THESIS

IMPACT OF TIMING OF PROTEIN INTAKE ON NITROGEN BALANCE IN EXERCISING OLDER INDIVIDUALS ON A HYPERCALORIC DIET

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ABSTRACT

IMPACT OF TIMING OF PROTEIN INTAKE ON NITROGEN BALANCE IN EXERCISING OLDER INDIVIDUALS ON HYPERCALORIC DIET

We have previously shown that in older adults, consumption of protein in the form of chocolate milk immediately after exercise enhances nitrogen balance (NBAL) when energy balance is maintained. Since it is known that hypercaloric diets increase nitrogen (N) retention, it is important to know if the timing of protein intake after aerobic exercise provides further increases in N retention compared to the consumption of carbohydrate only post exercise. **PURPOSE:** To investigate if consumption of protein and carbohydrate (PRO + CHO) immediately after exercise as opposed to earlier in the day can improve NBAL in older individuals consuming a hypercaloric diet. **METHODS:** In a randomized cross-over design, subjects completed two separate 3-day exercise and nutrition interventions. Exercise (60 minutes of stationary cycling at 55% of VO_2max) was performed daily at 4:30 PM. Diets were hypercaloric (calculated at +15% daily intake), with a PRO+CHO or carbohydrate only (CHO) drink consumed at 10 am and the opposite drink consumed after exercise (5:30 PM). Both diets (1.2 g protein/kg bodyweight, 30% fat, and balance as carbohydrate) were isonitrogenous and isocaloric with only the timing of the drinks differing. A 24 hour stay in a metabolic chamber

confirmed positive energy balance while 24-hour urine collections determined NBAL. **RESULTS:** The 3-day mean NBAL was not significantly different (p=.0881) (n=6) between the CHO trial (.970 ± .517 g N) and the PRO + CHO trial (1.659 ± .430 g N) although a trend toward increased NBAL with PRO+CHO was apparent. The mean energy balance was not significantly different (p=.2906) between the CHO trial (+13.09 ± 1.94%) and the PRO + CHO trial (+ 14.28 ± 1.75%). Further analyses comparing the positive energy balance cohort to previously completed negative, and even energy balance cohorts distinguished the role of energy balance and timing of nutrition effects. **CONCLUSION:** Older individuals in positive energy balance do not maintain a significantly more positive NBAL balance by consuming protein after aerobic exercise as opposed to earlier in the day although energy balance does change the effect of protein timing on NBAL.

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CHAPTER 1

INTRODUCTION

Sarcopenia is a prevalent condition as individuals age, described by a decrease in muscle mass, strength, and fatigue resistance (Jackman RW 2004). Sarcopenia is observed in 50% of individuals over the age of 80, and 13-24% or individuals under age 70 (Baumgartner RN 1998), with muscle cross sectional area (CSA) declining by approximately 40% from the age of 20 to 80 (Berger MJ 2010). With declines in muscle size, come declines in muscle strength (Fiatarone, Marks et al. 1990; Frontera, Hughes et al. 1991). Elderly populations in their seventh and eighth decade have an average strength decline of 20-40% in their knee extensors compared to their younger counterparts, with relative strength losses observed for muscles of both the upper and lower extremities (Berger MJ 2010). Age related decreases in muscle CSA and strength lead to an overall decrease in muscle quality (MQ), which refers to strength per unit of muscle mass. When corrected for a decline in absolute muscle mass, the amount of peak torque and strength per unit of muscle mass reduces with age (Lindle, Metter et al. 1997). Additionally, the age related declines in muscle size and strength can have negative implications on the independence of individuals as they age, with sarcopenic women having 3.6 times higher disability rates and men 4.1 times higher disability rates than those with greater levels of muscle mass (Baumgartner RN 1998).

There are a multitude of factors that contribute to sarcopenia. Some of these factors include changes in endocrine function (Balagopal, Proctor et al. 1997), increases in inflammatory

cytokines (Bruunsgaard and Pedersen 2003), mitochondrial dysfunction (Hiona and Leeuwenburgh 2008), and neurogenic changes (Vandervoort and McComas 1986; Berger MJ 2010). However, in the aging population it is argued that the major contributors to sarcopenia are lack of exercise or "disuse", as well as inadequate nutrition both in regards to caloric (Morley 2001) and/or protein intake (Campbell and Evans 1996; Campbell WW 2001). Regardless of mechanism, sarcopenia is a result of a negative net protein balance. Negative protein balance can occur as a result of decreased protein synthesis, increased muscle protein breakdown, or a combination of both.

A common measure of examining whole body protein balance is through nitrogen balance (NBAL). Since amino acids are the main source of nitrogen (N) in the body, analysis of N intake through protein consumption compared to N losses can be used in calculating total body NBAL (Gropper 2009). Thus, shifts in overall body NBAL are indicative of protein balance in the body. A negative NBAL indicates a net loss of body protein, whereas a positive NBAL is indicative of a net gain of body protein.

NBAL is largely dependent on both energy balance and protein intake. Energy restriction leads to a decreased NBAL (Todd, Butterfield et al. 1984; Friedlander, Braun et al. 2005), with increased amino acid oxidation occurring to meet energy needs. However, there is evidence that increased protein intake is helpful in maintaining NBAL. Specifically, 0.92 g of protein/kg bw has been shown to be superior than 0.45 g of protein/kg bw at maintaining NBAL, lean body mass (LBM), muscular strength, and immune function in elderly women over the course of 9 weeks (Castaneda, Charnley et al. 1995). However, even in the presence of adequate protein intake, inadequate energy intake results in lower NBAL than when energy balance is maintained (Calloway and Spector 1954). Conversely, increasing energy intake will

lead to an increased NBAL due to protein sparing effects (Munro 1951; Butterfield and Calloway 1984). Since protein synthesis is energetically costly and is dependent on ATP availability (Proud 2002), elderly individuals who increase their energy intake will reduce their dependence on amino acids for energy purposes so they can be used for synthetic pathways. Since NBAL is influenced largely by energy balance and protein intake, it seems an intuitive strategy for older individuals to focus on ingesting adequate calories and dietary protein to improve NBAL.

Exercise is another proposed method to increase protein balance. Although resistance training has been shown to increase muscle size and strength in older individuals (Frontera, Meredith et al. 1988), many older individuals are unable to perform resistance training due to prior injury, limited access to equipment, and lack of knowledge regarding proper technique. Fortunately, aerobic exercise (3 hrs @75 %VO_2max) has been shown to increase whole body protein synthesis (WBPS) following exercise, with whole body protein breakdown (WBPB) not differing from values at rest (Devlin, Brodsky et al. 1990). A long-term aerobic exercise program has been shown to increase basal whole protein turnover (WBPT) (Lamont, Patel et al. 1990), while decreasing leucine oxidation at rest (Gaine, Viesselman et al. 2005). Additionally, it has been shown that prolonged aerobic exercise can increase myofiber size in elderly women (Harber, Konopka et al. 2009), thus providing evidence of the benefits of aerobic exercise at increasing LBM in elderly individuals. Aerobic exercise's effects on NBAL are also largely dependent on energy balance. When individuals maintain energy balance, aerobic exercise increases NBAL over time (Todd, Butterfield et al. 1984; Gaine, Viesselman et al. 2005). However, the increases in NBAL observed from aerobic exercise are decreased when individuals are in a hypocaloric state (Todd, Butterfield et al. 1984). Regardless of energy status, in the absence of nutrient intake protein balance will remain negative for up to 3 hours post exercise. (Biolo, Maggi et al. 1995).

Combining exercise with proper nutritional strategies has been proposed to be more effective than either intervention alone in stimulating muscle protein synthesis (MPS) (Biolo, Tipton et al. 1997; Miller 2007), and in treating sarcopenia. Ingestion of amino acids with or without carbohydrate causes a shift towards increased protein balance post exercise (Biolo, Tipton et al. 1997; Miller, Tipton et al. 2003; Borsheim, Aarsland et al. 2004). However, by consuming amino acids with carbohydrates post exercise, net protein balance can be increased to levels greater than if the amino acids or carbohydrate were ingestion alone (Miller, Tipton et al. 2003; Borsheim, Aarsland et al. 2004). In the elderly, protein combined with carbohydrate (40g carbohydrate, 20g whey) leads to increases in WBPT in the elderly above those observed with an isoenergetic amount of carbohydrate (60g carbohydrate) (Murphy and Miller 2010).

The timing of amino acid and carbohydrate ingestion has an influence on the degree of increase in protein balance. The consumption of an oral supplement (10g protein, 8g carbohydrate, 3g fat) consumed immediately after aerobic exercise resulted in greater increases in MPS than if consumed 3 hours after exercise (Levenhagen, Gresham et al. 2001). Our lab has previously shown that in older individuals in energy balance, consumption of protein and carbohydrates (chocolate milk) immediately following moderate aerobic exercise at 55% VO_2max leads to greater NBAL than if consumed earlier in the day. Our findings were particularly significant due to the fact that both trials were isocaloric and isonitrogenous, with only the timing of the protein intake differing. Since increased energy intake also leads to increases in NBAL (Munro 1951; Butterfield and Calloway 1984), our lab wished to investigate the impact of the timing of protein intake on NBAL in exercising older individuals on a *hypercaloric* diet.

Statement of the Problem:

To determine if protein intake immediately following moderate intensity aerobic exercise increases NBAL to a greater degree than consuming the protein earlier in the day in hypercaloric older individuals.

Hypothesis:

While in positive energy balance, consumption of chocolate milk immediately after moderate aerobic exercise will increase 3-day NBAL more so than if consumed earlier in the day.

Delimitations, Limitations, and Assumptions:

The present study is delimited to 6 male (n=2) and female subjects (n=4) aged 55-75 years old. Subjects were recruited from Loveland and Fort Collins. Since milk consumption is a major component of the study all of the subjects had to be tolerant of lactose.

There are limitations of the NBAL technique, including the potential to overestimate NBAL (DM 1976). Despite this, the NBAL technique was used under both control and experimental conditions, so the same magnitude of error existed under both conditions.

It's assumed that subjects fast for 12-hours prior to resting metabolic rate testing in the pretesting portion of the study methods. During the lead-in and inpatient periods, it's assumed that subjects are consuming only what is provided to them by the study staff.

CHAPTER 2

LITERATATURE REVIEW

Section 1: Implications of Sarcopenia

Sarcopenia is a condition characterized by a decrease in strength and lean body mass as individuals age. Sarcopenia's prevalence is 13-24% in individuals under the age of 70 and more than 50% of individuals over the age of 80 (Baumgartner RN 1998). In Greek, "sarcx" means flesh, and "penia" means deficiency (IH 1989). The definition describes a decrease in muscle protein content, force production, fatigue resistance, and a corresponding decrease in muscle fiber diameter (Jackman RW 2004). Although there is no *universally accepted* definition of sarcopenia (Berger MJ 2010), some define sarcopenia as the loss of LBM greater than two standard deviations (SD) from the mean of young controls (Baumgartner RN 1998).

It has been shown that total muscle CSA declines by approximately 40% from the age of 20 to 80 (Berger MJ 2010). The correlation between muscle mass and strength has been shown in multiple studies (Fiatarone, Marks et al. 1990; Frontera, Hughes et al. 1991), such that the loss of muscle mass leads to an approximate 1.0% decrease in strength per year after the fourth decade of life (Doherty 2003). When motor nerves of elderly individuals are electrically stimulated during voluntary maximal contraction no additional torque is produced (Vandervoort and McComas 1986). Thus, declines in strength observed with aging seem to have more to do with muscle atrophy rather than the inability to stimulate motor neurons.

Elderly populations in their seventh and eighth decade exhibit an average strength decrease of 20-40% in their knee extensors when compared to younger populations, with relative strength losses similar for muscles of the upper and lower extremities (Berger MJ 2010). Although men and women vary little in their relative strength declines, men tend to have a larger absolute drop in strength since they tend to start at a higher baseline of strength than women (Berger MJ 2010). These age related decreases in muscle CSA and strength lead to an overall decrease in MQ, which refers to strength per unit of muscle mass. When corrected for a decline in absolute muscle mass, the amount of peak torque and strength per unit of muscle mass reduces with age (Lindle, Metter et al. 1997). This decline in peak torque and strength implies a decrease in MQ and efficiency throughout the aging process. The potential decrease in MQ is important since MQ is argued to be a better indicator of muscle function than just strength alone (Roubenoff and Hughes 2000).

Data clearly demonstrates that a decrease in muscular strength inhibits mobility, and increase risk for injury and dependence. Women and men with sarcopenia have 3.6 and 4.1, respectively, greater disability rates than younger controls (Baumgartner RN 1998). In addition, it has been shown in nursing home residents with two or more documented falls, that the "fallers" have weaker ankle and knee flexion than the "non-fallers" control group (Wolfson, Judge et al. 1995).

Sarcopenia also reduces resting metabolic rate (RMR). LBM is the largest factor contributing to an individual's RMR (Miller and Blyth 1953), therefore losses of LBM via the onset and progression of sarcopenia may contribute to a decline in RMR (Tzankoff and Norris 1977). Reduced RMR makes elderly individuals more susceptible to the numerous negative health implications associated with excess body fat. There is a decrease of 13-20% in RMR

between age 30 and age 80, with men having a larger decrease along with earlier onset of decline in RMR (Wilson MM 2003). Additional contributors to a declining RMR include a decrease in Na+/K+-ATPase activity and decreases in membrane permeability within the mitochondria (Wilson MM 2003). When exposed to overfeeding, older individuals also experience a smaller compensatory increase in their RMR than younger populations. By maintaining or attenuating age-related reductions in LBM, older individuals may be less susceptible to fat gain.

Later, the review of literature will discuss the mechanisms leading to sarcopenia. Regardless of mechanism, the underlying end result is a net loss in muscle protein because protein synthesis is exceeded by protein breakdown. To understand these processes, protein metabolism will be discussed first.

Section 2: Protein Metabolism

In Greek the protein root "proteos" means "primary", or "taking place first" (Gropper 2009). Forty percent of total body protein mass is found in skeletal muscle, 25% in body organs, and the rest primarily in skin and blood (Gropper 2009). The processes of protein synthesis and protein breakdown are termed protein turnover and will be discussed in further detail in the "Protein Turnover" section.

There are 20 different amino acids, which are used to build proteins. Nine of the amino acids are considered "essential", which means they must be obtained from dietary sources. The rest of the amino acids are considered "nonessential", which means they can be synthesized in the body (Houston 2006). All amino acids share the same type of basic structure, including a carbon (C), at least one carboxyl (acid) group (-COOH), and at least one amino group ($-NH_2$). The

differences between amino acids are due to their varying side chains, known as the R-group (Gropper 2009).

Amino acids are liberated during the digestion of dietary protein. Like carbohydrate, dietary protein provides ~4 kcal/g of physiological energy. Protein digestion begins in the stomach, where hydrogen and electrostatic bonds are broken by hydrochloric acid (HCI), causing an unfolding of the protein. Pepsin then hydrolyzes the peptide bonds of the protein, resulting in large polypeptides, oligopeptides, and free amino acids which then enter the small intestine. Once in the small intestine, proenzymes and zymogens are converted to active enzymes that further hydrolyze the peptide bonds. The final products of digestion are dipeptides, tripeptides, and amino acids, which are then absorbed across the brush border of the small intestine into enterocytes. If unused, the amino acids are then transported into the interstitial fluid where they enter the villi's capillaries and the portal vein on their way to the liver (Gropper 2009). The liver has the ability to synthesize nonessential amino acids that can be utilized in the liver or to keep a balance in the what is called the amino acid pool (Houston 2006).

The amino acid pool is used to make new proteins. The amino acid pool is supplied and regulated through protein intake, protein synthesis and protein breakdown of tissues, and the synthesis and release of amino acids by the liver (Houston 2006). The whole-body amino acid pool is about 150g. It is thought that endogenous amino acids are the primary source of amino acids taking part in protein synthesis (Gropper 2009). Amino acids in the pool that are in excess of what is required for protein synthesis of new proteins and non-protein N containing molecules are generally oxidized for energy (Gropper 2009).

When used for energy, amino acids first lose their amino group and other N atoms. The carbon skeleton can then be used to make glucose via gluconeogenesis in the liver, converted to

acetyl-CoA for entry into the citric acid cycle (TCA), or used to synthesize fatty acids via lipogenesis. The liver is responsible for removing the amino groups in a process called deamination. Skeletal muscle however, has a specific capacity to deaminate branched-chain amino acids (BCAA), which consist of leucine, isoleucine, and valine (Houston 2006). In deamination, the amino groups are removed without being transferred to another amino acid, which generates ATP. This process results in the creation of an α-keto acid. Oxidative deamination creates ammonia, which must be disposed of through the urea cycle, which will be covered in the "Nitrogen Balance" section. Transamination can transfer the amino group to a carbon skeleton or an existing α-keto acid, in order to synthesize a new non essential amino acid (Gropper 2009).

To summarize, the consumption of dietary protein yields amino acids, which enter an amino acid pool for the eventual synthesis of proteins, transamination, or production of energy.

Section 3: Protein Turnover

Protein turnover refers to the continuous process of the synthesis of new proteins from amino acids and degradation of proteins into individual amino acids. Protein turnover is necessary to preserve the function of body proteins. In terms of magnitude of contribution, skeletal muscle contributes slightly less than 30% of the whole body protein turnover (WBPT) rate (Nair 1995). It is energetically costly to make and breakdown proteins and WBPT has been shown to account for approximately 20% of an individual's RMR (Welle and Nair 1990).

There are many reasons for protein turnover in the body. Modified or wrongly translated proteins may be destroyed to prevent cellular damage. Additionally, when energy intake is deficient, proteins may be oxidized to meet energy requirements. Energy deficient states can be observed in both the post absorptive and fasting states, where protein breakdown

is elevated and protein synthesis is diminished. Changes in the rates of protein turnover also allow humans to adapt to an altered environmental or nutritional condition (Houston 2006). For example, increases in dietary protein intake have been shown to lead to increases in WBPT in elderly individuals (Pannemans, Halliday et al. 1995), with increases in WBPT and NBAL observed when dietary protein intake is increased in young subjects (Pannemans, Halliday et al. 1995). There is a need to further study WBPT in elderly individuals, since approximately 30% of total body protein can be attributed to skeletal muscle (Cohn, Vartsky et al. 1980), with the remainder incorporated into non-muscle tissues. Although sarcopenia is largely a result of decreased skeletal muscle protein turnover, the present study examines NBAL, a WBPT measure.

Wasting results from a negative net protein balance, which is a result of decreased protein synthesis, increased protein breakdown, or a combination of both. It has been shown that per kilogram (kg) of bodyweight (bw) that elderly individuals have diminished protein turnover rates compared to younger individuals (Morais, Gougeon et al. 1997). Differences in protein turnover per kg/bw may be due changes in body composition since no difference in protein turnover was seen when corrected per unit of fat free mass (FFM) (Morais, Gougeon et al. 1997). However, another study found that even when corrected for FFM, elderly individuals experience a 20% reduction in WBPT compared to their younger counterparts (Balagopal, Rooyackers et al. 1997).

Section 3.1: Protein Synthesis

The process of protein synthesis occurs in the cell's cytosol, and begins with messenger ribonucleic acid (mRNA) following post-transcriptional modifications of ribonucleic acid (RNA) from deoxyribonucleic acid (DNA) transcription. The mRNA's sequence of nucleotide bases

determines the amino acid sequence when forming a peptide chain. Amino acids are attached to specific transfer ribonucleic acid (tRNA). Every tRNA contains an anticodon and amino acid, which combines with codons on mRNA, providing the amino acid to the protein sequence. During translation initiation, the formation of a complete ribosomal subunit occurs at the start codon (AUG) on mRNA with tRNA and its amino acid (Houston 2006). A number of eukaryotic initiation factors (eIFs) play a role in this initiation process (Wang and Proud 2006), such as recruiting the mRNA to the 40S ribosomal subunit (Gingras, Raught et al. 1999), as well as recruiting methionyl-tRNA (Met-$tRNA_1$), which together inspect the 5'-untranslated region of the mRNA for the start codon (Wang and Proud 2006).

During elongation, tRNA arrives at the ribosomal complex, three bases at a time, with the associated coded amino acids and form peptide bonds with the preceding amino acid. The energy required for elongation comes from the hydrolysis of GTP to GDP and Pi. The process of elongation continues, with the growing of a peptide chain until the stop codon (UAG, UGA, or UAA) is reached on the mRNA molecule. Eukaryotic elongation factors (eEFs) participate in the elongation process, by assisting in the translocation of the tRNA to and along the ribosomal complex. The peptide chain is released once the ribosomal complex is removed from the mRNA using energy from GTP hydrolysis (Houston 2006).

Section 3.2: Protein Breakdown

Protein breakdown processes occur via proteinases, which are protein degrading enzymes. These enzymes act primarily through the ubiquitin-proteasome pathway, the lysosomal system, and the calpain system (Houston 2006).

The ubiquitin-proteasome pathway recognizes specific proteins for degradation. The covalent attachment of ubiquitin, a small protein, allows for this recognition to occur. Once

ubiquination occurs, proteins are broken down to polypeptides via a 26s proteasome complex. The polypeptides are broken down by proteases, which release small peptides that are then degraded to amino acids by cytosolic peptidases. During exercise there is a decrease in activity of the ubiquitin-proteasome pathway (Houston 2006). The decreased activity of the ubiquitin-proteasome pathway is important, since the pathway is thought to account primarily for the degradation of myofibrillar proteins (Taillandier, Combaret et al. 2004).

Lysosomes are organelles that contain cathepsins, which are protein degrading enzymes. Cathepsins break the peptide bonds on the interior of a protein molecule. Additionally, lysosomes possess proteinases that cleave individual amino acids. Proteins must enter the lysosomal system via endocytosis for degradation (Houston 2006).

The calpain system involves calcium-activated proteinases in the cell cytosol. These proteinases are activated by increases in the concentration of intracellular calcium. The proteinases are thought to be activated by the increased calcium concentrations from muscular contraction during exercise. The calpain system acts on structural proteins in muscle (Houston 2006), and contributes to muscle breakdown by cleaving cytoskeletal and myofibrillar proteins (Belcastro, Shewchuk et al. 1998).

Although muscle protein breakdown (MPB) is a large player in the protein balance equation, age is correlated with decreased MPB with a greater reduction seen in women (Morais, Gougeon et al. 1997). The decreased MPB observed in aging men and women provides evidence that a blunted MPS (Nair 1995) is primarily responsible for the effects of sarcopenia.

Section 3.3: Assessing Protein Turnover

There are a number of methods that assess protein turnover. These methods either take into consideration the whole body or the skeletal muscle specifically.

Both WBPT and MPS can be assessed using stable isotopes. By using labeled amino acids and introducing them into the plasma, isotopic ratios in the plasma can be assessed to determine WBPT (Garlick and Cersosimo 1997). MPS can be directly measured through the use of tracers such as leucine (Nair, Halliday et al. 1988; Welle, Thornton et al. 1995; Balagopal, Rooyackers et al. 1997; Miller, Olesen et al. 2005) or phenylalanine (Phillips, Tipton et al. 1997; Volpi, Mittendorfer et al. 1999; Volpi, Sheffield-Moore et al. 2001). These "precursor: product labeling" techniques work by allowing the tracer and amino acid to incorporate into the amino acid pool. This pool supplies amino acids to MPS, and the tracer and amino acid are incorporated into skeletal muscle (Gasier, Fluckey et al. 2010). Due to the necessity of a controlled environment for tracer infusion, and short term nature of the measurements, tracer methods are unable to be administered in a free-living conditions (Gasier, Fluckey et al. 2010).

In an attempt to directly measure protein synthesis in free-living conditions, the use of tracers such as $^{2}H_2O$ have also been used. By consuming $^{2}H_2O$, deuterium is incorporated into free amino acids through intermediary metabolism from body water. Like the previously described tracer techniques, these labeled amino acids are incorporated into skeletal muscle following MPS (Robinson, Turner et al. 2011). $^{2}H_2O$ offers a distinct advantage in that it can be consumed orally rather than needing to be infused (Belloto, Diraison et al. 2007). However, $^{2}H_2O$ is best suited for longer-term studies.

MPB is more difficult to directly measure, but techniques are available. MPB is commonly measured *in vivo* via 3-Methylhistidine (3-MeH) excretion in urine (Ballard and Tomas

1983). 3-MeH is a component of the myofibrillar proteins actin (Asatoor and Armstrong 1967), and myosin (Johnson, Harris et al. 1967). Since 3-MeH is unique to actin and myosin, increases in 3-MeH excretion is indicative of increased MPB *in vivo* (Ballard and Tomas 1983). Additionally, the use of tracers can be can be utilized and measured for "arterio-venous difference" to asses MPB as a component within the skeletal muscle protein turnover (Wagenmakers 1999). The "arterio-venous difference" method can be challenging since it requires a great degree of analytical precision and consistency in blood flow through the muscle tissue (Macdonald 1999).

Section 3.4: Nitrogen Balance

Measuring the body's NBAL is a common method to assess an individual's WBPT. Amino acids are the main source of N in the body, with N comprising approximately 16% of the average mass of most proteins (Gropper 2009). Therefore, shifts in overall body NBAL is indicative of protein balance in the body. A negative NBAL indicates a net loss of body protein, whereas a positive NBAL is indicative of a net gain of body protein. By subtracting N losses from N intake through diet, total body NBAL can be calculated (Gropper 2009). In order to calculate NBAL, N intake and N output must be measured. In terms of N intake, 6.25 g of protein is assumed to contain 1 g of N, thus a daily intake of 70 g of protein would provide 11.2 g of N (Gropper 2009). Measurement of N output is more complicated, and relies on some assumptions, which will be discussed.

In discussing the methods of measuring N output, the urea cycle must first be discussed. The urea cycle disposes of excess N. As mentioned previously, amino acids that are not used for protein synthesis are typically oxidized, liberating amino acids (Gropper 2009). The carbon skeletons of the amino acids can be used to either make glucose via gluconeogenesis in the liver,

be converted to acetyl coA for entry into the citric acid cycle (TCA cycle) or be used for fat synthesis. In the process of deamination, the amino group is transferred to α-ketoglutarate forming glutamate, or from glutamate to a keto acid, thus creating a new amino acid and new keto acid. The resulting amino groups are removed as urea in the liver. The amino group on glutamate is first transferred to the liver, and incorporated into the urea molecule, which contains two amino groups. The N from the glutamate is released as ammonia via the glutamate dehydrogenase reaction in the liver (Houston 2006). This oxidative deamination reaction is depicted below:

glutamate + H_2O + NAD^+ ←→ α-ketoglutarate + NADH + H^+ + $NH4^+$ (Houston 2006)

Although ammonia can exist safely in the form of the amino group on glutamate and in the side chain of glutamine, it is otherwise toxic, especially to the brain. Following urea formation, it is excreted from the kidney with formation of urine. The N in urea comes from protonated ammonia or from the amino group from aspartate. This ammonia comes from the blood via protein breakdown, from glutamine, or from glutamate in the glutamate dehydrogenase reaction discussed above. The aspartate, on the other hand, comes from the transamination reaction of oxaloacetate from glutamate, resulting in the formation of aspartate and α-ketoglutarate (Houston 2006).

The actual formation of urea consists of a number of enzymatic steps. These steps occur in both the mitochondrial matrix and cytosol of liver cells. The first step in the process occurs in the mitochondrial matrix and involves the formation of carbamoyl phosphate from ammonia and carbon dioxide. This step is catalyzed via carbamoyl phosphate synthetase, and is the rate limiting step in the formation of urea. When MPB is accelerated, carbamoyl phosphate synthetase is allosterically controlled by N-acetyl glutamate and increases flux through the urea

cycle. Once carbamoyl phosphate enters the urea cycle it joins with ornithine, forming citrulline via ornithine transcarbamoylase. Once aspartate enters the urea cycle it joins citrulline, forming argininosuccinate via argininosuccinatesynthetase in the mitochondrial matrix. This argininosuccinate is then split forming arginine and fumarate via argininosuccinatelysase. Finally, arginine is split via arginase and subsequently forms ornithine and urea. While the ornithine goes onto assist in the first step of the urea cycle, the urea leaves the liver through blood entering the kidneys (Houston 2006). Finally, the urea is excreted in urine and can be measured for N.

Of total N losses in the body, 80% of the N is lost in the urine under normal conditions. Other urinary sources of N include ammonia, creatinine, uric acid, with trace N losses also seen in amino acids, and hippuric acid. Losses of N are also seen in urea in sweat, as well as hair, skin, and fecal N losses (Gropper 2009). These losses are considered miscellaneous N losses.

The equation commonly used in calculating NBAL is:

NBAL (g)= N intake (calculated via protein consumption) - (fecal N + urinary N (directly measured) + misc. N loss)

Both fecal N and misc. N are estimates, of 2g/day (Calloway, Odell et al. 1971) and 5 mg/kg bw/day (Calloway, Odell et al. 1971), respectively. When adequate protein is ingested the N balance should be zero (equilibrium) under normal weight stable conditions.

There are drawbacks to the use of the NBAL technique when examining WBPT. The NBAL technique is not able to distinguish between relative contributions between protein synthesis and protein breakdown as it relates to increases or decreases in NBAL. Thus, it is impossible to know that an increase or decrease in N retention by the body is a result of

increased or decreased protein synthesis, decreased or increased protein breakdown, or a combination of both.

A second criticism is that NBAL overestimates N retention due to underestimations of N output (Kopple 1987). The exact amount of N output via fecal and miscellaneous N losses are commonly estimated due to impractical methods of collection. Additionally, N losses from brushing one's teeth, toilet paper, and exhaled ammonia are typically ignored (Calloway, Odell et al. 1971). With this number of miscellaneous N sources, there is a larger potential for variability. For example, N from sweat has shown in to be quite variable (Consolazio, Nelson et al. 1963; Consolazio, Matoush et al. 1966; Ashworth and Harrower 1967). Increasing protein intake also increases sweat N output (Cuthbertson and Guthrie 1934). However, these inaccuracies can be minimized by testing subjects under the same conditions when doing the intervention.

If the NBAL technique is employed it may be prudent to have individuals participate in a dietary lead in period. The lead in period allows NBAL to adjust to the new protein intake, since it has been shown to take approximately a week (2007). However, additional research has shown that acute changes in protein intake (within ± 4% of habitual intake) does not lead to significant changes in WBPB, MPS, or muscle proteolytic enzymes. These lack of significant changes provide evidence that a lead in period may not be as necessary as previously thought (Yarasheski, Castaneda-Sceppa et al. 2011).

Despite the limitations of the NBAL technique, it is easy to employ and provides valuable information regarding the whole body protein balance in the body. Unlike the leucine and phenylalanine tracer methods, the NBAL method can study WBPT over multiple days. Additionally, the NBAL method incorporates all activities of daily living in free living individuals.

Its noninvasive nature makes it an appealing method in studying the aging population. The NBAL technique can be used to determine adequate protein intakes, and changes in NBAL and WBPT can be analyzed with regards to the effects of exercise and nutritional interventions.

Section 4: Interrelationship between energy balance and protein intake on NBAL

NBAL is dependent on both energy balance and protein intake. Regarding energy balance, it is common for individuals to decrease their energy intake as they age (Morley and Silver 1988). The decreased energy intake can have negative implications, with energy restriction resulting in a decreased NBAL (Todd, Butterfield et al. 1984; Friedlander, Braun et al. 2005). Fasting for ≤4 days increases leucine oxidation (Knapik, Meredith et al. 1991), total leucine flux and proteolysis (Tsalikian, Howard et al. 1984), which can lead to losses in LBM. Amino acid oxidation is increased during fasting to meet energy demands, with skeletal muscle providing disposable AAs to maintain proteins in more vital organs. Specifically, it has been shown that dietary restricted rats preserve the protein synthetic rate of the heart, whereas MPS is reduced in both acute and chronic restriction scenarios (Yuan, Sharma et al. 2008). The decrease in MPS and increase in MPB with negative energy balance leads to losses of LBM (Friedlander, Braun et al. 2005).

Conversely, increased energy intake spares protein resulting in a more positive NBAL (Munro 1951; Butterfield and Calloway 1984). When additional carbohydrate or fat is added to an adequate energy and protein intake, NBAL will increase (Munro 1951). Supplying 700 non-protein calories has been shown to maximally spare N losses in the absence of dietary protein. However, when ample N is provided but there are inadequate non-protein calories, NBAL will not be maintained (Calloway and Spector 1954). Added energy intake will also increase NBAL if the added energy is balanced with an equal amount of energy expenditure (EE) through exercise

(Butterfield and Calloway 1984). Protein synthesis is dependent on ATP availability, and is extremely energetically costly (Proud 2002). Increasing energy intake reduces the dependence on amino acids for energy purposes so they can be used for synthesis of new proteins. Conversely, during an energy deficit, protein synthesis is down regulated due to its energetically costly nature.

Dietary protein intake also has an impact on NBAL. NBAL is extremely adaptive, with urinary N equilibrium occurring after an average of 8 days into a protein free diet (Rand, Young et al. 1976). Despite the ability of the body to adapt to reductions in dietary protein, insufficient dietary protein can lead to a number of negative consequences. Specifically, it has been shown that 0.45 g/kg bw is inferior to 0.92 g/kg bw in maintaining NBAL, LBM, muscular strength, and immune function in elderly women over the course of 9 weeks (Castaneda, Charnley et al. 1995). Additionally, it was shown that exercise in the presence of a protein intake below the Recommended Dietary Allowance (RDA) (0.57 g/kg bw), results in less N retention than if the person is consuming the RDA for protein (0.8 g/kg bw) (Todd, Butterfield et al. 1984). By increasing dietary protein intake individuals can increase NBAL in circumstances of both dietary (Calloway and Spector 1954), and exercise induced (Pikosky, Smith et al. 2008) energy deficits. Therefore, it appears that increases in protein intake have a compensatory effect on attenuating or eliminating the decreases in NBAL observed with negative energy balance. Maintaining protein intake will not totally negate the effects on NBAL observed with negative energy balance. Even if an individual is consuming their habitual protein intake, the presence of negative energy balance can result in a decrease in NBAL (Iyengar and Narasinga Rao 1979). Therefore, it is likely that maintaining adequate caloric intake is more important than consuming adequate protein in maintaining NBAL.

It is debated whether the RDA for protein is adequate for elderly adults. The current RDA for protein is 0.8 g/kg bw per day for both men and women aged 19 years and older (Campbell, Johnson et al. 2008). At the time the RDA was established, little data was available regarding protein requirements in the elderly. The present recommendation is based on NBAL data collected in younger subjects. Recently, when young and old individuals were compared during three 18 day trials at 0.5, 0.75, and 1.00 g protein/kg bw, there were no observed differences in NBAL between young and old individuals (Campbell, Johnson et al. 2008). An additional study examined older men and women consuming the RDA for protein while in energy balance for 30 days. An observed negative NBAL was seen in some of the subjects during the final 5 days of the month long study (Gersovitz, Motil et al. 1982), which indicated that longer-term interventions may be necessary to determine the adequacy of the protein RDA in older populations. One such long-term study provided 0.8 g protein/kg bw/day (RDA) paired with sufficient energy intake so that bodyweight was not changed over the course of 14 weeks in men and women aged 55-77 years old. The researchers found that urinary N excretion actually decreased throughout the study, providing evidence of NBAL adaptation (Campbell WW 2001). There were also no significant changes in protein turnover, oxidation, and synthesis over time. However, measurements of mid-thigh muscle area decreased significantly over the course of the 14 week study (Campbell WW 2001).

As discussed, NBAL is influenced largely by energy balance and protein intake. It seems an intuitive strategy for older individuals to focus on ingesting adequate calories and dietary protein due to improve NBAL, and attenuate the losses in LBM associated with aging.

Section 5: Anabolic Resistance in Elderly

As mentioned previously, the onset and progression of wasting can be attributed to a number of interrelated factors. However, of primary importance, is that aging individuals have a degree of "anabolic resistance" to stimuli that promote protein synthesis. Before discussing the observed anabolic resistance in the elderly, it is necessary to discuss the mammalian target of rapamycin-signaling pathway (mTOR) and its regulation on metabolism and growth.

Section 5.1: The mTOR Pathway

mTOR is a multidomain protein that plays a role in both cellular metabolism and hypertrophic growth through the control of protein synthesis. mTOR interacts with a number of protein partners that regulate its function. Two of the binding partners for mTOR are raptor and rictor, which assist in the forming of the mTOR1 and mTOR2 complexes, respectively. Raptor interacts with proteins regulated by mTOR in a rapamycin sensitive manner. These targets possess TOR signaling motifs (TOS motifs) , which contain 4E binding proteins (4E-BPs) and S6 kinase (S6K's) involved in the translation phase of protein synthesis (Wang and Proud 2006).

mRNA translation can be described in three steps: initiation, elongation, and termination. The mTOR pathway controls protein synthesis via the initiation and elongation phases of the synthetic process. Translation initiation is mediated through the actions of eIFs, as briefly discussed previously. Specifically, eIF4E is responsible for binding to the 5'-cap end of the mRNA, and recruiting the 40S ribosomal subunit and Met-tRNA to locate a start codon (Gingras, Raught et al. 1999). eIF4E also binds to eIF4G which binds to poly(A)-binding protein (PABP), circularizing the mRNA (Wang and Proud 2006). eIF4E binds to phosphoproteins called 4E binding proteins (4E-BPs). One particular 4E-BP, 4E-BP1, binds to the same region of eIF4E as

eIF4G, so the binding of 4E-BP1 to eIF4E prevents eIF4E binding to eIF4G, thus inhibiting translation initiation complexes (Gingras, Raught et al. 1999). 4E-BP1 phosphorylation can impair eIF4E binding, depending on the site of phosphorylation. mTOR is thought to be responsible for the phosphorylation of 4E-BP1, thus releasing eIF4E so it is able to bind to eIF4G for translation initiation. Another eIF, eIF4B, is a substrate for phosphorylation by mTOR regulated S6 kinases (S6K's) (Lawson, Lee et al. 1989; Raught, Peiretti et al. 2004). eIF4B stimulates RNA helicase activity through interaction with eIF4A (Lawson, Lee et al. 1989). Phosphorylation is thought to stimulate its function (Wang and Proud 2006), by recruiting ribosomes to the 5' end of the mRNA (Holz, Ballif et al. 2005), where eIF4B stimulated RNA unwinding can occur during translation initiation (Wang and Proud 2006).

Translation elongation requires elongation factors, eEF1 and eEF2, with the latter being controlled by signaling through the mTOR pathway. eEF2 promotes translocation, in which the ribosome moves along subsequent codons of the mRNA following the formation of peptide bonds (Wang and Proud 2006). Phosphorylation of eEF2 through eEF2 kinase inhibits eEF2 binding to the ribosome, impairing elongation (Carlberg, Nilsson et al. 1990). mTOR regulated S6K1 can phosphorylate eEF2 kinase , inhibiting eEF2 kinase action (Wang and Proud 2006).

Protein synthesis is dependent on ATP availability and is energetically costly (Proud 2002). Of particular importance regarding energy balance and protein synthesis is AMP kinase (AMPK). During an energy deficit, AMPK is activated due to a rise in AMP and a decrease in the ATP/AMP ratio. AMPK decreases the energetically costly process of protein synthesis by forming the tuberous sclerosis complex (TSC1/TSC2 complex), which is an inhibitor of the mTOR pathway (Inoki, Zhu et al. 2003). Conversely, mTOR can be stimulated by an energy surplus and the availability of ATP and AAs for protein synthesis. Following feeding, mTOR can be stimulated

both by insulin and by essential amino acids (EAAs). Insulin is believed to exert its effects by increasing phosphatidylinositol 3-kinase (P13K) and protein kinase B (Akt/PKB) activity. Akt phosphorylates the tuberous sclerosis complex (TSC2 complex), thus increasing mTOR activity (Fujita, Dreyer et al. 2007). It is unclear how exactly EAAs activate the mTOR pathway. However, it has been shown that EAAs, in the absence of insulin, are capable of rapidly stimulating S6K1 and 4E-BP1. In addition, there is evidence that EAAs stimulate upstream targets of mTOR such as human vacuolar protein sorting 34 (hVps34), which in turn drives S6K1 activation (Gulati and Thomas 2007). To conclude, mTOR is modulated by a number of nutritional signals and serves to either increase or decrease the rate of protein synthesis.

Section 5.2: Nutritional Anabolic Resistance in Elderly

The age related losses in LBM observed with aging can largely be attributed to a blunted response to nutritional anabolic stimuli. Some research shows no differences in postabsorptive basal muscle protein turnover between young and old individuals (Volpi, Mittendorfer et al. 1999; Volpi, Sheffield-Moore et al. 2001), however other research does demonstrate differences in basal MPS (Welle, Thornton et al. 1993; Yarasheski, Zachwieja et al. 1993). Mixed results regarding postabsorptive protein turnover led to research investigating if an anabolic resistance is present in the elderly after feeding, which may contribute to the losses in LBM associated with aging.

Essential amino acids up-regulate MPS in the postprandial state (Biolo, Tipton et al. 1997; Cuthbertson, Smith et al. 2005). It has since been observed that the rise in MPS observed from EAA ingestion without an associated increase in insulin signaling, is blunted in the elderly (Cuthbertson, Smith et al. 2005). Mechanistically, it is believed this anabolic resistance to EAAs is due to decreased activation of mTOR, 4E-BP1, S6K1, and eIF2B (Cuthbertson, Smith et al.

2005). Inhibition of mTOR mediated MPS in response to EAA feeding may result in a net loss of muscle protein and contribute to LBM reductions in the elderly.

Additional research has been done to elucidate insulin's role in MPS and MPB, as well as insulin's effects in the elderly. It is believed that amino acid availability is what drives MPS beyond an insulin concentration of ~10m IU.ml^{-1} (Cuthbertson, Smith et al. 2005). In support of this, insulin in the range of ~30-170 m IU.ml^{-1} has no effect on MPS (Greenhaff, Karagounis et al. 2008), with the presence of AAs appearing to be necessary for MPS to occur (Bell, Fujita et al. 2005). However, insulin protects against MPB, with no additional effects observed beyond 30 m IU.ml^{-1} in young subjects (Greenhaff, Karagounis et al. 2008). Although basal leg protein breakdown (LPB) appears to be similar between young and old, older subjects seem to be resistant to insulin's inhibitory effects on proteolysis (Wilkes, Selby et al. 2009). Specifically at plasma insulin concentrations of 5 m IU.ml^{-1}, younger subjects experienced a 35% greater reduction in LPB than older individuals (Wilkes, Selby et al. 2009).

In response to mixed meal ingestion, the postprandial myofibrillar protein fractional synthesis rate is blunted in the elderly as compared to younger subjects (Welle, Thornton et al. 1994). It's been shown that young individuals exposed to hyperaminoacidemia in conjunction with hyperinsulinemia experienced increases in MPS (Bennet, Connacher et al. 1990; Volpi, Mittendorfer et al. 2000), along with decreases in MPB (Volpi, Mittendorfer et al. 2000). However, older individuals exposed to the same protocol were shown to have a decreased anabolic response to hyperaminoacidemia and hyperinsulinemia compared to the younger individuals (Volpi, Mittendorfer et al. 2000). The decreased anabolic response was due to no significant increase in MPS in the older individuals, although decreases in MPB were seen in both young and old (Volpi, Mittendorfer et al. 2000). The decreases in MPB observed were

likely due to the hyperinsulinemic state, since amino acid administration in isolation has not been shown to reduce MPB in elderly individuals (Volpi, Ferrando et al. 1998). To conclude, the diminished anabolic response to feeding may be a primary driver in the progression of wasting due to decreased rates of protein synthesis and prolonged negative protein balance.

Section 6: Additional Contributing Factors to Sarcopenia

Beyond the anabolic resistance to feeding observed in the elderly, there are a number of other factors that contribute to the progression of age associated wasting. Intrinsic factors include hormonal influences, neuromuscular changes and muscle morphology, and mitochondrial dysfunction and oxidative stress. Extrinsic contributors include reductions in physical activity, as well as inadequate energy intake.

Section 6.1: Hormonal influences

There are multiple hormonal influences within body that provide additional anabolic stimuli beyond those observed with feeding. In an aging population it is common to see decreases in endogenous anabolic hormones such as testosterone (van den Beld 2000), growth hormone, and IGF-1 (Rudman, Feller et al. 1990).

Total testosterone decreases in individuals as they age. Additionally, a large amount of the total testosterone is bound to sex hormone binding globulin (SHBG) or albumin, resulting in less free testosterone for anabolic uses (Morley and Perry 2000). A 3% annual decline in free testosterone has been observed in males age 73-94, which appears to parallel the decreases in muscle size and strength observed in aging men (van den Beld 2000). Testosterone replacement in older hypogonadal men has led to increases in lean body mass (LBM) (Tenover 1992). However, there are negative consequences seen with exogenous testosterone administration

such as prostate growth, specific antigens increase, increased hematocrit, and negative effects on lipid profiles (Morley and Perry 2000). Postmenopausal women also experience decreases in free testosterone. Additionally, those having undergone hysterectomy with oophorectomy have 40% less total and free testosterone levels than those observed in the intact women (Laughlin, Barrett-Connor et al. 2000).

Growth hormone (GH) and Insulin-like Growth Factor 1 (IGF-1) also decrease with age (Rudman, Feller et al. 1990), and likely contribute to the age related declines in LBM. GH is secreted from the pituitary gland and leads to peripheral production of IGF-1. It has been shown in elderly males that exogenous GH administration to raise IGF-1 levels has a positive effect on increasing LBM along with decreases in fat mass (Rudman, Feller et al. 1990). However, in elderly populations GH administration has shown both a positive (Butterfield, Thompson et al. 1997) effect on MPS, as well as no effect (Welle, Thornton et al. 1996) on both whole body protein synthesis or MPS. It is speculated that individuals that are not deficient in GH observe no additional anabolic benefit from GH administration (Rennie 2003). Additionally, there is evidence that resistance exercise in older men combined with exogenous growth hormone does not lead to greater increases in muscle strength, compared to resistance training alone (Taaffe, Pruitt et al. 1994). IGF-1 administration can increase protein synthesis in the short-term (Butterfield, Thompson et al. 1997), however long-term administration does not lead to increases in LBM or strength in elderly women (Friedlander, Butterfield et al. 2001).

Although there is a decrease in anabolic hormones during the aging process, there is recent evidence that testosterone, growth hormone, and IGF-1 have no significant role in the anabolic processes resulting from an acute bout of resistance training (West, Burd et al. 2010). Two types of resistance exercise protocols were compared, with one leading to significant acute

increases in testosterone, growth hormone, IGF-1, while the other protocol did not lead to acute increases in these anabolic hormones. After 12-weeks, there was no significant difference in muscle CSA or strength between groups (West, Burd et al. 2010). The insignificant differences in muscle CSA and strength suggest that acute physiological increases in testosterone, growth hormone, and IGF-1 do not play a role in the anabolic response from resistance training.

Section 6.2: Changes in Neuromuscular Functioning and muscle morphology

Muscle denervation can lead to muscle atrophy. Elderly individuals over age 60 can have as little as 50% of the motor neurons as their young and middle aged counterparts (Tomlinson and Irving 1977; Brown, Strong et al. 1988). Following denervation, a muscle decreases its mass by over 50% within the first month (Gutmann 1962). This denervation is thought to be a result of changes in the functional and structural integrity of the neuromuscular junction (NMJ). Specifically, there is observed pre-terminal thinning, sprouting, and distention on motor axons associated with aging. Additionally, post synaptic endplates reduce in size, length, number, and density (Jang and Van Remmen 2011). Oxidative stress is thought to play a large role in the previously described degeneration of the NMJ. Specifically, the homozygous deletion of Sod1 (CuZnSOD) in mice led to accelerated changes in the NMJ compared to those observed with aging (Jang, Lustgarten et al. 2010). As a result, muscle contractile force is diminished in these Sod1knockout mice (Jang, Lustgarten et al. 2010). However, there is also evidence that neural dysfunction is not a major contributing factor in sarcopenia. As mentioned previously, when motor nerves of elderly individuals are electrically stimulated during voluntary maximal contraction no additional torque is produced (Vandervoort and McComas 1986). Thus, in humans, it appears that declines in strength observed with aging have more to do with muscle atrophy rather than the inability to stimulate motor neurons.

Sarcopenia also changes muscle morphology. Between the ages of 20 and 80 years, there is approximately a 50% reduction in total fiber number, with an accelerated decline after 50 years of age (Lexell, Taylor et al. 1988). The use of ATPase staining is able to analyze changes in muscle fiber types with aging. It has been demonstrated that Type II fibers show a greater degree of atrophy than Type I fibers with aging, with an associated decrease in the mRNA content of MHC 2a and MHC 2x with age (Balagopal, Schimke et al. 2001). Type II fibers have the greatest propensity for growth and produce more force when contracting than Type I fibers. Therefore, Type II fiber atrophy arguably has more negative implications than Type 1 fiber atrophy on mobility in the aging population. Additionally, it's been shown that as individuals age and experience Type II fiber atrophy there is a shift in proportion to more aerobic and oxidative myosin light chain (MLC) fiber types, with myosin heavy chain II (MLC II) isoforms increasing substantially (Klitgaard, Mantoni et al. 1990; Gannon J 2009). Sedentary elderly individuals show greater amounts of myosin heavy chain type I (MHC I) and myosin light chain II fibers (MLC II) than younger individuals, which may be induced through compensation of Type II fiber atrophy (Klitgaard, Mantoni et al. 1990).

Section 6.3: Mitochondrial Dysfunction and Oxidative Stress

Mitochondrial dysfunction and oxidative stress may contribute to the progression of sarcopenia. The free-radical theory of aging suggests that age-related increases in mutagenic oxygen radicals lead to cellular damage and ultimately cellular senescence (Harman 1956). These free radicals are primarily produced in the mitochondria via oxidative phosphorylation, and are termed reactive oxygen species (ROS). The increase in ROS is responsible for damage to mitochondrial DNA (mtDNA), leading to electron transport chain (ETC) protein defects (Greenlund LJ 2003; Johnston, De Lisio et al. 2008).

Disturbances within the mitochondria may mediate cellular apoptosis via the activation of caspase family of proteases (Green and Reed 1998). In aging rats it has been shown that mtDNA copy numbers in skeletal, but not cardiac muscle, reduce with the aging process (Barazzoni, Short et al. 2000). This decrease in skeletal muscle mtDNA suggests that skeletal muscle mtDNA seems to be less protected from degradation during the aging process or disuse.

Oxidative stress is known to contribute to protein breakdown. Increases in ROS activate nuclear factor-kB (NF-kB). NF-kB is a transcription factor that up-regulates catabolic inflammatory cytokines such as interleukin 6 (IL-6), interleukin 1β (IL-1β), and tumor necrosis factor-α (TNF-α). NF-kB tends to be elevated in elderly individuals contributing to sarcopenia (Bruunsgaard and Pedersen 2003), with elderly males having nearly a fourfold higher level of NF-kβ in their myofibers than younger males (Cuthbertson, Smith et al. 2004). More research still needs to be done to elucidate mitochondrial changes during aging, and their effects on the progression of sarcopenia. Even though oxidative stress is a known contributor to protein breakdown, it has been observed that MPB does not increase during the aging process (Volpi, Sheffield-Moore et al. 2001). Therefore, it appears that the decreases in LBM observed with aging are more so a result of decreases in MPS.

Section 6.4: Behavioral Factors

There are a number of behavioral factors that contribute to wasting, such as reduced physical activity, as well as reduced energy intake. In the United States, the recommended daily amount of physical activity is 30+ minutes of moderate physical activity 5 days per week. According to the 2009 Behavioral Risk Factor Surveillance System, 61% of individuals aged 18-24 get the recommended amount of daily physical activity, whereas only 40% of individuals over the age of 65 achieve the recommended amount (CDC 2009). Age associated muscle weakness,

increased fatigability, and endurance losses all contribute to the observed age-related decreases in physical activity (Greenlund LJ 2003). Decreases in endurance capacity are even observed in long-term (8 years) endurance trained older individuals, as evidenced by a 5.5% decrease in VO_2max per decade. However, those who were not endurance trained over 8 years experienced a 12% decline in VO_2max per decade (Rogers, Hagberg et al. 1990). The increased decline in VO_2max in non-endurance trained individuals provides evidence that endurance exercise can attenuate the declining rate in VO_2max as individuals age. Since resistance (Chesley, MacDougall et al. 1992; Biolo, Maggi et al. 1995) and endurance exercise (Sheffield-Moore, Yeckel et al. 2004) can also increase MPS, incorporating and maintaining an exercise regime could help prevent the losses in LBM.

It is common for older individuals to consume fewer calories than their younger counterparts. Although many older individuals have decreased physical activity, many older individuals are still in negative energy balance. This "anorexia of aging" is a common occurrence in the older population, and has a number of contributing factors. Increases in gastrointestinal satiety signals such as the hormone cholecystokinin (CCK) are observed with age (MacIntosh, Andrews et al. 1999), limiting the neural drive to consume additional calories. Additionally, age related decreases in taste, flavor, and palatability play a role in the decreased food intake (Morley 2001). Regardless of mechanism, negative energy balance increases AMPK, thus suppressing mTOR and MPS. The inhibition of mTOR and MPS can lead to the decreases in LBM observed with sarcopenia.

Section 7: Interventions

Although losses of LBM are inevitable throughout the aging process, there are behavioral modifications that can attenuate this wasting. Both exercise and proper nutritional practices seem to be promising strategies for preventing wasting.

Section 7.1: Exercise

Resistance and aerobic exercise can lead to a positive muscle protein balance. However, exercise's effects on WBPT are still unclear. Carraro et al. failed to demonstrate an effect of aerobic exercise (4 hrs @ 40% VO_2max) on WBPT both during and after the exercise bout (Carraro, Hartl et al. 1990). However, others have shown that aerobic exercise (3 hrs @75 %VO_2max) increases whole body protein synthesis following exercise, with WBPB not differing from values at rest (Devlin, Brodsky et al. 1990). Although there is mixed evidence regarding aerobic exercise's acute effects on WBPT, long-term aerobic exercise has been shown to increase basal WBPT (Lamont, Patel et al. 1990), while decreasing leucine oxidation at rest (Gaine, Viesselman et al. 2005). This indicates improved protein utilization as a result of exercise.

Aerobic exercise and its magnitude of effect on NBAL appears to be dependent on energy balance. When maintaining energy balance, aerobic exercise increases NBAL over time (Todd, Butterfield et al. 1984; Gaine, Viesselman et al. 2005). However, the increases in NBAL observed from aerobic exercise are decreased when individuals are in hypocaloric state (Todd, Butterfield et al. 1984). Additionally, if protein intake is adequate, a caloric deficit created by increased physical activity results in a greater NBAL than if the deficit is created through diet alone (Todd, Butterfield et al. 1984).

In rodents, acute aerobic exercise not only inhibits MPS during the bout of exercise (Dohm, Kasperek et al. 1980; Davis and Karl 1986), but also increases MPB (Dohm, Kasperek et al. 1980) as a result of increased amino acid utilization during exercise in order to meet the increased energy demand. The magnitude of decrease in MPS during aerobic exercise is positively correlated with the exercise intensity (Balon, Zorzano et al. 1990), and duration (Dohm, Kasperek et al. 1980). Additionally, it does not appear that prior endurance training attenuates the decrease in MPS observed with acute aerobic exercise (Davis and Karl 1986). The decrease in MPS and increase in MPB, results in an acute negative net protein balance during the exercise bout (Norton LE 2006).

Despite decreases in MPS and increases in MPB during exercise, physical activity has been shown to lead to the maintenance or increase in skeletal muscle, as well as an increased NBAL. This indicates a compensatory response within the post-exercise recovery period to increase MPS and/or decrease MPB. It is well known that MPS (Chesley, MacDougall et al. 1992; Biolo, Maggi et al. 1995) and mixed muscle fractional synthetic rate (FSR) (Phillips, Tipton et al. 1997) increase following resistance exercise, with elevations in MPS observed for up to 48 hr (Phillips, Tipton et al. 1997) or 72 hr (Miller, Olesen et al. 2005) following the resistance bout. Additionally, MPB (Biolo, Maggi et al. 1995) and fractional breakdown rate (FBR) (Phillips, Tipton et al. 1997) increase following resistance exercise, however MPB is elevated to a lesser extent than MPS (Biolo, Maggi et al. 1995). Muscle protein balance therefore improves (Biolo, Maggi et al. 1995; Phillips, Tipton et al. 1997), but does not reach a positive value (Biolo, Maggi et al. 1995). Additionally, there is evidence that chronic resistance training can increase resting myofibrillar FSR. After 10 weeks of chronic resistance training in the fed state, resting myofibrillar FSR increased 37% over prior resting conditions (Wilkinson, Phillips et al. 2008). Additionally, 4 months of aerobic (bicycle) training (up to 45 min at 80% max heart rate, 3-4

days/week) has been shown to increase steady-state mixed MPS 22% from baseline (Short, Vittone et al. 2004).

Regarding aerobic exercise, MPS increases following aerobic exercise at 40% VO_2Max (Sheffield-Moore, Yeckel et al. 2004) and at 72 ± 1% VO_2Max (Harber, Konopka et al. 2010). Additionally, increases in MPS following aerobic exercise have been observed in both the fasted and fed state (Harber, Konopka et al. 2010). However, there is evidence that increases in MPS resulting from aerobic exercise is balanced by equivalent increases in MPB (Sheffield-Moore, Yeckel et al. 2004).

Additional research has also been done comparing the response of both young and old populations to resistance and aerobic training programs. The fractional rate of MPS in the quadriceps of elderly individuals is lower than younger individuals prior to undergoing a resistance training program, but 2 weeks of daily resistance exercise was shown to increase FSR of muscle to the same extent seen in the younger individuals measured following the last bout of resistance exercise (Yarasheski, Zachwieja et al. 1993). Additionally, in the elderly group, there was no observed increase in MPB after 2 weeks of training, as measured following the last bout of resistance exercise (Yarasheski, Zachwieja et al. 1993). There is mixed evidence on whether elderly individuals experience a similar anabolic response to exercise as their younger counterparts. In one study, after an acute bout of moderate intensity aerobic exercise (40% VO_2max), mixed muscle FSR was not statistically elevated at time points measured beyond 10 minutes post-exercise in untrained older men (Sheffield-Moore, Yeckel et al. 2004). However, mixed muscle FSR was still elevated at 60 minutes post exercise in younger untrained men following the same protocol (Sheffield-Moore, Yeckel et al. 2004). Another study found no differences in mixed MPS between young and old men following 4 months of aerobic exercise

(up to 45 min @ 85% max heart rate for 3-4 days/week) (Short, Vittone et al. 2004). Additionally, it has been shown that prolonged aerobic exercise can increase myofiber size in elderly women (Harber, Konopka et al. 2009), thus providing evidence of the benefits of aerobic exercise increasing LBM in elderly individuals.

The benefits of aerobic exercise extend beyond its direct impact on protein turnover. As individuals age, insulin's ability to vasodilate decrease (Meneilly, Elliot et al. 1995). It is thought that insulin's blunted ability to stimulate MPS in older individuals is largely due to its reduced ability to induce vasodilation (Fujita S 2007). However, it's been shown in older subjects that a single bout of moderate aerobic exercise can improve endothelial function, by enhancing the vasodilating properties of infused insulin and promote MPS through insulin's action on phosphorylating Akt and mTOR/S6 kinase (Fujita S 2007). The control group who did not exercise experienced no increases in MPS via insulin infusion (Fujita S 2007).

Exercise has a number of benefits with regards to reducing the effects of wasting, serving as a stimulus that can lead to positive protein balance during the recovery period. Since there is evidence that in isolation neither resistance nor aerobic training induces a positive protein balance, the hypertrophic response following exercise must be dependent on additional factors such as feeding.

Section 7.2: Nutrition after exercise

In the absence of nutrient intake after exercise, net protein balance will remain negative (Biolo, Maggi et al. 1995). However, proper ingestion of amino acids with or without carbohydrate causes a shift towards increased protein balance in the post exercise period (Biolo, Tipton et al. 1997; Miller, Tipton et al. 2003; Borsheim, Aarsland et al. 2004). Although hyperinsulinemia at rest increases MPS, hyperinsulinemia post exercise provides no further

increases in MPS (Biolo, Williams et al. 1999). However, hyperinsulinemia's ability to decrease MPB is increased following exercise compared to rest (Biolo, Williams et al. 1999). Insulin's inability to stimulate MPS following exercise provides evidence to the importance of amino acids during the post exercise period to further increase MPS to create a positive protein balance.

It has been shown that in fasting humans at rest, infusion of amino acids increases MPS and NBAL, with no effects on MPB (Biolo, Tipton et al. 1997). However, the same amino acid infusion after a bout of resistance exercise results in greater levels of MPS, which suggests an additive effect of the exercise and the amino acids (Biolo, Tipton et al. 1997). An additional line of thought is that exercise serves as a stressor, and that EAA ingestion provides the nutritional substrates necessary to induce adaptation to this stress (Miller 2007). By consuming amino acids with carbohydrates post exercise, net protein balance can be increased to levels greater than if an amino acids or carbohydrate were ingestion alone (Miller, Tipton et al. 2003; Borsheim, Aarsland et al. 2004). Mechanistically, the acute increases in MPS that occur after post exercise feeding occur at the level of translation initiation, with the availability of eIF4E for 48S ribosomal complex formation increasing after a complete meal feeding post exercise (Gautsch, Anthony et al. 1998).

There is relatively limited data in the area of post aerobic exercise nutrition in the elderly population. When consumed post aerobic exercise, it has been shown that protein combined with carbohydrate (40g carbohydrate, 20g whey) leads to increases in WBPT in the elderly above that observed with an isoenergetic amount of carbohydrate (60g carbohydrate) (Murphy and Miller 2010).

Section 7.3: Nutrient Timing

The timing of amino acid and carbohydrate ingestion has an influence on the degree of increase in protein balance. In resistance trained older male subjects the consumption of a post-workout protein beverage (10g protein, 7g carbohydrate, 3 g fat) immediately after resistance exercise resulted in greater gains in muscular hypertrophy and isokinetic strength after 12 weeks, than if the beverage was consumed 2 hrs following resistance exercise (Esmarck, Andersen et al. 2001). In another study either 6g of EAA with 35g of sucrose or a placebo was consumed either 1 hour or 3 hours after a bout of resistance training (Rasmussen, Tipton et al. 2000). Although consumption of the beverage at both time points resulted in greater MPS than the placebo, there was no statistical difference in net protein balance when the drink was consumed 1h or 3h post exercise (Rasmussen, Tipton et al. 2000). MPB was also unchanged by the consumption of the drink vs. the placebo, indicating that the increase in anabolism was due to increased MPS (Rasmussen, Tipton et al. 2000). With regards to aerobic exercise, the consumption of an oral supplement (10g protein, 8g carbohydrate, 3g fat) consumed immediately after exercise resulted in greater increases in MPS than if consumed 3 hours after exercise (Levenhagen, Gresham et al. 2001). However, a longer duration study found that post aerobic exercise protein ingestion did not lead to increases in mixed MPS over the course of 6 weeks, providing evidence that the acute increases in MPS occurring after post exercise protein feeding do not persist long-term (Robinson, Turner et al. 2011).

In a study similar to ours, young female athletes following two seven-day trials of increased training volume, the post exercise consumption of a mixed-meal beverage led to an increased trend ($p=.06$), albeit insignificant, towards a more positive NBAL than if consumed earlier in the day. Similar to our study, the two trials were isocaloric and isonitrogenous, with

only the timing of the mixed-meal beverage differing between trials (Roy, Luttmer et al. 2002). As mentioned previously, our lab has also shown that the consumption of chocolate milk immediately following moderate aerobic exercise (55% VO2max) in older adults in energy balance, leads to greater NBAL than if it is consumed earlier in the day. The increase in NBAL is especially significant due to the fact that both trials were isocaloric and isonitrogenous, with the only difference being the timing of the milk intake (Jordan, Melanson et al. 2010).

Section 8: Leucine

The composition of amino acids ingested following exercise has a direct impact on increasing protein synthesis thus creating a shift towards a positive protein balance. EAAs are primarily responsible for stimulating MPS in the elderly (Volpi, Kobayashi et al. 2003), while non essential AA are not sufficient in initiating MPS in the elderly (Volpi, Kobayashi et al. 2003), or in young populations (Tipton, Gurkin et al. 1999). Thus, choosing a protein source rich in EAA, particularly leucine, is important for increasing MPS.

BCAAs, particularly leucine, are known regulators of MPS and translation initiation (Kimball and Jefferson 2001), and plays an important role in the protein synthetic response from feeding. Leucine itself, in the absence of other amino acids, is capable of initiating mRNA translation, exerting its effects on protein synthesis through the mTOR signaling pathway, including S6K, eIFs, and 4E-BP1 (Anthony, Lang et al. 2002; Crozier, Kimball et al. 2005). Additionally, leucine has the ability to stimulate MPS in a manner independent of mTOR activation. This mTOR independent stimulation of MPS occurs via the direct activation and phosphorylation of eIF4G, resulting in the ability of eIF4E to join with eIF4G to initiate MPS (Bolster, Vary et al. 2004).

Research has also examined the leucine content in meals in regards to plasma leucine and protein synthesis. Meals that are low in protein (10g/meal) with low leucine are inferior in increasing plasma leucine to meals higher in protein (30g/meal) with higher leucine levels (Layman DK 2003). Additionally, the quantity of leucine in isonitrogenous meals will directly affect the peak activation of protein synthesis, however it does not affect the duration (Norton LE 2009). Whey, a rich source of leucine, is able to stimulate MPS to a greater extent than an isonitrogenous amount of wheat protein, which has a lower comparative leucine content (Norton LE 2009). Additionally, consumption of whey hydrolysate both at rest and after a bout of acute resistance exercise increases blood leucine and MPS to a greater degree than an isonitrogenous amount of micellar casein or soy protein isolate (Tang, Moore et al. 2009). Leucine has therefore been proposed to be a key component of a meal with regards to increasing protein synthesis (Layman DK 2003).

Following an acute bout of endurance exercise, plasma leucine concentrations decrease (Paul, Rokusek et al. 1996). Post-exercise leucine ingestion has therefore been examined as an aid to the recovery process. Following exhaustive aerobic exercise in rats, the ingestion of a glucose and sucrose electrolyte beverage increases blood glucose, glycogen content, and insulin concentrations, but has no effect on increasing MPS (Gautsch, Anthony et al. 1998). However, post exercise ingestion of leucine alone (Anthony, Anthony et al. 1999) or a complete meal with protein (Gautsch, Anthony et al. 1998) increases MPS, indicating the importance of protein (and leucine) during the recovery period. As mentioned previously, consumption of whey hydrolysate (a rich source of leucine) results in greater MPS than micellar casein and soy protein isolate when consumed following a bout of resistance training (Tang, Moore et al. 2009). Additionally, leucine in addition to protein and carbohydrate has been shown to increase whole

body protein balance to a larger extent than carbohydrate with protein, or carbohydrate alone when consumed post exercise (Koopman, Wagenmakers et al. 2005).

Section 9: Milk as a postexercise beverage

While many studies have used customized blends of protein and carbohydrate beverages when studying the effects of protein and/or carbohydrate after exercise, relatively few studies have used a naturally occurring food source. An economical and easily accessible source of essential amino acids (particularly leucine) and carbohydrates is in the form of milk. Both fat free and whole milk have been shown to stimulate MPS after resistance exercise (Elliot, Cree et al. 2006). Skim milk has also been shown to result in greater protein balance and fractional synthesis rates over 3 h than an isocaloric, isonitrogenous soy-protein beverage when consumed after resistance training (Wilkinson SB 2007). As mentioned previously, our lab has shown that consumption of chocolate milk immediately following 1 hour of moderate intensity aerobic exercise, can lead to greater increases in NBAL in older individuals in energy balance than if the milk is consumed earlier in the day (Jordan, Melanson et al. 2010).

There are dietary sources of both “fast” and “slow” acting proteins. Both whey and casein are milk fractions, with fluid bovine milk containing approximately 80% casein and 20% whey by mass (Wilkinson SB 2007). Whey, considered a “fast” protein, digests rapidly and provides a rapid, although short lived rise in plasma AAs (Boirie, Dangin et al. 1997). Casein, considered a “slow” protein, provides a more moderate but prolonged rise in plasma AAs. Casein’s effects are thought to be due to delayed gastric emptying (Boirie, Dangin et al. 1997). Additionally, casein has been shown to reduce protein breakdown, whereas whey actually increases leucine oxidation (Boirie, Dangin et al. 1997). Since whey is more effective at

stimulating rapid increases in plasma AAs and protein synthesis, combining it with casein to reduce protein breakdown is a viable strategy to increase the anabolic environment.

Section 10: Conclusions

Wasting has a number of factors influencing the loss of muscle mass and strength as individuals age. However, both exercise and nutrition are within one's own control and can be manipulated to elicit a greater NBAL over time. Combining regular exercise with balanced daily caloric intake and proper post workout nutrition can attenuate the progression or onset of wasting. Our lab previously showed that in older individuals in *energy balance*, consumption of protein and carbohydrates (chocolate milk) immediately following moderate aerobic exercise at 55% VO_2max led to greater 3-day mean NBAL than if consumed earlier in the day. Because increased energy intake leads to increases in NBAL (Munro 1951; Butterfield and Calloway 1984), we wish to further investigate the impact of the timing of protein intake on NBAL in exercising older individuals on a *hypercaloric* diet.

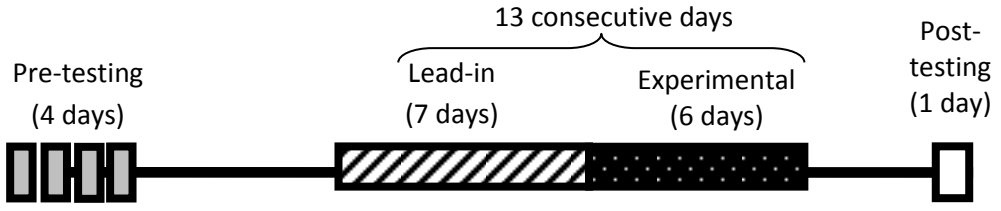
CHAPTER 3

METHODS AND PROCEDURES

Overview

The present study compared NBAL in older subjects in positive energy balance with chocolate milk consumption immediately following moderate aerobic exercise compared chocolate milk consumed earlier in the day. Two, three-day periods were used for each intervention and were isocaloric and isonitrogenous to one another. The only difference within the phases was the timing of the milk protein intake. There were four distinct periods within the duration of the study for each participant: the pre-experimental testing, a 7-day lead in diet, the 6-day experimental intervention consisting of 2, 3-day phases, and post testing (Figure 3.1). The study protocol was approved by the Colorado State University Institutional Review Board and the Colorado Multiple Institutional Review Board for human participants research.

Figure 3.1 Experimental timeline

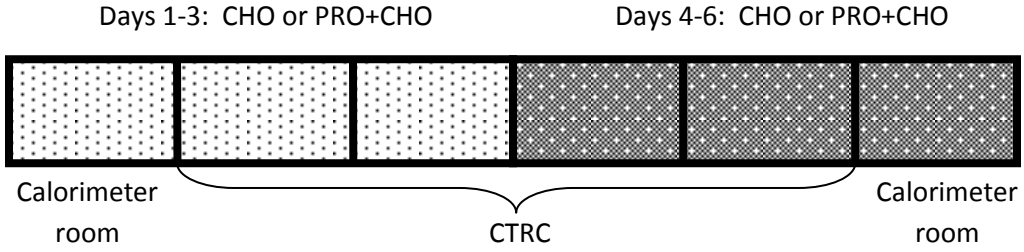


Pre-testing		Lead-in	Experimental		Post-testing
Day 1: GXT, diet log collection Day 2: RMR, DEXA Day 3: VO_{2max} Day 4: steady-state VO_2	2-3 wks break to allow for dietary planning and scheduling	Controlled diet, daily weight measurement, 2 days with activity monitor	Controlled diet, 1-hr daily exercise followed by test beverage, 24-hr urinary collection, daily weight measurement, activity monitor, 2 days in Cal Room	~1wk	DEXA, nutritional follow-up, subject payment, weight measurement

The pre-testing period allowed for planning of lead-in and controlled experimental diets. The lead-in diet allowed subjects to adapt to the level of protein intake they would be exposed to during the experimental portion of the study. This adaptation period may be necessary since protein kinetics can change with acute changes in protein intake (Rand, Young et al. 1976), although this is currently debated (Yarasheski, Castaneda-Sceppa et al. 2011). Regardless of whether a lead-in period is necessary for adaptation to changes in protein intake, we took a conservative approach to insure our subjects did not have changes in NBAL due solely to acute changes in protein intake.

During the experimental portion of the study, subjects underwent a 6-day inpatient stay at the University of Colorado Denver Clinical and Translational Research Center (CTRC). Each subject completed 2, 3-day trials in a randomized cross-over design (CHO and PRO+CHO). Days 1 and 6 of the patient's stay were spent in the whole room calorimeter to confirm positive energy balance (Figure 3.2).

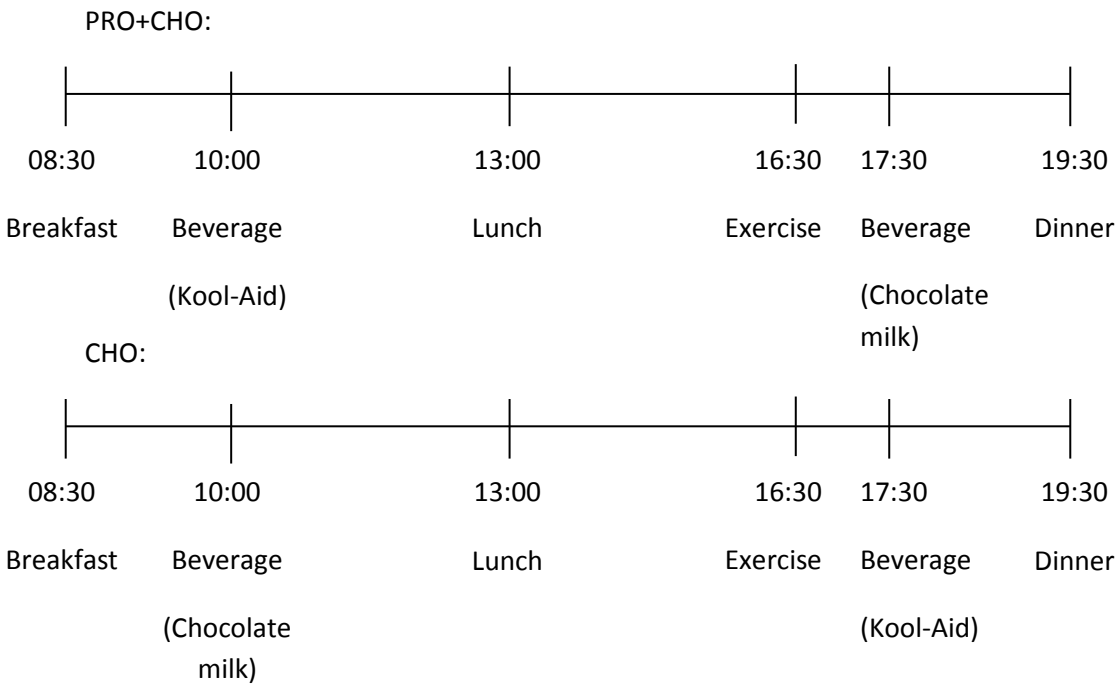
Figure 3.2 Experimental in-patient period



The subjects stayed in an in-patient hospital room at the CTRC for days 2-5, where they were permitted to leave for 30 minutes twice a day. Breakfast, lunch, and dinner were all served at the same times every day. From 4:30-5:30 pm each day, subjects performed one hour of cycling exercise @ 55% VO2 max. Immediately following the exercise, subjects consumed the

respective post-exercise beverage. During the PRO+CHO phase, subjects consumed a chocolate milk beverage immediately after exercise (5:30 pm), with a carbohydrate beverage consumed earlier in the day (10:00 am). During the CHO phase, subjects consumed the carbohydrate beverage immediately after exercise (5:30 pm), with the chocolate milk beverage consumed earlier in the day (10:00 am) in the CHO phase. Total energy, macronutrients, and food were identical for both conditions. The only difference between phases was the timing of the protein-containing beverage (Figure 3.3).

Figure 3.3 Timing of meals and consumption of beverages during in-patient stay



Participants

Six male (n=2) and female (n=4) subjects between 55 and 75 years old were recruited from the Fort Collins and Loveland, Colorado area. Individuals were required to be inactive, lactose tolerant, non-smokers, and not taking any medications. Additional exclusion criteria

included: obesity (BMI > 30), any orthopedic injury that impeded their ability to exercise, any conditions that affected food digestion, any thyroid condition (TSH <0.05 mu/mL or TSH>5.0 mu/mL), bleeding disorders, or any current illness and/or infection. Due to finances, we were limited to six subjects. See Table 3.1 for subject characteristics.

Table 3.1 Subject Characteristics

	Male (n=2)	Female (n=4)	All subjects (n=6)
	Mean ±SEM	Mean ±SEM	Mean ±SEM
Age	62.50 ± 5.50	66 ± 2.04	64.83 ± 2.06
Height (cm)	175.26 ± 2.54	160.02 ± 1.37	165.10 ± 3.39
Weight (kg)	84.68 ± 2.95	59.66 ± 3.16	68.00 ± 5.69
BMI (kg/m^2)	27.61 ± 1.76	23.34 ± 1.47	24.76 ± 1.37
Body fat (%)	24.70 ± 1.30	37.93 ± 4.21	33.52 ± 3.87
VO_{2max} ($ml \cdot kg^{-1} \cdot min^{-1}$)	32.30 ± .80	22.93 ± 1.88	26.05 ± 2.32

Screening

At the initial visit, subjects read and signed an informed consent, HIPAA-B Approval form, and a medical and exercise history questionnaires (Appendices A, B, and C). In addition, online food preference and food allergy questionnaires from the CTRC were completed and three-day diet logs were given to the subjects who were instructed to maintain normal eating habits and record all foods and beverages consumed for 2 week days and 1 weekend day. Subjects returned their completed three-day diet logs at the first pre-testing appointment. The diet logs were entered into NutritionistPro software (Axxya Systems, Stafford, TX) in order to analyze and determine habitual daily energy and macronutrient intakes. Subjects also completed a blood draw at Poudre Valley Hospital prior to the initial pre-testing appointment,

which was analyzed for blood lipid profile, and normal thyroid stimulating hormone concentrations.

Pre-Testing

Over the course of four separate days, subjects completed a series of pre-tests at the HPCRL in the Department of Health and Exercise Science at Colorado State University. On the initial visit, subjects completed a treadmill graded exercise test (GXT). The test began with subjects walking at 3.3 miles per hour (mph) at 0% grade. The treadmill speed remained at 3.3 mph for the duration of the test but the grade increased 2% after the first, and 1% every minute thereafter. Data collected during the test included: heart rate, rating of perceived exertion (RPE), blood pressure, and an electrocardiograph (EKG), all of which were recorded every three minutes. The test was terminated when subjects reached 85-100% of their predicted maximum heart rate (220-age) or when the subjects were too exhausted to continue. The GXT was supervised by an on-site cardiologist who excluded subjects if the test indicated an ischemic or hypertensive response to the exercise.

Subjects returned to the HPCRL on a second day, following an overnight fast. Resting metabolic rate (RMR) was measured (Parvomedics TrueOne 2400, Sandy, UT) in order to determine the subject's 24-hour resting caloric expenditure. During the test, subjects were instructed to rest in the supine position with the lights dimmed and all expired gases were collected. Subjects were asked to refrain from sleeping during the testing procedure. The flow rate was manually adjusted throughout testing to maintain $FECO_2$ levels between 0.9-1.0%. The testing procedure lasted a total of 45 minutes. The first 15 minutes of the test was used to achieve the appropriate flow rate and to allow subjects to become familiarized and comfortable with the experimental conditions. The final 30 minutes of data was used to measure the

subject's RMR using the Weir equation (Weir 1949). Reported values for daily energy expenditure were averaged with measurements outside ±2 standard deviations being omitted. Following the RMR test, subjects were administered a dual-energy X-ray absorptiometry (DEXA) scan (QDR 4500W, Hologic, Inc., Bedford MA) in order to determine body composition and bone mineral density.

On the third day of testing subjects underwent an incremental exercise test on a cycle ergometer (Monark Excalibur, Groningen, The Netherlands) with indirect calorimetry (ParvomedicsTrueOne 2400, Sandy, UT) to determine the subject's VO_2max. Subjects pedaled at 50 Watts for the first minute with subsequent 20 (female) or 30 (male) Watt increases every two minutes. During the test, subjects maintained a pedal rate between 70-90 rpm. The test was terminated once the following criteria were met: subjects reached volitional exhaustion, heart rate was within 10 beats of age predicted maximum heart rate, respiratory exchange ratio (RER) was greater than 1.1, and when VO_2 reached a plateau (Brooks GA 2005). Following the determination of subject's VO_2max, 55% of each subject's VO_2max was calculated and recorded. Equation 3.1 (ACSM 2010)was utilized in estimating the cycle ergometer rate that would correspond to a steady-state exercise intensity at 55% of VO_2max.

Equation 3.1 ACSM leg cycle ergometry equation

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

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

Subjects returned to the HPCRL on a third day for a submaximal steady-state cycling test. Subjects began cycling at their calculated workload of 55% VO_2max (from equation 3.1).

Once VO_2 stabilized subjects continued cycling for an additional 30 minutes. Using indirect calorimetry, exercise energy expenditure was determined by averaging the energy expenditure from the final 30 minutes of cycling. The calculated energy expenditure from the 30 minutes of cycling was used to calculate the subject's energy expenditure for the one hour of cycling that would occur daily during the inpatient period.

7-day lead in diet period

Two to three weeks following the pretesting period, subjects completed a controlled, 7-day lead-in diet immediately prior to their inpatient stay and the intervention/experimental period. The purpose of the lead-in diet was to habituate subjects to the protein intake of the inpatient intervention (1.2g/kg bw). Exposure to this protein intake ruled out the possibility that NBAL data during the inpatient intervention period was due to an adaptation to a new level of protein ingestion.

Both the lead-in and in-patient study diets were constructed using Pronutra software (Viocare, Inc., Princeton, NJ) by a licensed dietician. In addition to the 1.2 g/kg bw of protein, 30% of caloric intake was in the form of dietary fat, with the remaining balance coming from carbohydrate. During the lead-in period subjects were kept in energy balance. Lead-in diet energy intake was calculated by using their calculated RMR multiplied by an activity factor of 1.5, as in our previous study (Jordan, Melanson et al. 2010).

During the lead-in diet subjects were instructed to arrive every morning to the Colorado State University Nutrition Center. Food for the day was prepared with breakfast being consumed in front of study staff. Food for the rest of day was sent home with the subjects in a cooler with instructions to eat only the contents within the cooler. Subjects returned the

following day for the next days' worth of food. Subjects were instructed to undergo their typical daily activities throughout the duration of the 7-day lead-in period.

On the evening of the 7th day of the lead-in period, subjects were admitted to the University of Colorado-Denver CTRC, where they were fed dinner. The following day started the 6-day in-patient intervention/experimental period of the study.

6-day experimental intervention

The experimental intervention involved a 6-day stay at the University of Colorado-Denver CTRC, with subjects following an inpatient diet and 24-hour urine collection. Both the first and last day of the inpatient stay were spent in a 12' x 12' whole room calorimeter, while days 2-5 were spent in a regular inpatient room at the CTRC. During days 2-5, subjects were permitted to leave their room for 30 minutes, twice a day. Subjects were only allowed to eat only what was provided to them and were instructed to refrain from exercise outside of the 1 hr of moderate intensity cycling that occurred each day. Subjects were allowed to consume water ad libitum, but were allowed to request additional non-caloric, non-caffeinated beverages as well. Subjects were weighed in their hospital gowns at the same time, on the same scale each morning.

Subjects each completed two 3-day trials in a randomized crossover design. Every day at 4:30 pm, subjects participated in 1 hr of cycling exercise at 55% VO_2max. All exercise was performed on a Lode Corival bicycle ergometer (Lode, Groningen, The Netherlands). Subjects recorded their heart rate every 15 minutes during exercise using a heart rate monitor (Polar FS1, Lake Success, NY). The exercise was intended to be of approximately the same intensity as a brisk walk, and was tolerated well by all subjects.

Following the daily exercise bout, a post-exercise beverage was immediately consumed at 5:30 pm. During the PRO + CHO phase, the beverage consumed immediately after exercise was a 248kcal chocolate milk drink which contained 15.3 g PRO, 43.6 g CHO, and 1.3 g FAT (330g skim milk, 4 g whey protein, and 42 g chocolate syrup). During the CHO phase, the beverage consumed immediately after exercise was a 247kcal Kool-Aid beverage which contained 0 g PRO, 63.51 g CHO, .06 g FAT. During each respective phase, the opposing drink (Kool-Aid for the PRO + CHO phase, Chocolate milk drink for CHO phase) was consumed as a snack at 10:00 am. The order in which subjects completed each phase was randomized.

Within each of the 3-day trials the patient's diets were reproduced and identical in terms of caloric intake, macronutrient profile, and foods consumed. Specifically, the diet plans for day 1, 2, and 3 were repeated on days 6, 4, and 5, respectively (Reference Table 3.2 on next page for sample in-patient diet plan). The only difference between phases was the timing of the intake of the chocolate milk drink. Similar to the lead-in diet, the in-patient diet plan was composed of 1.2g of protein/kg bw, 30% of calories from dietary fat, and the balance as carbohydrate. Both the lead-in and inpatient diets corresponded with the USDA's acceptable macronutrient distribution ranges (AMDRs) (10-35% PRO, 20-35% FAT, and 45-65% CHO)(Services 2005).

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 S- chocolate milk L-salad S- Kool-Aid D-spaghetti
2 (CHO)	1988	B-toast S- chocolate milk L-soup S- Kool-Aid D-stir-fry
3 (CHO)	1972	B-eggs S-chocolate milk L-sandwich S- Kool-Aid D-steak/potato
4(PRO+CHO)	1988	B-toast S- Kool-Aid L-soup S- chocolate milk D-stir fry
5(PRO+CHO)	1972	B-eggs S- Kool-Aid L-sandwich S- chocolate milk D-steak/potato
6(PRO+CHO) Cal room	1896	B-oatmeal S- Kool-Aid L-salad S- chocolate milk D-spaghetti

Since the goal was to have subjects in 15% caloric excess, estimated daily energy expenditure (EE) was calculated prior to the in-patient diet. In the original energy balance study the subject's RMR was multiplied by an activity factor of 1.35 for the calorimeter days, and by 1.45 for the non-calorimeter days in order account for additional activity outside of the calorimeter room (Melanson, unpublished data). However, it was determined that subject's actual energy balance was higher than estimated in the energy balance study. Therefore, for this study the subjects RMR was multiplied by 1.30 for the calorimeter days, and by 1.40 for the non-calorimeter days. Then, steady state VO_2 data was used to estimate energy expenditure (EE) during exercise with an additional 20% factored in for excessive post exercise oxygen consumption (EPOC) ((Jordan, Melanson et al. 2010), Melanson, unpublished data). Equation

3.2 was used to predict total daily energy expenditure (TDEE) for calorimeter room and inpatient days.

Equation 3.2 Estimation of TDEE

EE= (Activity factor (variable) x RMR) + (exercise EE + (.2 x exercise EE)) (Gersovitz, Motil et al. 1982).

The calorimeter room was calibrated with ambient air (O_2 = 20.93% and CO_2=0.03%) and a known gas mixture (O_2 =20.1% and CO_2 =0.9%). The dimensions of the room were 12' x 12', and contained a bed, sink, toilet, bicycle ergometer, computer, and television for subject use. Subjects entered the calorimeter room at 7:45 am on Days 1 and 6, and exited at 7:15 am on the following morning. While in the calorimeter room, subjects were not permitted to nap or perform any exercise other than the one hour of cycling from 4:30-5:30 pm. To prevent air from escaping the calorimeter room, all meals were passed through an air lock that could not be simultaneously opened from the outside or inside of the room. Daily energy expenditure and substrate oxidation were calculated using the difference in the O_2 and CO_2 gas content that was entering and exiting the room. To prevent air from escaping, all meals were passed through an air lock that could not be simultaneously opened from the inside and outside. Daily energy expenditure and substrate oxidation were calculated by the difference in gas content that was entering and exiting the room. Specifically, the difference in the incurrent and excurrent airstream's gas content was determined by using a differential infrared carbon dioxide analyzer (ABB Advance Optima Uras 14 NDIR CO2 Analyzer; ABB, Zurich, Switzerland) as well as a differential paramagnetic oxygen analyzer (Siemens Oxymat 6E Oxygen Gas Analyzer; Siemens, Houston, TX). All analyzed gas values were further corrected for relative humidity, barometric pressure, and temperature (Melanson, Ingebrigtsen et al. 2010). Total energy expenditure and substrate oxidation were calculated by using oxygen consumption and respiratory quotient (RQ)

(Jequier, Acheson et al. 1987). All measurements reported as one-minute averages in the data file.

During the inpatient stay (including calorimeter days), subjects wore an accelerometer (Actigraph GT1M, Pensacola, FL), which was removed during sleep, showering, and exercise. Using the accelerometer data, energy expenditure from non-exercise daily activity was estimated. The Freedson equation (Equation 3.3) was used to estimate EE for activity counts >1,952 per minute (Freedson, Melanson et al. 1998). For activity counts ≤ 1,952 per minute, EE was estimated using the work energy theorem (Equation 3.4).

Equation 3.3 Freedson equation

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

Equation 3.4 Work-energy theorem

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

TDEE on calorimeter days was directly measured and non-calorimeter days was estimated by using Equation 3.5.

Equation 3.5 TDEE on non calorimeter days

TDEE= RMR + non-exercise EE (from accelerometer) + exercise EE (measured as average of 2 days in calorimeter room) + dietary induced thermogenesis (measured as average of two days in calorimeter room)

Energy balance for the inpatient period was determined using Equation 3.6, with energy intake calculated from the dietician.

Equation 3.6 Daily Energy Balance

EB= caloric intake- TDEE.

Daily physical activity level (PAL) was calculated using Equation 3.7, while non-exercise PAL was calculated using Equation 3.8. The value for exercise energy expenditure was multiplied by 1.2 to account for the additional energy expenditure resulting from post exercise oxygen consumption (EPOC) (Melanson, unpublished observations, (Jordan, Melanson et al. 2010)).

Equation 3.7 Physical activity level (PAL)

PAL=TDEE/RMR

Equation 3.8 Non-exercise PAL.

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

Nitrogen Balance Data Collection

NBAL was determined through continuous 24 hr urine collection from subjects. Urine was analyzed for urinary N using the chemiluminescent technique. Specifically, N was analyzed by using an Antek 7000 Elemental Nitrogen Analyzer (PAC, Houston, TX). N intake was calculated as g of protein intake divided by 6.25, since 6.25 g of protein contains approximately 1g of N. Fecal N was estimated at 2g/day, while miscellaneous N loss was estimated at 5mg/kg bw (Calloway, Odell et al. 1971). NBAL was calculated using equation 3.8.

Equation 3.8 Nitrogen Balance

NBAL (g)= N intake (calculated via protein consumption) - (fecal N + urinary N(directly measured) + misc. N loss)

Both 3 day trials of NBAL data were averaged to compare both the CHO, and the PRO + CHO phase with regards to 3-day mean NBAL.

Post testing

Following the inpatient intervention/experimental period, subjects reported back to Colorado State University for a final visit. This visit typically occurred within a week of completion of the inpatient stay. Subjects received a follow up DEXA scan, as well as the analysis of their 3-day food logs which they completed as part of the pre-testing procedures. Lastly, the subjects were compensated for their participation.

Statistical Analysis

All statistical analysis were performed using GraphPad Prism (version 4.00 for Macintosh, Graphpad Software, San Diego, California). NBAL is the primary dependent variable in this study. NBAL data, energy balance data, as well as pretest/posttest bodyweight and body fat data were analyzed using student's paired *t* tests. Any correlations between NBAL and energy balance, or NBAL and relative protein intake (g/kg bw) were determined using Pearson's "r". One-way repeated measures ANOVA and multiple regressions analyzed all diet related variables within the free-living, lead-in diet, inpatient stay. Differences that occurred within the diet related variables were determined via the Student Newman-Keuls post hoc test. All variables tested have a $p<.05$. All data is presented as the mean ± standard error of mean (SEM).

CHAPTER 4

RESULTS

Energy and macronutrient intake

The study diets followed USDA Nutritional guidelines and foods selected were typical for each subject. Figure 4.1 depicts the average energy intake for subjects under the free-living, lead-in, and inpatient phases of the study. Additionally, Figure 4.2 depicts the average macronutrient intakes for subjects under the free-living, lead-in, and inpatient phases of the study. The increase in energy intake during the inpatient phase of the diet was designed to place the subjects in approximately + 15% energy balance.

Figure 4.1 Energy intake for free-living, lead-in, and inpatient phases of the study.*****Significantly different than free-living conditions ($p<0.05$).

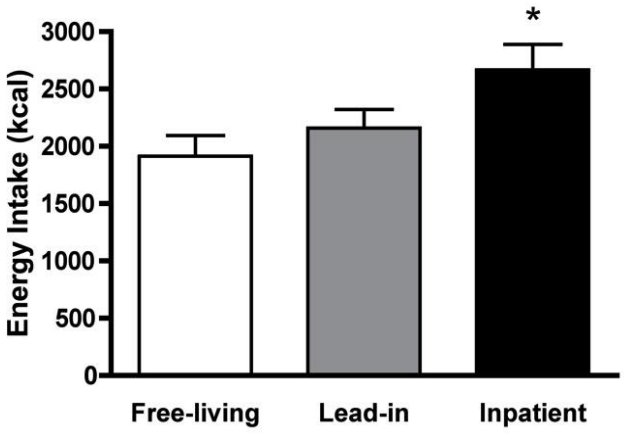
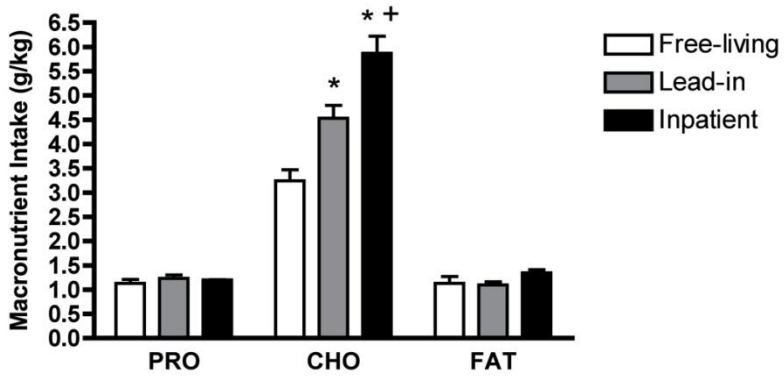


Figure 4.2 Macronutrient intake for the free-living, lead-in, and inpatient phases of the study. *****Significantly different than free-living conditions ($p<0.0001$ for Inpatient, $p<.01$ for Lead-in). **+** Significantly different than lead-in conditions ($p<0.01$).



Energy expenditure

PAL and Non-exercise PAL levels did not differ between the CHO and PRO + CHO trials, as depicted in Figure 4.3 and Figure 4.4, respectively.

Figure 4.3 PAL during inpatient CHO and PRO + CHO trials

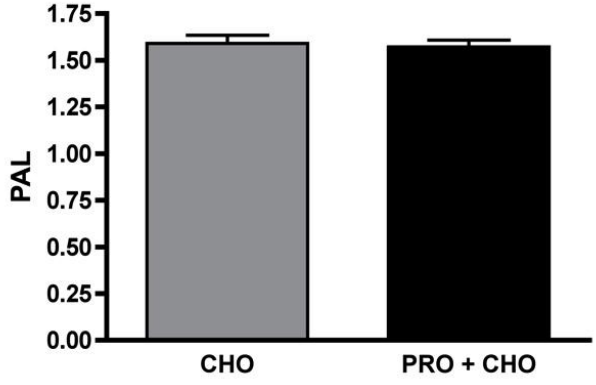
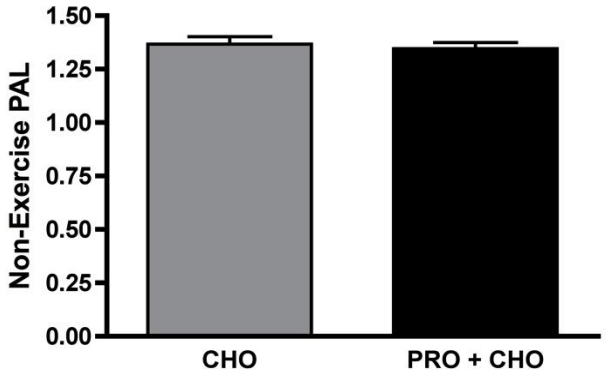


Figure 4.4 Non-exercise PAL during inpatient CHO and PRO + CHO trials



Energy balance

During the lead-in diet, estimated mean daily positive energy balance was positive by 15% compared to each subjects estimated maintenance caloric intake. Subjects were in positive energy balance during the inpatient stay, with a mean daily positive energy balance of + 278.1 ± 40.70 kcal for the CHO trial, and + 313.1 ± 37.46 kcal for PRO + CHO trial as depicted in Figure 4.5 (p=.2654). Additionally, there was no significant difference in energy balance between Days 1-3 and Days 4-6 regardless of whether subjects were in the CHO or PRO + CHO trial as depicted in Figure 4.6 (p=.3127).

Figure 4.5 Energy balance during CHO and PRO + CHO trials

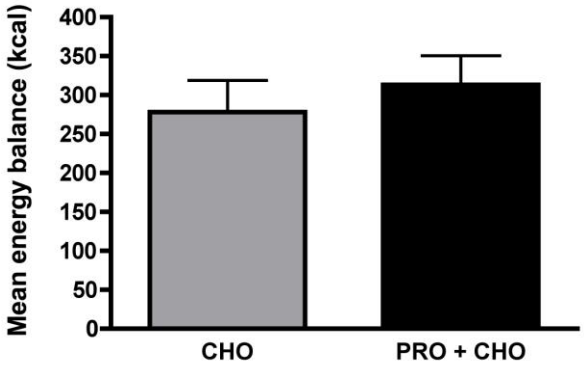
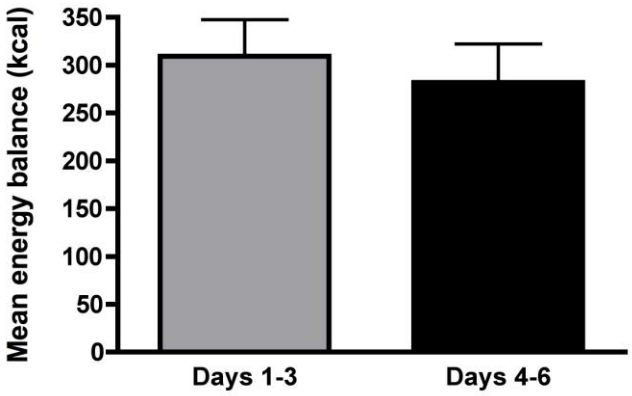


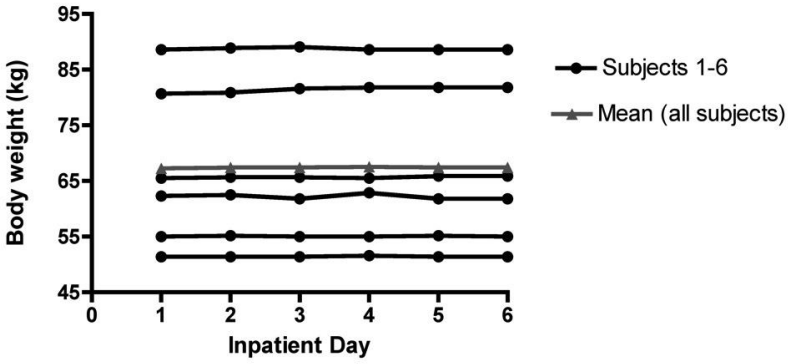
Figure 4.6 Mean inpatient energy balance of Days 1-3 and Days 4-6



Weight and body composition

Body weight did not change from pre DEXA scan (67.2±5.9 kg) to post DEXA scan (67.6±6.0 kg) (p=0.41). There were no significant changes in body fat percentage (p=.23) from pre (33.52±3.87%) to post (32.85±3.82%) along with no significant changes is FFM (p=.24) from pre (45.71 ± 5.83 kg) to post (46.07 ± 5.61 kg). The tracking of individual and group mean daily body weight during the inpatient period is shown in Figure 4.7. Mean body weight did not change during the inpatient stay, however changes in bodyweight would have likely been observed over a longer duration due to positive energy balance.

Figure 4.7 Daily in-patient bodyweight for individual subjects and group mean



Nitrogen Balance (NBAL)

There was a trend for an increase in 3-day average NBAL in the PRO + CHO versus CHO as depicted in Figure 4.8 (p=0.0881). The mean NBAL for the CHO trial was 0.9699 ± 0.5171 g N, and 1.659 ± .4296 g N in the PRO + CHO trial. However, a calculation of the number of subjects needed to show a significant difference at alpha less than 0.05 at a power of 0.80 was determined to be 35 subjects. If we added 2 or 4 subjects to bring our n up to 8 or 10, the power was determined to be 0.30 and 0.36, respectively (calculated using STPLAN V4.3, The University of Texas M. D. Anderson Cancer Center). Mean NBAL data during CHO and PRO + CHO trial for individual subjects is presented in Figure 4.9. Importantly all subjects were in positive NBAL during PRO + CHO and only one subject had a negative NBAL in the CHO trial. There was no significant difference in NBAL between Days 1-3 and Days 4-6 regardless of whether subjects were in the CHO or PRO + CHO trial as depicted in Figure 4.10 (p=0.3488).

Figure 4.8 3-day mean NBAL during CHO and PRO + CHO trials (p=.0881)

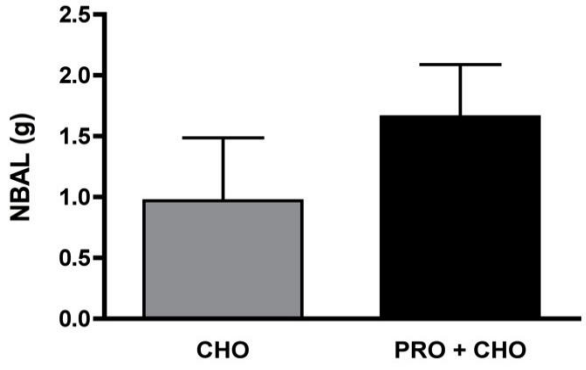


Figure 4.9 Mean NBAL of individual subjects and group mean during CHO and PRO + CHO trials

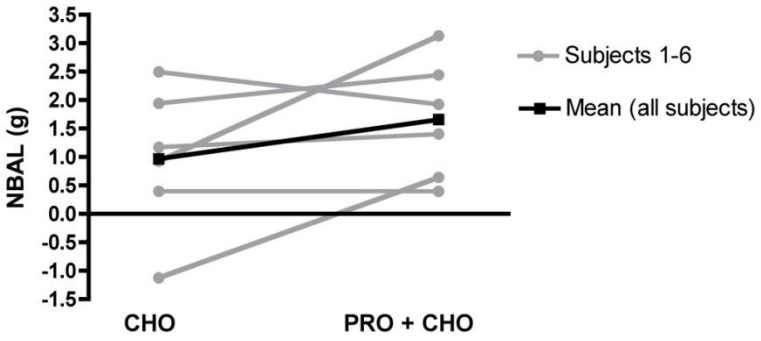
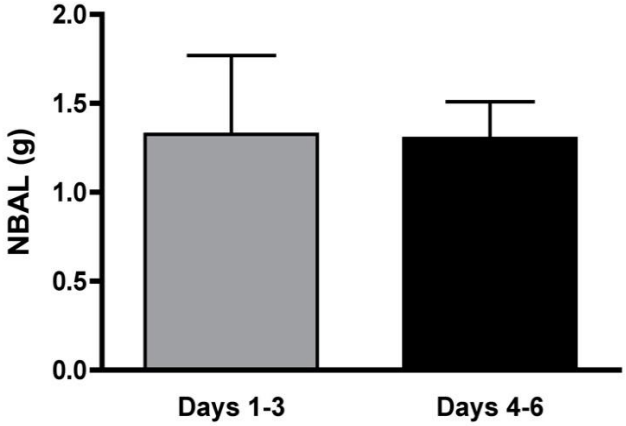


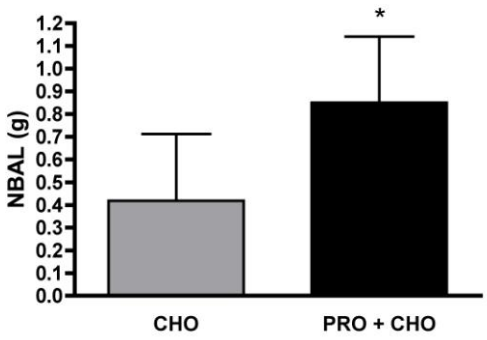
Figure 4.10 Mean NBAL during Days 1-3 and Days 4-6 (p=0.4775)



Combined data for negative, even, and positive energy balance studies

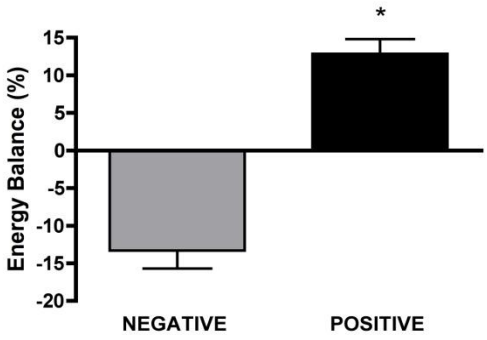
By using data from all three energy status cohorts of the study (negative, even, and positive), further comparisons for NBAL with regards to the timing of protein intake and/or energy balance were made. When combining all of the data from the negative, even, and positive energy balance studies, significant differences were observed for mean NBAL between the CHO and PRO + CHO trial (.4188 ± .2941 g N in the CHO trial, and .8505 ± .2908 g N in the PRO + CHO trial)(p=.01) as depicted in Figure 4.11.

Figure 4.11 Mean NBAL in CHO and PRO + CHO trials in all studies combined (negative, even, and positive energy balance)



Data from all three studies was then stratified so that the 6-day mean energy balance percentages <0 were placed in a negative group, and 6-day mean energy balance percentages >0 were placed in a positive group. Average energy balance in the adjusted model was -13.39 ± 2.41 % for the negative group, and 12.85 ± 1.96% for the positive group ($p<0.0001$) as depicted in Figure 4.12.

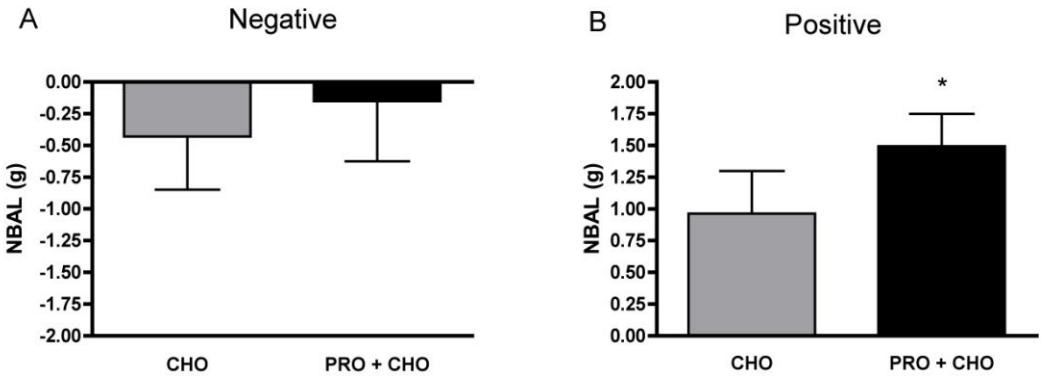
Figure 4.12 Mean energy balance in adjusted negative and positive groups ($p<0.0001$)



Differences in mean NBAL for the CHO and PRO + CHO trials in both the adjusted negative and positive energy balance groups were analyzed. There was no significant difference in mean NBAL between the CHO and PRO + CHO trial in the adjusted negative energy balance

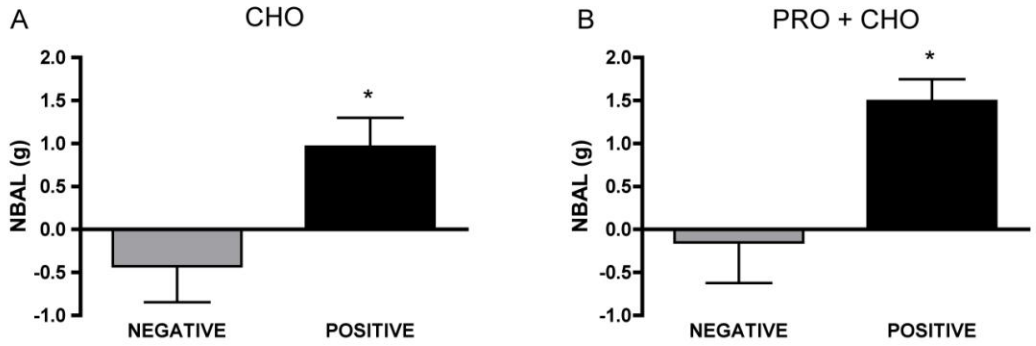
group (p=.1782) as depicted in Figure 4.13. However, significant differences were observed between the CHO and PRO + CHO trial in the adjusted positive energy balance group (.9637 ± .3359 g N in the CHO trial, and 1.494 ± .2555 g N in the PRO + CHO trial)(p=.0158) as also depicted in Figure 4.13.

Figure 4.13 Mean NBAL in CHO and PRO + CHO trials in adjusted negative and positive energy balance groups (p=.1782 for Negative, p=.0158 for Positive)



When comparing the mean *change* in NBAL going from the *CHO* to the *PRO + CHO* trial in the newly adjusted negative and positive energy balance groups, the differences remained insignificant (p=.2435). However, when splitting the newly adjusted negative and postive groups up by trial, differences in mean NBAL were significant in both the in CHO trial (p=.0085), and the PRO + CHO trial (p=.0016) as depicted in Figure 4.14. Specifically, the mean NBAL values for the CHO trial were -0.4288 ± 0.4184 g N for the negative group, and 0.9637 ± 0.3359 g N for the positive group. The mean NBAL values for the PRO + CHO trial were -.1507 ± .4728 g N for the negative group, and 1.494 ± .2555 g N for the positive group.

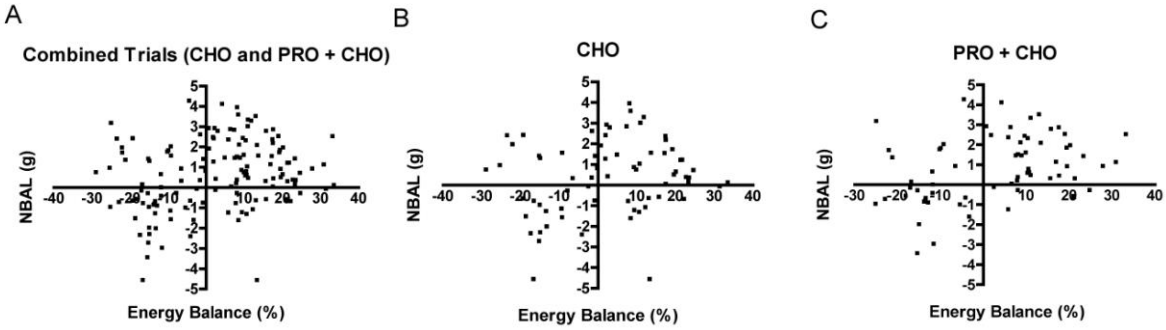
Figure 4.14 Mean NBAL in adjusted negative vs positive groups during CHO trial (p=.0085), and PRO + CHO trial (p=.0016)



Energy balance and Nitrogen balance

A significant correlation was observed between all of the subjects daily energy balance and their respective daily NBAL (R^2=0.065,p=.003) as depicted in Figure 4.15. Additionally, when seperating the daily energy balance and NBAL by trial (CHO and PRO+CHO), no significant correlation was observed between daily EB and daily NBAL for the CHO trial (R^2=.03450, p=.1265) as also depicted in Figure 4.15. However, daily EB was significantly correlated with daily NBAL in the PRO + CHO trial (R^2=.1149, p= 0.0044) as also depicted in Figure 4.15.

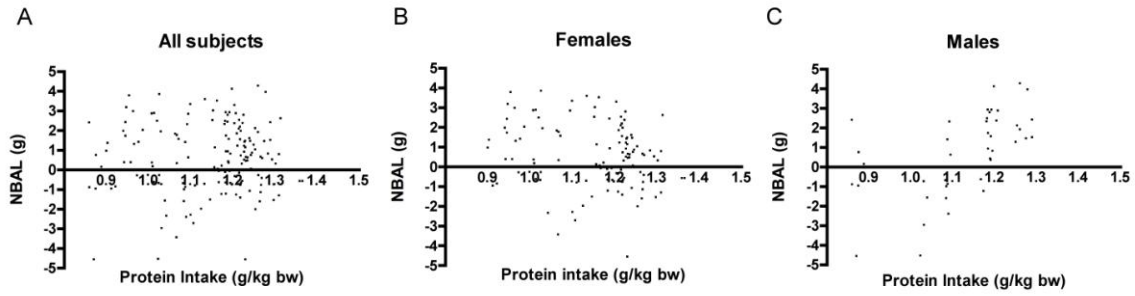
Figure 4.15 Correlation between daily energy balance (%) and daily NBAL in Combined Trials (p=.003), CHO trial (p=.1265), and PRO + CHO trial (p=.0044)



Protein intake and Nitrogen balance

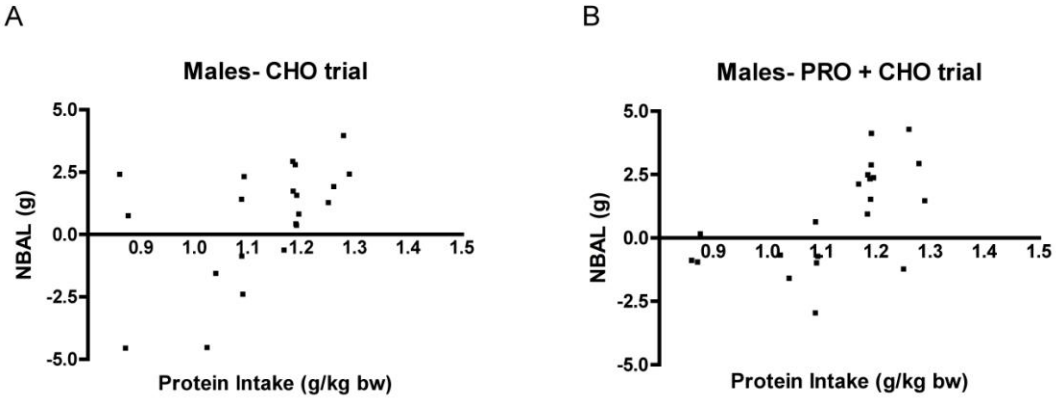
When correlating all subjects daily protein intake (g/kg bw) with daily NBAL no significant correlation was observed (R^2=.0031, p=0.49) as depicted in Figure 4.16. However, once dividing the data by sex, significant correlations between daily protein intake (g/kg bw) and daily NBAL were observed for both females (R^2=.04219, p=.0284), and males (R^2=.3279, p=<.0001) as also depicted in Figure 4.16.

Figure 4.16 Correlation between daily protein intake (g/kg bw) and daily NBAL for all subjects (p=0.49), females (p=0.0284), and males (p=<.0001)



Further correlations were then made by separating both women and men into *CHO* and *PRO + CHO* trials. In women, daily protein intake (g/kg bw) was not significantly correlated to daily NBAL in either the *CHO* (R^2=.05099, p=.0912), or the *PRO + CHO* trial (R^2=.03524, p=.1620). However, in men daily protein intake (g/kg bw) was significantly correlated to daily NBAL in both the *CHO* (R^2=.2515, p=0.0205), and the *PRO + CHO* trial (R^2=.4346, p=0.0012), as depicted in Figure 4.17.

Figure 4.17 Correlation between daily protein intake (g/kg bw) and daily NBAL for men during CHO (p=0.0205), and PRO + CHO trials (p=0.0012)



DISCUSSION

The present study examined the effects of the timing of protein in relation to a bout of aerobic exercise on NBAL in older individuals on a hypercaloric diet. In this study, subjects completed two, three-day trials that were isocaloric and isonitrogenous, where only the timing of a protein beverage differed with regards to a bout of aerobic exercise. Previous research in our lab has examined the effects of the timing of protein intake in relation to aerobic exercise in older individuals in *energy balance.* The original *even* energy balance data showed that timing protein intake immediately after exercise resulted in greater NBAL than if the same protein dose was consumed earlier in the day (Jordan, Melanson et al. 2010). This led to the question of whether the timing of protein would have a similar effect when subjects were placed on a hypercaloric diet, since increasing caloric content increases NBAL in itself (Munro 1951; Butterfield and Calloway 1984).

Contrary to data from the *even* energy balance study, the results of the current study showed that when older individuals consume protein immediately following exercise (PRO + CHO) on a hypercaloric diet, there was no significant increase in 3-day average NBAL than if the protein was consumed earlier in the day (CHO). Although p=.0881, a calculation of the number of subjects needed to show a significant difference at alpha less than 0.05 at a power of 0.80 was determined to be 35 subjects. If we added 2 or 4 subjects to bring our “n” up to 8 or 10, the power was determined to be 0.30 and 0.36, respectively.

If the degree of positive NBAL in the *positive* energy balance group was maintained over the long-term, consuming protein immediately after exercise *may* lead to more significant gains in whole body protein than if the protein was consumed earlier in the day. Specifically, when extrapolating the results of the current study over a one-month period, the CHO trial (.970 ± .517 g N) would result a net protein accretion of 182 g (0.970 g N·day^{-1} x 6.25 g PRO ·g N^{-1} x 30 days), while the PRO + CHO trial (1.659 ± .430 g N) would result in a net protein accretion of 311 g (1.659 g N·day^{-1} x 6.25 g PRO ·g N^{-1} x 30 days). If extrapolating the 3-day mean NBAL results over the span of an entire year 2.2 kg of body protein would be gained in the CHO trial, with 3.78 kg of body protein being gained in the PRO + CHO trial. These gains are theoretically feasible given that 1.5 kg of FFM can be accrued in women over 60 years of age during a 24 week resistance training program (Nichols, Omizo et al. 1993).

Our studies were unique in that they were the first studies of their kind examining the timing of protein intake in relation to exercise on NBAL in older individuals. The effects of energy balance on NBAL have been previously elucidated. Specifically, energy restriction results in a decreased NBAL (Todd, Butterfield et al. 1984; Friedlander, Braun et al. 2005), while increasing caloric content leads to increased NBAL (Munro 1951; Butterfield and Calloway 1984). However, these studies did not strictly examine an older population or the timing of protein with regards to exercise on NBAL. Additionally, studies by Campbell et al., have evaluated the effects variable protein intakes on NBAL (Campbell and Evans 1996; Campbell WW 2001; Campbell, Johnson et al. 2008). However, Campbell's studies did not examine the timing of protein intake in relation to exercise on NBAL. One study similar in design to ours was done by Roy et al., who examined the effects of the timing of mixed meal ingestion with regards to exercise on NBAL in young female athletes following two seven-day trials of increased training volume. Similar to our study, the trials only differed with the timing of the mixed-meal beverage

(Roy, Luttmer et al. 2002). However, the subjects in the Roy study were young female athletes, not older individuals at risk for wasting. Our study did not include increased training volume, but used the same level of moderate intensity aerobic exercise each day which emulated the intensity of a brisk walk. This is novel, since walking is the most common form of exercise in the elderly population (McPhillips, Pellettera et al. 1989). Additionally, subjects in the Roy study were in an exercise induced negative energy balance, whereas our subjects were in positive energy balance.

Data from our three studies: Negative, Even, and Positive Energy Balance

By examining data from all three studies our lab performed, we are better able to understand the relationship between energy balance and timing of protein intake on NBAL. When combining the data from all three studies (negative, even, and positive energy balance), there was a significantly greater mean NBAL observed when protein *and* carbohydrates were consumed following exercise, as opposed to an isoenergetic amount of strictly carbohydrate. Since energy balance can change day to day in "real life" conditions, timing protein *and* carbohydrate after exercise could be considered a valid strategy at increasing NBAL in older individuals.

Additionally, we decided to stratify subjects into either a negative (energy balance <0 %) or positive (energy balance >0 %) group. We then were able to examine differences in mean NBAL between the CHO and PRO + CHO trial in both the adjusted negative and positive energy balance groups.

No significant differences in mean NBAL were observed between trials in the adjusted negative energy balance group. The lack of significant differences (p=.1782) in NBAL between trials in the adjusted negative group somewhat contradict previous findings by Roy and

colleagues who found that consuming a mixed meal beverage immediately following exercise resulted in a strong trend (p=.06), albeit insignificant, towards a more positive NBAL than if consuming the same beverage earlier in the day while in an exercise induced negative energy balance state (Roy, Luttmer et al. 2002). Although subjects in the Roy study consumed their habitual energy intake, calories from exercise were not replaced which led to subjects being in negative energy balance, as evidence by weight loss in both trials. Although impossible to pinpoint, the differences between our findings and Roy's may be attributed to a potentially smaller caloric deficit achieved by subjects in the Roy study, as energy balance was not directly determined in the Roy study.

Contrary to subjects in negative energy balance, significant differences in mean NBAL were observed between trials in the adjusted positive energy balance group. Therefore our data indicates that timing protein intake after exercise has a greater impact on improving NBAL when in positive energy balance than when in negative energy balance.

When *comparing* these newly formed positive and negative groups, we found that significant differences existed in mean NBAL in both the CHO and PRO + CHO trials of each study. However, the difference in mean NBAL between negative and positive energy balance groups was more pronounced in the PRO + CHO trial than the CHO trial. Although significant differences in mean NBAL existed between energy balance groups for the CHO trial, these differences may likely be attributed to the impact of energy balance alone, since increasing energy intake increases NBAL in itself (Munro 1951; Butterfield and Calloway 1984). Since greater differences in mean NBAL were observed between energy balance groups for the PRO + CHO trial than the CHO trial, timing protein intake after exercise appears to have an additional benefit in increasing NBAL above increasing energy balance alone.

The issue of energy balance is often ignored in studies examining protein turnover. Specifically, due to the short term nature measurements of protein turnover using tracer methods, whole day energy balance is often ignored. Since "real life" scenarios involve multiple periods of fasting and feeding throughout the day, the issue of whole day energy balance cannot be ignored. By using the NBAL method, we were able to evaluate the effects of the timing of protein intake with regards to exercise on whole body protein turnover over the span of an entire day. Our findings indicated that in combination with timing protein intake after exercise, maintaining a positive energy balance will yield greater increases in NBAL than timing protein intake after exercise while in negative energy balance. Together, this data suggests that energy balance is likely more important than the timing of protein intake with regards to NBAL in older individuals.

NBAL has been shown to acutely increase but diminish over time when subjects are subjected to exercise and overfeeding, indicating NBAL adaptation (Butterfield and Calloway 1984). However, the adjusted positive model shows that consuming protein immediately after exercise (PRO +CHO) is still more beneficial at increasing NBAL than consuming the same protein beverage earlier in the day (CHO trial). Therefore, by consuming a hypercaloric diet combined with protein consumption immediately following moderate aerobic exercise, older adults may be able to reduce the losses in LBM associated with aging. However, prolonged exposure to a hypercaloric diet is not recommended due to the negative health consequences associated with weight gain. Rather, older individuals in negative energy balance would benefit from increasing caloric intake and timing protein intake after exercise in order to increase NBAL.

Our studies were the first of their kind to evaluate the combined effects of variable energy balance states and the timing of protein intake with regards exercise on NBAL in an older

population. When in negative energy balance, timing protein *and* carbohydrates (PRO + CHO) after exercise led to no significant increases in NBAL than if consumed earlier in the day. The lack of significant differences in NBAL while in negative energy balance may be attributed to the fact that, regardless of timing, more amino acids are being oxidized for energy with less being available for the synthesis of new proteins. Conversely, when in positive energy balance, timing protein *and* carbohydrates (PRO + CHO) after exercise led to significant increases in NBAL more so than if consumed earlier in the day. Since increased caloric intake spares amino acid oxidation, more amino acids are available for the synthesis of new proteins. Specifically, the amino acids consumed after an exercise stimulus are likely utilized for anabolic processes since they are not required for energy production.

Energy Balance and Nitrogen Balance

Significant correlations were observed with daily energy balance and daily NBAL when both CHO and PRO + CHO trials were combined. However, when seperating by trial, the correlation between relative energy balance and NBAL was only significant in the PRO + CHO trial. Therefore, it appears that energy balance may be more determinant of NBAL when protein *and* carbohydrates are consumed immediately following exercise, rather than an isoenergetic amount of strictly carbohydrates. Our findings demonstrated that in a hypercaloric state, consuming protein *and* carbhoydrates after exercise resulted in a greater NBAL than if consumed before exercise. By consuming protein *and* carbohydrates after exercise, amino acids are likely utilized for anabolic processes rather than being oxidized for energy.

Protein intake and Nitrogen Balance

No significant correlations were observed with subjects' daily protein intake (g/kg bw) and daily NBAL. However, once seperating subjects by sex we found significant correlations for

protein intake (g/kg bw) and NBAL in both women and men. This data indicates that relative protein intake is predictive of NBAL in men and women when seperated by sex. Once we further divided the sex based groups up by CHO and PRO + CHO trial we found a strong correlation between daily protein intake (g/kg bw) and daily NBAL in *men only* (R^2=.2515, p=.0205 for CHO,R^2=.4346, p=.0012 for PRO + CHO). Therefore, in older men, relative protein intake is more predictive of NBAL when protein and carbohydrates are consumed immediately following aerobic exercise, rather than an isoenergetic amount of strictly carbohydrates. To our knowledge this is the first evidence showing any type of sex related differences regarding relative protein intake and protein timing, on NBAL. It's possible that since the men had a greater average exercise workload, that they better utilized the amino acids for synthetic purposes after exercise. Additionaly, the more anabolic endocrine profile of men may allow them to use more of the ingested amino acids for synthetic processes than women. Sex differences with regards to protein intake's contributive effects on NBAL should be further explored.

NBAL method

Since the NBAL method used in our three studies does not elucidate rates of protein synthesis, or protein breakdown, we are unable to determine with certainty how net protein balance amongst all of the studies was achieved. However, there is evidence that MPB actually decreases with age (Morais, Gougeon et al. 1997), which would indicate that changes in NBAL may be *partially* attributed partially to changes in MPS. In addition, it is impossible to be certain where protein is being deposited using the NBAL technique. By design, our studies employed the NBAL technique since we were concerned with WBPT in the elderly. Worth noting is that prior research has shown that skeletal muscle contribute just under 30% of the WBPT rate (Nair

1995). Therefore, increases in NBAL are likely correlated with increases in skeletal muscle, however overall increases in *whole body* protein was the primary concern of our study.

Maintaining and/or increasing whole body N retention is extremely important during the aging process. As mentioned previously, outside of skeletal muscle, 25% of protein is found in body organs, and approximately 35% found in the skin and blood (Gropper 2009). Therefore, it is intuitive that decreases in whole body protein would have adverse effects on overall health and wellbeing.

Limitations

The NBAL technique may overestimate NBAL values (Kopple 1987). Specifically, N intake can be overestimated if food is not completely consumed, which would result in a higher calculated NBAL. However, subjects were instructed to consume all foods given to them, with meal consumption closely monitored during the inpatient period to ensure that all foods and beverages were ingested. Additionally, N intake is estimated based on total protein intake, with 6.25 grams of protein containing approximately 1 gram of N. However, the idea that 6.25 grams of protein contains 1 g of N is an average based on protein quality. Additionally, inadequate collection of any excreted N can also contribute to the overestimation NBAL. Directly calculating miscellaneous N losses can be quite challenging, and therefore relies on estimates. Therefore, there is some possibility that NBAL was overestimated. However, any measurement errors would occur during both conditions to the same extent. Therefore, the difference in observed NBAL between trials would remain constant despite the possibility to overestimate NBAL.

Despite the limitations to the NBAL technique, it is easy to employ and provides information regarding the WBPT. The noninvasive nature of the NBAL method makes it a useful

technique for studying protein turnover in the aging population. Additionally the NBAL method allows researchers to study WBPT over an extended period of time.

During the *even energy balance study*, an extra 160 calories (in the form of CHO) was provided during the inpatient diets. These extra calories were due to an error in the nutritional information for the Kool-Aid beverage, which means that the post-exercise beverages in the *even energy balance study* were not isocaloric. However, since the protein synthetic effect of carbohydrate is dependent on the amount ingested, the NBAL values in the CHO condition were likely more positive than they would have been if the Kool-Aid was isocaloric with the chocolate milk.

Protein and exercise recommendations for older adults

There have been a multitude of exercise and nutritional interventions studied with regards to their impact on attenuating wasting. Our studies examined the combined effects of exercise and the timing of protein on NBAL under variable energy balance states. Our *combined* data shows that consuming protein after exercise results in greater NBAL than if consumed earlier in the day while in positive energy balance. By timing the milk intake after exercise, NBAL likely increased due to the combined effects of protein and carbohydrate at increasing MPS following exercise (Howarth, Moreau et al. 2009). Since more amino acids are utilized for synthetic processes, fewer amino acids are oxidized or excreted. Therefore, milk consumption immediately following moderate intensity aerobic exercise provides a possible method for attenuating sarcopenia by increasing NBAL.

However, our data also suggests that energy balance is more important than the timing of protein intake with regards to improving NBAL. Therefore, older individuals should focus on

consuming adequate calories first and foremost with the timing of protein intake a secondary concern.

Additionally, although increasing protein intake has shown mixed results at increasing NBAL (Gersovitz, Motil et al. 1982; Castaneda, Charnley et al. 1995; Campbell WW 2001), research has shown that meals with at least 25-30g protein/meal (~10g EAA) maximally stimulate protein synthesis (Paddon-Jones D 2009). Although our study diets were not based around this idea, older individuals may be further able to increase postprandial protein synthesis and attenuate wasting by consuming 25-30 g protein/meal.

Future directions

In the future, a longer term intervention examining milk intake after exercise with regards to NBAL should be explored. Although likely difficult to administer, a longitudinal cohort could evaluate whether consuming milk after exercise could *prevent* wasting in subjects starting the intervention around ages 45-50 years before symptoms typically surface.

Additionally, longer-term research evaluating sex related differences in the correlation between relative protein intake and whole body protein balance should be explored. These studies should also examine age related sex differences in NBAL and relative protein intake.

Other research in our lab has shown that post exercise protein supplementation does not increase MPS over the course of 6 weeks (Robinson, Turner et al. 2011). Thus, it may be possible that the timing of protein intake does not make a difference in *long-term* MPS. However, to our knowledge, long-term NBAL studies examining the timing of protein intake have not been carried out. By examining the timing of protein intake on long-term NBAL, we

may be able to elucidate if protein timing has differential effects on protein kinetics at the whole body, and muscle level.

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APPENDIX I

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 exerciseintensity 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 slightly more (approximately 200 kcal) than 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 is720-848-6662.

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 II

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.

1. I have to cancel it in writing.

2. 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.

3. If I cancel my authorization, I may no longer be able to be in the study.

4. 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.

6. 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.

9. I have to cancel it in writing.

10. 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.

11. If I cancel my authorization, I may no longer be able to be in the study.

12. 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.

14. 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 III

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_____

_____

_____

_____