THESIS

MILK CONSUMPTION AFTER MODERATE INTENSITY AEROBIC EXERCISE IMPROVES NITROGEN BALANCE IN OLDER ADULTS

Submitted by

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WE HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER OUR SUPERVISION BY LEORA JORDAN ENTITLED MILK CONSUMPTION AFTER MODERATE INTENSITY AEROBIC EXERCISE IMPROVES NITROGEN BALANCE IN OLDER ADULTS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE.

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ABSTRACT OF THESIS

MILK CONSUMPTION AFTER MODERATE INTENSITY AEROBIC EXERCISE IMPROVES NITROGEN BALANCE IN OLDER ADULTS

Sarcopenia is the progressive decline in muscle mass that happens with age and can lead to falls, fractures, and inability to maintain activities of daily living. Simplistically, sarcopenia results from a disequilibrium in the rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB), with a net negative protein and nitrogen balance over time. This study was designed to determine whether subjects in energy balance can increase nitrogen balance by timing milk intake immediately after exercise. In a randomized cross-over design, ten older individuals (64.5±2.0 y) completed two 3-day trials that were isocaloric and isonitrogenous. During the two trials, subjects consumed either chocolate milk (PRO+CHO) or a Kool-Aid type beverage (CHO) following a daily bout of cycling exercise (60 minutes at 55% of VO_{2max}). Study diet was repeated across trials and controlled for protein/nitrogen content (1.2 g/kg and 14% of total kcal) and energy balance (within 200 kcal). For all trial days, 24-hour urine collections were completed and analyzed for nitrogen content. Mean energy balance (CHO+PRO: 202±36 kcal; CHO: 191±44 kcal; p=0.68) and mean physical activity level (PAL) (CHO+PRO: 1.61±0.04; CHO: 1.60±0.04; p=0.60) did not differ between trials, but mean nitrogen balance was significantly more positive in the PRO+CHO (1.2 g N±0.32) trial than the CHO trial (0.8 g N ±0.45) ($p<0.05$).

Thus, under conditions of energy balance, nitrogen/protein balance becomes more positive when chocolate milk is consumed immediately after moderate intensity aerobic exercise compared to a CHO-only beverage.

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CHAPTER 1

INTRODUCTION

Sarcopenia is the age-related decline in muscle mass and function, which can lead to falls, fractures, and inability to maintain activities of daily living. Although all men and women experience sarcopenia to some degree, a clinical definition is a loss of appendicular muscle mass exceeding two standard deviations below the average of healthy young adults (1). The prevalence of clinically significant sarcopenia is 13-24% in 65-70 year olds and increases to more than 50% in individuals over 80 years old (1). Age-related muscle loss is a gradual process, yet the total cross sectional area (CSA) of muscle can decrease by 40% between the ages of 20 and 80 (2). Type II muscle fibers generally atrophy more than Type I fibers, with decrements of 25-50% and 1-25%, respectively (3). Lost muscle mass, particularly Type II fibers, causes the strength decrements experienced with age (4). Muscle weakness leads to accidents and disability, as sarcopenic individuals are more prone to falling and have a greater likelihood of using a cane or walker (1). The mechanisms underlying sarcopenia are complex, but if muscle protein is lost over time, the overall rate of muscle protein breakdown (MPB) must exceed that of muscle protein synthesis (MPS).

As the size of the elderly population continues to grow, interventions for the treatment and prevention of sarcopenia are of increasing importance. Current proposals for maintaining muscle protein mass with age include increased protein intake, exercise training, and combinations thereof. While certain pharmacological treatments have also been proposed,

they will not be discussed here. Importantly, protein balance cannot be considered in the absence of energy balance. Even when protein intake is adequate, caloric restriction results in negative nitrogen balance and declining muscle mass (5). Thus, energy balance must be considered during any intervention aiming to preserve protein balance and muscle mass.

It is equivocal whether increasing protein intake beyond the current adult recommendation of 0.8 g PRO·kg body weight^{-1}·day^{-1} is necessary for the elderly to maintain nitrogen balance and thus, body protein mass. Loss of midthigh muscle area when the protein RDA is consumed suggests that the recommended amount might be insufficient for older populations to maintain muscle mass (6). Conversely, a recent long term study confirms that the current recommendation is adequate, as nitrogen balance is maintained when 0.8 g PRO·kg^{-1} is consumed (7). Some researchers stipulate that rather than increasing total daily protein intake, attention should be paid to distributing protein equally among meals (8). Paddon-Jones and Rasmussen speculate that including 25-30 grams of quality protein at each meal to maximally stimulate protein synthesis will enhance protein building at numerous time points during the day, with the potential to cause a chronically positive protein balance (8). Although increasing the absolute protein requirements for elderly individuals is questionable, nutritional strategies to maintain muscle mass represent a viable option for elderly individuals who are unable to perform exercise.

Seemingly, the most effective intervention for stimulating muscle hypertrophy and improving strength in older individuals is long-term resistance training (9). However, weightlifting programs generally require special equipment and knowledge of proper technique to avoid injury. Since walking is the most popular form of exercise in elderly populations (10), aerobic exercise provides a preferred and easy to access alternative to weight training. In terms of physiological benefit, both aerobic and resistance type activities stimulate mixed muscle

protein synthesis (11). Aerobic exercise also increases insulin sensitivity (12), which may improve the MPS response of older individuals to nutrition.

Finally, combined nutrition and exercise strategies have been proposed to prevent or treat sarcopenia, possibly being more effective than either intervention alone. As long as protein quantity is sufficient, increasing the total amount of daily protein does not augment the strength or fat free mass gains observed with resistance training (13). There is conflicting evidence as to whether timing a protein supplement intake after resistance training results in long-term muscle adaptations that are superior to those resulting from only the exercise intervention (14, 15). However, exercise certainly stimulates an anabolic period, and the timing and type of nutrition provided post-exercise can optimize the acute metabolic response of muscle protein synthesis (16). For example, timing protein intake immediately after exercise instead of three hours after increases muscle protein synthesis and whole body protein synthesis by threefold and 12%, respectively (17). Essential amino acids (EAAs), particularly leucine, are potent stimulators of MPS following exercise (18). Thus, when energy balance is maintained, timing the ingestion of a leucine-rich protein source immediately after a daily bout of moderate intensity exercise is a possible means to increase whole body protein balance to a positive value. Since sarcopenia is a gradual process, a strategy that slightly elevates body protein balance over time may be sufficient to maintain muscle protein mass.

Statement of the Problem:

This study aims to investigate the effect of milk intake immediately after moderate intensity aerobic exercise on nitrogen balance while maintaining subject energy balance.

Hypothesis:

In subjects in energy balance, timing the ingestion of a protein drink (milk) immediately after exercise will increase three-day nitrogen balance as compared to a carbohydrate drink (Kool-Aid).

Delimitations, Limitations, and Assumptions:

This study was delimited to 10 male and female subjects between the ages of 55-75 years old. All subjects were recruited from the Fort Collins and Loveland area and were Caucasian, except one Hispanic participant. It was necessary for all participants to be lactose tolerant since milk was consumed as the study intervention.

There are inherent limitations of the nitrogen balance technique, including a likely overestimation of nitrogen balance (19). However, the same magnitude of error would be present during both conditions. Thus, it is reasonable to compare the two conditions since nitrogen balance would be overestimated equally. The limitations of the nitrogen balance method will be discussed further in the Literature Review.

It was assumed that subjects followed all study instructions, including a 12-hour fast prior to blood and RMR screenings and consumption of only meals provided by study staff during the lead-in and inpatient periods.

CHAPTER 2

LITERATURE REVIEW

Many studies have explored the cause and prevention of sarcopenia. Although the exact etiology of this inevitable condition is unknown, there is an overall imbalance in protein metabolism that acts through or in concert with changes in muscle characteristics, motor neurons, endocrine function, mitochondrial function, nutrition, and physical activity. When considering the underlying mechanisms of this disease, an understanding of protein metabolism is helpful. Therefore, this review aims to first introduce some fundamental concepts of protein metabolism. Then, the underlying mechanisms of declining muscle mass will be addressed as they relate to protein turnover. Next, the nitrogen balance method will be introduced as a means of measuring protein balance in the body. This section will detail the urea cycle, factors affecting nitrogen balance, and the limitations of the method. The next section will cover nutrition after exercise, explaining the mechanistic pathways involved, the timing and type of nutrition provided, and comparing the response of older and younger individuals to nutrition. Finally, strategies for the prevention of sarcopenia will be discussed, with particular attention to current nutritional recommendations for older individuals.

PROTEIN METABOLISM

Amino acids (AAs) are the building blocks of protein, and are thus vital to the structure and function of all body tissues. Structurally, AAs contain a central carbon atom, one or more amino groups (--NH_2), one or more carboxyl groups (--COOH), and a unique side chain (R group). AAs can be classified as essential (must be derived from the diet) or nonessential (can be formed endogenously). The digestion of proteins begins in the stomach, where pepsin hydrolyzes the peptide bonds between AAs. Protein digestion is subsequently completed by numerous enzymes in the small intestine, after which the free AAs are absorbed across the brush border into intestinal enterocytes. Any AAs that are not used by the intestinal cells are transported into interstitial fluid and eventually reach splanchnic circulation via villi capillaries (20).

Free AAs exist in the human body as a pool that can be drawn from mainly for the making of proteins, although AAs are also used to form some nonprotein compounds. When protein synthesis occurs, AAs are linked together by peptide bonds to form the primary structure of proteins. During protein breakdown, alternatively, multiple proteases act to degrade proteins into constituent AAs either through lysosomal or non-lysosomal pathways (21). Protein synthesis increases during the fed state with the availability of AAs and conversely, protein degradation increases during fasting. The existence of a free AA pool allows demands for AAs be met during times of fasting or a nutritional deficit. Skeletal muscle plays an important role in maintaining the free AA pool because unlike the liver or heart, its proteins can be broken down extensively without threatening survival. However, if a nutritional deficit is prolonged, the AA pool will be depleted which is followed by adverse health consequences like lost muscle mass and suppressed immunity (22). Thus, a constant input of energy and protein is necessary to maintain the body's AA and protein pools.

When body proteins are degraded, most of the derived AAs are reused for protein synthesis. There are several fates for the AAs that exceed the quantity needed for making proteins or maintaining the free AA pool, the size of which remains relatively constant. First, the AAs must either undergo deamination (removal of the amino group to form an α-keto acid) or transamination (transfer of the amino group to an already existing AA carbon skeleton or α-keto acid) (20). Upon transamination, nonessential AAs are formed from essential AAs or other nonessential AAs. Meanwhile, when AAs undergo oxidative deamination, the ATP generated can help meet energy demands during a caloric deficit. The carbon skeleton/α-keto acids can also be used in gluconeogenesis (glucose formed from a non-carbohydrate source) or to form cholesterol or fatty acids (20). The nitrogen-containing ammonia produced from oxidative deamination is excreted through the urine in urea, a process that will be discussed more thoroughly in the "Nitrogen Balance" section of this review. To conclude, AAs are used mainly to synthesize proteins and are reutilized in a cyclical fashion following protein breakdown. The amino groups of any leftover AAs are removed and form either urea or another AA; the resultant α-keto acids can be used for energy or glucose production.

Whole Body Protein Turnover

Whole body protein turnover (WBPT) is a process that is vital for maintaining the quality of proteins used for tissue structure, function, and regulatory proteins like hormones and enzymes. WBPT is comprised of the degradation of proteins that may be damaged or needed to replenish the AA pool, and the re-synthesis of body proteins. While individual proteins have different turnover rates, WBPT represents the turnover of all of the body's proteins. In a 70 kg man with 12 kg of protein, about 300 g of protein is degraded and replaced each day (23), which accounts for about 20% of resting metabolic rate (24). WBPT rates are different at various times

during the day depending on nutritional or exercise status. During an energetic stress (like fasting or exercise), the degradation of the body's proteins replenishes the free AA pools in the muscle, liver, blood, interstitial fluid, and intracellular water. Therefore, the protein breakdown rate exceeds the protein synthesis rate. Conversely, during the fed state when total energy and protein intake are adequate, proteolysis is suppressed and whole body protein synthesis is stimulated. WBPT becomes more positive or negative throughout the day in response to various stimuli with an overall fractional rate of about 2.5%/day, assuming that 300 g of 12 kg of total body protein is degraded and replaced (23). The turnover of protein is an energetically expensive, yet vital, process for maintaining the integrity of the body's protein mass.

Protein Synthesis

The process of protein synthesis has been well characterized in the literature and begins with the transcription of the genes contained in the DNA that encode a specific messenger RNA (mRNA) sequence. Following transcription in the nucleus, the mature mRNA , which encodes a single protein, is transported to the cytoplasm for translation. Next, during translation initiation, several eukaryotic initiation factors (eIFs) regulate the attachment of the mRNA to the initiation complex on a ribosome, which scans the mRNA for the initiator codon AUG (methionine) and forms an attachment.

During elongation, AAs are systematically added to the initiated peptide chain. During this step, synthetases form aminoacyl-transfer RNAs (tRNAs) and these molecules deliver the AAs that correspond to each codon in the mRNA sequence. The amino group on each new AA is attached by a peptide bond to the carboxyl group on the last AA in the growing peptide chain. Several eukaryotic elongation factors (eEFs) facilitate this process, which is eventually stopped when the ribosome detects a termination codon and a eukaryotic releasing factor (eRF) cleaves

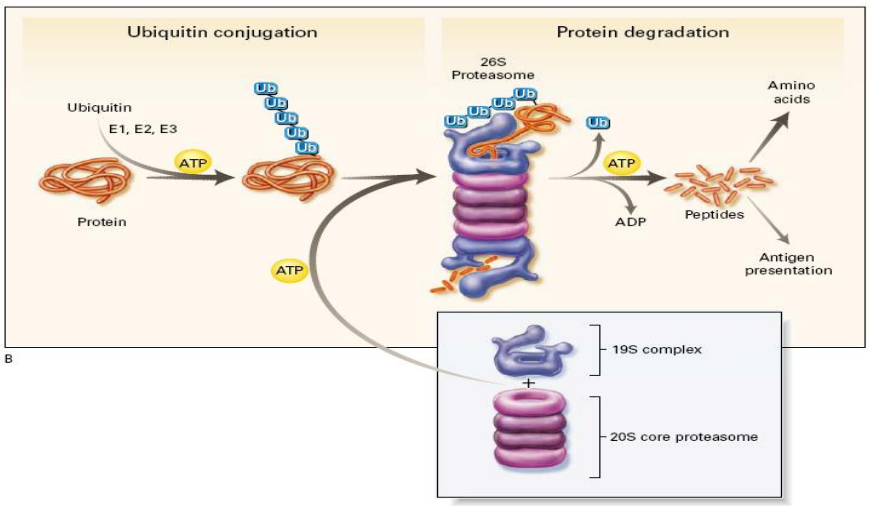
the completed peptide. Lastly, post-translational modifications occur to affect the higher-level structure of the peptide. These modifications include breakage of some peptide bonds, alterations in side groups and terminal amino and carboxyl groups, and assembling the correct 3-dimensional structure to render a fully functional protein. The synthesis of proteins from AAs occurs solely through the described pathway of DNA transcription, mRNA translation, elongation of the peptide chain, and post-translational modifications.

Protein Breakdown

Protein breakdown, or proteolysis, is less understood than protein synthesis, but can be divided into lysosomal and non-lysosomal pathways. Lysosomal mechanisms are classified as heterophagic or autophagic; the heterophagic process degrades proteins from either the extracellular matrix or from other cells, while autophagic processes involve the breakdown of intracellular proteins. Lysosomal protein degradation becomes more prominent during nutrient deprivation and contributes minimally to the turnover of most cellular proteins (21).

The non-lysosomal ubiquitin-proteasomal system (Figure 2.1) appears to be the most

Figure 2.1. The Ubiquitin Proteasomal Pathway for Proteoloysis. From Mitch and Goldberg (21).



common route for proteins to undergo proteolysis (21). In this ATP-dependent pathway, the small protein cofactor ubiquitin attaches to lysine residues on proteins that are meant to be broken down. This ubiquitination is regulated by the enzymes E1, E2, and E3 and is repeated to form a chain of ubiquitin molecules. Once proteins have been ubiquitinated, they are degraded within the 26S proteasomes by protein subunits comprising the machinery for proteolysis. These particles are cylinder-shaped with a hollow core and can be found in the cytoplasm and nucleus. The 19S subunit serves as a binding site for the ubiquitin chains and also dismantles the protein's structure, delivers the modified protein to the proteolytic 20S proteasome, and stimulates peptidase activity within the 20S. The protein is further broken down in the 20S and the resulting peptides are released into the cytosol. The final step involves the hydrolyzation of the peptides into AAs by exopeptidases (25). While there are multiple regulators and initiators of proteolysis, the ubiquitin proteasome system is the body's main pathway for protein breakdown.

Net Protein Balance

Whole body protein turnover undulates throughout the day in response to feeding and exercise, characterized by periods of increased synthesis followed by increased breakdown. The rate-limiting step of protein breakdown appears to be the initiation of proteolysis, where the E1-E3 enzymes ubiquitinate proteins to mark them for destruction (26). Protein synthesis, meanwhile, is mainly regulated at the step of translation initiation by the eIFs that are present in low concentrations or are prone to phosphorylation (27). The synthesis and breakdown rates of specific proteins depend on cell type, nutritional status, and status of breakdown regulators (28). However, the orchestration of synthetic and degradative machinery allows maintenance of

total body protein and free AA pools. Therefore, whole body net protein balance over 24 hours is usually zero (29), meaning that protein synthesis is equal to protein breakdown.

Skeletal Muscle Protein Turnover

Using tracer methods, the turnover rate of skeletal muscle and specific proteins can be determined. Although skeletal muscle contains half or more of the body's protein mass, it only comprises about 30% of WBPT rate, indicating that skeletal muscle turns over more slowly than other tissues (30). The fractional synthetic rate (FSR), or the percentage of the existing pool that becomes synthesized during a given time period, is about 1.15%/day for mixed skeletal muscle (30, 31) and ~12%/day for liver (32), as measured using continuous tracer infusion methods. However, skeletal muscle serves as an important site for the interchange between bodily protein and AA pools. Not only does muscle contain about 60% of the body's free AAs, there are large amounts of AAs stored in the 7-kg of muscle protein contained in the average human (23). Also, the skeletal muscle protein reservoir can sustain considerable losses without becoming a life-threatening situation (33). Thus, during times of fasting, the degradation of muscle proteins releases free AAs to be used by tissues that turn over more rapidly and whose protein content is essential for survival (e.g. heart, brain, and liver). Conversely, in the fed state, muscle protein synthesis is stimulated by ingested proteins and allows maintenance of muscle mass. Specifically, the FSR of skeletal muscle increases with the ingestion of EAAs that must be derived from dietary sources (34). Overall, skeletal muscle is sensitive to the body's nutritional status, allowing for maintenance of the free AA pools during fasting and the replenishment of its own protein stores during feeding.

Measuring muscle-specific protein turnover rates helps elucidate potential underlying causes of muscle wasting in older people. For example, the FSR of myosin heavy chain (MHC),

declines with age, suggesting that older people have a reduced capacity to rebuild important contractile components in muscle (31). Additionally, older individuals have reduced rates of mitochondrial protein synthesis with possible implications being diminished muscular endurance and ATP availability to aid in synthesizing other proteins (35). The overall synthetic response of muscle proteins to EAAs is also diminished in the elderly compared to younger counterparts (36). Since there is no increase in muscle protein breakdown with age (37), impaired synthesis of new proteins is often identified as the culprit behind lost muscle mass. In the next section, some of the proposed mechanisms for sarcopenia will be detailed with emphasis on their relation to muscle protein turnover.

MECHANISMS OF SARCOPENIA

Declining muscle mass with age is a multifactorial process, with an overall increase in catabolic stimuli and withdrawal of anabolic stimuli (38). Although there are numerous potential pathways through which sarcopenia occurs, the end result is a net negative muscle protein balance that gradually develops over time from disequilibrium in the rates of MPS and MPB. Oxidative stress appears to be one factor contributing to a net catabolic state. In the free radical theory of aging, this stress is caused by an increase in free radical reactions (39), particularly those involving reactive oxygen species (ROS) produced by the electron transport chain (ETC) (40, 41). Relevant to muscle protein metabolism, ROS activate nuclear factor-κB (NF-κB) (40, 41), which is a proinflammatory transcription factor that can lead to MPB through several pathways (42). First, NF-κB activates proteins in the ubiquitin proteasome pathway like MuRF1 E3 ubiquitin ligase, which plays a role in MPB (43). NF-κB also mediates the actions of the cytokine tumor necrosis factor-α (TNF-α), including degradation of protein in the myotubes (44) and connections between the extracellular matrix and cytoskeleton by matrix

metalloproteinase enzyme- 9 (MMP-9) (45). NF-κB activation resulting from oxidative stress certainly contributes to muscle protein degradation and the high levels of the activated transcription factor present in older individuals (36) may contribute to negative protein balance over time.

Oxidative damage to DNA is also proposed as a contributor to the aging process (39). Since the ETC and the mitochondrial DNA (mtDNA) are in close proximity, mtDNA is particularly susceptible to the damaging effects of free radicals. Deletions in the mtDNA occur more frequently in muscle and nerve tissue (46) and eventually lead to muscle fiber loss through derangement in the encoding of ETC chain components. First, since mtDNA encodes several of the cytochrome *c* oxidase (COX)/Complex IV subunits, deletions in the mitochondrial genetic code suppress COX activity in the final step of the ETC. The activity of nuclear-encoded succinate dehydrogenase (SDH)/Complex II often increases to stimulate mitochondrial biogenesis in compensation for decreased ETC energy output (47). This so-called ragged red fiber COX^{-}/SDH^{++} phenotype is associated with muscle fiber atrophy and breakage (48). In older humans (>80 years old), up to 70% of the mitochondrial genome can be affected by deletions, concomitant with decreased activity of complexes III and IV (49). Thus, ROS exert additional catabolic effects on muscle through damage to the mitochondrial DNA. However, the mitochondrial/free radical theory of aging has not gone unopposed, as accelerated aging occurs in rats with mtDNA dysfunction regardless of levels of oxidative stress (50).

Concurrent with these catabolic mechanisms is a retraction of anabolic stimuli such as decreased neuromuscular, hormonal, and nutritional signaling. There is also interplay among these factors and age-associated decreases in physical activity, nutritional intake, and protein intake. First, the function of muscle satellite cells, which are vital to skeletal muscle regeneration, is impaired in the elderly (51). Depending on the muscle and model studied, there

is conflicting evidence about whether satellite cell abundance declines with age (51). Yet even a reduced satellite cell population appears to maintain its regenerative capacity (52). Additionally, the proliferation and regeneration of aged satellite cells are restored when exposed to a young microenvironment (53), suggesting that extrinsic factors are more important to the functionality of satellite cells than population size or intrinsic factors. Along these lines, aging is associated with increased inhibitor of differentiation (Id) proteins (54). Id proteins interfere with myogenic regulatory factors (MRFs), transcription factors that facilitate the various stages of myogenic differentiation. Id proteins impede MRFs in several ways, including inhibition of binding to DNA (55). Related to inflammation and ROS, TNF-α may stimulate Id protein production (56), reducing satellite cell differentiation by MRFs. Overall, reduced muscle satellite cell function due to the effects of extrinsic factors may blunt MPS.

Another factor contributing to sarcopenia may be impaired input from α-motor neurons, resulting from decreased neuron quantity and conduction. While the quantity of motor neurons decreases during aging, this cannot entirely account for the impaired synaptic function characteristic of old age (57). Neuroaxonal dystrophy (NAD), an enlargement of the axon terminals and eventual accumulation of plaque-like material, may account for the remaining neural shortfalls. This process is gradual and likely related to ROS, as glutathione (an oxidative stress indicator) is present in higher levels within axons undergoing NAD (58). Oxidative stress may negatively impact protein synthesis by decreasing innervation of newly forming muscle cells, which prevents complete differentiation. High levels of embryonic myosin heavy chain (eMHC) in sarcopenic muscle fibers substantiate such a process, as eMHC indicates incomplete state of differentiation (59).

Blunted MPS in the elderly may also be related to the suppressed synthetic rates of specific muscle proteins. The FSR of myosin heavy chain (MHC) protein, which works with actin

to hydrolyze adenosine triphosphate (ATP), diminishes by about 40% with increasing age (31) and muscular strength decreases concurrently (31). Mitochondrial protein synthesis also declines by 40% into middle age (~54 years old) and remains reduced to the same degree into older age (~73 years old) (35). However, mitochondrial enzyme activity continues to decline into advancing age (35). Reduced ATP availability caused by declining ETC function may also impair the synthesis of other proteins. Yet again, oxidative stress is a suggested mechanism for these observations, as ROS damage to the mtDNA hinders transcription of ETC proteins and enzymes (49).

Withdrawal of anabolic stimuli may occur through alterations in hormonal signaling, with age-related declines in growth hormone (GH), insulin-like growth factor I (IGF-I), and sex hormones (60). GH and IGF-I have been proposed as potential contributors to the body composition changes that occur with aging. In the short-term, MPS has alternately been shown to increase (61) or remain unaffected (62) when GH is replaced in elderly individuals. Long-term effects are also equivocal, as muscle mass and strength was increased (62) or unchanged (63) after three months of GH treatment. Meanwhile, IGF-1 administration increases MPS in the short-term (61, 64), but does not increase lean body mass or strength following one year of treatment (65).

Next, testosterone production declines with age with a corresponding decrement in muscle function (66). Replacement of the hormone increases muscle protein synthesis and strength in healthy older men (67). Similarly, post-menopausal women experience declining muscle function as hormone production decreases, which may or may not be attenuated by hormone replacement therapy (68, 69). Decreased hormonal signaling may attenuate MPS, although a causative role between the decline of any hormone and sarcopenia has yet to be

established. Further, androgen therapy does not improve age-related decrements in MPS in either men or women (70).

Behavioral factors such as decreased physical activity and decreased energy and protein intake are also likely contributors to sarcopenia. From the 2007 Behavioral Risk Factor Survey, only 39% of individuals over the age of 65 achieved the recommended amount of physical activity (5 days per week of 30+ minutes of moderate exercise) compared to 60% of 18-24 year-olds (71). While the fat-free mass of active and inactive older individuals is not different, exercise training enhances muscle function and possibly allows the elderly to maintain activities of daily living (72). Related to protein metabolism, exercise increases myofibrillar protein synthesis for prolonged periods (up to 72 hours) (73), so maintaining a regular exercise program has the potential to chronically make protein balance more positive.

A more sedentary lifestyle, among other factors, also leads to reduced nutritional intake in the elderly. Energy intake, and consequently individual macronutrient intake, decreases progressively through each decade of life (74). Inadequate protein intake (<0.8 g protein·kg body weight^{-1}·day^{-1}), in particular, leads to a net catabolic state in muscle through reduced stimulation of MPS (22, 75). The retraction of nutritional and exercise-induced anabolic stimuli may also contribute to the negative protein balance that occurs in sarcopenia. This results in a downward spiral, where any muscle-related physical disability leads to even less physical activity.

Overall, sarcopenia has a complex pathophysiology and there is likely more than one mechanism leading to the condition. The list of potential factors provided is certainly not exhaustive, but emphasizes some of the more commonly identified contributors to age-related muscle loss, including behavioral changes as well as disordered nutritional, hormonal, and neuromuscular signaling. Viewed simply, sarcopenia results from a disparity between MPS and

MPB, where oxidative stress, catabolism, and suppressed anabolism cause a slightly negative muscle protein balance which results in lost muscle mass over time.

NITROGEN BALANCE

A common method of measuring whole body protein balance is the nitrogen balance (NBAL) technique. Through the measurement of nitrogen intake and output, nitrogen balance and its response to varying nutritional and exercise interventions can be calculated, most commonly to determine adequate protein intake for various populations. The premise of this method is that since protein is the body's main nitrogen-containing compound, a fluctuation in nitrogen reflects a change in body protein mass (76). Nitrogen balance is defined by Equation 2.1.

Equation 2.1. Nitrogen balance.

NBAL (g) = Nitrogen intake – (urinary N + fecal N losses + miscellaneous N losses)

Nitrogen intake is determined by the amount of protein ingested, with 6.25 grams of protein containing an average of 1 gram of nitrogen. Nitrogen output is measured by the various routes through which nitrogen exits the body. Urine nitrogen is mainly in the form of urea, but AAs, creatinine, ammonia, and uric acid in the urine also contain nitrogen (20). Feces eliminate approximately 2 grams/day (77) of nitrogen-containing AAs and ammonia (20). Finally, miscellaneous N losses are approximated at 5 mg/kg body weight/day and occur through sweat and sloughing off of hair, skin, and nails (77). Dietary protein intake is considered sufficient when nitrogen balance is zero (i.e. intake=output), a situation where protein synthesis and breakdown are also in equilibrium. A changing nitrogen balance, on the other hand, reflects a more positive or negative protein balance.

Disposal of nitrogen through the urea cycle

The main function of the urea cycle is to maintain nitrogen equilibrium in the body. The cycle proceeds rapidly and automatically, such that it always has the ability to eliminate excess nitrogen in the form of ammonia (78). Ammonia in the body can come from food sources, bacterial lysis of urea or AAs in the GI tract, or from deamination of AAs in the liver (20), which will be described next. AAs that are in excess of the body's ability to synthesize proteins undergo oxidative deamination or transamination. Although there are multiple ways these processes can occur, the most common initial route for the nitrogen-containing group of an AA to be removed is through transamination with α-ketoglutarate to form glutamate (79). The glutamate can then: 1) be oxidized by NAD^+ through the glutamate dehydrogenase reaction, forming α-ketoglutarate and an ammonium ion (NH_4^+), or 2) be transaminated by the action of several enzymes, including glutamate-pyruvate transaminase (GPT) and glutamate-oxaloacetate transaminase (GOT) to form the AAs alanine and aspartate, respectively, as well as α-ketoglutarate (a tricarboxylic acid (TCA) cycle intermediate). Oxidative deamination occurs in the liver, while the transaminase reactions are the means for exchanging nitrogen in most other tissues. Most of the nitrogen eventually excreted from the body goes through the formation of aspartate, an intermediate in the urea cycle (79).

Since oxidative deamination and the urea cycle both occur in the liver, the liver is considered the major site for the elimination of ammonia (79). Carbamoyl phosphate synthetase, the initiating enzyme in the urea cycle, forms carbamoyl phosphate from ammonia (NH_3 formed from the NH_4^+ of oxidative deamination) and carbon dioxide (CO_2). In the mitochondria of the periportal hepatocytes, carbamoyl phosphate and ornithine react to form citrulline. The next step is rate-limiting: Aspartate brings additional nitrogen to be eliminated by the cycle and forms argininosuccinate (20). Fumarate (a TCA cycle intermediate) is subsequently

formed during the conversion of argininosuccinate to arginine. In the final step, urea is formed, released into the blood, and is extracted by the kidneys or sweat glands for excretion (79). The elimination of nitrogen is a vital process because the buildup of nitrogen in the body becomes toxic, with the potential to cause brain damage and coma (20). Since most of the nitrogen from the oxidative deamination of AAs is eliminated as urea, urine urea levels are sometimes used to measure protein oxidation. Moreover, since 70-90% of excreted nitrogen is in the form of urea, total nitrogen excretion gives a close approximation of protein oxidation through AA deamination and allows inferences to be made about whole body protein metabolism (28).

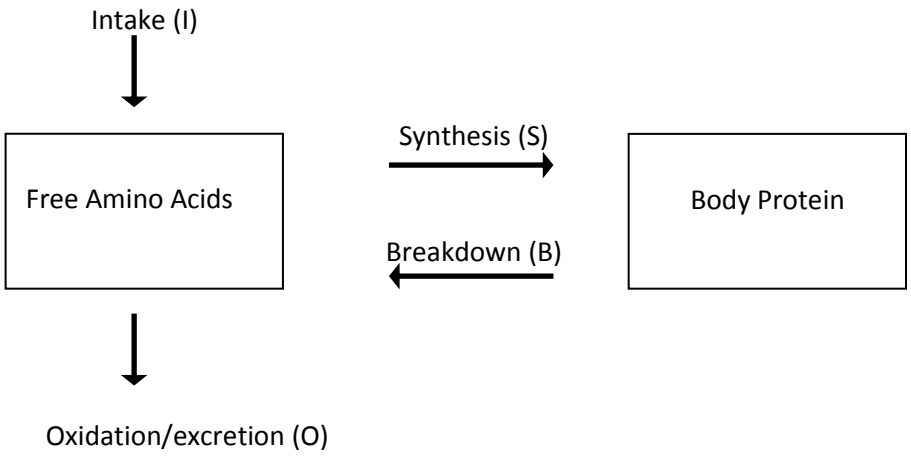
Limitations of the nitrogen balance method

Since the urine nitrogen coming from sources besides urea is relatively minor, urine N is assumed to reflect AA oxidation coming from proteins in the NBAL method. For an individual to be in nitrogen balance, the amount of nitrogen excreted through all routes must equal the amount of nitrogen consumed as dietary protein. The free AA pool serves as a connection between nitrogen balance and protein balance (Equation 2.2, Figure 2.2).

Equation 2.2. Turnover or flux (Q) of the free AA pool.

Q = S (synthesis) + O (oxidation/excretion) = B (breakdown) + intake (I).

Figure 2.2 Simplified model of protein metabolism. Adapted from Wagenmakers et al. (23).



Since the size of the pool remains relatively constant, the equation can be rearranged to show that when N intake and N excretion are in balance, so are protein synthesis and breakdown: (I - O) = (S - B) (78). In this simplified model, if nitrogen balance becomes more positive, protein balance must have also changed to become more positive. This can either be achieved through stimulation of protein synthesis or suppression of protein breakdown.

Related to Equation 2.2, one major limitation of the NBAL method is that even though changes in the body's protein mass are being measured, the mechanism by which the change occurs remains unknown. For example, if a more positive nitrogen balance is measured, it cannot be concluded with any certainty whether the change resulted from increased synthesis or decreased breakdown. Without a confirmed change in protein kinetics, there is less certainty whether there was indeed an altered protein balance or whether an error occurred in the NBAL measurement.

Along these lines, another limitation of the NBAL technique is that incomplete measurements of routes of excretion result in an artificially positive nitrogen balance. For total accuracy, it is necessary to collect all forms of N excretion, including urine, feces, skin, sweat, and hair. Due to the impractical nature of such collections, fecal N and other miscellaneous N losses are often approximated (77). However, interindividual variability in N loss through the various routes might result in a miscalculation of N balance when the standard values are used. Inaccuracy in the measurement of N intake may also contribute to the overestimation of N balance. If any of the food provided is not consumed, even scrapings left on plates and utensils, the calculated intake will appear higher than the actual intake. In addition, study diets may not contain the standard 1 g nitrogen per 6.25 g of protein, a problem that can be solved by analyzing aliquots of the food for nitrogen content. Since the precision of nitrogen balance measurement is within tenths of a gram, the method should be able to detect changes in the

body's protein mass as little as 0.1%. However, due to measurement issues, NBAL is commonly overestimated (19).

Lastly, nitrogen balance requires a period of time to adapt to an altered diet. In response to a new protein intake, NBAL generally takes 5-7 days to adapt (76). Thus, before an experimental period begins, a sufficient lead-in period is necessary to eliminate any acute adaptations to dietary stimuli that can affect nitrogen balance. Despite the commonly identified limitations of the NBAL method, it is a relatively easily implemented technique for detecting changes in protein balance. In addition to being the classical method to establish adequate protein intakes for specific populations, NBAL can be used to measure the whole body protein metabolism response to varying nutrition or exercise interventions.

Energy balance, protein intake, and nitrogen balance

Several lifestyle factors can cause NBAL to become more positive or negative, including energy intake, protein intake, and physical activity. NBAL is closely related to energy intake, such that if energy intake is inadequate, a negative nitrogen balance and loss of lean body mass will be the result (5). Conversely, when energy balance becomes more positive, nitrogen losses are ameliorated (80). Calloway and Spector specified 700 kcal as the amount of non-protein calories necessary to diminish nitrogen loss with a protein-free diet (80). On the other hand, when energy intake is restricted to 500 kcal/day, nitrogen balance is maintained with a higher than recommended protein intake of 1.5 $g \cdot kg^{-1}$ (81). The dynamic relationship between energy and nitrogen balance were further elucidated by Todd et al., who demonstrated that nitrogen balance was better maintained when the energy deficit resulted from exercise as opposed to caloric restriction. In fact, with the addition of one hour of exercise during energy balance, an anabolic effect was observed where subjects spared 2.5 mg N per kg body weight (82).

During a caloric deficit, the body oxidizes more AAs as fuel and the availability of AAs to build proteins becomes limited. Skeletal muscle is able to withstand substantial losses in AAs without becoming life threatening (33), so the AAs from the skeletal muscle reservoir are used to help maintain the function of vital organs. In support of this, during chronic caloric restriction in rats, the protein synthetic rate of the heart is maintained while that of skeletal muscle is diminished (83). Since building proteins is costly from an energetic standpoint (84), it follows that muscle protein synthesis will be downregulated during an energy crisis. AMP-activated protein kinase (AMPK) is stimulated by a shortage of energy and inhibits the action of mammalian target of rapamycin (mTOR), a stimulatory regulator of protein synthesis (85). The AMPK/mTOR relationship will be discussed further in a later section, but in general, insufficient energy halts protein synthesis beginning in areas like muscle that can withstand AA losses. In this situation (refer to Figure 2.2), protein breakdown exceeds synthesis (B>S) and unless a compensatory amount of protein is ingested, AA oxidation will exceed AA intake (O>I). The nitrogen from the oxidized AAs is excreted and nitrogen balance consequently becomes more negative.

Urinary nitrogen also declines in response to a decreased protein intake, characterized by a steep initial decline and subsequent stabilization; urinary nitrogen equilibrates to a protein-free diet after approximately eight days (86). Despite the body's ability to adapt to a marginal dietary protein intake, adverse health consequences occur during prolonged protein deprivation. When groups of elderly women were provided either 0.45 g or 0.92 g protein ·kg body weight^{-1}·day^{-1} for nine weeks, the low protein group had decrements in fat-free mass as well as immune and muscle dysfunction (22). Similar to caloric restriction, protein deficiency limits the amount of AAs that are available for muscle protein synthesis and nitrogen loss will occur unless surfeit energy is provided.

Overall, nitrogen balance is sensitive to both energy and protein intake. During a shortage of either energy or protein, supplementation with the other ameliorates excretory losses of nitrogen. In healthy adults consuming adequate energy and protein, nitrogen balance should be zero. During aging, however, there is a loss of fat free mass and resultantly, nitrogen. Although the rate of N loss that is likely characteristic of aging (5 g/yr or 0.02 g/day) is too slight to be detected by the NBAL method (28), interventions that chronically cause a slight positive protein/nitrogen balance may be sufficient to prevent the loss of muscle mass. Since inadequate nutrition can become problematic in the elderly (74), effective interventions must incorporate both adequate caloric and protein intake due to the link to nitrogen balance.

Exercise and nitrogen balance

Similar to the adaptation of nitrogen balance to altered protein intake, there may be an adjustment period of nitrogen balance after an exercise program is started. Some research suggests that in response to beginning a daily aerobic exercise program, NBAL initially becomes acutely negative and then returns toward equilibrium (87). In this case, NBAL values would expectedly be more negative during the adaptation period and more positive thereafter. However, if energy intake is not adjusted to account for the energy expenditure of exercise, negative NBAL may reflect negative energy balance rather than any exercise effect on protein metabolism. When energy balance is maintained, moderate intensity exercise can cause NBAL to become more positive by increasing nitrogen retention (82, 88). Thus, moderate intensity exercise represents a strategy to cause a more positive protein balance under circumstances of sufficient energy. Overall, there is an adaptation period during which NBAL adjusts to new protein and energy intakes and possibly new exercise programs. However, NBAL may increase immediately with moderate exercise as long as energy and protein intake are sufficient.

NUTRITION AFTER EXERCISE

mTOR pathway in modulating muscle protein synthesis

The mammalian target of rapamycin (mTOR) is a protein that integrates various stimuli like nutrition and exercise to regulate MPS. The effects of mTOR on MPS are facilitated by the action of three regulatory proteins: p70 ribosomal S6 kinase ($p70^{s6k}$), eukaryotic initiation factor 4E-binding protein 1 (4E-BP1) (89), and eukaryotic initiation factor 4G (eIF4G) (90). These regulators enhance protein synthesis, acting at different points of the translation process. 4E-BP1 affects the release of eIF4E (91), which forms a complex with eIF4G, a necessary step for translation initiation (92). Meanwhile, $p70^{s6k}$ is active in the expression and subsequent activity of eukaryotic elongation factor 2 (eEF2) (93), an important factor in the elongation phase of protein translation. Although mTOR also exerts an effect on transcription (94), it mainly stimulates MPS through factors active in the translation process.

The process of MPS is relatively energetically expensive and is therefore sensitive to ATP availability (84). mTOR integrates the signal of energy status from AMPK with signaling from AAs and insulin to either stimulate or inhibit MPS (95). AMPK is activated when the AMP/ATP ratio increases and cellular energy is sparse. To prevent ATP consumption by MPS, AMPK inhibits mTOR indirectly by phosphorylating tuberous sclerosis complex 2 (TSC2) (85). Similarly, when AA availability is low, the cell conserves ATP by avoiding MPS. When AAs become available, mTOR is stimulated and MPS proceeds. Although the exact mechanism by which this occurs is unclear, theories include AA interaction with proteins upstream of mTOR or the direct action of AAs on mTOR (96). EAAs are particularly potent for stimulating MPS. For example, a flooding dose of EAAs increases FSR in skeletal muscle, whereas a flood of nonessential AAs does not (34). MPS is contingent on the availability of energy and AAs (particularly EAAs), with mTOR modulating both signals within the cell.

Insulin, a hormone released when blood glucose is elevated, also exerts an effect on mTOR signaling. This is an important consideration since most meals are consumed as a combination of protein, carbohydrate, and fat. It has been suggested that a minimal amount of insulin is necessary to permit MPS to proceed and thereafter insulin exerts no further effect on MPS (36). Insulin's impact on mTOR is mediated through several molecules, with Akt/PKB (protein kinase B) eventually activating mTOR. An oral EAA/carbohydrate bolus boosts mTOR and Akt/PKB phosphorylation along with MPS. At the same time, AMPK phosphorylation diminishes upstream from mTOR and S6 kinase 1 (S6K1) and 4E-binding protein 1 (4E-BP1) increases downstream from mTOR (97). These data demonstrate the coordination of a permissive amount of insulin and AA signaling by mTOR after mixed meal feeding to modulate MPS.

Lastly, exercise also works mainly through the mTOR cascade to augment MPS and in combination with nutrition, mTOR activation is optimized. Physical exertion may stimulate MPS and muscle hypertrophy through PKB, GSK3 (downstream from PKB) or a direct effect on mTOR (98). Whatever the mechanism, aerobic and resistance type exercise comparably activate MPS in untrained individuals through phosphorylation of mTOR pathway proteins (11). With administration of EAAs after resistance exercise, the phosphorylation of $p70^{s6k}$ is enhanced significantly when compared to a placebo (99). mTOR integrates numerous signals within the cell and represents a mechanism of control for the synergistic effect of nutrition and exercise on MPS.

Nutrition optimizes anabolic stimulation of exercise

It has been established that both resistance and non-resistance exercise stimulates an anabolic period in which MPS surpasses MPB. In older and younger men, the synthesis of mixed

muscle and plasma proteins increases similarly after 45 minutes of moderate walking at 40% of VO_{2peak} (100). This effect also occurs following resistance-type exercise, with the elevation in mixed muscle FSR lasting up to 48 hours (101). The impact of exercise on whole body protein kinetics is less clear. Some research suggests that moderate intensity aerobic exercise does not acutely affect WBPT (102). However, there is evidence that whole body protein synthesis increases acutely following vigorous aerobic exercise (103) and that longer term aerobic training increases basal WBPT (104, 105). Even if WBPT rates are not affected acutely by exercise, the increase in synthesis of individual proteins, like those in muscle, may conserve nitrogen from protein breakdown and allow whole body protein balance to become more positive (102).

Nutrition takes advantage of the anabolic period after exercise, as MPS is amplified to a greater extent when AAs are ingested than during fasting post exercise (106). MPB is stimulated concurrently with MPS following exercise (100, 101), but the rate of MPB remains relatively constant even when nutrition is provided. Thus, the administration of AAs exploits the period of post-exercise anabolism and allows net positive protein balance mainly through an augmentation of MPS (106). Primarily EAAs are responsible for the enhanced MPS, as the addition of nonessential AAs does not further the response (106). Lastly, combining exercise and nutrition prolongs the heightened MPS response of either nutrition or exercise alone. When nutrition and physical activity are combined, MPS remains elevated for up to 72-hr (73) as compared to just a few hours for exercise (107) or feeding (108) alone. Nutrition, particularly in the form of EAAs, optimizes the anabolic stimulus of exercise, a different viewpoint than simply an additive response of the two stimuli (16). Additionally, this combination elicits an extended response, with potential implications for more positive protein balance over time.

Timing of nutrition

The timing of nutrient consumption immediately after an exercise bout is important for an optimal protein-building response. In one study, older men completed a 12-week resistance training program and consumed a protein supplement either immediately (PO) or 2 hours after exercise (P2). Muscular strength and hypertrophy were enhanced further in the PO group than the P2 group, demonstrating that early post-exercise nutrition is most effective for creating a situation of net MPS over time (109). There is some conflict about whether long-term protein supplementation immediately after exercise augments the gains in strength and muscle mass observed from resistance training alone. Twelve weeks of protein supplementation in elderly men before and after exercise does not enhance skeletal muscle hypertrophy or strength gains compared to a placebo (15). Meanwhile, in older women, protein supplementation augments the response of muscular strength and mass and bone development to a 24-week weightlifting program (14). The reason for the conflicting data is not entirely clear, but it is possible that the study in men was not long enough to elicit a response, as there was no significant hypertrophy in the study on women after the first 12 weeks (14). If adding nutrition after exercise only causes a slightly positive protein balance, a longer time period would be necessary to detect differences in muscle accretion.

With regard to muscle protein metabolism, a mixed nutrient oral supplement taken immediately after one hour of moderate aerobic exercise causes whole body and leg protein balance to become more positive than when taken three hours post-exercise (17). Contrary to this, the MPS response to a carbohydrate/AA supplement is similar when consumed either one or three hours after resistance exercise, yet is still superior to a placebo treatment (110). On the whole, timing protein consumption as soon as possible after exercise is most effective for

stimulating protein synthesis. Regular use of this strategy to combine exercise and nutrition also has the potential to enhance longer term muscle mass and strength gains.

Type of nutrition

In addition to timing considerations, the type of protein ingested can impact the extent of MPS stimulation after exercise and the resulting gains in lean mass. Milk protein is a more effective stimulus for MPS and causes a more positive muscle protein balance than soy protein when consumed after resistance exercise (111). The heightened anabolic response to milk protein translates into greater muscle mass gains when skim milk rather than soy milk is consumed following each weightlifting bout during a 12-week program (112). Augmented MPS responses to milk may be due to the fact that milk is a rich source of leucine. This EAA is particularly effective at stimulating MPS, acting through the mTOR pathway via both insulin dependent (113) and independent (114) mechanisms. Koopman et al. examined the response of muscle and whole body protein metabolism to three different post exercise nutrient combinations: carbohydrate only (CHO), carbohydrate and protein (CHO+PRO), and leucine added to protein and carbohydrate (leucine+CHO+PRO) (115). Following 45 minutes of resistance exercise, the leucine+CHO+PRO combination stimulated MPS significantly above CHO. Additionally, whole body net protein balance was more positive in leucine+CHO+PRO compared to both other treatments.

Bovine milk contains ~20% whey protein and ~80% casein protein by mass (111) and each fraction contributes differently to the effect of milk on protein metabolism. Whey and casein are referred to as fast and slow proteins, respectively, because of the rate and time course of plasma AA stimulation (116). Whey proteins are digested more quickly and stimulate a steep rise in plasma AAs, thus acutely inducing protein synthesis and having a short term

effect on protein metabolism. Meanwhile, casein is digested more slowly and the rise in plasma AAs is slower and more sustained, possibly due to gastric emptying. Casein stimulates protein synthesis to a lesser extent than whey, but also inhibits protein breakdown by about 34% (116). The combination of the two types of protein in milk seemingly produces an ideal response in terms of protein metabolism, as the whey acutely stimulates MPS and the casein inhibits MPB. Indeed, milk protein stimulates a modest initial rise and a more prolonged increase in plasma AAs as compared to the quick rise and fall of AAs in response to soy protein (a "fast" protein) (117). The sustained increase in AAs in response to milk translates into greater lean mass accretion, as previously mentioned (111, 112). Due to its leucine content and whey and casein fractions, milk ingestion may be an ideal intervention to prevent muscle loss.

Old vs. young: protein metabolism in response to nutrition

Blunting of MPS by nutritional signaling may be an underlying cause of sarcopenia. Studies of AA and protein metabolism in the postabsorptive state demonstrate that basal muscle protein metabolism is not different between younger and older subjects (37, 118). Such evidence led Cuthbertson et al. (36) to hypothesize that declining muscle mass is instead related to a blunted anabolic response to AA feeding in the elderly. Indeed, myofibrillar and sarcoplasmic protein synthesis stimulation by oral EAA administration is suppressed in older subjects. The underlying mechanism appears to be decreased activation and expression of mTOR, $p70^{S6k}$, 4EBP-1, and eIF2B. The suppression of the mTOR pathway translates into decreased MPS in the old subjects (36). Thus, the diminished mTOR-signaled MPS response after feeding may result in a more negative protein balance in older individuals.

There is also evidence that elderly muscle is less sensitive to the anabolic effects of insulin during mixed meal nutrition (119). Recent work by Fujita et al. suggests that age

associated insulin resistance may account for the diminished meal response in older individuals. In healthy elderly muscle, MPS and the corresponding mechanisms in the mTOR pathway are only augmented with the provision of a supraphysiological insulin dose (120). However, there was no provision of AAs in this study and AAs are a primary determinant of MPS with insulin providing a permissive effect. In the old and young, only a minimal threshold of insulin (~10 m $IU.ml^{-1}$) must be reached to allow MPS to proceed, beyond which EAA is the main stimulus of anabolism (36). Also, MPS does not increase when insulin and glucose are provided in the absence of AAs (121). The precise effect of insulin on muscle protein metabolism is unclear. Certainly, higher levels of AAs stimulate an optimal MPS response and increased energy (122) and potentially insulin (123) enhance the response.

Despite the potential for the muscles of elderly individuals to be less responsive to nutrition, certain types of nutritional interventions can improve MPS levels to match those of younger counterparts. Combining carbohydrate with protein and leucine stimulates MPS equivalently in older and younger subjects and causes whole body protein balance to become positive (124). In fact, a high proportion of leucine (41%) in a 6.7 g EAA supplement stimulates MPS above rates induced by a lower proportion (26%) (125). Shortcomings in anabolic signaling in elderly muscle by AAs and insulin may contribute to sarcopenia via diminished MPS and negative protein balance over time. However, the muscle of older individuals has the potential to be maximally stimulated, particularly when a high proportion of leucine is provided.

Prevention of sarcopenia through nutrition and exercise

Decreased protein intake results in decreased EAAs, which are potent stimulators of muscle protein synthesis. Increasing the absolute amount of protein above the recommended level has been proposed as a potential strategy in the prevention of sarcopenia, yet the results

of such studies are inconsistent. In a study of healthy men and women aged 55-77 years, 14 weeks of a eucaloric diet providing 0.8 $g \bullet kg^{-1} \bullet day^{-1}$ of protein resulted in decreased mid-thigh muscle area, which was associated with a decrease in urine nitrogen excretion (6). Similar conclusions of the inadequacy of this intake were made in other studies (126, 127). However, in a recent long-term study, researchers concluded that the dietary protein recommendation is not different between older and younger adults. The study by Campbell et al. eliminated some of the weaknesses of previous studies by including only healthy older subjects, allowing sufficient study time periods, using young subjects as a control group, and following a valid NBAL protocol (7).

Regardless of the debatable issue of dietary protein adequacy, exercise appears to be a promising strategy for the prevention of sarcopenia, with nutritional supplementation also of potential benefit. Short-term resistance-type exercise stimulated MHC, as well as mixed muscle protein synthesis in healthy elderly subjects (128). The increase in synthesis translates into muscular strength gains in the long term, as older subjects who underwent a 10-week progressive resistance training program increased their 1-Repetition Maximum (RM) strength by 113 +/-8% compared to 3+/-9% in the non-exercising controls. In the same study, nutritional supplementation with or without the resistance training did not impact strength gains made by the subjects (129). Similar results have emerged in other studies, where nutritional supplementation does not further augment the effects of resistance training on muscle mass and strength gains (15, 130). On the other hand, some studies have demonstrated that exercise stimulates a period of anabolism, which can be taken advantage of through optimal timing of nutritional supplementation (14, 110, 131, 132).

Sarcopenia occurs in all older individuals to a certain extent, yet few strategies for prevention have been identified. The underlying causes are complex and are likely different

across individuals. However, aging is generally accompanied by an increase in catabolic signals like oxidative stress and a relative withdrawal of anabolic signals like those from nutrition. Within the muscle, there is an overall shift toward breakdown, leading to loss of protein and thus, nitrogen. There is not an apparent increase in the rate of muscle protein breakdown in the elderly, so a diminished rate of synthesis has been proposed as the cause of the protein imbalance. Since the rate of nitrogen loss through sarcopenia is very small, prevention of muscle loss might only require an intervention that elevates protein synthesis and allows nitrogen to be spared. Using exercise to stimulate an anabolic period and optimizing the protein synthetic response with the proper timing and type of nutrition is one such strategy. With this in mind, this study aims to determine if timing the intake of a chocolate milk beverage immediately after exercise can cause nitrogen balance to become more positive and represent a realistic strategy for older individuals to prevent sarcopenia.

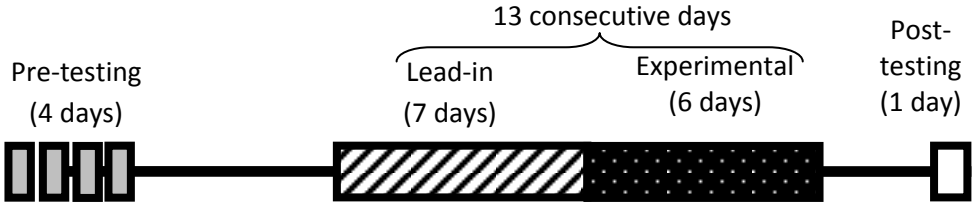
CHAPTER 3

METHODS AND PROCEDURES

STUDY OVERVIEW

The aim of this study was to compare nitrogen balance in subjects in energy balance when milk consumption was timed immediately after aerobic exercise compared to some other time during the day. By design the two treatment diets were isocaloric and isonitrogenous with only the timing of the milk protein intake varying. The study consisted of four distinct periods: pre-experimental testing, a seven-day lead-in period, a six-day inpatient/experimental period, and post-testing (Figure 3.1).

Figure 3.1. Study timeline.

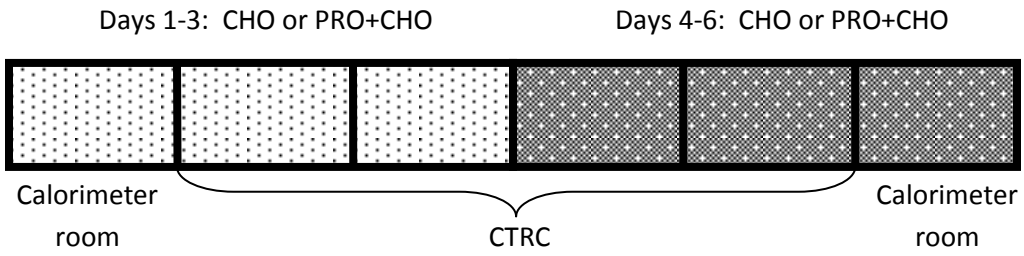


Pre-testing		Lead-in (outpatient)	Experimental (inpatient)		Post-testing
Day 1: GXT Day 2: Blood draw, DEXA, RMR Day 3: VO_{2max} Day 4: steady-state VO_2, diet log collection	2-3 wks to allow diet planning and preparation	Controlled diet, daily weight measurement, 2 days with accelerometer	Controlled diet, daily exercise followed by test beverage, 24-hr urine collection, daily weight measurement, accelerometer, 2 days in Cal room	<1 wk	DEXA

The metabolic information obtained during pre-testing was used to plan the lead-in and experimental diets. The purpose of a seven-day lead-in diet was to allow a period for each subject to adapt to the level of protein provided during the inpatient/experimental period since nitrogen balance is sensitive to changes in protein intake (86). The time required for adaptation is about eight days when subjects are switched to an essentially protein-free diet (less than 0.1 $g \cdot kg^{-1}$) (86), but a timeframe of 5-7 days is generally deemed sufficient to allow adaptation to changes of smaller magnitude (76). We used a seven-day lead-in diet since protein intake during the study was close to the habitual intake of the subjects.

The experimental protocol was implemented during a six-day inpatient stay at the University of Colorado Denver Clinical and Translational Research Center (CTRC) in which NBAL was continuously monitored. We used a randomized cross-over design (Figure 3.2) during which each subject completed two three-day trials, PRO+CHO and CHO. Additionally, one day during each condition (Inpatient Day 1 and Day 6) was spent in a whole room calorimeter to confirm energy balance.

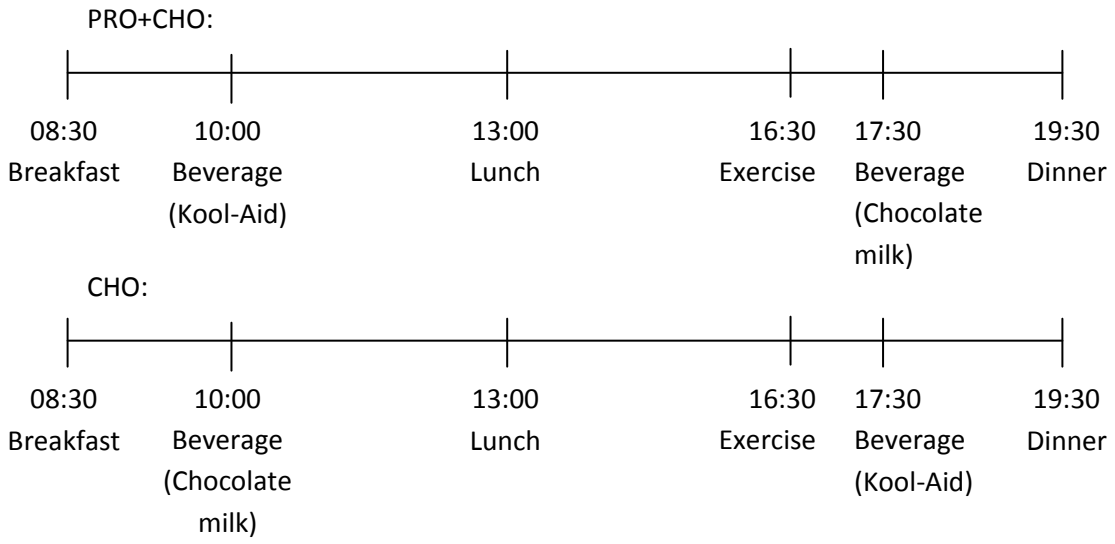
Figure 3.2. Inpatient/experimental period.



Subjects were given a regular hospital room on the CTRC during the remaining inpatient days (Days 2-5). Meals were delivered at specific times and one hour of cycling exercise was performed each afternoon. During the PRO+CHO condition (Figure 3.3), subjects consumed a chocolate milk beverage after exercise. During CHO, a Kool-Aid beverage was consumed post-

exercise. Three-day diets were identical between the two conditions, so that energy, macronutrients and foods consumed were reproduced. The only difference between trials was the timing of the protein-containing beverage in relation to the exercise bout.

Figure 3.3 Timing of meals and consumption of beverages during experimental trials.



PROCEDURES

Screening

During the initial screening appointment, subjects completed an informed consent, a HIPAA-B Approval form, and a medical and exercise history questionnaire (Appendices A, B, and C). An online food preference and allergy questionnaire from the CTRC was also completed by each subject so that the study diets would contain acceptable food choices. Only non-smoking, inactive individuals who were not taking medications were included in the study. Since milk was used as an experimental beverage, it was required that the subjects were not self-reported to be lactose intolerant. Additional exclusionary criteria included: Obesity (BMI>30), recent orthopedic injuries that would impede the ability to exercise, conditions affecting food

absorption or digestion, undiagnosed hypo- or hyper-parathyroidism (TSH<0.05 uU/mL or TSH>5.0 uU/ml), a bleeding disorder, or a current illness or infection.

Subjects

Ten healthy sedentary male (n=3) and female (n=7) subjects between the ages of 55-75 years were recruited to participate in this protocol, which was approved by the Colorado State University Institutional Review Board (IRB) and the Colorado Multiple Institutional Review Board (COMIRB) for human subjects research. The data from one subject was excluded from the analysis because an incorrect activity factor was used to estimate energy expenditure (EE) and as a result the subject was calorically restricted by about 35%. This exclusion was justified because of the established relationship between caloric restriction and nitrogen balance (5) and because all other subjects were kept near energy balance. Therefore the final sample size was n=9, with two male and seven female participants. See Table 3.2 for subject characteristics.

Table 3.2. Subject characteristics.

	Male (n=2)	Female (n=7)	All subjects (n=9)
	Mean ±SE	Mean ±SE	Mean ±SE
Age	62±7.0	66±2	65±2
Height (cm)	182±0.5	157±1.0	162±4.0
Weight (kg)	88.8±2.2	57.3±2.8	64.3±5.1
BMI (kg/m^2)	26.8±0.8	23.3±1.1	24.1±1.0
Body fat (%)	22.2±0.5	34.4±2.4	31.7±2.5
VO_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	30.2±0.4	23.6±1.5	25.0±1.5

Pre-Testing

Each subject underwent a series of pre-experimental testing, both to ensure their eligibility and to determine various metabolic and physical characteristics. On the first day of testing, a graded exercise test (GXT) was completed on the treadmill under the supervision of a

cardiologist. Using the Balke protocol, the treadmill speed remained the same throughout the test (3.3 mph), with grade increasing by 2% after the first minute and 1% every minute thereafter. Every three minutes, heart rate (HR), rating of perceived exertion (RPE), blood pressure (BP), and an electrocardiograph (EKG) was recorded. Termination of the GXT occurred either by voluntary subject termination or if the subject had reached 85-100% of their predicted maximum heart rate (MHR = 220-age). Individuals were excluded if the test indicated a hypertensive or ischemic response to exercise as determined by the supervising cardiologist.

Subjects returned to the laboratory on a separate morning after abstaining from exercise for 24 hours and completing an overnight fast. A blood sample was collected followed by a dual-energy X-ray absorptiometry (DEXA) scan (QDR 4500W, Hologic, Inc., Bedford MA). Resting metabolic rate (RMR) was then measured to estimate 24-hour resting caloric expenditure (Parvomedics TrueOne 2400, Sandy, UT). During this test, a hood was placed over the head of the subject and expired gases were collected for 45 minutes. The first 15 minutes were treated as an adaptive period to allow the subject to become accustomed to the experimental setup and to determine the appropriate flow rate. The lights were dimmed and the flow rate was adjusted so that $FECO_2$ levels were maintained between 0.9-1.0%. The data from the final 30 minutes predicted RMR using the Weir equation, which calculates caloric output based on the volume of exhaled oxygen (133). The values for daily energy expenditure were averaged and any outlier measurements (greater than ±2 standard deviations) were eliminated.

On another day during the same week, subjects completed an incremental exercise test on a cycle ergometer (Monark Excalibur, Groningen, The Netherlands) with indirect calorimetry (Parvomedics TrueOne 2400, Sandy, UT) to determine VO_{2max}. The maximal cycling protocol began at 50 Watts for the first minute, increasing by 20 Watts for females and 30 Watts for

males every two minutes thereafter. The ergometer adjusted resistance depending on pedal rate, so the subjects were instructed to maintain a pedal rate of 70-90 RPMs throughout the protocol, since at lower pedal rates, the resistance becomes difficult to work against particularly for sedentary individuals. The test was terminated when subjects reached volitional exhaustion and the following common criteria were used to determine if true maximal level was reached: Exercise HR came within 10 beats of predicted MHR, respiratory exchange ratio (RER) was greater than 1.1, and VO_2 values appeared to reach a plateau (79).

After determination of VO_{2max}, 55% of each subject's VO_{2max} was calculated to target an exercise intensity that simulates a brisk walking pace. Equation 3.1 (134) was used to estimate the cycle ergometer work rate that would produce a steady-state exercise level at 55% of maximal VO_2.

Equation 3.1. ACSM leg cycle ergometry equation

$VO_2\,(ml \cdot kg^{-1} \cdot min^{-1}) = 1.8\,(\text{work rate})/\,(BM)^{*} + \text{resting } VO_2\,(3.5\ ml \cdot kg^{-1} \cdot min^{-1}) +$

$\text{unloaded cycling}\ \ (3.5\ ml \cdot kg^{-1} \cdot min^{-1})$

*work rate=$kg \cdot m \cdot min^{-1}$ and BM=body mass (kg)

On the final day of testing, subjects began cycling at the estimated workload and once a stable VO_2 was reached, they cycled for another 30 minutes. By indirect calorimetry, EE in kcal/min was determined as an average of the EE values from the 30 minutes. This value was used to estimate the exercise EE during the study's inpatient daily exercise protocol (one hour of cycling at 55% of VO_{2max}).

During the pre-testing period, subjects also completed a diet log by recording all foods and beverages that were ingested during three consecutive days. The record was collected to provide an indication of normal dietary habits of the subjects. Study investigators did not control diet on these days. Participants were instructed to include two weekdays and one

weekend day and to be as specific as possible about their diet. Subjects provided food labels for any specialty items and gave investigators information about the foods that were eaten, portion sizes, and how foods were prepared. All foods were entered into Food Intake Analysis System software (FIAS 3.99, Houston, TX) and analyzed. The data from the three days were averaged, providing an indication of habitual energy, macronutrient, and fiber intake. This record allowed investigators to compare free-living diets to those that were provided during the lead-in and inpatient periods.

Lead-in Diet

Several weeks after completing the pre-testing, each participant completed a seven day lead-in diet immediately followed by a six day inpatient stay at the University of Colorado-Denver CTRC (refer to figure 3.1). During the seven day lead-in diet, subjects reported each morning to the CSU Nutrition Center for weight measurement and breakfast. Subjects were instructed to arrive in the same light-weight clothing each day and were weighed without shoes on the same scale (Detecto, Webb City, MO) each day. Breakfast was eaten at CSU under the supervision of study staff and the remainder of each day's food was prepared and placed in a soft cooler. Besides coming to the Nutrition Center each morning, participants were allowed to maintain their normal everyday activities. Thus, lunch, dinner, and snacks were consumed either at the subject's home or place of work.

All study diets (outpatient and inpatient) followed USDA nutritional guidelines and were created using Pronutra software (Viocare, Inc., Princeton, NJ). The macronutrient breakdown of the lead-in diet was as follows: 15% protein, 30% fat, and 55% carbohydrate taken as a percentage of total calories. Breakfast, lunch, and dinner comprised 30%, 30%, and 40% of calories, respectively. Subjects were instructed to eat only the food that was provided to them

by study staff and to eat everything in the cooler. If they were unable to eat any food items, they were asked to return the food the following day so that it could be weighed. Otherwise, it was assumed that the subjects ate all foods provided to them and nothing else.

Subjects were kept in energy balance (energy intake= energy expenditure) for the entire study period, with caloric intake planned based on the subject's estimated caloric expenditure. For the lead-in diet, each subject's RMR was multiplied by an activity factor of 1.55-1.60, a range of activity factors that approximates those of free-living sedentary elderly individuals (135). The lead-in diet was intended to provide an equal amount of protein to that of the inpatient diet, so that nitrogen balance measurements made during the inpatient stay would not be a reflection of an acute adaptation to a new level of protein intake.

During the first two days of the lead-in period, subjects wore an accelerometer (Actigraph GT1M, Pensacola, FL) to estimate calories expended during activities of daily living. Accelerometers detect motion in both the vertical and horizontal axis and record data in activity counts per minute. If activity counts were less than or equal to 1952 per minute, then the work-energy theorem (Equation 3.2) was used to estimate EE. Meanwhile, the Freedson equation (Equation 3.3) was used to estimate EE when activity counts were greater than 1952 per minute. Subjects were instructed to wear the activity monitor on their waistband at all times except during sleeping or bathing.

Equation 3.2. Work-energy theorem

Kcal/min = 0.0000191*counts/minute*body mass (kg)

Equation 3.3. Freedson equation (136)

Kcal/min = 0.00094*counts/minute + 0.1346*body mass (kg) – 7.37418

Free-living physical activity level (PAL) was calculated using Equation 3.4. Total daily energy expenditure (TEE) was estimated using the concept that the major components of

energy expenditure are resting metabolic rate, diet-induced thermogenesis (DIT), and activity (exercise and non-exercise) thermogenesis (137). Since subjects were sedentary, the EE predicted by the accelerometer was used for activity thermogenesis. The measured value for RMR was used and DIT was estimated to be 10-15% of TEE (138).

Equation 3.4: Physical activity level (PAL).

PAL = Total energy expenditure (TEE)/ Resting metabolic rate (RMR)

TEE = RMR + DIT + activity thermogenesis

Free-living energy balance was calculated using Equation 3.5, with energy intake determined from the diet plans created by the study dietician.

Equation 3.5. Energy balance

Energy balance= (Energy intake – TEE)

Experimental Protocol (Inpatient period)

On the evening before the six-day inpatient study period, subjects were admitted to the CTRC and provided dinner as the final meal of their lead-in diet. The study began the next morning with the start of the study diet and 24-hour urine collection. The first and last days were spent in a whole room calorimeter (Cal room) and the rest of the days were spent in an inpatient room on the CTRC. During the non-Cal room days, subjects were allowed to leave the unit two times during the day for 30 minutes. Subjects were asked to consume only the diet provided to them by the CTRC staff and to exercise only during the one-hour cycling bout prescribed as part of the study protocol. The study diets contained only non-caffeinated beverages and subjects were allowed to request non-caloric non-caffeinated beverages and to consume water ad libitum. Fasted blood draws were obtained on Day 1, Day 3, and Day 6 and daily gowned-weight measurements were taken on the same digital scale every morning.

The study design was randomized crossover, where subjects completed two trials (refer to figure 3.3). Each day, the subjects completed one hour of cycling exercise at an intensity corresponding to 55% of VO_{2max} (as determined by the previously discussed steady state exercise test). The exercise was completed on a Lode Corival bicycle ergometer (Lode, Groningen, The Netherlands) and the average workload of the bouts was 44±8.7 Watts. Subjects wore a heart rate (HR) monitor (Polar FS1, Lake Success, NY) and recorded their own HR every 15 minutes during the exercise bout. The average measured HR during the cycling was 106±8.5 beats per minute. The exercise bout was intended to simulate a brisk walking pace and was tolerated well by all subjects except for one, who had some difficulty completing the full hour of exercise.

A post-exercise beverage was consumed immediately after the cycling exercise. The beverage was either a 248-kcal chocolate milk drink containing 15.3 g PRO, 43.6 g CHO, and 1.3 g FAT (330 g skim milk, 4 g whey protein, and 42 g of chocolate syrup) or a 407-kcal Kool-Aid beverage containing 100 g CHO (57 g polycose, 50 g Kool-Aid, and 350 g water). During the CHO condition, the chocolate milk beverage was consumed at 10:30 and the Kool-Aid beverage was consumed post-exercise at 17:30. The order of the drinks was switched during PRO+CHO, with Kool-Aid consumed at 10:30 and chocolate milk consumed post-exercise at 17:30. The order in which each subject completed the trials was randomized.

Besides the timing of the different beverage types in relation to the exercise bout, the diets during the two trials were exactly the same. The diet plans for Day 1, 2, and 3 were repeated on Day 6, 4, and 5, respectively. Table 3.2 provides a sample inpatient diet plan. The inpatient diet contained 13.5% PRO, 21% FAT, and 65.5% CHO as a percentage of total calories. It was originally intended for the inpatient diet to provide the same macronutrient content as the lead-in diet (15% PRO, 30% fat, and 55% CHO). However, an error in the nutritional information for the Kool-Aid beverage resulted in the provision of an additional 159

grams of CHO daily for each subject. The diets still aligned with the USDA's acceptable macronutrient distribution ranges (AMDRs) [45-65% CHO, 20-35% FAT, 10-35% PRO] (139), but provided additional energy and caused the post-exercise beverages to no longer be isocaloric, as stipulated by the study design. Since the stimulatory effect of carbohydrate depends on the amount ingested (122), the NBAL values in the CHO condition may have been more positive than they would have been if the Kool-Aid were isocaloric with the chocolate milk.

To maintain energy balance, energy intake must be equal to TEE. Therefore, TEE was approximated first and intake was planned based on the estimate. The estimate for TEE in the Cal room was lower than non-Cal room days because ambulatory activity levels are lower when restricted to the Cal room. To determine EE on the Cal room days, RMR was multiplied by 1.35, an activity factor previously used by the Cal room investigators (Melanson, unpublished observations). Then, exercise EE was estimated from steady-state VO_2 results and an additional 20% of exercise EE calories were added to account for excessive post-exercise oxygen consumption (EPOC) (Melanson, unpublished observations). Equation 3.6 approximates TEE during the Cal room days. A higher activity factor of 1.45 was used to estimate TEE on the days not spent in the Cal room, as subjects were able to move around the CTRC and the inpatient building. Equation 3.7 represents EE on non-Cal room days. Thus, intake was higher on non-Cal room days (see Table 3.2).

Equation 3.6. Estimation of energy expenditure in the calorimeter room

EE= (1.35*RMR) + (exercise EE + 0.2*exercise EE)

Equation 3.7. Estimation of energy expenditure on non-calorimeter room days

EE= (1.45*RMR) + (exercise EE + 0.2*exercise EE)

Table 3.2. Sample inpatient diet plan.

Day	Energy intake (kcal)	Diet: Breakfast (B), Snacks (S), Lunch (L), Dinner (D)
1 (CHO) Cal room	1896	B- Oatmeal , strawberries, almonds, toast and butter S- Chocolate milk L-Salad, grilled chicken, cheese , crackers, orange S- Kool-Aid D-Salmon stir fry, rice, cantaloupe
2 (CHO)	1988	B-English muffin, butter, honey, strawberries S- Chocolate milk L-Turkey sandwich, baby carrots, chips, apple S- Kool-Aid D-Chicken breast, baked potato, salad, crackers
3 (CHO)	1972	B-Toast, peanut butter, honey, strawberries S-Chocolate milk L-Egg salad sandwich, crackers, baby carrots, grapes S- Kool-Aid D-Spaghetti, meat sauce, salad, canataloupe
4(PRO+CHO)	1988	B- English muffin, butter, honey, strawberries S- Kool-Aid L- Turkey sandwich, baby carrots, chips, apple S- chocolate milk D- Chicken breast, baked potato, salad, crackers
5(PRO+CHO)	1972	B- Toast, peanut butter, honey, strawberries S- Kool-Aid L- Egg salad sandwich, crackers, baby carrots, grapes S- chocolate milk D- Spaghetti, meat sauce, salad, canataloupe
6(PRO+CHO) Cal room	1896	B-Oatmeal , strawberries, almonds, toast and butter S- Kool-Aid L- Salad, grilled chicken, cheese , crackers, orange S- Chocolate milk D- Salmon stir fry, rice, cantaloupe

Prior to each day spent in the Cal room, the O_2 and CO_2 analyzers were calibrated using ambient air (CO_2 = 0.03% and O_2 = 20.93%) and a known gas mixture (CO_2 =0.9%, O_2 =20.1%). Subjects entered the room at 07:45 on the morning of Days 1 and 6 and exited at 07:15 the following morning. The 12'x12' room contained a bed, toilet and sink, computer desk, television, and bicycle ergometer. Subjects were instructed not to nap during the day as standard protocol for the calorimeter room. To prevent air from escaping, meals were passed through an air lock that could not be simultaneously opened from the inside and outside of the Cal room. Daily EE and substrate oxidation were determined by the difference in gas content (O_2 and CO_2) that was entering and exiting the room. Ambient air was constantly drawn into the room. The air exiting the room was comprised of the gases consumed and expelled by the resident subject. An infrared analyzer (ABB, Houston, TX) was used to measure CO_2, while a paramagnetic analyzer was used to measure O_2 (Siemens, Norcross, GA). These values were corrected for temperature, relative humidity, and barometric pressure. Urine N was used to calculate 24-hour protein oxidation, with 1 g of urine N reflecting 6.25 g of oxidized protein. Equations from Jequier et al. were used to calculate TEE and substrate oxidation from oxygen consumption and the respiratory quotient (RQ) (140). All measurements were taken as one-minute averages and recorded to a data file.

Subjects also wore an accelerometer (Actigraph GT1M, Pensacola, FL) during the inpatient stay, which was removed during cycling exercise, sleep, and showering. Data from the accelerometer allowed estimation of EE from activity (see Equations 3.2 and 3.3). Twenty-four hour EE of non-calorimeter days was estimated using activity EE, previously measured RMR, and EE from both DIT and exercise as measured in the Cal room (values taken as an average of the two days spent in the room). Physical activity level (PAL) and non-exercise PAL were calculated

using Equations 3.4 (see above) and 3.8, respectively. The value for exercise EE was multiplied by 1.2 to account for the additional energy of EPOC (20% of exercise EE).

Equation 3.8: Non-exercise PAL.

Non-exercise PAL = (Daily EE - Exercise EE*1.2)/RMR

Energy balance for the inpatient period was determined using Equation 3.5. Again, energy intake was determined from the diet plans created by the study dietician. If a subject left any study foods unconsumed, CTRC staff weighed the leftovers and the value was reported to the dietician to allow accurate diet analyses. TEE was measured on the Cal room days and estimated on the non-Cal room days as described above.

Nitrogen Balance

To determine urinary nitrogen during the inpatient stay, 24-hour urine samples were collected, beginning with the first voiding on Day 1. Urine was collected in acid, total volume was measured, and two 10-mL aliquots were frozen and stored for later analysis. The samples were analyzed for nitrogen by a chemiluminescent technique (141) using an Antek 7000 Elemental Nitrogen Analyzer (PAC, Houston, TX). Nitrogen balance was calculated using the method described by Friedlander et al. (5), using Equation 2.1. Since 6.25 grams of protein contain one gram of nitrogen, N intake was calculated as [protein intake (g)/6.25]. Daily miscellaneous N losses and fecal N losses were estimated at 5 mg·kg body weight^{-1} and 2 g, respectively (77). The NBAL during the PRO+CHO and CHO conditions was compared to test the hypothesis that timing milk after aerobic exercise improves nitrogen balance in older adults. Urinary creatinine was analyzed to measure completeness of urine collections. Collection was considered complete if daily creatinine was within 2 standard deviations of each subject's mean value.

Post-testing

Within one week after the inpatient period, subjects reported to CSU and underwent a DEXA scan and a final height and weight measurement.

Statistical Analysis

Nitrogen balance data, Cal room oxidation data, and pre and post body weight/body composition measurements were analyzed using student's paired *t*-tests. Pearson's correlation coefficients (r) were used to determine any correlations between NBAL and energy balance or NBAL and PALs; shared variance (R^2) was also calculated for the correlations. Diet-related variables (free-living, lead-in, inpatient energy and macronutrient intake) and longitudinal body weight were analyzed using one-way repeated measures ANOVA and multiple regressions, respectively. Then, the Student Newman-Keuls post-hoc test was used to determine where differences occurred. Variables were tested at a level of significance of $p<0.05$, which allows only 5% of the measured difference to be accounted for by random chance and indicates that a true difference exists. Data is presented as mean±standard error of the mean (SEM) unless indicated otherwise.

CHAPTER 4

RESULTS AND DISCUSSION

Energy and macronutrient intake

The study diets were intended to be representative of USDA Nutritional Guidelines (139) and relatively normal for subjects in terms of types of food provided. Total calories and macronutrient intake during free-living (from 3-day self-reported diet records), lead-in, and inpatient diets are provided in Figure 4.1 and 4.2. The increase in energy during the inpatient diet was to account for the energy expenditure of exercise. However, the average measured exercise EE was only 256±41 kcal, representing a net caloric cost of 204±37 kcal. A surplus of 160 kilocalories (in the form of CHO) provided during the inpatient diets was due to an error in the nutritional information for the Kool-Aid beverage. The error increased absolute and relative carbohydrate intake during the inpatient stay.

Figure 4.1. Energy intake during free-living, lead-in, and inpatient diets. *****Significantly different than free-living ($p<0.001$). **†**Significantly different than lead-in ($p<0.01$).

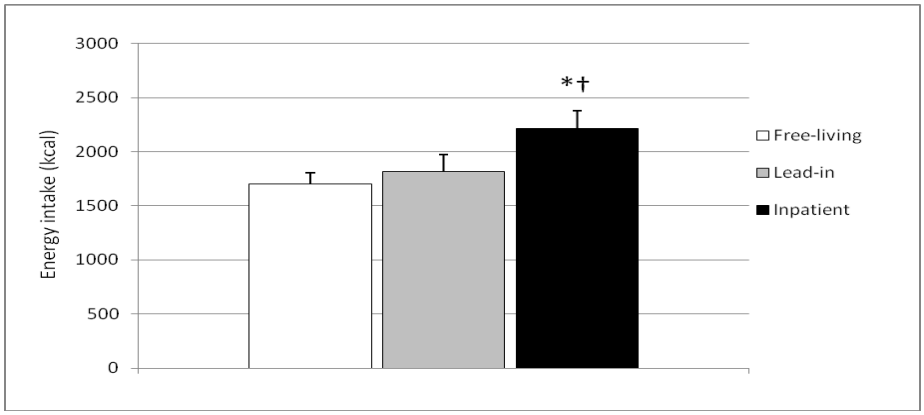
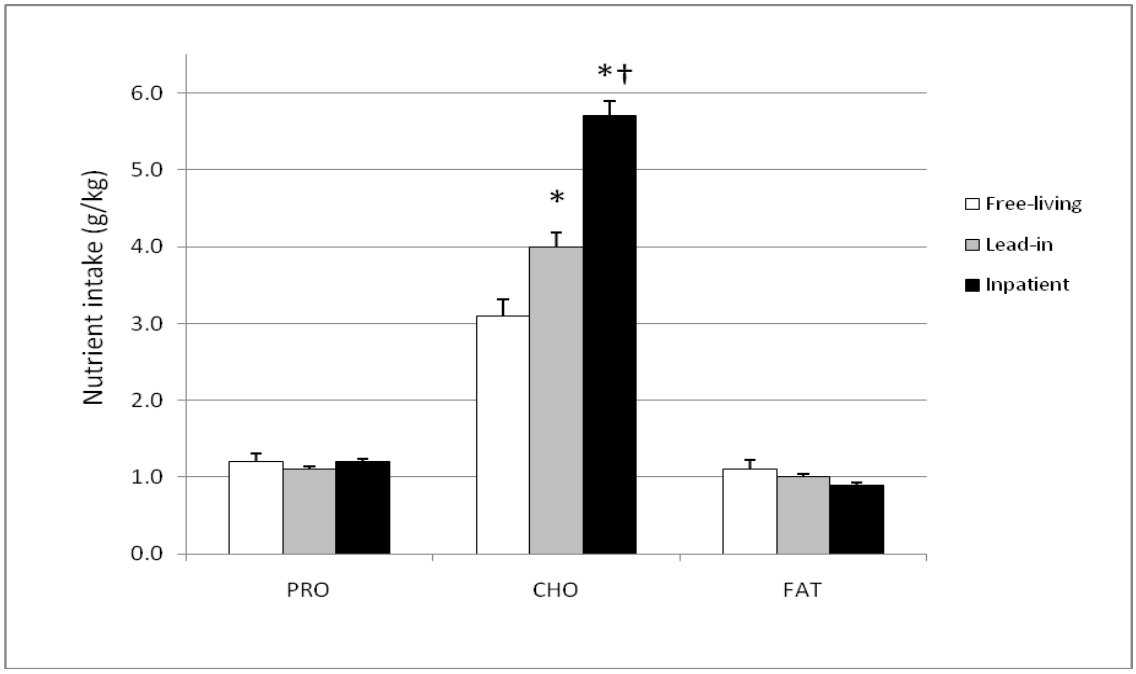


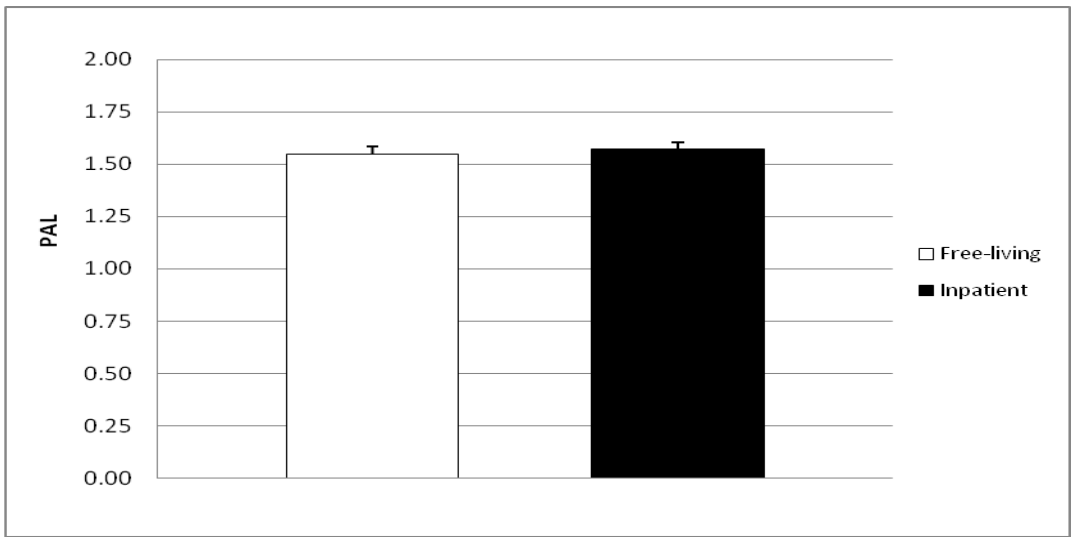
Figure 4.2. Macronutrient intake during free-living, lead-in, and inpatient diets. *Significantly different than free-living ($p<0.01$). †Significantly different than lead-in ($p<0.01$).



Energy expenditure

Free-living PAL and inpatient PAL were not significantly different (Figure 4.3).

Figure 4.3. PAL during free-living and inpatient periods.

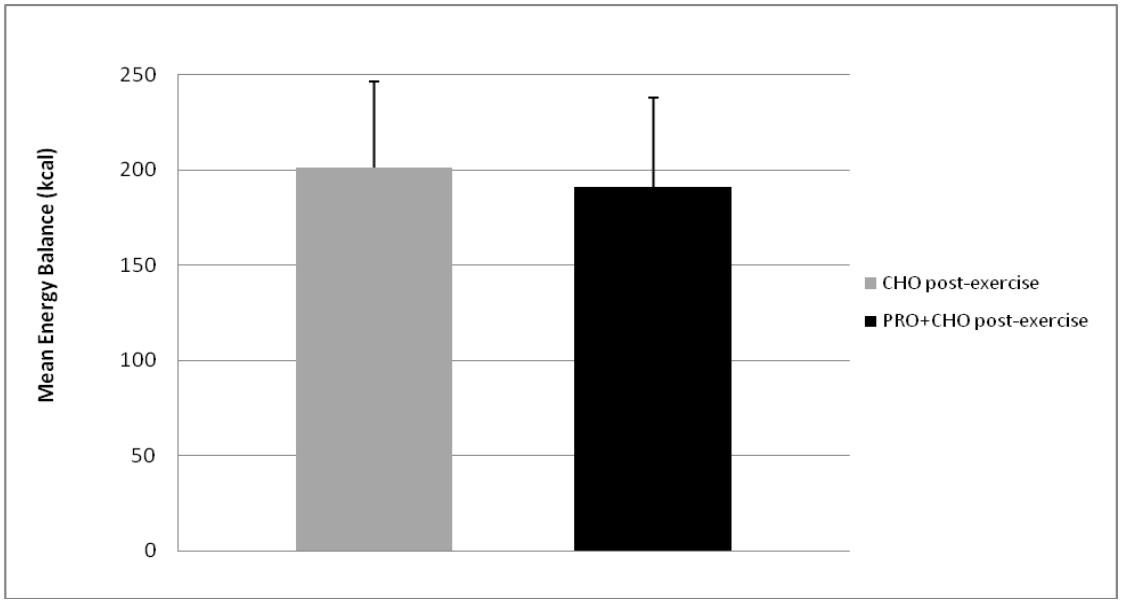


During the inpatient stay, PAL was consistent across conditions (1.60±0.04 in CHO and 1.61±0.04 in PRO+CHO, p=0.60). The same was true for non-exercise PAL (1.36±0.02 in CHO and 1.37±0.02 in PRO+CHO, p=0.60).

Energy balance

During the lead-in diet, estimated mean daily energy balance was -103±66.4 kcal. Subjects were near energy balance during the inpatient stay, with a mean daily energy balance of 196±21 kcal. Additionally, energy balance was consistent between the PRO+CHO and CHO trials (Figure 4.4). There was no correlation between energy and nitrogen balance (data not shown).

Figure 4.4. Three-day mean energy balance during PRO+CHO and CHO.

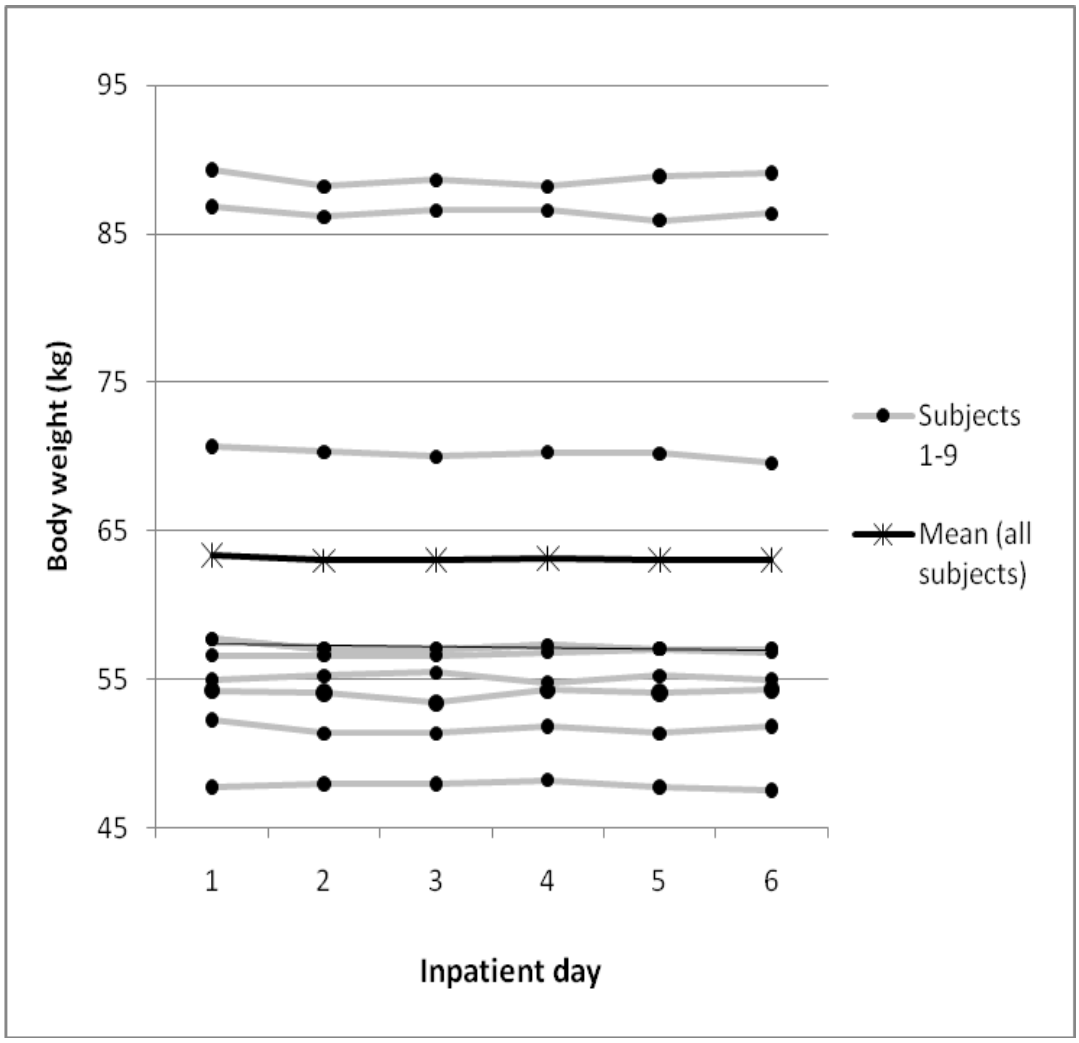


Weight and body composition

Body weight did not change from pre (69.5±6.9 kg) to post (69.2±6.9 kg) (p=0.33). However, body fat percentage decreased slightly from pre (31.7±2.4%) to post (30.6±2.1%)

along with an increase in FFM (pre: 43.4±4.4 kg and post: 43.9±4.5 kg) ($p<0.05$). The tracking of body weight during the inpatient period is shown in Figure 4.5. Mean body weight did not change during the inpatient stay, indicating that energy balance was maintained.

Figure 4.5. Daily body weight during inpatient period for individual subjects and group.



Nitrogen Balance (NBAL)

Three-day average NBAL was 57% higher during the CHO+PRO condition than the CHO condition (Figure 4.6). Mean nitrogen balance data of all subjects is presented in Figure 4.7.

Figure 4.6. Three-day mean nitrogen balance during PRO+CHO and CHO trials *(p<0.05).

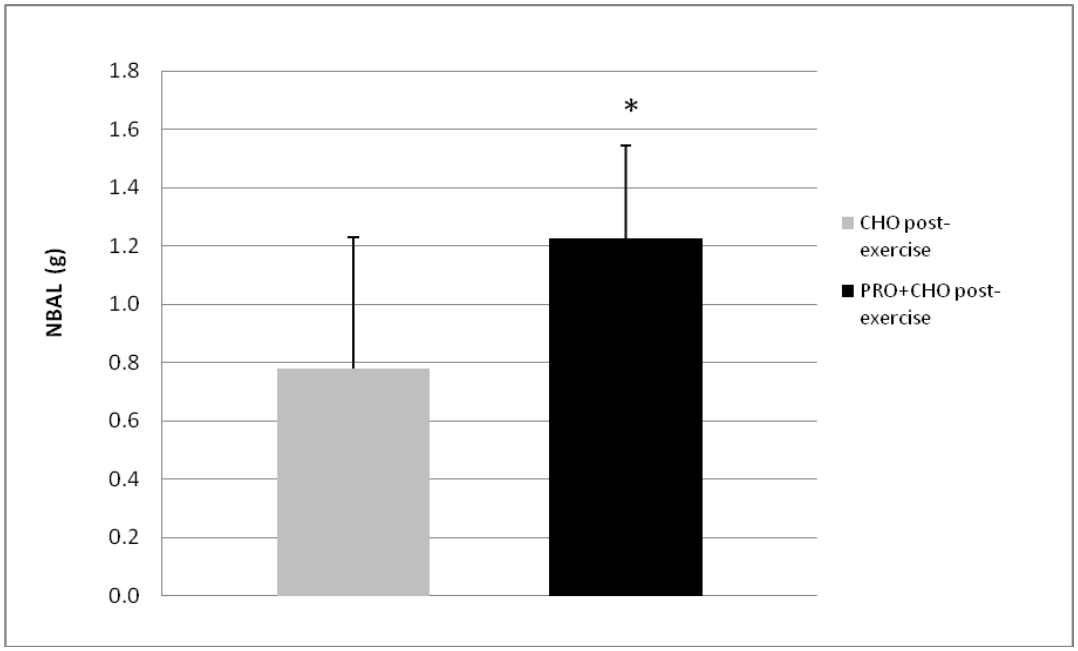
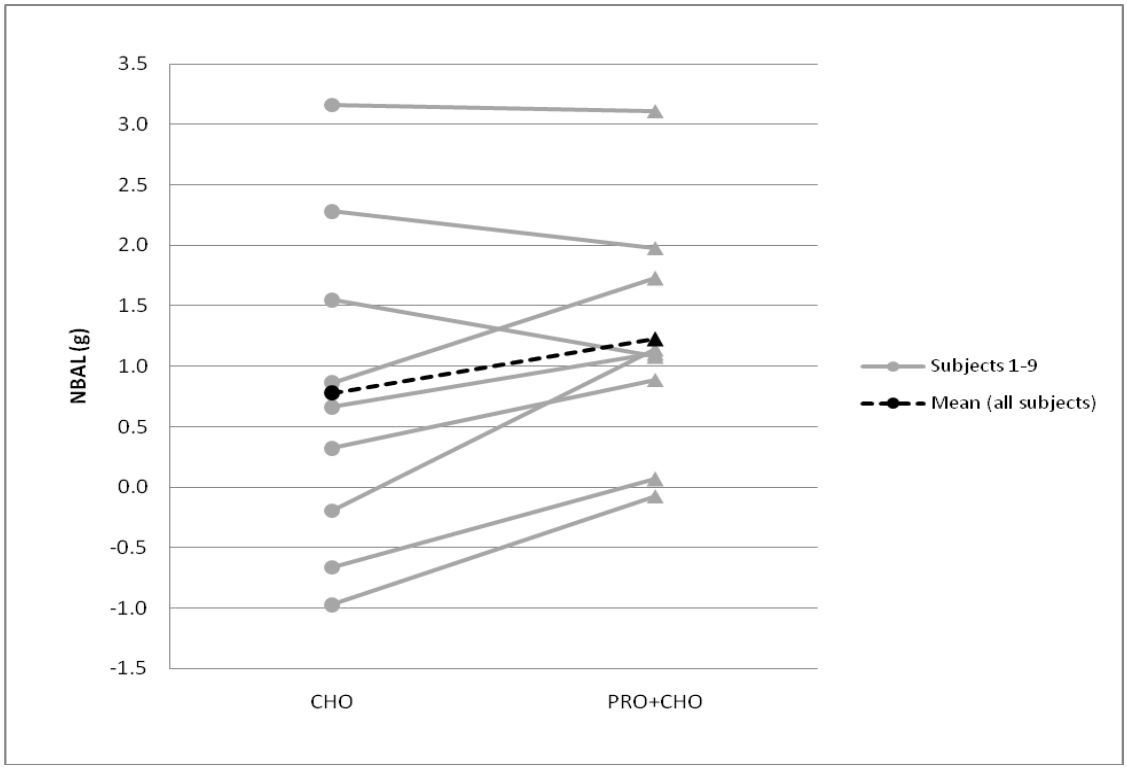
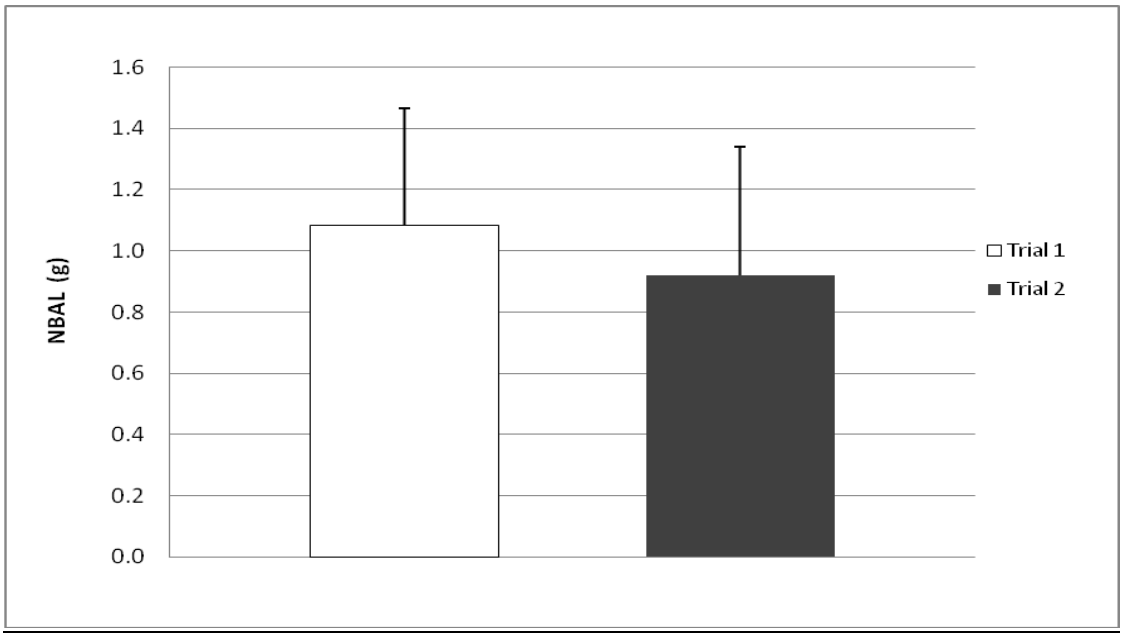


Figure 4.7. Three-day mean nitrogen balance of individual subjects and group during PRO+CHO and CHO.



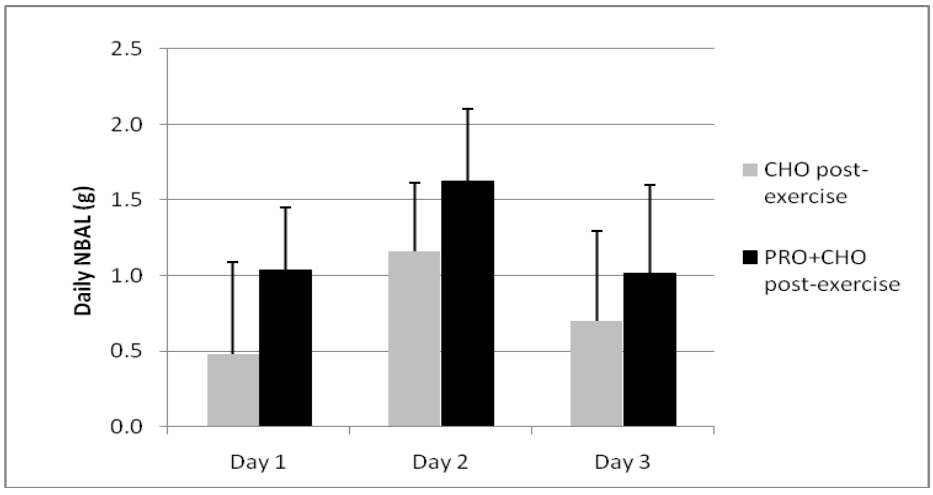
Nitrogen balance was not different between the time-ordered first and second trials completed (Figure 4.8).

Figure 4.8. Three-day mean NBAL of Trial 1 (inpatient Days 1-3) and Trial 2 (inpatient Days 4-6).



Average NBAL on Day 1, Day 2, and Day 3 of the PRO+CHO condition was not significantly different than average NBAL on Day 1, Day 2, and Day 3 of the CHO condition, respectively (Figure 4.9). However, when taken as a three-day mean, NBAL was significantly higher in the PRO+CHO condition (shown in Figure 4.6).

Figure 4.9 Mean NBAL on day 1, 2, and 3 of each trial



Urinary Creatinine

Daily urinary creatinine values were within two standard deviations of each subject's mean (Table 4.1). Thus, urinary collections were considered complete for all subjects.

Table 4.1 Daily and mean urinary creatinine for all subjects

Subject #	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Mean	Standard deviation
1	920	819	819	911	812	853	856	49
2	788	888	814	660	783	776	785	74
3	1472	1891	1870	1525	1754	1774	1714	176
4	953	966	1088	1101	675	516	883	236
5	766	910	901	1136	666	756	856	166
6	751	839	780	802	866	742	797	49
7	1851	1593	1732	1798	1788	1846	1768	96
8	826	819	837	800	740	792	802	35
9	Missing	898	929	608	850	876	832	129

Calorimeter room oxidation

TEE and energy balance in the calorimeter room were not different between the two trials. Energy expenditure from individual macronutrients was consistent across trials (Figure 4.10). The daily respiratory quotient (RQ) in PRO+CHO was 0.90±0.02, which did not differ significantly from 0.91±0.02, the RQ in CHO. There were also no significant differences in the balance (balance = intake-oxidation) of individual macronutrients between the two trials (Figure 4.11).

Figure 4.10. One-day mean oxidation of macronutrients in Cal room during PRO+CHO and CHO.

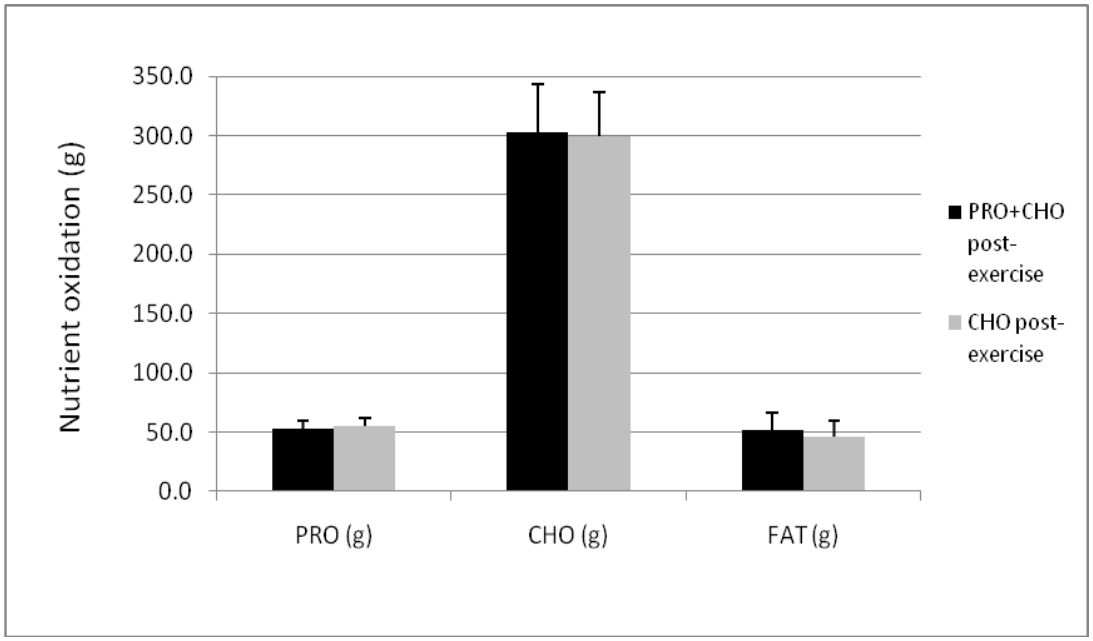
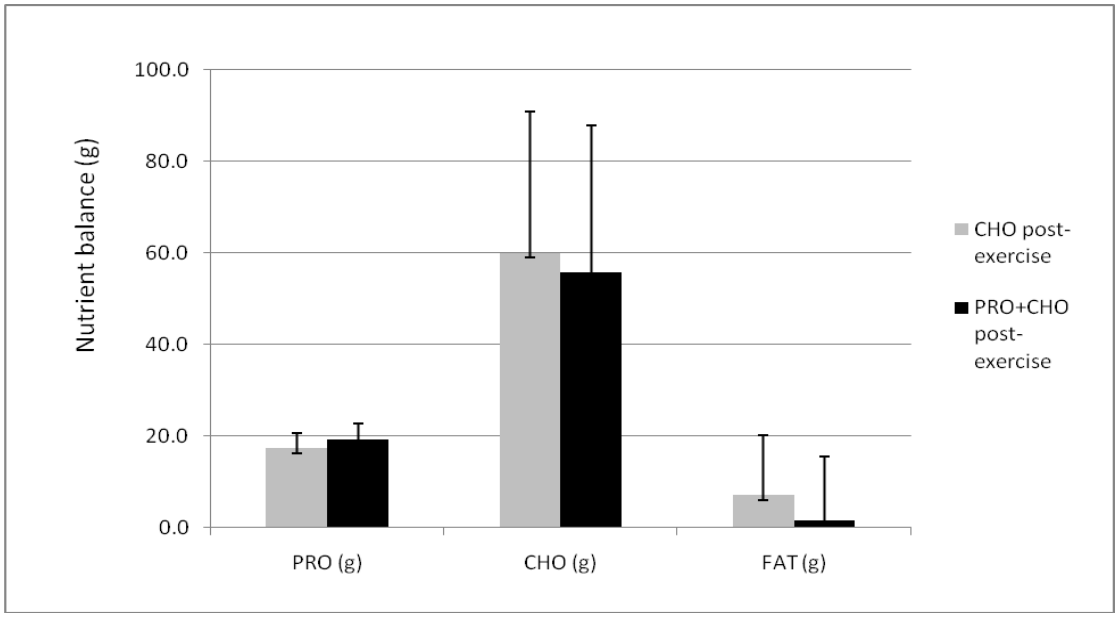


Figure 4.11. One-day macronutrient balance during PRO+CHO and CHO in Cal room.



DISCUSSION

In the current study, older subjects completed two, three-day periods in which an isocaloric and isonitrogenous diet was consumed with only the timing of protein consumption in relation to an exercise bout differing between the three-day periods. The primary finding of this study was that when protein was consumed immediately after moderate-intensity exercise rather than earlier in the day, NBAL was more positive. These results indicate that the timing of protein nutrition is as important as quantity of dietary protein.

The premise of the NBAL method is that since protein is the main nitrogen-containing compound in the body, positive NBAL reflects positive protein balance and conversely, a negative NBAL reflects negative protein balance. When chocolate milk (PRO+CHO) was consumed post-exercise, protein balance was more positive than when the chocolate milk was taken in the morning and Kool-Aid (CHO) was consumed post-exercise. Thus, greater accretion of body protein occurred when PRO+CHO ingestion was timed immediately after exercise, aligning with previous research on protein supplementation (14, 109). If the magnitude of positive NBAL remained consistent in the long term, protein accretion would continue, albeit at a lesser rate in CHO than PRO+CHO. Over a one month period, the daily NBAL during the CHO (0.8 ±0.32 g N) trial would result in a protein accretion of 150 g (0.8 g N·day^{-1} x 6.25 g PRO ·g N^{-1} x 30 days). Meanwhile, the NBAL of the PRO+CHO trial (1.2 ±0.45 g N) would theoretically translate into a body protein accretion of 234 g (1.2 g N·day^{-1} x 6.25 g PRO ·g N^{-1} x 30 days). During one year under the same conditions, 1.8 kg and 2.7 kg of body protein would be gained with CHO and PRO+CHO treatment, respectively. Such a gain seems reasonable, considering that 1.5 kg of FFM can be gained after 24 weeks of resistance training in women over the age of 60 (142).

However, body protein mass does not continue growing indefinitely and NBAL adapts when a ceiling for body tissue mass is reached (88). For example, during conditions of strenuous exercise and a 30% energy surplus, NBAL becomes acutely positive and then diminishes over time (88). Still, based on the current study, there is greater potential for NBAL to remain positive when chocolate milk is consumed immediately after exercise. For a gradual process like sarcopenia, preventing muscle loss may be as important as building muscle mass. Maintaining slight positive protein balance over time may be enough to prevent the gradual loss of body protein characteristic of aging.

Because no direct measurements of protein synthesis or breakdown were made during this study, it cannot be concluded whether the positive protein balance resulted from increased synthesis, decreased breakdown, or a combination thereof. Also, conclusions cannot be drawn about where the protein accrual occurred. However, based on previous research, it is surmised that muscle protein synthesis increased in response to the exercise stimulus (100) and the consumption of leucine-containing chocolate milk optimized this response by increasing MPS beyond the level reached with carbohydrate alone (115). Short-term positive muscle protein balance acutely stimulated by exercise and EAA ingestion reflects the 24-hour response (143). If muscle FSR remains heightened throughout the day (143), nitrogen-containing AAs continue to be utilized for MPS and less AA oxidation and nitrogen excretion should occur. From the NBAL equation (Equation 2.1), decreased nitrogen excretion results in a more positive NBAL.

The study diets followed USDA AMDRs for the percentage of total kilocalories that should come from each macronutrient: 45-65% CHO, 20-35% FAT, 10-35% PRO (139). Inpatient protein intake was 1.2 $g \cdot kg^{-1}$, which is above the 0.8 $g \cdot kg^{-1}$ recommendation for older individuals, but representative of a typical protein intake for this population (144). Indeed, mean self-reported habitual protein intake was 1.2±0.1 $g \cdot kg^{-1}$ and was maintained by the study diet. Thus,

the lead-in period might have been unnecessary for some subjects, but allowed an adaptation period for those with higher or lower free-living protein intakes.

Whether protein recommendations for older adults should be higher than 0.8 $g \cdot kg^{-1}$ remains controversial (6, 7, 126, 127). Regardless, older individuals are less sensitive to AA nutrition with blunted MPS and muscle protein accretion when provided the same amount of EAAs as their younger counterparts (36, 145). Certain types of protein nutrition can improve the anabolic response of muscle and overall protein balance. The chocolate milk beverage was chosen as a protein source for this study because milk is a rich source of the EAA leucine, which enhances MPS through the mTOR pathway (146). Elderly muscle is resistant to MPS stimulation by EAAs in the postabsorptive state (36), but adding a higher proportion of leucine allows MPS to be maximally stimulated in older individuals (125). Additionally, milk contains both fast and slow acting proteins, with the potential to acutely increase MPS with whey protein and to stimulate a longer-lasting suppression of MPB with casein (116). The milk beverage in our study consisted of 11.3 g PRO in skim milk, supplemented by 4 grams of whey protein. Based on the EAA and leucine content of previously used milk (111) and whey protein (147) interventions, this beverage contained approximately 6.8 g EAAs with 1.7 g leucine. Although ~7 g EAAs does not maximally stimulate MPS in older individuals (145, 148), the combination of a leucine-containing protein source with exercise was a sufficient stimulus to make protein balance more positive when diet provided adequate protein and energy.

The daily exercise bout completed by subjects was 60 minutes of moderate intensity (55% VO_{2max}) cycling, fulfilling ACSM recommendations for cardiorespiratory exercise (134). The exercise was generally well-tolerated and was realistic in terms of its contribution to total daily energy expenditure, as free-living and inpatient PAL were similar and characteristic of older populations (135). Exercise at 55% of VO_{2max} simulates a brisk walking pace and provides an

effective anabolic stimulus, as aerobic exercise at only 40% of VO_{2max} spurs increased MPS (100). Over the long term, progressive endurance training programs stimulate muscle hypertrophy (149), indicating that protein balance has become more positive. Indeed, aerobic exercise causes more positive NBAL due to increased N retention during energy balance (82, 88). The exercise bout in the study was potent enough to provide an anabolic stimulus, but attainable in terms of time, intensity, and expenditure. Also, since older individuals most commonly choose walking as a form of exercise, the proposed exercise intervention is a practical strategy for this population.

Although exercise and protein nutrition have independent effects on muscle protein kinetics and NBAL, this study was concerned with the combination of the two, with particular attention paid to the timing of the protein intake. MPS is stimulated when milk is ingested after resistance exercise (150). Additionally, MPS remains elevated longer when EAAs and exercise are combined (73) compared to either treatment alone (107, 108). The positive NBAL that resulted from timing milk intake just after exercise in this study reflected such a synthetic response. The influence of timed post-exercise nutrition on NBAL was elucidated by Roy et al. (151). Young female athletes underwent two seven-day trials in which they increased their training volume and either consumed a mixed-meal beverage (POST) or a non-caloric placebo beverage (CON) after exercise. As in our study, diet was replicated during the two trials, so the timing of the beverage was the only difference. Intake was not adjusted for the energy expenditure of exercise, so subjects lost weight over both study periods. Even with the caloric deficit, there was a strong trend ($p=0.06$) for NBAL (measured only on Day 5 and Day 6 of each trial) to be more positive in POST than CON. Also, body mass declined by only 0.7±0.2 kg in POST compared to 1.4±0.4 in CON. This shows that increased exercise levels combined with post-exercise mixed-meal nutrition can influence NBAL to become more positive and preserve

body mass, even in a small timeframe (151). Such short-term changes can carry into the long term, as muscle-building adaptations are optimized by immediate post-exercise ingestion of protein nutrition (109).

While carbohydrate alone after exercise increases MPS (152), protein and carbohydrate together augments the stimulus (153). The stimulatory effect of carbohydrate alone depends on the amount of carbohydrate provided (122). Thus, the unintended additional carbohydrate provided by the Kool-Aid beverage might have stimulated MPS in the CON condition above the level that would have been observed if the Kool-Aid was isocaloric with the chocolate milk. NBAL would expectantly be less positive in CHO if the Kool-Aid beverage contained fewer calories. Despite the additional energy immediately post-exercise during CHO, nitrogen balance was still significantly more positive in PRO+CHO.

In terms of energy balance, the 159 surplus kilocalories provided by the Kool-Aid beverage caused a slight positive energy balance, yet subjects were still within 200 kcal of equilibrium. Generally, positive energy balance results in more positive nitrogen balance (80). If each additional kcal of energy results in retention of 1.21 mg N as suggested by Butterfield and Calloway (88), the NBAL in PRO+CHO and CHO would have been 1.0 mg N and 0.5 mg N, respectively, without the excess energy. Thus, NBAL would remain more positive in PRO+CHO than CHO. However, our results conflict with those of Butterfield and Calloway, as there was no relationship between energy balance and NBAL ($r=-0.15$, not significant). Even if this weak relationship was statistically significant, its direction was opposite of what would be expected (i.e. nitrogen balance becomes more positive concomitantly with energy balance). Seemingly, subjects in the study were so consistently near energy balance that nitrogen balance remained unaffected, or there was not enough scatter to determine a significant relationship.

Calorimeter room oxidation

The calorimeter room data allowed for exact energy balance measurement on one day during each condition, while energy balance on the remainder of the inpatient days was estimated. The calorimeter room oxidation of total energy in PRO+CHO was not different from CHO, so we were able to conclude with greater confidence that energy balance was consistent across conditions. Oxidation of individual macronutrients also remained constant, so substrate utilization did not change across Cal room days.

Limitations

NBAL requires an adaptation period in response to certain lifestyle changes, so the interpretation of short-term studies is sometimes questioned (154). However, the use of a lead-in period ensures that equilibration has already occurred. It was intended for the inpatient diet to contain the same macronutrient breakdown as the lead-in diet, but due to the error with the Kool-Aid beverage, the absolute and relative amount of carbohydrate was higher during the inpatient diet. Importantly, absolute protein intake of the lead-in and inpatient diets was not different, so the lead-in period was successful. Also, the calculation of mean NBAL values accounts for inter-individual variation in daily NBAL (144) and short-term NBAL results can predict long-term nitrogen status (154). Generally, a 3-5 day study period for each condition is long enough to yield stable NBAL results (155).

Next, there may have been some errors in the calculated NBAL values. Since N intake was consistent between the two trials and constants were used for fecal (2 $g \cdot day^{-1}$) and miscellaneous (5 $mg \cdot kg$ body $weight^{-1} \cdot day^{-1}$) excretion, urine N excretion was the only variable that changed NBAL values across conditions. Some subjects might have had higher or lower fecal and miscellaneous excretions. However, the constants were used due to the difficulty of

complete measurement of these excretions. The validity of the values is established (77) and the constants have been used by other researchers for NBAL measurement (5).

Another potential source of error in the NBAL calculations was the N intake provided by the study diets. The standard of 1 g N for every 6.25 g protein was used to determine N intake. For example, if a subject consumed 65 g PRO, then N intake= 65/6.25=10.4 g N. Sometimes the N content of food varies, so N intake might differ from the calculated value. It is also possible that study foods were not completely consumed, like scrapings on plates and utensils. However, subjects were instructed to ingest all foods and the inpatient diet was monitored closely to ensure that all food and beverage was consumed.

Study conditions in each trial were consistent, including study diet, exercise bouts, PAL, and energy balance. Therefore, all sources of error related to NBAL would be the same across conditions and makes it reasonable to compare the PRO+CHO and CHO trials.

Protein and exercise recommendations for older adults

Numerous exercise and nutrition interventions to delay or diminish muscle wasting have been studied with varying results. Increasing protein recommendations for the elderly has been proposed for the prevention of sarcopenia (156). However, it is inconclusive whether elevating protein intake above current guidelines is necessary to maintain muscle mass. Varying responses to the recommended 0.8 $g \cdot kg^{-1}$ protein intake have been observed, with NBAL either declining (6, 126) or being maintained (7). Rather than recommending an elevated total protein intake, Paddon-Jones and Rasmussen surmise that ingesting 20-30 grams of protein at each meal will preserve muscle mass by maximally stimulating MPS at multiple times during the day (8). The current intervention proposes combined nutrition and exercise to stimulate a longer-

lasting anabolic response. Protein and carbohydrate-containing nutrition after exercise elevates MPS, sparing body protein/nitrogen (153).

As previously mentioned, strategies to prevent the loss of body protein may be sufficient to thwart the effects of sarcopenia, particularly if implemented early in aging populations. Since sarcopenia is such a gradual process, more subtle strategies were examined for increasing protein balance and maintaining (not necessarily increasing) muscle mass over time. This study confirmed that the consumption of milk after moderate intensity aerobic exercise improves nitrogen and protein balance. The hour-long cycling exercise bout (55% of VO_{2max}) was generally well-tolerated and could be replicated in a free-living setting simply by going out for a brisk walk. Important for exercise adherence is the fact that walking is the most popular form of exercise in the elderly (10). Also, milk is a widely available and commonly used source of protein. The proposed intervention utilizes readily available forms of exercise and protein nutrition, specifying only that the protein source be consumed immediately following the exercise bout.

CHAPTER 5

CONCLUSION AND RECOMMENDATIONS

This study examined how timing a protein source in relation to an exercise bout affected whole body protein balance. It was hypothesized that in subjects in energy balance, timing a protein-containing milk beverage after moderate intensity aerobic exercise would increase nitrogen balance compared to a carbohydrate-only Kool-Aid beverage. Indeed, nitrogen balance was 57% higher when milk was consumed post-exercise. Thus, a rethinking of protein recommendations for older adults is proposed that goes beyond absolute quantity. Due to the potential for long term positive protein balance and maintenance of muscle mass, the timing of a high quality protein source following exercise should be included as part of current protein guidelines for the elderly. This strategy likely works by optimally increasing muscle protein synthesis through exercise and immediate consumption of nutrition containing EAAs (particularly leucine). If more nitrogen-containing AAs are utilized for MPS, then fewer will undergo oxidation and excretion. Therefore, milk consumption after exercise provides a possible means for preventing sarcopenia by increasing nitrogen and protein balance, particularly in the muscle.

In the future, it would be prudent to study whether positive nitrogen balance can be maintained long term when milk is consumed after exercise. Since nitrogen balance may begin to equilibrate with aerobic training, variables like exercise intensity and duration can be manipulated to spur further adaptations. Future studies could also examine if timing protein

after exercise ameliorates the negative nitrogen balance associated with negative energy balance or inadequate protein intake. The nutrition intervention could also be changed to include higher whey/leucine content. A longitudinal cohort study would be ideal to test whether implementing the proposed intervention around the age of 50 could actually prevent symptoms of sarcopenia, but such a study would be difficult to administer. Nonetheless, milk consumption after aerobic exercise represents an easy to follow strategy for older individuals to improve nitrogen balance, with possible implications for preserving muscle mass.

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APPENDIX A

Consent Form Approval

Date: _____ Valid For Use Through:_____

SUBJECT CONSENT FORM

COLORADO MULTIPLE INSTITUTIONAL REVIEW BOARD
Protocol #08-0640

And CSU IRB
Protocol # 08-187H

"Consumption of milk after physical activity - rethinking protein recommendations in older individuals"

PRINCIPAL INVESTIGATOR: Edward L. Melanson, Ph. D. and Benjamin F Miller, PhD

Version #1
Last Updated: June 12, 2008

You are being asked to be in a research study. This form provides you with information about the study. A member of the research team will describe this study to you and answer all of your questions. Please read the information below and ask questions about anything you don't understand before deciding whether or not to take part.

Why is this study being done?

This study plans to learn more about how to prevent muscles from wasting with the aging process. We would like to use a simple strategy using exercise and

nutrition. What this study seeks to determine is when you have protein can be just as important as how much protein you receive. In the two study periods what you eat will be the same, but when you eat it will be different.

You are being asked to be in this research study because you are a healthy individual aged 55-75.

Other people in this study

Up to 40 people from your area will participate in the study.

What happens if I join this study?

If you join the study, you will first complete a physical screening including a screening for heart disease at Colorado State University. We will ask you to answer some questions about your past and current participation in exercise. Your body weight, height, and body composition will be measured by laying in a bed and being scanned. Your gender and date of birth will also be recorded. A 3 tsp sample of blood will be taken for screening purposes. Before undergoing the sampling procedures, you will be asked a few questions relating to your present state of health, current medication and past medical history. This is to exclude the presence of any condition or medication that might prolong your bleeding time or make the blood sampling unsafe for you. This visit will take approximately 30 minutes.

You will also undergo cardiac screening in the presence of a cardiologist. This will involve the placement of ten collecting electrodes on your chest that will be connected to an electrocardiogram (ECG). You will then be asked to walk on a treadmill, slowly at first and progressively faster until the cardiologist asks you to stop. This test will take approximately 45 minutes. If anything adverse is found in any of the medical screening, you will be advised.

In the seven-day period leading up to the study, we will provide you with all of your food from the Department of Food Science and Human Nutrition at CSU. The food will be normal food tailored to your diet, but we ask that you only consume this food.

For the study, you will report to the general clinical research center (GCRC) at the Colorado Health Sciences Center the night before the start of the study and sleep over night. You will then remain in the GCRC for six days. During this stay you will be asked to exercise once per day for one hour on a bike. The exercise

intensity will be slightly more than that required to complete a brisk walk. During the entire stay you will also be fed a controlled diet that will be similar to your normal calorie and protein intake and you will wear a monitor to measure the amount of activity. You will receive a protein supplement either in the morning or after your exercise bout and when you receive the protein supplement will switch half way through the stay. Throughout the study all your urine will be collected for analysis. In addition, we will collect a small blood sample (2 tsp) at three different times. For a 24 hr period at the beginning of the study and at the end of the study, you will reside in a room that will measure the rate your body is using energy. This room contains everything you will need for normal living, but is specially designed for our measurements. After you exit the special room on the last day, you will receive one more body scan for body composition.

In total your commitment to the study is a half-day for screening, seven days with normal living and food provision, and a six-day (seven-night) stay at the GCRC.

What are the possible discomforts or risks?

In this study we will need to get a total of about 9 teaspoons of blood from you. We will get blood by putting a needle into one of your veins and letting the blood flow into a glass tube. You may feel some pain when the needle goes into your vein. A day or two later, you may have a small bruise where the needle went under the skin. If you have not participated in a regular exercise program before, you may experience some discomfort with the exercise bout including muscle soreness or labored breathing. You may experience discomfort (boredom) associated with a prolonged stay in our facility and special room. However, all efforts will be made to ensure your comfort including access to television, internet, and daily exercise.

Other possible risks include a small risk (less than 1 in 10 000) of death due to a cardiac event during exercise screening. There are also risks of fatigue and muscle strains. You may experience temporary breathlessness or dizziness towards the end of the test. These feelings are transient and pass once the test is finished. During your daily exercise there is also a risk of cardiac complications but in individuals with good cardiac health this risk is extremely low (1 in 1 000 000).

As part of this study we will perform two DEXA scans of your body. DEXA is a way of looking inside the body by using X-rays. X-rays are a type of radiation. Your natural environment has some radiation in it. This DEXA will give you about the same amount of radiation that you would get from your environment in four days.

This study may include risks that are unknown at this time

What are the possible benefits of the study?

This study is designed for the researcher to learn more about potential non-pharmaceutical treatments for the prevention of muscle wasting with aging. We want to incorporate easy-to-follow strategies for improved muscular health. You will receive a medical and cardiac screen and will obtain information on your body composition.

This study is not designed to treat any illness or to improve your health. Also, there may be risks, as discussed in the section describing the discomforts or risks.

Who is paying for this study?

This research is being funded by the Colorado Agricultural Experiment Station with the mission of increasing the quality of foods in Colorado.

Will I be paid for being in the study?

You will not be paid for the screening tests or travel expenditures, but you will be paid $300 for completion of the GCRC stay. If you leave the study early, or if we have to take you out of the study, you will be paid $40 for each overnight stay.

It is important to know that payments for participation in a study are taxable income.

Will I have to pay for anything?

It will not cost you anything to be in the study.

Is my participation voluntary?

Taking part in this study is voluntary. You have the right to choose not to take part in this study. If you choose to take part, you have the right to stop at any time. If you refuse or decide to withdraw later, you will not lose any benefits or rights to which you are entitled.

Can I be removed from this study?

The study nurse, Rebecca Benson PA/RN, or her supervising physician, Robert Eckel, MD, may decide to stop your participation without your permission if the

study nurse/doctor thinks that being in the study may cause you harm, or for any other reason.

What happens if I am injured or hurt during the study?

You should inform your care provider(s) if you decide to participate in this research study. If you have questions about injury related to the research, you may call the study coordinator, Edward Melanson, Ph.D. at (303) 724-0935 and/or your private physician. Edward Melanson, Ph.D. should be informed about any injury you experience while you take part in this study. If you are hurt by this research, we will give you medical care of you want it, but you will have to pay for the care that is needed.

Who do I call if I have questions?

The researchers carrying out this study are Dr. Ed Melanson and Dr. Benjamin Miller. You may ask any questions you have now. If you have questions later, you may call Dr. Melanson at 303-724-0935 or Dr. Miller at 970-491-3291. You will be given a copy of this form to keep.

You may have questions about your rights as someone in this study. You can call Dr. Melanson at 303-724-0935 or Dr. Miller at 970-491-3291 with questions. You can also call the Colorado Multiple Institutional Review Board (COMIRB). You can call them at 303-724-1055.

The main person to talk to if you have questions about this study is Dr. Melanson at 303-724-0935 or Dr. Miller at 970-491-3291. You can also talk to a Subject Advocate at the General Clinical Research Center (GCRC)/ the Clinical Translation Research Center (CTRC). The phone number there is 720-848-6662.

Who will see my research information?

We will do our best to keep your research records private. But there are some people and agencies who will be allowed to see them. These include:

- Federal offices such as the Food and Drug Administration (FDA) that protect research subjects like you.
- People at the Colorado Multiple Institutional Review Board (COMIRB)
- The study doctor and his/her team of researchers.
- Officials at Colorado State University or the Colorado Health Sciences Center who are in charge of making sure that we follow all of the rules for research

We might talk about this research study at meetings. We might also print the results of this research study in relevant journals. But we will always keep the names of the research subjects, like you, private.

We will ask you to sign a different form that talks about who can see your research records. That form is called a HIPAA form. It will mention companies and universities who will see your research records.

You have the right to request access to your personal health information from the Investigator. [To ensure proper evaluation of test results, your access to these study results may not be allowed until after the study has been completed – if applicable].

This HIPAA authorization does not expire. However, you may withdraw this authorization for use and disclosure of your personal health information by providing written request to the Investigator. If you withdraw this authorization, the Institution, the Investigator, the research staff, and the research Sponsor will no longer be able to use or disclose your personal health information from this study, except so far as that they have already relied on this information to conduct the study.

Agreement to be in this study

I have read this paper about the study or it was read to me. I understand the possible risks and benefits of this study. I know that being in this study is voluntary. I choose to be in this study: I will get a copy of this consent form.

Signature:_____ Date:_____

Print Name:_____

Consent form explained by:_____ Date:_____

Print Name: _____

Investigator:_____ Date:_____

APPENDIX B

Authorization To Use or Release Health Information About Me For Research Purposes *Authorization B: Enrollment into Research*	Study Title: Consumption of milk after physical activity - rethinking protein recommendations in older individuals COMIRB Number: 08-0460

I _____ *(Subject's Full Name)* **authorize**

_____ *(PI or Physician Name)* **and staff members of**

_____ *(Facility Name)* **working for him/her to use the following health information about me <u>for research</u>:** *(Please check the appropriate boxes. NOTE: If a category is checked "yes" and a line follows the category, you MUST describe the type of the procedures done.)*

No	**Yes**	
☐	☐	Name and/or phone number
☐	☐	Demographic information (age, sex, ethnicity, address, etc.)
☐	☐	Diagnosis(es)

☐ ☐ History and/or Physical

☐ ☐ Laboratory or Tissue Studies: _____

☐ ☐ Radiology Studies: _____

☐ ☐ Testing for or Infection with Human Immunodeficiency Virus (HIV) (or results)

☐ ☐ Procedure results: _____

☐ ☐ Psychological tests: _____

☐ ☐ Survey/Questionnaire: _____

☐ ☐ Research Visit records

☐ ☐ Portions of previous Medical Records that are relevant to this study

☐ ☐ Billing or financial information

☐ ☐ Drug Abuse

☐ ☐ Alcoholism or Alcohol abuse

☐ ☐ Sickle Cell Anemia

☐ ☐ Other (Specify): _____

For the Specific Purpose of
☐ Collecting data for this research project

☐ Other* _____

**Cannot say "for any and all research", "for any purpose", etc.*

If my health information that identifies me is also going to be given out to others outside the facility, the recipients are described on the next page(s).

☐ No personally identifiable health information about me will be disclosed to others

The PI (or staff acting on behalf of the PI) will also make the following health information about me available to: (check all that apply and **describe the type of the procedures** done where applicable)

Recipient (name of person or group) _____

No Yes

☐ ☐ All Research Data Collected in this Study (if you check this box Yes, no other boxes need to be checked in this section)

☐ ☐ All Research Data Collected in this Study except for name, phone number, and/or address (if you check this box Yes, no other boxes need to be checked in this section)

☐ ☐ Name and phone number

☐ ☐ Demographic information (age, sex, ethnicity, address, etc.)

☐ ☐ Diagnosis(es)

☐ ☐ History and Physical

☐ ☐ Laboratory or Tissue Studies: _____

☐ ☐ Radiology Studies: _____

☐ ☐ Testing for or Infection with Human Immunodeficiency Virus (HIV) (or results)

☐ ☐ Procedure results: _____

☐ ☐ Psychological tests: _____

☐ ☐ Questionnaire/Survey: _____

☐ ☐ Research Visit records

☐ ☐ Portions of previous Medical Records that are relevant to this study

☐ ☐ Billing/Charges

☐ ☐ Drug Abuse

☐ ☐ Alcoholism or Alcohol

☐ ☐ Sickle Cell Anemia

☐ ☐ Other (Specify): _____

For the Specific Purpose of

☐ Evaluation of this research project

☐ Evaluation of laboratory/tissue samples

☐ Data management

☐ Data analysis

☐ Other*: _____

**Cannot say "for any and all research", "for any purpose", etc.*

For additional Recipients, copy this page as needed.

I give my authorization knowing that:

- I do not have to sign this authorization. But if I do not sign it the researcher has the right to not let me be in the research study.
- I can cancel this authorization any time.
  - I have to cancel it in writing.
  - If I cancel it, the researchers and the people the information was given to will still be able to use it because I had given them my permission, but they won't get any

more information about me.
    - If I cancel my authorization, I may no longer be able to be in the study.
    - I can read the Notice of Privacy Practices at the facility where the research is being conducted to find out how to cancel my authorization.
- The records given out to other people may be given out by them and might no longer be protected.
- I will be given a copy of this form after I have signed and dated it.

This authorization will expire on**:** _____ (Date) OR

☐ The end of the research study

☐ Will not expire

☐ _____

(Describe dates or circumstances under which the authorization will expire.)

ADDITIONAL INFORMATION: _____

_____

_____

_____

Subject's Signature Date

_____

Signature of Legal Representative (If applicable) Date

_____

Name of Legal Representative (please print

_____

Description of Legal Authority to Act on Behalf of Patient

Site: Colorado State University, Dr. Benjamin Miller

I give my authorization knowing that:

- I do not have to sign this authorization. But if I do not sign it the researcher has the right to not let me be in the research study.
- I can cancel this authorization any time.
  - I have to cancel it in writing.
  - If I cancel it, the researchers and the people the information was given to will still be able to use it because I had given them my permission, but they won't get any more information about me.
  - If I cancel my authorization, I may no longer be able to be in the study.
  - I can read the Notice of Privacy Practices at the facility where the research is being conducted to find out how to cancel my authorization.
- The records given out to other people may be given out by them and might no longer be protected.
- I will be given a copy of this form after I have signed and dated it.

This authorization will expire on**:** _____ (Date) OR

☐ The end of the research study

☐ Will not expire

☐ _____

(Describe dates or circumstances under which the authorization will expire.)

ADDITIONAL INFORMATION: _____

_____

_____

Subject's Signature Date
Signature of Legal Representative (If applicable) Date
Name of Legal Representative (please print)
Description of Legal Authority to Act on Behalf of Patient

APPENDIX C

DEPARTMENT OF HEALTH AND EXERCISE SCIENCE

MEDICAL AND EXERCISE HISTORY

NAME_____ GENDER_____ DATE_____

BIRTHDATE_____ AGE_____ HEIGHT_____ WEIGHT_____

ADDRESS_____

TELEPHONE_____ EMAIL_____

1. How often do you exercise? _____ times/week

2. Describe the intensity of your exercise (circle one)
   1 = none
   2 = light (e.g. casual walking, golf)
   3 = moderate (e.g. brisk walking, jogging, cycling, swimming)
   4 = heavy (e.g. running, high intensity sport activity)

3. What types of exercise do you engage in and how much do you do each session? (circle all that apply)
   1 = none
   2 = walking _____ km or minutes
   3 = jogging/running _____ km or minutes
   4 = swimming _____ meters or minutes
   5 = cycling _____ km or minutes
   6 = team sports (rugby, cricket, soccer, etc.) _____minutes _____ intensity
   7 = racquet sports _____minutes
   8 = weight training _____minutes _____# reps _____# sets
   9 = other _____

4. How much time per week do you spend exercising? _____ hours/week

5. Do you measure your heart rate during exercise? _____
   If yes:
   a. How high does it get during your typical workout? _____ beats/min
   b. What heart rate is maintained throughout most of your workout? _____beats/min

6. How long have you had a regular exercise program?_____

7. What condition or shape do you consider yourself to be in now (in terms of physical fitness)?
   1 = poor
   2 = fair
   3 = good
   4 = excellent

8. Do you or have you ever smoked? _____
   If yes: How long ago?_____ For how many years?_____ How many packs/day?_____

9. How much and what type of alcohol do you consume in an average week?
   _____
   _____
   _____

10. Has a close blood relative had or died from heart disease or related disorders (Heart Attack, Stroke, High Blood Pressure, Diabetes etc.)?
    1=Mother
    2=Father
    3=Brother - Sister
    4=Aunt - Uncle
    5=Grandmother - Grandfather
    6=None
    If yes- Give ages at which they died or had the event and the problem they had.
    _____
    _____
    _____

11. Have you ever had your cholesterol measured?
    1=yes
    2=no

    If yes- write the date and value (or if it was normal or abnormal)
    _____

12. Indicate which of the following apply to you (circle all that apply).
    1 = high blood pressure
    2 = high blood fats or cholesterol
    3 = cigarette smoking
    4 = known heart disease or abnormalities
    5 = family history of heart disease (parents or siblings before age 50)
    6 = sedentary lifestyle
    7 = stressful lifestyle at home or at work
    8 = diabetes mellitus
    9 = gout (high uric acid)
    10 = obesity

13. Any medical complaints now (illness, injury, limitations)?
    1 = yes If yes, describe completely_____
    2 = no _____
    _____

14. Any major illness in the past?
    1 = yes If yes, describe completely_____
    2 = no _____

_____
_____

15. Any surgery or hospitalization in the past?
1 = yes If yes, describe completely_____
2 = no _____
_____
_____

16. Are you currently taking any medications (prescription or over-the-counter: including birth control)?
1 = yes If yes, list drugs and dosages _____
2 = no _____
_____
_____

17. Are you allergic to any medications?
1 = yes If yes, list medications_____
2 = no _____
_____
_____

18. Have you ever had any neurological problems?
1 = yes If yes, describe completely_____
2 = no _____
_____

19. Do you now have, or have you ever had, any of the following? (circle all that apply)
1 = heart murmurs
2 = any chest pain at rest
3 = any chest pain upon exertion
4 = pain in left arm, jaw, neck
5 = any palpitations
6 = fainting or dizziness
7 = daily coughing
8 = difficulty breathing at rest or during exercise
9 = any known respiratory diseases

Please describe fully any items you circled_____
_____
_____
_____

20. Do you now have, or have you ever had, any of the following? (circle all that apply)
1 = any bone or joint injuries
2 = any muscular injuries
3 = muscle or joint pain following exercise
4 = limited flexibility
5 = any musculoskeletal problems which might limit your ability to exercise

Please describe fully any items you circled_____
_____
_____
_____