blood pressure and standing blood pressure were measured manually with a stethoscope and Diagnostix 703 sphygmomanometer (American Diagnostic Corporation, Hauppauge, NY). Those whose blood pressure dropped by more than 10 mm Hg, either systolic or diastolic, from resting to standing during the orthostatic hypotension test were excluded (n = 0). Following the orthostatic hypotension test, anthropometric variables were measured. Age (years) was self-reported. Height, to the nearest mm, was measured using a stadiometer (Seca 213, Chino, CA) and body mass, to the nearest 0.1 kg, was recorded using a digital balance scale (Denver Instrument DA-150, Arvada, CO). Waist and hip circumferences were completed using a Gulick (Fitness Mart Division of Country Technology Inc., Gays Mills, WI) spring-loaded measuring tape to the nearest mm.

5.3.2. Performance Measures

Prior to performance testing, participants completed a light, self-paced, five-minute warm-up on a cycle ergometer. To optimize performance, research staff encouraged participants to employ “all-out effort” during tests of muscle strength and endurance. Handgrip strength (kg) was assessed first using an analog Jamar Handheld Dynamometer (Bolingbrook, IL). Each participant was instructed to grasp the dynamometer in her dominant hand and to keep her elbow at her side with a 90° bend between the upper arm and forearm while standing. Participants were told to squeeze the dynamometer as hard as possible for two to three seconds. Each participant performed three maximal attempts; the highest grip strength was used. Gait speed was then measured using a Brower TCi system (Draper, UT). Participants were instructed to walk at their normal pace over a 10 m distance. Timing gates were placed 6 m apart. Gait speed was recorded

three times, and mean time was used in analyses. Participants then performed a 30 second (30s) chair stand test on a 43 cm chair. All trials were performed with participants' arms crossed and feet at a comfortable distance apart (i.e., about hip to shoulder width). Participants were instructed to fully sit down and stand up for each repetition, and practice repetitions were performed to ensure adequate performance during the test. The total number of repetitions completed in 30s was recorded. Participants were seated, and the 30s period began when participants started to rise.

After these three assessments, muscle strength and endurance of the lower body were tested using isokinetic dynamometry on a Biodex Pro IV System (Biodex Medical Systems, Shirley, NY). Lower body muscular strength was assessed using peak torque performed during a three-repetition test at 60° per second for knee extension-flexion and a three-repetition test at 30° per second for plantar-dorsiflexion. Similar to others' work,[279] lower body muscular endurance was evaluated using the total amount of work performed during a 21-repetition test at 180° per second for knee extension-flexion and 60° per second for plantar-dorsiflexion. Muscular strength and then endurance were first assessed in the upper leg (i.e., knee extension-flexion) and then in the lower leg (i.e., plantar-dorsiflexion). A warm-up set was completed before each lower-body strength test (i.e., knee extension-flexion, and plantar-dorsiflexion); participants were instructed to perform three repetitions at <75% of their perceived maximal effort. Thirty seconds of rest was given between all extension-flexion tests. One minute of rest was provided between plantar-dorsiflexion tests. To better capture muscular performance of the entire right leg, peak torques from the isokinetic strength test and total work from the isokinetic endurance test were added together to create summed peak torque and summed total work (i.e., knee extension + knee flexion + plantarflexion + dorsiflexion).

5.3.3. Physical Activity Assessment

Following performance testing, accelerometers, three-day food diaries, and FFQs were given to participants. Physical activity was recorded using Actigraph (Pensacola, FL) GT9X accelerometers worn on the non-dominant wrist for seven consecutive days. Participants were instructed to wear the accelerometer during all waking hours except activities involving water (e.g., bathing or swimming). The raw acceleration data were collected at 80 Hz and processed in R software using the GGIR package (version 1.10-10).[224] A sleep log was provided to help delineate non-wear time from time spent sleeping. Non-wear time was defined as intervals of at least 90 minutes of zero counts with allowance of two-minute interval of non-zero counts within a 30 minute window,[280] thus only valid time during waking hours of each day was included for statistical analyses. The minimum number of wear days was four, including one weekend or one non-routine day, over the weeklong collection period, with a minimum wear time of 10 hours per day. Due to its beneficial,[282,301,302] but in this case, also confounding effect on muscle and performance, moderate to vigorous physical activity (MVPA) was included in all analyses as a covariate.

5.3.4. Nutrition Analysis

Participants were given both three-day food diaries and a 153-item FFQ[286] and received training on how to record dietary intakes by a member of the research team. Participants were also required to watch a prerecorded training video provided by the study's registered dietitians. Dietary intakes from three-day food diaries, including nutritional supplements, were entered into Food Processor Nutrition Analysis Software (ESHA Research, Salem, OR) which uses Food Data Central (i.e., the USDA Nutrient Data Base),[283] by trained research assistants. Data entry was then line-by-line verified by a registered dietitian. As the three-day food diary asked

participants to record their intakes in real-time and the FFQ asked participants about their intake over the last 90 days, the two methods do not assess the same nutritional variables; the former represents immediate intake, whereas the latter represents some level of historical intake. Nonetheless, as this project lacked criterion validity for dietary intake (i.e., an objective measure of dietary intake was not performed),[303] the data from the FFQ was used as to verify estimates from three-day food diaries.[153,304]

5.3.5. Follow-up Visit

After 7 to 14 days, participants returned to the lab to turn in accelerometers, food diaries, and food frequency questionnaires, have their body composition measured, and give a blood sample. Body composition was measured using DXA on a Lunar Prodigy, model #8915 (GE Healthcare, Waukesha, WI), with enCORE software.

5.3.6. Statistical Analyses

A total of 192 women completed both a three-day food diary and the FFQ and wore an accelerometer for at least 10 hours a day for four or more days. Three participants were excluded from all analyses because they failed to wear the accelerometer as directed. Thus, all analyses have at most 192 participants. Total and relative intakes, including the percent of energy from each of the macronutrients, were verified using paired t-tests between data from the three-day food diary and the FFQ.

To examine the effects of the evenness of protein intake distribution, data collected from three-day food diaries were first blocked into three periods: waking to 11:30 (breakfast), afternoon 11:31 to 16:30 (lunch), and evening after 16:30 (dinner). Protein intakes were averaged for each period across all three days that the food diary was recorded. Even protein intake distribution was then defined using two methods: a relative intake methodology (i.e., 0.24

or 0.4 g per kg body weight or more per period) and a total intake methodology (i.e., 25 g or more per period). Mean relative protein intakes of at least 0.24 g per kg of body weight per period for younger adults (<60 years) and 0.4 g per kg body weight per period for older adults (≥60 years), respectively, were the cut-points for the relative intake method,[50,258] whereas greater than or equal to 25 g per period was the cut-point for total intake method;[176] consuming an average of protein equal to or greater than these cut-points during one of these periods were recorded as "1"s, and these were summed to create two ordinal variables each with four levels, achieving greater than 0.24/0.4 g per kg body weight per period or 25 g per period at 0, 1, 2, or 3 periods. These ordinal variables were entered into separate multiple linear regression models each controlling for age, body mass index (BMI), MVPA, relative energy intake, and percent of energy from protein.

5.4. Results

Table 10 displays descriptive statistics for the 192 women included in this work.

Table 10. Descriptive statistics of the 192 women included in this work.

Variable	Mean ± SEM
Age (years)	41.9 ± 1.3
Height (cm)	164.8 ± 0.5
Body mass (kg)	70.0 ± 1.0
BMI (kg/m^2)	25.7 ± 0.3
MVPA (min/day)	89.3 ± 2.2

BMI = body mass index. MVPA= moderate to vigorous physical activity. SEM = standard error of the mean.

All analyses except for that of the 30s chair stand test had 192 participants; the chair stand test had 191 participants. Age ranged from 18 to 79 years. A total of 147 participants (76.6%) were less than 60 years of age, and 45 (24.4%) were 60 years or older. The minimum BMI was 15.3 and the maximum was 41.9 kg/m^2. According to BMI, two participants (1.0%)

were underweight with BMIs less than 18.5 kg/m^2, 96 (50%) had BMIs between 18.5 and less than 25, 63 (32.8%) were overweight with BMIs between 25 and less than 30 kg/m^2, 20 (10.4%) had BMIs between 30 and less than 35 kg/m^2, nine (4.7%) had BMIs between 35 and less than 40 kg/m^2, and two had (1.0%) had BMIs of 40 or greater kg/m^2; thus, 31 participants (16.1%) were considered obese according to BMI. A total of 171 (89.1%) of participants wore accelerometers for at least seven days with greater than or equal to 10 hours of wear time on each day (a valid wear day was considered to have to 10 hours of wear time); one participant (0.5%) only had four days, three (1.6%) had five days, and 17 (8.9%) had six days with at least 10 hours of wear time. Time spent in MVPA ranged from a minimum of 18.8 and a maximum of 185.9 minutes per day.

The results of the paired t-test analyses of dietary intake data comparing the three-day food diary and the FFQ are shown in Table 11. Total and relative intakes of energy, protein, and carbohydrates were not significantly different, showing convergent validity; only total ($p = 0.006$) and relative fat ($p = 0.003$) intakes were significantly different. When expressed as percentages of energy intake, fat ($p < 0.001$) and carbohydrate ($p < 0.001$) intakes were significantly different and protein intake was not.

Table 11. Paired comparison of dietary intake data from three-day food diaries and the food frequency questionnaire.

Variable	Three-Day Diary Mean ± SEM	FQQ Mean ± SEM	Paired Difference Mean ± SEM	P
Total Energy (kcal/day)	2022 ± 40	2004 ± 63	18 ± 58	0.758
Total Protein (g/day)	85.3 ± 1.8	85.2 ± 2.9	0.0 ± 2.6	0.989
Total Fat (g/day)	84.5 ± 2.0	77.4 ± 2.6	7.1 ± 2.5	0.006
Total Carbohydrate (g/day)	230.9 ± 5.7	243.5 ± 8.5	-12.6 ± 7.6	0.099
Relative Energy (kcal/kg/day)	29.736 ± 0.686	29.378 ± 0.977	0.359 ± 0.858	0.676
Relative Protein (g/kg/day)	1.262 ± 0.033	1.245 ± 0.044	0.016 ± 0.038	0.669
Relative Fat (g/kg/day)	1.238 ± 0.033	1.128 ± 0.039	0.110 ± 0.037	0.003
Relative Carbohydrate (g/kg/day)	3.401 ± 0.095	3.587 ± 0.133	-0.187 ± 0.113	0.100
Protein Percent Energy (%)	17.3 ± 0.3	17.1 ± 0.2	0.1 ± 0.3	0.622
Fat Percent Energy (%)	37.2 ± 0.5	34.8 ± 0.5	2.4 ± 0.4	<0.001
Carbohydrate Percent Energy (%)	45.0 ± 0.6	48.4 ± 0.6	-3.3 ± 0.6	<0.001

FFQ = food frequency questionnaire.[286] SEM = standard error of the mean.

One of the goals of this work was to control for both energy and protein intakes when investigating dietary protein distribution, as recommended by others,[50,248] yet relative energy and protein intakes are related,[245] potentially biasing estimates when entered into the same statistical model.[246] In models evaluating dietary protein intake distribution, energy intake was expressed as relative energy intake (i.e., kcal per kg body weight per day) and protein intake as a percentage of energy intake. As percent of energy from carbohydrates and fats were different according to paired t-test analyses of data from the three-day food diaries and the FFQs, these variables were not used as covariates in regression models.

The distribution of dietary protein intake is described in Table 12. Intakes were greatest in the evening or dinner period and lowest during the morning or breakfast period. Of the 147 participants less than 60 years of age, 67 (45.6%), 116 (78.9%), and 143 (97.3%) consumed an average of at least 0.24 g of protein per kg body weight during the breakfast, lunch, and dinner periods, respectively. Of the 45 participants 60 years and older, 13 (28.9%), 22 (48.9%), and 33 (73.3%) consumed an average of at least 0.4 g of protein per kg body weight during the

breakfast, lunch, and dinner periods, respectively. For the relative protein intake per period summed ordinal variable, nine (4.7%) participants had a score of 0 (< 60 years = 2; ≥ 60 years = 7), 34 (17.7%) had a score of 1 (< 60 years = 19; ≥ 60 years = 15), 87 (45.3%) had a score of 2 (< 60 years = 71; ≥ 60 years = 16), and 62 (32.3%) had a score of 3 (< 60 years = 55; ≥ 60 years = 7). Although the total cut-off (i.e., 25 g/period) did not vary for those younger than 60 years and those 60 years and older, data for the total protein intake per period method are presented for these two populations separately for comparison with the relative cut-point method. At the morning or breakfast period, 44 (22.9%) participants consumed 25 g of protein or more (< 60 years = 32; ≥ 60 years = 12), at the midday or lunch period 98 (51.0%) participants, consumed 25 g of protein or more (< 60 years = 78; ≥60 years = 20), and at the evening or dinner period 159 (82.8%) participants consumed 25 g of protein or more (< 60 years = 120; ≥60 years = 39). For the total protein intake per period summed ordinal variable which counts how many periods participants consumed a mean protein intake of equal to or greater than 25 g, 17 (8.9%) participants had a score of 0 (< 60 years = 14; ≥ 60 years = 3), 73 (38.0%) had a score of 1 (< 60 years = 56; ≥ 60 years = 17), 78 (40.6%) had a score of 2 (< 60 years = 57; ≥ 60 years = 21), and 24 (12.5%) had a score of 3 (< 60 years = 20; ≥ 60 years = 4).

Table 12. Distribution of dietary protein intake with unadjusted 95% confidence intervals for comparison.

Variable	Period			
	Breakfast	Lunch	Dinner	Total
	Mean ± SEM	Mean ± SEM	Mean ± SEM	Mean ± SEM
	[95% CI]	[95% CI]	[95% CI]	[95% CI]
Total Protein (g)	17.4 ± 0.8	28.1 ± 0.9	39.8 ± 1.1	85.3 ± 1.8
	[15.9, 18.9]	[26.3, 29.8]	[37.7, 42.0]	[81.6, 88.9]
Relative Protein (g/kg)	0.255 ± 0.012	0.418 ± 0.015	0.588 ± 0.018	1.262 ± 0.033
	[0.232, 0.278]	[0.388, 0.448]	[0.553, 0.623]	[1.197, 1.326]
Percent of Energy (%)	3.5 ± 0.2	5.7 ± 0.2	8.0 ± 0.2	17.3 ± 0.3
	[3.2, 3.8]	[5.4, 6.0]	[7.7, 8.4]	[16.6, 17.9]
Percent of Total Protein (%)	20.0 ± 0.7	33.2 ± 0.8	46.8 ± 0.8	100*
	[18.6, 21.4]	[31.6, 34.7]	[45.2, 48.4]	

95% CI = 95% confidence interval. SEM = standard error of the mean.

* Standard error and 95% confidence interval could not be calculated as all values were 100.

The results of separate multiple linear regression models evaluating the relationship between these two summed ordinal variables with lean body mass (kg) and body composition assessed via DXA (% fat), and handgrip strength (kg), 30s chair stand test (repetitions), mean gait speed (s), and summed lower-body strength (Nm) and endurance performance (J) are presented in Table 13. All models were significant (all $p < 0.05$). Both methods used to define evenness of dietary protein intake distribution were related to total lean body mass and maximal handgrip strength. Neither method was related to 30s chair stand or gait speed performance, although the relative (i.e., 0.24/0.4 g/kg/period) intake per period method approached significance ($p = 0.063$) for gait speed. Intakes of ≥ 25 g of protein per period were related to lower-body strength ($p = 0.004$), whereas intakes of 0.24 or 0.4 g of protein per kg body weight per period were associated with lower-body endurance ($p = 0.018$).

Table 13. Model summaries of separate multiple linear regression models and coefficients evaluating two different methods of defining protein intake distribution when controlling for age, BMI, MVPA, relative energy intake, and percent of energy from protein.

Outcome	Protein Intake Variable*	Model			Coefficient	
		R	$R^2_{adj.}$	P	B ± SE	P
Lean Body Mass (kg)	≥25 g/period	0.710	0.489	<0.001	1.067 ± 0.273	<0.001
	0.24/0.4† g/kg/period	0.700	0.474	<0.001	0.754 ± 0.244	0.002
Body Composition (% fat)	≥25 g/period	0.835	0.687	<0.001	-0.715 ± 0.563	0.205
	0.24/0.4 g/kg/period	0.833	0.684	<0.001	-0.033 ± 0.497	0.948
Maximal Handgrip Strength (kg)	≥25 g/period	0.517	0.243	<0.001	3.274 ± 0.737	<0.001
	0.24/0.4 g/kg/period	0.495	0.221	<0.001	2.451 ± 0.658	<0.001
Thirty-Second Chair Stand Test (repetitions)	≥25 g/period	0.306	0.064	0.006	0.348 ± 0.588	0.555
	0.24/0.4 g/kg/period	0.303	0.062	0.006	0.07 ± 0.519	0.893
Mean 6m Gait Speed (s)	≥25 g/period	0.359	0.100	<0.001	0.007 ± 0.073	0.927
	0.24/0.4 g/kg/period	0.380	0.117	<0.001	-0.119 ± 0.064	0.063
Summed Lower-Body Peak Torque (Nm)	≥25 g/period	0.583	0.319	<0.001	22.858 ± 7.918	0.004
	0.24/0.4 g/kg/period	0.561	0.293	<0.001	8.019 ± 7.099	0.260
Summed Lower-Body Endurance (J)	≥25 g/period	0.544	0.273	<0.001	170.522 ± 88.159	0.055
	0.24/0.4 g/kg/period	0.551	0.303	<0.001	184.852 ± 77.185	0.018

BMI = body mass index; MVPA= moderate-to-vigorous physical activty; SE = standard error.

*Mean protein intakes during three periods, waking to 11:30 (breakfast), afternoon (lunch) 11:31 to 16:30, and evening after 16:30 (dinner), equal to or greater than the listed cut-offs were coded as "1s" and were then summed to create ordinal levels with 4 levels, meeting the cut-off at 0, 1, 2, or 3 periods.

†For those 60 and under 0.24 g/kg/period; for those 60 and over 0.4 g/kg/period.

5.5. Discussion

Consistent with the results of others[251,255,259] and the conclusion of Jespersen and Agergaard who wrote the review regarding the evenness of dietary protein intake and muscle mass, strength, and protein turnover,[50] we found that evenness of protein intake distribution was related to lean body mass using both the 25 g per period and the 0.24/0.4 g per kg body weight per period cut-points. This finding further supports the hypothesis that achieving sufficient protein intake at each meal increases net protein balance, resulting in higher levels of lean mass.

However, we found that the evenness of dietary protein intake was not related to body composition, in contrast to the findings of another cross-sectional study with similar methods (i.e., three-day food diaries and DXA) which reported that those who ate more than an average of 0.24 g per kg body weight per meal for all three meals had better body composition than those who did not.[259] That experimental group, though, was significantly younger consisting of college-aged participants only, and those authors did not use BMI as a covariate in their statistical models.[259] Of course, BMI and body composition are related, but BMI is not an accurate estimate of body fat percentage, often misclassifying people as overweight or obese.[305,306] Despite the association of BMI with lean mass and body composition,[305,306] we found that lean mass was related whereas body composition was not related to the evenness of dietary protein intake, indicating that the evenness of protein intake is important for preserving or increasing lean mass but not for losing or preventing gains in fat mass.

Our work does show that evenness of dietary protein intake was positively associated with muscle strength. More specifically, mean intakes of at least 25 g per period were significantly associated with both upper (i.e., handgrip) and lower-body strength, whereas intakes of 0.24 or 0.4 g per kg body weight per period were only related to handgrip strength. We do not believe that this disparity is the result of relative per meal metrics (i.e., 0.24 or 0.4 g/kg/period) being generally less informative than total per meal metrics (i.e., 25 g/period). Rather, the cut-points for this relative method, 0.24/0.4 g per kg body weight per period, are based on one work in young (i.e., 18 – 37 years) and older men (i.e., 65 – 80 years) and did not include middle-aged men.[258] As our work evaluated women across much of the adult lifespan (i.e., 18 to 79 years), the true relative cut-points needed for muscle protein synthesis are likely different for our sample. Although sex does not affect muscle protein synthesis at fasting[307] or

after a meal (i.e., postabsorptive state)[308] in younger populations, the anabolic effects of a meal are blunted in older women compared to men.[309] Thus, older women would likely need to achieve protein intakes greater than what was indicated in older men, which is 0.4 g per kg of body weight per meal.[258] Future studies should examine the relative amount of protein needed at one meal to stimulate muscle protein synthesis in women, particularly older women.

Additionally, our results indicate that evenness of dietary protein intake distribution is related to lower-body endurance. However, there was a discrepancy between our findings for lower-body endurance and lower-body strength when examining the two methods of defining even protein intake distribution. In contrast to our findings for lower-body strength, the relative method of expressing the evenness of protein intake distribution was positively associated with lower-body endurance performance, whereas the total method was not. Yet, the total method, as opposed to that of the relative intake method in the case of the lower-body strength model, approached significance and was closer to the estimate of the relative method than the two methods were for lower-body strength. Thus, we do not view this difference as incongruent with our results. In order to have a relative intake of exactly 0.24 g per kg of body weight per period when eating 25 g of protein, one would need to have a body mass of approximately 104 kg. The mean body mass of participants was 70 kg for this study and is 77.5 kg for women 20 years and older in the United States.[310] Thus, 25 g per period is greater than the relative 0.24 g per kg body weight per period cut-point for 95.9% of the 147 women under 60 years of age included in this study and for the average woman in the United States. Our results suggest that relative intakes greater than 0.24 g per kg of body weight per meal or period are likely needed for women under age 60 to see benefit in lower-body strength, but intakes of 0.24 may be sufficient to benefit lower-body endurance.

Evenness of dietary protein intake was not related to functional ability in our sample. The relative intake method approached significance for mean 6 m gait speed, suggesting some benefit with increased evenness of intake. These measures of functional ability, though, may not be related to performance in younger or middle-aged healthy adults. In the context of the European Working Group's revised consensus,[37] for instance, measures of physical performance (i.e., functional ability) are intended to differentiate between those with sarcopenia and those with "severe sarcopenia." In support of this, others,[28] using a cross-sectional sample of 409 adults aged 60 to 96 years, reported no relationship between isokinetic leg strength and gait speed in stronger older adults, whereas leg strength was related to gait speed in weaker older adults, when using a quadratic regression model. We hypothesize that given an older population, associations between the evenness of dietary protein intake and functional ability would be observed, as protein intakes of ≥ 0.25 g/kg/meal were associated with decreased odds of self-reported functional disability.[311]

This study had some limitations. It was a cross-sectional study incapable of establishing causality. The participants may not be representative of the larger population, as convenience recruiting methods were used, and only healthy women were allowed to participate. In addition, subjective, self-reported tools measured dietary intake. We, however, used two subjective dietary tools, a three-day food diary and an FFQ,[286] to verify our results. This is a key strength of our work relative to many others who have only used subjective assessments of dietary intake, as using two subjective tools to measure dietary intake is considered a best practice when lacking criterion validity.[153,303,304] In addition to this strength, we included an objective measure of physical activity in our statistical models. Lastly, unlike other groups who have investigated the evenness of dietary protein intake,[245,249,250,260] we controlled for both energy and protein intakes.

In conclusion, we find further support for the relationship between the evenness of dietary protein intake and lean body mass. We also present compelling cross-sectional data that the evenness of dietary protein intake is positively associated with muscle strength and endurance, even when controlling for physical activity and energy and protein intakes. Future research needs to establish a relative per meal threshold for women as the current 0.24 and 0.4 g per kg body per meal recommendations[50] reflect data from men.[258]

6. ANIMAL-BASED PROTEIN INTAKE IS POSITIVELY ASSOCIATED WITH LOWER-BODY STRENGTH AND FUNCTIONAL ABILITY IN OLDER, BUT NOT YOUNGER WOMEN

6.1. Abstract

Background: As women age, their anabolic response to a consumed meal and dietary protein consumed is decreased. Animal- vs. plant-based dietary proteins are generally considered more anabolic and may benefit older women. **Objectives:** The association of animal-based protein intake with lean mass, muscle strength and endurance, and functional ability was determined among healthy women across the lifespan. **Design:** This was a cross-sectional study. **Setting:** Data were collected at a research university in the upper midwestern United States. **Participants:** 192 healthy women, aged 18-79 years, mean ± SEM 41.9 ± 1.3, completed the study, and were split into four cohorts based on self-reported age. **Measurements**: Dietary intake, differentiating animal- and plant-based dietary protein intake, energy, and other macro-nutrients, was assessed using three-day food diaries, and intakes of energy and all macronutrients were verified with food frequency questionnaires. Animal-based protein intake was adjusted for energy and protein intakes. Lower-body muscle strength and endurance were determined using isokinetic dynamometry. Upper-body muscle strength was measured with maximal handgrip strength. Functional ability was assessed using 6-meter gait speed and 30-second chair stand tests. Accelerometry measured physical activity. **Results:** Adjusted animal-based protein intake was unrelated to lean body mass and performance in women 60 years and younger. When controlling for age, body mass index, and physical activity, energy and protein adjusted animal-based protein intake was related to increased lower-body strength (β ± SE; 14.834 ± 7.287 Nm, p = 0.049) and faster gait speed (β ± SE; -0.177 ± 0.087 s, p = 0.049) in women older than 60 years

of age. **Conclusions:** Dietary protein quality is important for lower-body muscle strength and functional ability in older women. Older women should strive to eat more protein from animal sources.

Keywords: Protein distribution, dietary protein, muscle endurance, and muscle strength

6.2. Introduction

Dietary intake is a modifiable lifestyle factor that has profound effects on muscle health.[88,90,91,175] Many nutrients contribute to muscle health,[91,172] but dietary protein uniquely benefits muscle by stimulating muscle protein synthesis[159,174–176] via the mammalian target of rapamycin complex 1.[174,177] It is not just the amount of protein eaten[175,209,229,230] that affects muscle health; the distribution of protein intake[50,249,250] and the quality of protein consumed also affect skeletal muscle tissue.[175,204,261]

Historically, protein quality has been determined using the Protein Digestibility Corrected Amino Acid Score (PDCAAS), which has been used extensively since its adoption by the World Health Organization in 1989 as the organization's preferred method to calculate protein quality.[262,263] In 2011, another method to calculate protein quality, the Digestible Indispensable Amino Acid Score (DIAAS), was proposed by the Food and Agriculture Organization of the United Nations to replace PDCAAS,[264,265] as there were concerns about the PDCAAS overestimating the amount of amino acids absorbed by the body,[266] minimizing differences in protein quality between high and low quality proteins. Later, another group confirmed this discrepancy. These authors reported significantly higher protein quality for pea protein concentrate, soya isolate, soya flour, and wheat when using various forms of the PDCAAS compared to DIAAS.[52] Animal-based proteins (i.e., proteins from animals) such as egg (PDCAAS = 118), cow's milk (PDCAAS = 121), and beef (PDCAAS = 92) have greater protein

quality than plant-based proteins such as soy (PDCAAS = 91) and wheat (PDCAAS = 42).[51] These differences in protein quality between animal and plant-based foods are further magnified when the more appropriate DIAAS is used to measure protein quality. For example, the DIAAS of soy protein isolate was 84 and its PDCAAS was equal to 93, whereas whey protein isolate had a PDCAAS of 99 and a DIAAS of 100, resulting in a difference of 6 when using the PDCAAS and of 16 when using the DIAAS.[52] Regardless of whether the PDCAAS or the DIAAS is used, animal-based proteins tend to have better protein quality than plant-based proteins, spurring some researchers to investigate the effects of proteins from either animal or plant sources on muscle health.

Works that elevated the effects of animal- or plant-based dietary protein generally report that animal-based proteins are more closely associated with muscle health than plant-based proteins,[53,54] supporting the notion that eating proteins with greater DIAAS/PDCAAS result in greater lean body mass and improved performance. Using isotopic amino acid tracers, one group reported significantly greater increases in net protein balance following an egg breakfast compared to a cereal breakfast in a crossover sample of 12 older adults aged 57 to 74 years.[159] Another group of authors utilizing nine years of longitudinal nutrition and physical activity data from the "Framingham Offspring Study," reported that animal-based proteins were related to increased muscle mass even in those with lower physical activity levels, whereas greater physical activity levels were needed for participants' plant-based protein intake to be related to increased muscle mass.[96] In a cross-sectional sample of 1,853 Italian adults, those in highest tertile of animal-based protein intake had greater arm and calf circumferences and handgrip strength compared to those in the lowest tertile when controlling for a variety of covariates.[273]

Due to the higher DIAAS observed in animal-based foods[52] and previous indications that foods with better protein quality are more anabolic,[53,54] we sought to determine the association of animal-based protein intake with lean body mass, body composition, and muscle strength, muscle endurance, and functional ability. In addition, as older,[309] but not younger,[308] women's response to dietary protein is affected, we specifically sought to determine these associations in older women, as increased protein quality is most likely to benefit them.

6.3. Methods

This project was conducted in the North Dakota State University (Fargo, ND) Healthy Aging Research Lab from October 2017 to December 2019. A total of 195 women from the local community were recruited using e-mail, flyers, and word-of-mouth to visit the research lab for two sessions. During the first session, anthropometric and performance variables were measured, and accelerometers, three-day food diaries, and food frequency questionnaires (FFQ)[286] were provided. Within 7 to 14 days later, participants returned to the lab to return their accelerometers, food diaries, and FFQs and have a full-body dual energy x-ray absorptiometry (DXA) scan performed. Participants were between 18 and 80 years of age, not currently using any nicotine products, free of any untreated or nonresponsive diseases or conditions, ambulatory without any assistance, and had to include both animal-based and plant-based foods in their diets. Those who reported working during the night were excluded. Participants were eligibility screened using the 2017 Physical Activity Readiness Questionnaire,[285] a more detailed health history questionnaire, and an orthostatic hypotension test. The study was approved by the North Dakota State University Institutional Review Board (#HE18010) and complied with the Helsinki Declaration of 2013. All participants completed an informed consent document.

6.3.1. Participant Health Screening and Anthropometric Measures

To screen participants for orthostatic hypotension, related to regulatory and safety concerns set forth by the Institutional Review Board, resting blood pressure and standing blood pressure were measured manually with a stethoscope and Diagnostix 703 sphygmomanometer (American Diagnostic Corporation, Hauppauge, NY). Those whose blood pressure dropped by more than 10 mm Hg, either systolic or diastolic, from resting to standing during the orthostatic hypotension test were excluded (n = 0). Following the orthostatic hypotension test, anthropometric variables were measured. Age (years) was self-reported. Height, to the nearest mm, was measured using a stadiometer (Seca 213, Chino, CA) and body mass, to the nearest 0.1 kg, was recorded using a digital balance scale (Denver Instrument DA-150, Arvada, CO). Waist and hip circumferences were completed using a Gulick (Fitness Mart Division of Country Technology Inc., Gays Mills, WI) spring-loaded measuring tape to the nearest mm.

6.3.2. Performance Measures

Prior to performance testing, participants completed a light, self-paced, five-minute warm-up on a cycle ergometer. To optimize performance, research staff encouraged participants to employ "all-out effort" during tests of muscle strength and endurance. Handgrip strength (kg) was assessed first using an analog Jamar Handheld Dynamometer (Bolingbrook, IL). Each participant was instructed to grasp the dynamometer in her dominant hand and to keep her elbow at her side with a 90° bend between the upper arm and forearm while standing. Participants were told to squeeze the dynamometer as hard as possible for two to three seconds. Each participant performed three maximal attempts; the highest grip strength was used. Gait speed was then measured using a Brower TCi system (Draper, UT). Participants were instructed to walk at their normal pace over a 10 m distance. Timing gates were placed 6 m apart. Gait speed was recorded

three times, and mean time was used in analyses. Participants then performed a 30 second (30s) chair stand test on a 43 cm chair. All trials were performed with participants' arms crossed and feet at a comfortable distance apart (i.e., about hip to shoulder width). Participants were instructed to fully sit down and stand up for each repetition, and practice repetitions were performed to ensure adequate performance during the test. The total number of repetitions completed in 30s was recorded. Participants were seated, and the 30s period began when participants started to rise.

After these three assessments, muscle strength and endurance of the lower body were tested using isokinetic dynamometry on a Biodex Pro IV System (Biodex Medical Systems, Shirley, NY). Lower body muscular strength was assessed using peak torque performed during a three-repetition test at 60° per second for knee extension-flexion and a three-repetition test at 30° per second for plantar-dorsiflexion. Similar to others' work,[279] lower body muscular endurance was evaluated using the total amount of work performed during a 21-repetition test at 180° per second for knee extension-flexion and 60° per second for plantar-dorsiflexion. Muscular strength and then endurance were first assessed in the upper leg (i.e., knee extension-flexion) and then in the lower leg (i.e., plantar-dorsiflexion). A warm-up set was completed before each lower-body strength test (i.e., knee extension-flexion, and plantar-dorsiflexion); participants were instructed to perform three repetitions at <75% of their perceived maximal effort. Thirty seconds of rest was given between all extension-flexion tests. One minute of rest was provided between plantar-dorsiflexion tests. To better capture muscular performance of the entire right leg, peak torques from the isokinetic strength test and total work from the isokinetic endurance test were added together to create summed peak torque and summed total work (i.e., knee extension + knee flexion + plantarflexion + dorsiflexion).

6.3.3. Physical Activity Assessment

Following performance testing, accelerometers, three-day food diaries, and FFQs were given to participants. Physical activity was recorded using Actigraph (Pensacola, FL) GT9X accelerometers worn on the non-dominant wrist for seven consecutive days. Participants were instructed to wear the accelerometer during all waking hours except activities involving water (e.g., bathing or swimming). The raw acceleration data were collected at 80 Hz, and processed in R software using the GGIR package (version 1.10-10).[224] A sleep log was provided to help delineate non-wear time from time spent sleeping. Non-wear time was defined as intervals of at least 90 minutes of zero counts with allowance of two-minute interval of non-zero counts within a 30 minute window,[280] thus only valid time during waking hours of each day was included for statistical analyses. The minimum number of wear days was four, including one weekend or one non-routine day, over the weeklong collection period, with a minimum wear time of 10 hours per day. Due to its beneficial,[282,301,302] but in this case, also confounding effect on muscle and performance, moderate to vigorous physical activity (MVPA) was included in all analyses as a covariate.

6.3.4. Nutrition Analysis

Participants were given both three-day food diaries and a 153-item FFQ[286] and received training on how to record dietary intakes by a member of the research team. Participants were also required to watch a prerecorded training video provided by the study's registered dietitians. Dietary intakes from three-day food diaries, including nutritional supplements, were entered into Food Processor Nutrition Analysis Software (ESHA Research, Salem, OR) which uses Food Data Central (i.e., the USDA Nutrient Data Base)[283] by trained research assistants. Data entry was then line-by-line verified by a registered dietitian. As the three-day food diary asked

participants to record their intakes in real-time and the FFQ asked participants about their intake over the last 90 days, the two methods do not assess the same nutritional variables. The former represents immediate intake, whereas the latter represents some level of historical intake. Nonetheless, as this project lacked a measure of criterion validity for dietary intake (i.e., an objective measure of dietary intake was not performed), the data from the FFQ was used as a comparison measure to verify estimates from three-day food diaries.[153]

Animal-based protein intakes, included meat, fish and seafood, dairy, eggs, poultry, and wild game, were estimated using a line-by-line examination of dietary intake from the three-day food diaries by the study's registered dietitians. Foods with both animal- and plant-based protein (i.e., mixed foods) were split according to their ingredients list to distinguish protein sources, and foods that contained less than 1 g of protein were excluded from these calculations.

6.3.5. Follow-up Visit

After 7 to 14 days, participants returned to the lab to turn in accelerometers, food diaries, and food frequency questionnaires, have their body composition measured, and give a blood sample. Body composition was measured using DXA on a Lunar Prodigy, model #8915 (GE Healthcare, Waukesha, WI), with enCORE software.

6.3.6. Statistical Analyses

A total of 192 women completed both a three-day food diary and the FFQ and wore an accelerometer for at least 10 hours a day for four or more days. Three participants were excluded from all analyses because they failed to wear the accelerometer as directed. Thus, all analyses have 192 participants, except for those related to 30s chair-stand test which have 191. As older,[309] but not younger[308] women's anabolic response to dietary protein is blunted, there is greater potential for the anabolic effects of protein to be observed in older as opposed to

younger women. Thus, to investigate the role of animal-based protein intake in muscle health, this cross-sectional sample was subdivided into four cohorts based on self-reported age: college-aged women (18 – 24), younger women (25 – 44), middle-aged women (45 – 60 years), and older women (61 – 79). Previous research regarding the anabolic response of muscle tissue to a meal used women 65 to 80 years if age;[309] in order to increase our sample size, we slightly expanded this age range and included women aged 61 to 79 years as older women in our work. The other cut-points between 24 and 25 and 44 and 45 years were chosen arbitrarily to form more even numbers of participants in each cohort.

First, total and relative intakes of energy and macronutrients were verified using paired t-tests between data from the three-day food diary and the FFQ for each cohort and the entire sample. Multiple linear regression models determined the association of animal-based protein intake for each cohort and in aggregate when controlling for a variety of covariates.

To specifically evaluate the role of animal-based protein intake while controlling for both energy and protein intakes, mean relative animal-based protein intake (g/kg/day) was first regressed using both relative energy intake[248] (kcal/kg/day) and relative protein intake (g/kg/day) as independent variables for the each cohort. Standardized residuals from these multiple linear regression models were saved and used in other multiple linear regression models to investigate the association between animal-based protein intake and muscle mass and performance. Those with greater standardized residuals had greater relative intakes of animal-based protein given their relative energy and protein intakes. Thus, the results of the regression models show the unique contribution of animal-based protein to muscle mass and performance without the confounding effects of energy or protein intakes. In addition to controlling for energy and total protein intakes using this standardized residual methodology, age, and MVPA were included in

all multiple linear regression models evaluating the relationship between animal-based protein intake and lean body mass and performance as covariates, and BMI was also included in all regression models investigating performance variables. As decreases in circulating sex hormones affect muscle mass and strength,[312] self-reported menopause was included as a categorical covariate in all aggregated models, where participants were not subdivided by self-reported age.

6.4. Results

Of 192 women included in analyses, a total of 50 women were 18 to 24 years (i.e., "college-aged"), 52 were 25 to 44 years (i.e., "young"), 49 were 45 to 60 years (i.e., "middle-aged"), and 41 were 61 to 79 years (i.e., "older"). Table 14 shows participant age, BMI, mean time spent in MVPA from accelerometry, and lean body mass and body composition assessed using DXA, listed by cohort and for the entire sample with unadjusted 95% confidence intervals for comparison.

Table 14. Descriptive statisics for the 192 women sample by aggregate and overall.

		Mean ± SEM	95% Confidence Interval	Minimum	Maximum
Age (Years)	18-24	20.32 ± 0.264	[19.79, 20.85]	18	24
	25-44	33.08 ± 0.842	[31.39, 34.77]	25	44
	45-60	52.53 ± 0.677	[51.17, 53.89]	45	60
	61-79	66.54 ± 0.778	[64.96, 68.11]	61	79
	Total	41.86 ± 1.299	[39.30, 44.43]	18	79
Body Mass Index (kg/m^2)	18-24	24.54 ± 0.56	[23.42, 25.66]	18.71	39.59
	25-44	26.63 ± 0.81	[25.01, 28.26]	17.38	41.87
	45-60	25.83 ± 0.68	[24.47, 27.18]	19.05	39.28
	61-79	25.87 ± 0.67	[24.51, 27.22]	15.35	36.20
	Total	25.72 ± 0.35	[25.03, 26.40]	15.35	41.87
Moderate-to-vigorous Physical Activity (minutes/day)*	18-24	89.24 ± 3.52	[82.18, 96.31]	44.69	134.85
	25-44	88.95 ± 3.98	[80.96, 96.94]	18.78	179.42
	45-60	97.90 ± 4.34	[89.17, 106.62]	48.67	185.94
	61-79	79.50 ± 5.78	[67.83, 91.18]	34.92	176.55
	Total	89.29 ± 2.21	[84.94, 93.65]	18.78	185.94
Lean Body Mass (kg)†	18-24	43.35 ± 0.90	[41.53, 45.17]	30.73	59.50
	25-44	44.62 ± 1.03	[42.56, 46.68]	33.00	68.27
	45-60	43.00 ± 0.65	[41.68, 44.31]	35.95	52.55
	61-79	40.27 ± 0.80	[38.65, 41.89]	30.54	51.27
	Total	42.94 ± 0.45	[42.07, 43.82]	30.54	68.27
Total Body Composition (% Fat)†	18-24	31.45 ± 0.92	[29.60, 33.29]	20.40	47.10
	25-44	34.77 ± 1.06	[32.65, 36.89]	18.00	50.60
	45-60	35.82 ±1.07	[33.67, 37.96]	20.50	51.20
	61-79	35.80 ± 1.13	[33.51, 38.08]	20.20	52.30
	Total	34.39 ± 0.53	[33.34, 35.44]	18.00	52.30

*Assessed using accelerometry.
†Determined using dual x-ray absorptiometry.

Mean intakes of total energy, proteins, carbohydrates, and fats and relative intakes of energy, proteins, carbohydrates, and fats with unadjusted 95% confidence intervals for comparison, and the results of the paired t-test analyses of dietary intake data comparing the three-day food diary and the FFQ for both cohort and overall are shown in Table 15.

Table 15. Mean intakes of energy and macronutreints from three-day food diaries and food frequency questionnaires with paired t-tests to determine convergent validity of energy and macronutrient intakes.

		Three-Day Food Diary		Food Frequency Questionnaire		Mean Paired Difference*	
		Mean ± SEM	95% Confidence Interval	Mean ± SEM	95% Confidence Interval	Mean ± SEM	P
Total Energy (kcal/day)	18-24	2090.7 ± 87.6	[1914.6, 2266.7]	2266.8 ± 118.5	[2028.7, 2504.8]	-176.1 ± 124.4	= 0.163
	25-44	2071.4 ± 73.3	[1924.3, 2218.5]	2099.3 ± 123.2	[1851.9, 2346.7]	-27.91 ± 111.50	= 0.803
	45-60	2006.6 ± 70.7	[1864.6, 2148.7]	1726.6 ± 98.4	[1528.6, 1924.5]	**280.08 ± 107.06**	**= 0.012**
	61-79	1891.8 ± 84.4	[1721.3, 2062.4]	1892.4 ± 153.3	[1582.6, 2202.2]	-0.54 ± 112.24	= 0.996
	Total	2021.5 ± 39.6	[1943.4, 2099.7]	2003.6 ± 62.7	[1879.9, 2127.3]	17.9 ± 58.1	= 0.758
Relative Energy (kcal/kg/day)	18-24	31.93 ± 1.55	[28.81, 35.04]	34.16 ± 1.87	[30.40, 37.91]	-2.23 ± 1.91	= 0.248
	25-44	29.24 ± 1.21	[26.81, 31.66]	29.41 ± 1.71	[25.97, 32.84]	-0.17 ± 1.57	= 0.915
	45-60	28.63 ± 1.15	[26.33, 30.94]	24.77 ± 1.52	[21.72, 27.82]	**3.86 ± 1.55**	**= 0.016**
	61-79	29.02 ± 1.58	[25.83, 32.20]	29.02 ± 2.60	[23.75, 34.28]	0.00 ± 1.74	= 0.998
	Total	29.74 ± 0.69	[28.38, 31.09]	29.38 ± 0.98	[27.45, 31.30]	0.36 ± 0.86	= 0.676
Total Fat (g/day)	18-24	82.04 ± 4.00	[74.00, 90.08]	84.41 ± 4.57	[75.23, 93.59]	-2.37 ± 5.17	= 0.649
	25-44	87.61 ± 3.84	[79.90, 95.33]	83.33 ± 5.24	[72.80, 93.85]	4.29 ± 5.54	= 0.443
	45-60	85.58 ± 3.65	[78.24, 92.91]	66.61 ± 3.78	[59.01, 74.22]	**18.96 ± 4.42**	**< 0.001**
	61-79	82.14 ± 4.88	[72.27, 92.00]	74.19 ± 7.19	[59.66, 88.73]	7.94 ± 4.53	= 0.087
	Total	84.47 ± 2.02	[80.49, 88.46]	77.39 ± 2.63	[72.21, 82.57]	**7.08 ± 2.55**	**= 0.006**
Relative Fat (g/kg/day)	18-24	1.25 ± 0.07	[1.11, 1.38]	1.26 ± 0.06	[1.13, 1.39]	-0.01 ± 0.08	= 0.852
	25-44	1.23 ± 0.06	[1.11, 1.36]	1.16 ± 0.07	[1.02, 1.31]	-0.07 ± 0.08	= 0.371
	45-60	1.23 ± 0.06	[1.11, 1.34]	0.96 ± 0.06	[0.84, 1.08]	**0.27 ± 0.06**	**< 0.001**
	61-79	1.24 ± 0.08	[1.09, 1.40]	1.12 ± 0.11	[0.89, 1.35]	0.12 ± 0.07	= 0.088
	Total	1.24 ± 0.03	[1.17, 1.30]	1.13 ± 0.04	[1.05, 1.20]	**0.11 ± 0.04**	**= 0.003**
Total Carbohydrate (g/day)	18-24	249.92 ± 12.18	[225.44, 274.41]	276.81 ± 15.11	[246.43, 307.18]	-26.88 ± 15.60	= 0.091
	25-44	236.51 ± 11.18	[214.06, 258.96]	252.46 ± 16.89	[218.57, 286.36]	-15.96 ± 13.79	= 0.253
	45-60	227.15 ± 10.86	[205.31, 248.99]	211.71 ± 15.29	[180.98, 242.45]	15.44 ± 15.42	= 0.322
	61-79	205.19 ± 10.81	[183.35, 227.03]	229.48 ± 19.82	[189.42, 269.55]	-24.30 ± 15.52	= 0.125
	Total	230.92 ± 5.75	[219.59, 242.26]	243.50 ± 8.46	[226.80, 260.19]	-12.57 ± 7.59	= 0.099
Relative Carbohydrate (g/kg/day)	18-24	3.83 ± 0.22	[3.40, 4.27]	4.20 ± 0.25	[3.69, 4.70]	-0.36 ± 0.25	= 0.144
	25-44	3.33 ± 0.16	[3.00, 3.66]	3.54 ± 0.23	[3.08, 4.00]	-0.21 ± 0.19	= 0.269
	45-60	3.22 ± 0.16	[2.89, 3.55]	3.03 ± 0.23	[2.57, 3.50]	0.18 ± 0.22	= 0.412
	61-79	3.18 ± 0.20	[2.77, 3.59]	3.56 ± 0.34	[2.88, 4.25]	-0.38 ± 0.24	= 0.122
	Total	3.40 ± 0.09	[3.21, 3.59]	3.59 ± 0.13	[3.33, 3.85]	-0.19 ± 0.11	= 0.100
Total Protein (g/day)	18-24	89.78 ± 4.58	[80.56, 98.99]	101.19 ± 6.79	[87.54, 114.85]	**-11.42 ± 5.46**	**= 0.042**
	25-44	84.64 ± 3.19	[78.24, 91.05]	86.52 ± 4.82	[76.84, 96.19]	-1.87 ± 5.09	= 0.714
	45-60	82.79 ± 2.95	[76.85, 88.72]	71.05 ± 3.78	[63.45, 78.64]	**11.74 ± 4.22**	**= 0.008**
	61-79	83.46 ± 3.84	[75.69, 91.23]	81.02 ± 7.20	[66.46, 95.58]	2.44 ± 5.76	= 0.674
	Total	85.25 ± 1.84	[81.62, 88.89]	85.22 ± 2.94	[79.42, 91.01]	0.04 ± 2.62	= 0.989
Relative Protein (g/kg/day)	18-24	1.37 ± 0.08	[1.22, 1.52]	1.52 ± 0.10	[1.32, 1.73]	-0.15 ± 0.08	= 0.067
	25-44	1.21 ± 0.06	[1.09, 1.32]	1.20 ± 0.06	[1.08, 1.33]	0.00 ± 0.07	= 0.975
	45-60	1.19 ± 0.05	[1.08, 1.30]	1.02 ± 0.06	[0.90, 1.13]	**0.17 ± 0.06**	**= 0.009**
	61-79	1.28 ± 0.07	[1.14, 1.43]	1.23 ± 0.11	[1.00, 1.46]	0.05 ± 0.08	= 0.517
	Total	1.26 ± 0.03	[1.20, 1.33]	1.25 ± 0.04	[1.16, 1.33]	0.02 ± 0.04	= 0.669

*Paired differences are equal to values from three-day food diaries minus the value from the food frequency questionaries.[286]

Overall, total and relative intakes of energy, carbohydrate, and protein were not significantly different, displaying reasonable concurrent validity. Total ($p = 0.006$) and relative ($p = 0.003$) fat intakes were different for the overall sample. Both the older (i.e., 61-79 years) and younger (i.e., 25-44 years) cohorts did not have any significant differences between data from the three-diary and the FFQ. Data from the college-aged cohort (i.e., 18-24 years) were almost as good, with only total protein intake being significantly different ($p = 0.042$), according to paired t-tests. Convergent validity of data from the three-day food diary and the FFQ was poor for middle-aged women (i.e., 45-60 years), as estimates of total energy ($p = 0.012$), fat ($p < 0.001$), and protein ($p = 0.008$) intakes, and relative energy ($p = 0.016$), fat ($p < 0.001$), and protein ($p = 0.009$) intakes were all significantly different.

Table 16 displays the results of multiple linear models where age, MVPA, and animal-based protein intake adjusted for energy and protein intakes were entered as predictors and BMI, lean body mass, appendicular lean body mass, and body composition were outcome variables.

Table 16. The results of multiple linear regression models examining the assocation of energy and protein adjusted animal-based protein intake with BMI, lean body mass, appendicular lean body mass, and body composition when controlling for age and moderate-to-vigorous physical activity.

		Model		Animal-Based Protein Intake*	
		R	P	β ± SE	P
Body Mass Index (kg/m^2)	18-24	0.228	= 0.477	0.762 ± 0.582	= 0.197
	25-44	**0.409**	**= 0.031**	0.489 ± 0.789	= 0.538
	45-60	0.289	= 0.265	0.819 ± 0.722	= 0.262
	61-79	0.172	= 0.770	-0.444 ± 0.715	= 0.538
	Total†	0.186	= 0.157	0.409 ± 0.357	= 0.253
Lean Body Mass (kg)	18-24	**0.473**	**= 0.008**	0.811 ± 0.854	= 0.348
	25-44	0.241	= 0.406	0.399 ± 1.061	= 0.709
	45-60	0.112	= 0.903	-0.376 ± 0.726	= 0.607
	61-79	0.273	= 0.406	0.149 ± 0.836	= 0.859
	Total†	0.212	= 0.070	0.347 ± 0.456	= 0.448
Appendicular Lean Body Mass (kg)	18-24	**0.456**	**= 0.013**	0.346 ± 0.476	= 0.471
	25-44	0.220	= 0.491	0.063 ± 0.587	= 0.915
	45-60	0.154	= 0.780	-0.319 ± 0.377	= 0.402
	61-79	0.282	= 0.376	0.097 ± 0.392	= 0.806
	Total†	**0.239**	**= 0.025**	0.087 ± 0.246	= 0.723
Total Body Composition (% Fat)	18-24	0.295	= 0.239	1.184 ± 0.939	= 0.214
	25-44	**0.523**	**= 0.001**	0.769 ± 0.959	= 0.427
	45-60	**0.464**	**= 0.012**	1.890 ± 1.055	= 0.080
	61-79	0.265	= 0.437	-0.286 ± 1.182	= 0.810
	Total†	**0.355**	**< 0.001**	0.783 ± 0.521	= 0.135

*Relative animal-based protein intake was regressed according to relative energy and protein intakes for each cohort; standardized residuals were saved and entered into multiple linear regression models including age, and moderate-to-vigorous physical activity as covariates.
†Self-reported menopause status was included as a covariate for the total sample.

Only a few regression models were significant, and animal-based protein intake, adjusted for energy and protein intakes, was not related to BMI, whole-body or appendicular lean body mass, or body composition.

The results of multiple linear regression models evaluating the association between animal-based protein intake and muscle strength, muscle endurance, and functional ability are shown in Table 17.

Table 17. The results of multiple linear regression models examining the assocation of energy and protein adjusted animal-based protein intake with handgirp strength, lower-body strength and endurance, and 30-second chair stand and 6-meter gait speed performance, when controlling for age, body mass index, and moderate-to-vigorous physical activity.

		Model		Animal-Based Protein Intake*	
		R	P	β ± SE	P
Handgrip Strength (kg)	18-24	0.536	= 0.004	-0.985 ± 0.751	= 0.196
	25-44	**0.489**	**= 0.011**	0.388 ± 0.678	= 0.570
	45-60	0.284	= 0.779	-0.592 ± 1.008	= 0.560
	61-79	**0.478**	**= 0.048**	0.396 ± 0.836	= 0.610
	Total†	**0.410**	**< 0.001**	-0.101 ± 0.431	= 0.814
Summed Lower-body Strength (Nm)	18-24	0.584	= 0.001	2.151 ± 9.169	= 0.816
	25-44	0.390	= 0.095	-12.780 ± 9.548	= 0.187
	45-60	0.333	= 0.258	-0.366 ± 7.769	= 0.963
	61-79	**0.664**	**< 0.001**	**14.834 ± 7.287**	**= 0.049**
	Total†	**0.552**	**< 0.001**	0.713 ± 4.463	= 0.873
Summed Lower-body Endurance (J)	18-24	0.388	= 0.111	-19.267 ± 117.203	= 0.870
	25-44	**0.508**	**= 0.006**	-61.266 ± 98.071	= 0.535
	45-60	0.235	= 0.634	-56.518 ± 73.135	= 0.444
	61-79	**0.653**	**< 0.001**	-31.097 ± 85.571	= 0.806
	Total†	**0.525**	**< 0.001**	-42.121 ± 49.081	= 0.392
Thirty Second Chair Stand Test (Repetitions)	18-24	0.151	= 0.901	0.548 ± 0.796	= 0.495
	25-44	**0.479**	**= 0.014**	-0.356 ± 0.447	= 0.430
	45-60	0.186	= 0.819	-0.517 ± 0.735	= 0.486
	61-79	0.414	= 0.138	-0.472 ± 1.704	= 0.507
	Total†	**0.259**	**= 0.024**	-0.164 ± 0.326	= 0.616
Six Meter Gait Speed (s)	18-24	0.351	= 0.196	**0.172 ± 0.077**	**= 0.030**
	25-44	**0.551**	**= 0.002**	0.123 ± 0.072	= 0.092
	45-60	0.334	= 0.255	0.003 ± 0.081	= 0.972
	61-79	**0.579**	**= 0.004**	**-0.177 ± 0.087**	**= 0.049**
	Total†	**0.370**	**< 0.001**	0.051 ± 0.040	= 0.206

*Relative animal-based protein intake was regressed according to relative energy and protein intakes for each cohort; standardized residuals were saved and entered into multiple linear regression models including age, and moderate-to-vigorous physical activty as covariates.
†Self-reported menopause status was included as a covariate for the total sample.

For the large part, animal-based protein intake was unrelated to performance. Animal-based protein was only significant to three regression models evaluating performance variables, and only two of these models were themselves significant; both of these models were in the older women cohort, as hypothesized, and likely due to decreased anabolic response to dietary protein in older[309] but not younger women.[308] Greater animal-based protein intakes relative to one's energy and total protein intake was related to greater lower-body strength and faster gait speed in women 61 to 79 years of age, when controlling for age, BMI, and MVPA.

6.5. Discussion

For the sample as a whole and generally across cohorts, we found animal-based protein intake adjusted for energy and protein intakes was unassociated with lean body mass, body composition, and performance. We did find that increased intake of animal-based proteins, which generally have better protein quality than plant-based proteins,[52,204] is associated with greater lower body strength and faster gait speed in women 61 to 79 years of age. Although it has been shown that animal-based proteins are more anabolic than plant-based proteins,[53,54] we did not find an association between animal-based protein intake and lean body mass for any cohort. Nonetheless, our findings in older women support the hypothesis that dietary protein quality becomes more important during aging in women, as older women[309] do not respond as strongly to dietary protein compared to younger women.[308]

Although unrelated to our hypothesis regarding dietary protein quality, we report that convergent validity of energy and macronutrient data from three-day food diaries and from a FFQ[286] was poor for women 45 to 60 years of age, but was good for other age groups. In support of this, others using a sample of 80 middle-aged women, 40 to 65 years of age, found social desirability led to underestimation of energy intake from a FFQ.[149] Food diaries, unlike recalls and FFQs, demand that participants record their intake in real-time as they eat, and therefore, do not rely on long-term memory, an advantage of the method.[152]

In our view, data from food diaries are more accurate than data from FFQs due to recall bias. In fact, food diaries, collected for a period of four days, explained a larger proportion of energy intake and protein intake measured using two biomarkers, doubly-labeled water and urinary nitrogen, than a 24-hour recall in a sample of 450 older women, 50 to 79 years of age.[157] Another group of researchers found that nutrient intakes from 3-day food diaries were more

closely related to nutrient intakes from three other 3-day food diaries assessed at different times in the year than intakes from a population specific FFQ.[167,168] Some works have even used food diaries to validate other self-report methods.[167–170] In addition, food diaries can be used across contexts, so long as their instructions are clearly translated and participants can write. Nonetheless, food diaries are reactionary and thus are still biased;[152,158] participants are recording foods as they consume them, and therefore are more aware of their food choices, which affects which foods participants eat and how much they eat. Beyond this limitation, all self-report measures rely on nutrient databases that may have their own errors or omissions.[152] In sum, self-reported assessments are biased to some extent,[148–151,158] but food diaries seem to be the best as they limit errors due to recall.[157,158,168] For these reasons, we determined animal-based protein intake from three-day food diaries.

Despite our unique approaches of determining animal-based protein intake from three-day food diaries and regressing animal-protein intake relative to participants' energy and protein intakes, this study had some limitations. The data is only cross-sectional and cannot be used to determine causality. The sample consisted of healthy women who were recruited using convenience recruiting methods, such as word-of-mouth, e-mails, and flyers, and therefore, the sample is not representative. Also, aside from the cut-point for older women, other cut-points between cohorts were arbitrarily chosen in an effort to create equal group sizes. We did, however, control for both energy and protein intakes when evaluating animal-based protein intake, and we included an objective measure of physical activity as a covariate in analyses. Moreover, although we did not objectively measure dietary intake, we determined convergent validity of our subjective measures of dietary intake by using two different subjective methods to access dietary intake.[153,303,304]

In conclusion, we report that animal-based protein intake was largely unrelated to muscle mass, strength and endurance, body composition, and functional ability in a cross-sectional sample of women. We found that animal-based protein intake, adjusted for energy and protein intakes, was related to greater lower-body strength and faster gait speed in women 61 to 79 years of age, supporting the notion that dietary protein quality is more important to older women as their anabolic response to meal is decreased.[309]

7. CONCLUSION

7.1. Echogenicity and Specific Force

There is no agreed upon definition of muscle quality, but the measurement of muscle quality by researchers investigating sarcopenia is advocated for by experts.[37] Echogenicity[21] and specific force[47] are both considered measures of muscle quality, yet they have been infrequently compared to one another.[26,48,49] Echogenicity and specific force were unrelated in our sample, indicating that one measure is a not specified to determine muscle quality. As echogenicity was related to lower-body strength in our sample, specific force is likely not a true measure of muscle quality. Experts in health, aging and muscle, should come to consensus about how to determine muscle quality.

7.2. Protein Distribution and Muscle Strength, Quantity, and Quality

The link between the evenness of protein intake distribution and muscle mass is well established, but the relationship between the evenness of protein intake distribution and strength and physical performance is more tenuous.[50] Achieving of at least 25 grams of protein at each meal was related to greater amounts of lean body mass, handgrip strength, and lower-body strength and approached significance for lower-body endurance when controlling for age, MVPA, BMI, relative energy intake, and percent energy from protein. Relative intakes of at least 0.24 for those younger than 60 and 0.4 g per kg body weight per day for those 60 years and older were related to increased lean body mass, handgrip strength and lower-body endurance, controlling for the same covariates. Spreading protein intake throughout the day maximizes the anabolic response to dietary protein benefiting muscle mass and strength.

7.3. Protein Quality and Muscle Strength, Quantity, and Quality

Proteins from animal sources (i.e., animal-based proteins) have better protein quality[51,52] and are thought to stimulate muscle protein synthesis to a greater extent than lower quality plant-based proteins.[53,54] In a mixed-sex sample of middle-aged men and women, those with greater intakes of animal-based protein had greater lower-body strength and endurance and handgrip strength when controlling for sex, age, BMI, MVPA, relative energy intake and percent energy from the macronutrients. Increased intake of animal-based protein relative to participants' energy and protein intakes was related to greater lower-body strength and faster gait speed in older, but not younger women. Results indicate that dietary protein quality is more important during aging to mitigate losses in muscle mass and strength.

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APPENDIX A. IRB APPROVAL "BEEF PROTEIN INTAKE, PHYSICAL ACTIVITY, AND MUSCLE QUALITY IN MIDDLE-AGED WOMEN"

NDSU NORTH DAKOTA STATE UNIVERSITY

August 26, 2016

Dr. Sherri Stastny
Department of Health, Nutrition & Exercise Sciences

IRB Approval of Protocol #HE16219, "Beef protein intake, physical activity, and muscle quality in middle-aged women"
Co-investigator(s) and research team: Kyle Hackney, Shannon David, Wonwoo Byun, Chris Kotarsky, Rachel Iverson Dewey, Allison Barry, Kara Stone

Approval period: 8/26/2016 to 8/25/2017
Continuing Review Report Due: 7/1/2017

Research site(s): NDSU
Funding Agency: National Cattlemen's Beef Association (FAR0026153)
Review Type: Expedited category # 4, 7
IRB approval is based on the revised protocol submission (received 8/23/2016).

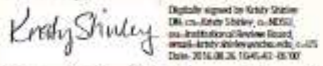
Additional approval from the IRB is required:
o Prior to implementation of any changes to the protocol (Protocol Amendment Request Form).
o For continuation of the project beyond the approval period (Continuing Review/Completion Report Form). A reminder is typically sent approximately 4 weeks prior to the expiration date; timely submission of the report the responsibility of the PI. To avoid a lapse in approval, suspension of recruitment, and/or data collection, a report must be received, and the protocol reviewed and approved prior to the expiration date.

A report is required for:
o Any research-related injuries, adverse events, or other unanticipated problems involving risks to participants or others within 72 hours of known occurrence (Report of Unanticipated Problem or Serious Adverse Event Form).
o Any significant new findings that may affect risks to participants.
o Closure of the project (Continuing Review/Completion Report Form).

Research records are subject to random or directed audits at any time to verify compliance with human subjects protection regulations and NDSU policies.

Thank you for cooperating with NDSU IRB procedures, and best wishes for a successful study.

Sincerely,



Kristy Shirley, CIP, Research Compliance Administrator

For more information regarding IRB Office submissions and guidelines, please consult www.ndsu.edu/irb. This Institution has an approved FederalWide Assurance with the Department of Health and Human Services: FWA00002439.

INSTITUTIONAL REVIEW BOARD
NDSU Dept 4000 | PO Box 6050 | Fargo ND 58108-6050 | 701.231.8995 | Fax 701.231.8098 | ndsu.edu/irb
Shipping address: Research 1, 1735 NDSU Research Park Drive, Fargo ND 58102

APPENDIX B. IRB APPROVAL "BEEF PROTEIN INTAKE, PHYSICAL ACTIVITY, AND MUSCLE QUALITY IN MIDDLE-AGED MEN"

Date Received
5/27/2017

NDSU NORTH DAKOTA STATE UNIVERSITY

INSTITUTIONAL REVIEW BOARD
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Continuing Review or Completion Report Form

Use this form to: 1) request a continuation of IRB approval if a project is currently active (recruiting subjects, collecting data, or analysis of identifiable data), or 2) report completion of a project.

Protocol Information

Protocol #: **HE16219** Original approval date*: **8/26/2016**

Title: **Beef protein intake, physical activity, and muscle quality in middle-aged women**

Principal investigator: **Sherri Stastny** 7/31/2015	Co-investigator: Kyle Hackney, Wonwoo Byun 11/30/2016 9/29/2016
Department: HNES	Department: HNES, HNES
E-Mail/Campus Address: sherri.stastny@Ndsu.edu	E-Mail/Campus Address: kyle.hackney@ndsu.edu; w.byun@ndsu.edu

* *Complete and submit an updated protocol form & relevant attachments every 5 years following approval. Protocol records must be updated every 5 years by completing a new protocol form and any relevant attachments, and including it with this report. Use the most recent version of the forms on the IRB website at: http://www.ndsu.nodak.edu/research/institutional_review_board/forms.html.*

Project Status

☒ Ongoing and currently active, Expected end date of research: **December, 2018**

☐ Complete, abandoned or inactive

Source of current funding: FAR# 0026153 ends Dec, 2017; FAR0026929 starts June 2017 and May 31, 2018 ☐ Not funded

Current Funding period: Start date: June 1, 2017 End Date: May 31, 2018

Has a progress report been filed with the funding agency since last review?
☐ **No** ☒ **Yes,** ▮▮ **Attach copy of final grant application(s), and/or recent report to funding agency.**

Research team: *List all individuals involved in the research (project design/oversight, recruiting participants, obtaining informed consent, intervening or interacting with participants to obtain information/data, and/or handling identifiable information for research purposes). May provide as a separate attachment.*

Name, dept. or affiliati	Specify role in research	Email Address	Training date: (IRB Use only)
Chris Kotarsky, HN graduate student	subject testing	christopher.kotarsky@ndsu.ed.edu	10/30/2014
Kara Stone, HNES greaduate student	subject testing	kara.stone@ndsu.edu	8/17/2015

Project Summary

1. Brief summary of results to date:

We have finished testing 50 women with funding from the National Cattlemen's Beef Commission; we have new funding to test 50 men from MN Beef Commission for the coming year and seek permission to continue with our study. Dr David will continue as PI for collected data only (will not continue with testing of new 50 subjects). Also, Rachel Iverson will graduate summer 2017 and Allison Barry is working on a completely different project and no longer is involved in this research.

2. List research site(s):

BBFH Room 14

3. List presentations or publications that have resulted from this research since the last review:

Stastny, S. (2017). How much leucine is in your food? [Abstract]. Journal of Frailty and Aging. P126.

Stastny, S., Kotarsky, C.J., Hackney, K.J., Iverson Dewey, R., (2017). Influence of beef protein intake and hand grip strength on muscle strength and cross sectional area in middle aged-women [Abstract]. Journal of Frailty and Aging. OC55.

Participants:

1. How many participants have completed the study since last review: 50.

2. How many participants have completed the study since first review: 50.

3. Will more participants be recruited?
   - ☐ No
   - ☒ Yes* – Indicate approximately how many: 50

Attach a copy of current consent form(s), and any recruitment materials.

4. Informed Consent: A copy of the approved informed consent form has been signed by each of the participants in the study, and retained for your records. Has this requirement been met?
   - ☒ Yes
   - ☐ N/A, waiver approved
   - ☐ No – explain:

5. Have any potential participants declined to participate, or withdrawn from the research?
   ☒ No
   ☐ Yes - explain:

6. Summarize any complaints about the research (and their resolution) since the last review?
   none

Risk/Benefit Ratio:

1. Summarize any unanticipated problems (even if previously reported) or adverse events that have occurred since the last review:
   none

Unanticipated problem: an unanticipated problem that involves risks to subjects or others is any incident, experience, or outcome that meets all the following criteria:

- *is unexpected (in terms of nature, severity, or frequency) given the characteristics of the subject population and the research as described in the IRB approved protocol and consent document(s)*
- *is related, or possibly related to participation in the research*
- *suggests the research places subjects or others at greater risk of harm (physical, psychological, economic, or social harm) that previously known or recognized*
- *may not have resulted in actual harm to subjects, but may only represent increased risk of harm (ie., physical, psychological, social, economic, legal).*

Adverse event: any untoward or unfavorable medical occurrence (physical or psychological) in a human subject, including any abnormal sign, symptom, or disease, temporally associated with the subject's participation in the research, whether or not considered related to their research participation. Such events may have already been expected to occur with a certain frequency and severity, and previously identified as potential risks in the protocol form, and consent document(s).

2. Has any new information resulted from the study or any literature, that would affect the risk/benefit ratio for new subjects (or for those currently or previously enrolled)?
   ☒ No
   ☐ Yes –explain, and indicate how this has been/will be addressed with future, current, or previously enrolled participants:

Investigator's Assurance

The signature below certifies that:

- information provided in this report is complete and accurate

- each individual involved as a member of the research team possesses the necessary experience for conducting research activities in their assigned role, and is aware of and will abide by NDSU policies and procedures for the protection of research participants
- the research will be conducted according to the approved protocol
- changes will receive IRB approval prior to implementation, unless necessary to prevent immediate serious harm to participants
- all unanticipated problems involving risks to participants or others will be promptly reported to the IRB.

Sherri Stastny (email) 5/27/2017

Principal Investigator signature, date

In lieu of a written signature, submission of this report via the Principal Investigator's NDSU email constitutes an acceptable electronic signature.

----------FOR IRB USE ONLY--------

Project is: ☒ Approved for continuation ☐ Complete/Inactive ☐ Archive after _____

IRB Signature: Kristy Shirley Date: 6/7/2017

Reviewed by: ☐ Full Board - meeting date _____ ☒ Expedited review, category # 4,7

Current approval period expires: 6/6/2018

Next Continuing Review/Completion Report due*: 5/1/2018

Note that the IRB office will typically remind the investigator a few weeks prior to the due date; however, timely submission of the report is the PI's responsibility.

APPENDIX C. DXA SCREENER

Developed by Diane Thériault for the Canadian Panel, International Society for Clinical Densitometry, April, 2004

Patient Questionnaire

Name (print):_____ Date: _____

Is there a chance that you are pregnant? Yes No
Have you had a barium X-ray in the last 2 weeks? Yes No
Have you had a nuclear medicine scan or injection of an X-ray dye in the last week? Yes No
Have you had hyperparathyroidism or a high calcium level in your blood? Yes No

If you answered yes to any of the above, speak to our receptionist right away.

1. Your: Age: ____ Sex: Male Female

2. Your ethnicity (check one):
__Caucasian (White) __Black __Aboriginal __Asian __Hispanic __Other
Your country of birth: _____

3. Have you ever had a bone density test? Yes No
If YES, when and where? _____

4. Have you had a recent weight change? Yes No
If YES, tell us about it: _____

5. Your tallest height (late teens or young adult): _____

6. Have you ever broken a bone? Yes No

Bone broken	Simple fall?	If not a simple fall, please describe the circumstances	Age when this occurred

7. Has a parent or sibling had a broken hip from a simple fall or bump? Yes No

8. Has a parent or sibling had any other type of broken bone from a simple fall or bump? Yes No

9. How many times have you fallen in the last year? _____

10. Have you ever had surgery of the spine, hips, legs or arms? Yes No
If YES, describe what type of surgery you had and which side was affected
_____
_____

11. Are you currently receiving or have you previously received prednisone pills (cortisone)?
Yes, currently ____ Yes, previously _____ No ____
If YES, for how long? _____ What is your dose? _____mg or _____ pills each day

12. List any chronic medical conditions that you have:
_____ _____
_____ _____
_____ _____

/Users/ginaschimek/Desktop/Research articles/Appendix A DEXA screening (1).doc

13. Are you currently receiving or have you previously received any of the following medications?

	No	Yes	For how long?
Medication for seizures or epilepsy			
Chemotherapy for cancer			
Medication for prostate cancer			
Medication to prevent organ transplant rejection			

14. Have you been treated with any of the following medications?

Medication	Ever?	Currently?	If current, how long?
Hormone replacement therapy (Estrogen)			
Tamoxifen			
Raloxifene (Evista)			
Testosterone			
Etidronate (Didronel/Didrocal)			
Alendronate (Fosamax)			
Risedronate (Actonel)			
Intravenous pamidronate (Aredia)			
Clodronate (Bonefos, Ostac)			
Calcitonin (Miacalcin nasal spray)			
PTH (Forteo)			
Zoledronic acid (Zometa)			
Sodium fluoride (Fluotic)			

15. How many servings of the following do you eat/drink per day (on average)?

	Milk (full cup)	Orange juice fortified with calcium (full cup)	Yogurt (small container or ½ cup)	Cheese
Number of servings				

16. Do you take any calcium supplements (including TUMS)? Yes No

17. Do you take any vitamin D supplements (including multivitamins and halibut liver oil)? Yes No

18. Do you smoke? Yes No

For women only…

19. Are you still having menstrual periods? Yes No

20. Before menopause, have you ever missed your periods for 6 months or more, besides during pregnancy? Yes No

21. Have you had your menopause? Yes No
If yes, at what age? _____

22. Have you had a hysterectomy? Yes No
If YES, at what age? _____
Have you had both of your ovaries removed? Yes No
If YES, at what age? _____

APPENDIX D. IRB APPROVAL "THE INFLUENCE OF ANIMAL-BASED PROTEIN AND BEEF CONSUMPTION ON ABILITY TO PERFORM FUNCTIONAL ACTIVITIES, MUSCLE QUALITY AND BONE MINERAL DENSITY AMONG ADOLESCENT TO OLDER FEMALES"

NDSU NORTH DAKOTA STATE UNIVERSITY

September 14, 2017

Sherri N. Stastny
Department of Health, Nutrition & Exercise Science

IRB Approval of Protocol #HE18010, "The influence of animal-based protein and beef consumption on ability to perform functional activities, muscle quality and bone mineral density among adolescent to older females"
Co-investigator(s) and research team: Kyle J. Hackney, Christopher Kotarsky, Kara Stone, Nathan Dicks, Regina Schimek, Bailee Sawyer, Madison Millner, Nathaniel Johnson

Approval period: 9/14/2017 to 9/13/2018 Continuing Review Report Due: 8/1/2018

Research site(s): NDSU Funding agency: ND Beef Commission (FAR0027460)
Review Type: Full Board, meeting date – 9/8/2017
Risk Level: A minor increase over minimal risk
IRB approval is based on the revised protocol submission (received 9/13/2017).

Additional approval is required:
o prior to implementation of any proposed changes to the protocol (Protocol Amendment Request Form).
o for continuation of the project beyond the approval period (Continuing Review/Completion Report Form). A reminder is typically sent two months prior to the expiration date; timely submission of the report is your responsibility. To avoid a lapse in approval, suspension of recruitment, and/or data collection, a report must be received, and the protocol reviewed and approved prior to the expiration date.

A report is required for:
o any research-related injuries, adverse events, or other unanticipated problems involving risks to participants or others within 72 hours of known occurrence (Report of Unanticipated Problem or Serious Adverse Event Form).
o any significant new findings that may affect risks to participants.
o closure of the project (Continuing Review/Completion Report Form).

Research records are subject to random or directed audits at any time to verify compliance with IRB regulations and NDSU policies.

Thank you for cooperating with NDSU IRB procedures, and best wishes for a successful study.

Sincerely,
Kristy Shirley
Kristy Shirley, CIP
Research Compliance Administrator

For more information regarding IRB Office submissions and guidelines, please consult www.ndsu.edu/irb. This Institution has an approved FederalWide Assurance with the Department of Health and Human Services: FWA00002439.

INSTITUTIONAL REVIEW BOARD
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