REGULATION OF POSTPRANDIAL PROTEIN METABOLISM AFTER FOOD INGESTION AND EXERCISE

BY

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DISSERTATION

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ABSTRACT

Whole body and skeletal muscle mass is regulated by counterbalanced fluctuations between protein synthesis and breakdown rates. Protein ingestion has been shown to stimulate protein synthesis rates and thus is an important variable within the net protein balance equation dictating a positive balance. However, postprandial protein metabolism has been almost exclusively characterized on the ingestion of isolated protein fractions (e.g., dairy-based whey and casein and plant-based soy) in liquid beverages, while the majority of protein in the diet is obtained from whole food sources with other macronutrients available. The purpose of this dissertation was to improve the understanding of postprandial protein metabolism after the ingestion of protein-dense whole foods during resting and post-exercise recovery conditions in both healthy and diseased individuals. Previous work suggested a short lived stimulation of postprandial muscle protein synthesis rates after protein ingestion. However, these findings were made after ingestion of fast-digested whey. In study 1, we showed that ingestion of a natural matrix of milk protein (20 % whey/80 % casein), resulted in a sustained release of dietary amino acids in circulation that coincided with prolonged postprandial elevations in intramuscular anabolic signaling and rates of muscle protein synthesis. In study 2, we demonstrated that production of labeled food proteins can be done in a cost-effective manner by supplementing the feed of laying hens with an amino acid tracer. The labeled eggs were used as a research tool in studies 3 and 4 to assess protein digestion and absorption kinetics after nutritional stimuli in health and disease. In study 3, we found that the ingestion of egg whites resulted in more rapid appearance of dietary amino acids in circulation when compared to isonitrogenous whole egg ingestion, with no differences in total dietary amino acid availability in circulation between the egg conditions. However, we did observe that whole egg ingestion resulted in a more potent postprandial muscle protein synthetic response when

compared with egg white ingestion. In study 4, we assessed the postprandial protein metabolic responses to mixed meal ingestion in maintenance hemodialysis (MHD) patients. We observed that basal rates of muscle protein synthesis were ~ 2.5 fold elevated in MHD patients when compared to controls; suggesting prolonged post-dialysis hypercatabolism. Furthermore, protein-dense mixed meal ingestion failed to stimulate postprandial rates of muscle protein synthesis in MHD patients. This observation may be attributed to reduced dietary amino acid availability after eating a meal. Overall, the studies performed as part this dissertation show that protein dense food ingestion is effective to stimulate the postprandial muscle protein synthetic response in healthy individuals. Moreover, it seems that clinical strategies to improve skeletal muscle health should involve a combination of increased physical activity and protein-dense whole food ingestion to maximize the muscle anabolic response with health and disease.

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CHAPTER 1: INTRODUCTION

1.1 Introduction to protein turnover

The turnover over of proteins is an essential biological process to all living organisms, including bacteria (78), plants (143), and animals/humans (222). Their turnover is necessary as proteins become dysfunctional/damaged over time and need to be replaced with new functional proteins. This cycle ultimately allows for a healthy functioning organism and its tissue. The first study to assess protein turnover directly in humans was conducted by Sprinson and Rittenberger in 1949 (193). The authors provided a single dose of the isotopically-labeled amino acid ^{15}N-glycine and measured the excretion of labeled nitrogen in urine for up to 72 h. The continued urinary excretion of labeled nitrogen indicated that *1)* the body protein pools undergo constant turnover and that *2)* any nitrogen (amino acid) losses need to be replaced by consuming protein in the diet (193). The first indication that rates of protein turnover may be altered across the lifespan was made nearly a decade later by Sharp et al. (185), who revealed that whole-body protein turnover was reduced in the elderly when compared to younger individuals. In the years following, numerous studies have demonstrated that whole-body protein turnover is (negatively) altered under a variety of clinical states such malnutrition (70, 190, 191), physical trauma (16), cancer (98), diabetes (140), obesity (140), chronic gastrointestinal disorders (138, 162) and diseases of the liver (136, 146), kidneys (12), and cardiovascular system (12).

Over the last four decades, the focus of protein metabolic research has largely turned towards the skeletal muscle as this tissue represents the largest depot of amino acids in the body. The first work to measure skeletal muscle protein synthesis was published in 1975 by Halliday and McKeran (79). Biopsies were obtained from the vastus lateralis between 14h and 30h of an unprimed continuous infusion of ^{15}N-lysine and a rate of muscle protein synthesis was calculated

between the sampling points. The authors were the first to show that rates of muscle protein synthesis contributed to about half of total rates of whole-body protein synthesis (79), thereby demonstrating the important role of skeletal muscle tissue in whole-body protein turnover. A few years later, Rennie et al. (178) suggested that skeletal muscle protein synthesis is particularly sensitive to protein ingestion, and concluded that the increase in whole-body protein synthesis rates after mixed meal ingestion largely reflects changes of protein synthesis rates at the level of the muscle, which may double after feeding. Marking the advancement in stable isotope research methodology, the work by Rennie et al. (178) published in 1982 set the stage for many research groups to examine skeletal muscle protein turnover in a variety of clinical states (55, 149, 177) as well as after physical activity and/or nutritional manipulations (6, 133, 160, 204) using metabolic tracers.

It is now well established that skeletal muscle tissue is characterized by a high degree of plasticity and can be altered drastically with advancing age (56), disease/physical trauma (87) (including chronic kidney disease (4, 166)) and various lifestyle factors such as diet (in particular protein intake) (233) and physical activity (86, 198). These alterations in skeletal muscle can have positive and/or negative metabolic consequences that can lead to improvements or decrements in health (231). For these reasons the study of protein turnover, with special reference to skeletal muscle, continues to be of great interest to researchers. This thesis will focus on the effects of diet and/or acute performance of physical activity to improve whole body and muscle protein turnover rates in health and diseased populations. In particular, special emphasis is placed on postprandial protein metabolism after whole food ingestion in a wide variety of adult participants, including both healthy and maintenance hemodialysis patients.

1.2 Methods to assess protein turnover in humans

The *in vivo* assessment of the whole-body or muscle protein metabolic response to divergent acute nutritional and/or exercise stimuli is commonly studied with isotopically-labeled amino acid tracers. An example of a commonly used stable isotope tracer to assess protein metabolism is ^{13}C-leucine (215). The additional neutron(s) of ^{13}C-leucine makes it 'heavier' than the most abundant form of the amino acid (i.e. the tracee), which is ^{12}C-leucine (often simply referred to as leucine). The tracer (e.g ^{13}C) is chemically and functionally identical to the tracee (e.g. ^{12}C) amino acid, but its additional mass enables precise detection in a biological sample with mass spectrometry. Other stable isotope amino acid tracers include ^{2}H, ^{15}N, and ^{18}O, however labeled carbon (e.g. ^{13}C) and/or hydrogen (e.g. ^{2}H) are most commonly used for to assess human protein metabolism due to greater predictability of *in vivo* metabolic flux when compared to nitrogen (^{15}N) and oxygen (^{18}O) (229).

A widely employed protocol to assess *in vivo* human protein metabolism acutely involves the primed-continuous intravenous (IV) infusion of one or more labeled amino acid(s) (232). Protein metabolism is often studied during a post absorptive period (first 3 h) to provide a control measurement and subsequently followed by measurements during a postprandial and/or post-exercise period (subsequent 5-7 h) (**Figure 1.1**).

The information gained from these acute studies (29, 46, 90, 179, 224, 227) provides insight into whole body and muscle protein remodeling. The findings from these acute studies can subsequently be used to reasonably 'predict' the long-term phenotypic outcome to chronic exposure of the respective nutritional and/or physical activity stimuli in healthy and diseased populations (59, 81, 89, 131, 189, 223). The sections below will describe the measurements of whole-body and muscle protein turnover in detail and highlights the important information gained from human metabolic studies pertinent to this thesis.

1.3 Whole body protein turnover in healthy individuals

Measurements of whole-body protein turnover are made by frequent sampling of plasma and/or breath (221). The classic model of studying whole-body protein turnover involves the use of ^{13}C-leucine (215) as the IV infused tracer, although other amino acid tracers have certainly been used to assess whole body protein turnover (71, 72, 91, 102, 103, 153, 205, 216). The measurement of protein turnover in stable isotope studies generally assumes a simplified two-pool model (**Figure 1.2**), although models with three or more pools have been used and described (14, 71, 213).

The two-pool model consists of an amino acid pool (e.g. the plasma or intracellular free) and a protein pool. The amino acids in the plasma or intracellular free pool are from protein breakdown and/or the ingestion of dietary protein plus intravenous tracer infusion and serve as the 'precursors' for synthesis of body protein or are irreversible lost from the free amino acid pool through oxidation. The determination of sample isotopic enrichment (in plasma and exhaled CO_2), in addition to measures of plasma amino acid concentrations and total CO_2 production, allows for the modeling of whole-body protein synthesis, breakdown, and amino acid oxidation rates (232).

Measuring whole-body protein turnover is relatively non-invasive and provides the researcher with important information on the *in vivo* turnover of body proteins that collectively maintain proper function, structure and regulation of the whole body. Perhaps less appreciated in recent times, measures of whole-body protein turnover have provided most of our initial understanding on the regulation of protein turnover in response to nutritional and/or physical activity manipulations *in vivo* in humans.

For example, the 24 h infusions in the 1980s by Clugston and Garlick (38) were amongst the first to reveal that fasted losses are off-set by fed gains so that overall body protein mass essentially

remains in balance over the course of a day. This observation was subsequently termed diurnal cycling of protein (38). Several experiments performed by the Millward group revealed that the magnitude of diurnal protein cycling is remarkably labile and is modulated by habitual dietary protein intake and physical activity levels (128, 130). These findings led to the development of a metabolic demand model suggesting that there is *1)* an obligatory need for nitrogen and amino acids, which remains more or less constant irrespective of habitual dietary protein intake and physical activity level, and 2) an adaptive need for nitrogen and amino acids, which varies depending on habitual protein intake and physical activity level of the individual (128).

These early studies provided the evidence that changes in protein balance are regulated by changes in protein synthesis and breakdown rates in the response to the fasting-feeding cycle. In particular, protein feeding results in an increase in whole-body protein synthesis and/or a decrease in breakdown while the postabsorptive state is marked by an increase in breakdown and/or decrease in synthesis (38, 84, 123, 144). There appears to be discrepancy on the relative contributions of protein synthesis and breakdown to the net protein balance, in particular as it relates to the fed-state. The early work performed by Clugston and Garlick (38) concluded that improvements in postprandial net protein balance mainly resulted from an increase in synthesis rather than suppression of breakdown. However, later work by the same group suggested that recycling of the tracer during the 24h infusion period likely confounded these findings and resulted in an overestimation of postprandial whole-body protein synthesis (123). Indeed, shorter infusions lasting 8-10 h (where tracer recycling is not of great concern) show that improvements in whole-body protein balance after feeding are generally the result of decreases in breakdown alone (10, 19, 84, 123, 137, 144), a combination of both (37, 68, 77, 103, 152, 153), or in some instances synthesis alone (32, 100). The apparent discrepancies may relate to the source of protein/amino

acids that are ingested and/or methodological differences in determining whole-body protein turnover between laboratories.

The ingestion of isolated protein sources, in particular isolated dairy proteins and free amino acids, appear to improve net balance through changes in whole-body protein synthesis (32, 100) and/or through equal contribution of breakdown and synthesis (37, 68, 77, 103, 152, 153). In the context of whole food ingestion, net protein balance appears to be modulated mainly by changes in breakdown rather than synthesis rates (26, 68, 103).

A possible explanation for the higher rates of whole-body protein synthesis with 'fast' digested isolated proteins and free amino acids maybe attributed to the increased stimulation of protein synthesis in the splanchnic bed; an observation likely explained by the role of the liver in preventing an excessive influx of amino acids in the plasma (109, 145). A further explanation may relate to the insulinotropic properties of isolated food sources, in particular dairy proteins (26), and free amino acids (208) as the rapid rise in insulinemina coupled with a postprandial rise in plasma amino acids is shown to result in increased gut tissue protein synthesis (45), and thus a large rise in whole body protein synthesis (32). Whole foods on the other hand are more slowly digested leading to a more gradual release of dietary amino acids into circulation (26, 209). The slower splanchnic release rates of dietary amino acids appear to improve net protein balance mainly by inhibition of breakdown as opposed to stimulation of synthesis (17, 42).

Some evidence suggests a dose-dependent relationship between dietary protein intake and protein breakdown. Early work by the Millward group showed that increases in protein intake were inversely associated with suppression of protein breakdown in healthy adults (68, 148). Recent work by Kim et al. (103) confirmed these findings and showed that whole food ingestion in the form of beef patty containing 70 g of protein resulted in greater suppression of whole-body protein

breakdown when compared to a patty containing 40 g of protein. Moreover, the ingestion of this whole food source (e.g. beef) modulated net protein balance mainly by changes in protein breakdown rather than synthesis (103, 153). Similar findings (68, 93, 148, 153) have led Wolfe and Deutz (47) to suggest that, while synthesis may become saturated as such high intakes of protein, there is no theoretical upper limit to the suppression of protein breakdown with increasing amounts of dietary protein. While there may be an inverse relationship between protein ingestion and suppression of breakdown in the fed state (68, 93, 103, 148, 153), such high intakes of dietary protein per meal may not per se translate into a greater daily net protein balance. In particular, several studies indicate that such high intakes of protein are simply matched by greater protein losses in the fasted state, as evidenced by greater postabsortive use of amino acids as a fuel (148, 165, 217). In addition, such high intakes of protein are unrealistic for the majority of the population, which generally consume 15-30 g of protein per meal (2). Therefore, strategies that involve the improvement of the anabolic response to more realistic feeding paradigms should be developed. Of particular interest are strategies that involve whole food ingestion as this is the manner in which protein is ingested within a normal dietary pattern. Accordingly, a major component of this thesis research was to improve our understanding of whole-body protein turnover after whole food ingestion.

1.4 Whole body protein turnover in dialysis patients

A further focus of this thesis was to study whole body protein turnover in MHD patients, and compare these outcomes to healthy controls. It is well-established that clinical states are characterized by a high degree of protein breakdown that is not compensated for with synthesis (176). In particular, clinical states such as malnutrition (70, 190, 191), physical trauma (16), cancer

(98), chronic gastrointestinal disorders (138, 162) and diseases of the liver (136, 146), kidneys (12), and cardiovascular system (12) have all been demonstrated to reduce net protein balance, either through insufficient rates of protein synthesis and/or exceedingly high rates of protein breakdown.

Body protein wasting is especially prevalent in MHD patients, with annual losses of lean mass as high as 1-3 kg (164). Moreover, the loss in lean mass is one of the strongest predictors of mortality in this patient population (31, 96). The potential underlying causes for rapid protein loss in MHD patients is likely multifaceted and includes contributing factors such as: inflammation, insulin resistance, decreased nutrient intake, metabolic acidosis, hormonal imbalances, increased amino acid oxidation, and loss of amino acids into the dialysate (219). These factors have all been independently shown to reduce body protein mass (21, 35, 95, 167, 170, 188, 237), which illustrates the challenges to improving defects in protein turnover to support muscle mass maintenance in MHD patients.

In particular, measurement of whole body protein turnover in the fasted state prior to dialysis revealed that whole body protein breakdown is increased when compared to healthy controls with no differences in protein synthesis (166). During dialysis, protein breakdown further increased, resulting in doubling of whole body net protein losses during dialysis when compared to pre-dialysis (95). In the same work, Ikizler et al. (95) demonstrated that this increase in whole body protein breakdown persisted for at least 2 h after the dialysis period, suggesting that the deleterious effects of dialysis are maintained beyond the dialysis period. Moreover, it was suggested that the losses in whole body net protein are mainly the results of losses on the level of the muscle (95). Given that MHD patients dialyze almost every other day for a total of 4-6 h/day, it is important to develop strategies that can mitigate some of the losses in whole body- and skeletal muscle protein.

Work by Veeneman et al. (211) provides insight in the time-course of the increased negative net protein balance after dialysis. In particular, the workers compared to fasted whole body turnover on dialysis and non-dialysis days and showed that whole body net balance is more negative during dialysis then when measured the next morning after afternoon dialysis (211). A further finding of interest made by the workers is that protein feeding increased whole body net protein balance during dialysis and non-dialysis days (211) through stimulation of protein synthesis and reduction in breakdown.

However, a limitation of this study is that protein was provided as small repeated meals and that whole body modeling was done under the assumption that first-pass absorption of dietary amino acids was only 20% (211). This practice of ingesting of small repeated meals was done for methodological purposes and is not representative of normal eating behavior (2). In addition, the value of first-pass extraction is likely an underestimate for MHD patients (7). As such the effect of meal consumption as a strategy to improve protein turnover remains to be firmly established in MHD patients. To properly model whole body kinetics, a novel and innovate research tool in the form of intrinsically-labeled food proteins allows the researcher to properly model whole body protein turnover with bolus feeding in a variety of populations. One of the goals of this thesis was to use this research tool to better understand postprandial protein metabolism in MHD patients and ultimately provide clinicians and dieticians with essential information on how to improve standard care for this population

1.5 Intrinsically labeled foods to improve the measurement of whole body protein turnover

When exogenous amino acids appear in the plasma pool after food intake, the measurement of whole body protein turnover becomes more challenging. In the fasted state, the only amino acids appearing in the plasma pool, other than the IV infused amino acid tracer, are endogenous. Therefore, any disturbance of IV infused tracer enrichment in the plasma pool is the result of protein breakdown. However, in the fed state tracer 'dilution' from appearance of unlabeled amino acids are the result of both endogenous protein breakdown and exogenous dietary protein-derived amino acids. Many researchers 'correct' the measurement of whole body protein breakdown by simply estimating the amount of amino acids appearing in the circulation as a result of the dietary protein ingestion (102, 103, 211) or by feeding protein as small repeated boluses to minimize tracer disturbances (38, 68, 148, 215). While the former incorrectly assumes uniformity between protein sources and populations in term of protein digestion and absorption kinetics (17, 19, 74), the latter is not representative of normal eating behavior (2). As such, workers in the field have developed an innovative research tool in the form of intrinsically-labeled dietary proteins to reliably model the exogenous appearance of dietary-derived amino acids and whole body protein kinetics in response to food ingestion (18, 207, 209).

Intrinsically-labeled dietary proteins have the amino acid tracer incorporated into their natural protein matrix. This allows the tracer to share the same metabolic fate as the representative unlabeled amino acid (i.e. the tracee) present in the food (20). The ingestion of intrinsically-labeled (e.g. ^{2}H leucine) dietary protein during primed-continuous IV infusion with a different tracer of same amino acid (e.g. ^{13}C leucine) allows the researcher to determine total leucine rates of appearance and subsequently model protein digestion and amino acid absorption rates and total plasma dietary-derived availability in circulation after the ingestion of a protein source. In other

words, measurement of plasma isotopic enrichment of the dietary-protein derived tracer allows for the reliable measurement of the exogenous 'share' (*Exo* R_a) of total amino acid flux (*Total* R_a). The use of labeled food proteins in primed-continuous IV infusion protocols have offered an important advantage over the classic study of whole-body protein turnover with unlabeled foods/amino acids, as the exogenous influx of amino acids (*Exo* R_a) with ingestion is reliably determined rather than estimated (18). As a result, the overall model of whole body protein turnover is improved as measures of protein breakdown can also be made more reliable (18). As the only routes of amino acid entry in the plasma pool are from dietary protein ingestion, IV infusion, and breakdown of intact proteins; the portion of total amino acid flux that remains after subtracting the *Exo* R_a (as determined with the labeled food source) plus infused tracer (F_{iv}) is endogenous (*Endo* R_a) thus coming from the release of amino acids from protein breakdown. Modified Steele equations (20, 42) were developed for the calculation of turnover under non-steady state kinetics (i.e. after feeding), which are described in detail in the supplemental methods of **Chapter 4**.

Using intrinsically labeled proteins it has been demonstrated that the rate of protein digestion and the pattern of postprandial dietary-derived amino acid availability is affected by several factors including protein source (17, 42, 104, 152, 209), texture (154), and macronutrient co-ingestion (37, 71, 72), in addition to consumer characteristics such as the habitual dietary protein intake (74) and age (19). Taken together, these data suggest that the measurement rather than estimation of exogenous influx of dietary-derived amino acids is preferred when modeling whole-body protein turnover.

Credited with the development of intrinsically labeled food proteins is the group of Yves Boirie in France. Through their endeavors of infusing large amounts (80-90g) of stable isotope amino

acid tracer into two lactating dairy cows, this group successfully produced intrinsically labeled 1-[^{13}C]leucine labeled milk (18). Over a decade later, Luc van Loon's research unit at Maastricht University in the Netherlands successfully produced intrinsically labeled 1-[^{13}C]phenylalanine labeled milk by infusing two lactating dairy cows for 48 h (207). 24h after cessation of the tracer infusion, this group also butchered and harvested the meat of the cows, consequently providing them with the opportunity to also use the meat to study protein digestion and absorption kinetics in humans (157). Using these labeled milk and meat proteins, it is now well established that the rate of protein digestion and amino acid absorption is an independent and important factor that controls whole-body and muscle protein metabolism after the ingestion of a protein containing meal (42, 104). While the examples above highlight the success of producing intrinsically labeled proteins by IV infusion of tracer to lactating dairy cows, large amounts of people-power and financial resources are required for the production of labeled food protein in this fashion. Previously, Evenepoel et al. (57) suggested a simple, non-invasive method of producing intrinsically labeled protein foods by supplementing the feed of laying hens with an amino acid tracer. However, the potential of using labeled eggs to study model to study protein digestion and absorption kinetics remained to be fully established. Therefore a major focus of this thesis was to produce labeled eggs and employ this innovative research tool in subsequent work assessing postprandial protein metabolism in healthy individuals and MHD patients.

Assumptions of the model of measuring whole-body protein turnover with intrinsically-labeled food proteins

While the use of labeled food proteins during IV tracer infusions allows for more accurate depiction of whole-body protein metabolism under non-steady kinetics (i.e. with protein feeding) there are several assumptions/limitations of the model that warrant discussion.

An important assumption is that the *in vivo* metabolic fate of the two different labels (e.g. ^{13}C and ^{2}H) used to trace the same amino acid (e.g. leucine) is similar. While this assumption has not been tested using intrinsically labeled proteins, a series of clever experiments by performed by Matthews et al. (121) may indicate that these two tracers generally provide similar flux values. In this work, subjects received simultaneous infusions of ^{13}C- and ^{2}H-leucine; one administrated intravenously (IV) and one intragastrically (IG). Halfway through the 7h protocol the IV infusion was switched to IG infusion and vice versa. Therefore, subjects received both IV and IG infusion of the same tracer on the same day. Comparison of fluxes with plasma ^{13}C- and ^{2}H-leucine enrichments as precursors did not reveal any differences between the two IV infused tracers. Splanchnic extraction, as determined by the IG route, was shown not be different between tracers either (121).

A further assumption of intrinsically-labeled proteins is that a single amino acid tracer (e.g. leucine or phenylalanine) represents postprandial dietary-derived availability of all amino acids. Data by Matthews et al. (121) published nearly 35 years ago had already revealed that splanchnic extraction of ^{2}H-phenylalanine is higher when compared to ^{13}C- and ^{2}H-leucine. Indeed, we (209) and others (42, 43) have used leucine-labeled food proteins and report ~ 10-20 % higher 5-6h postprandial dietary-derived amino acid availability than comparable data using phenylalanine-labeled foods ((26, 71, 72, 91, 104-106, 152-155, 157, 205, 207, 223). Thus some caution is necessary when comparing data on dietary-derived amino acid availability from different studies that have used different tracers to intrinsically label the food.

A further assumption pertinent to both labeled food proteins and IV tracer infusions is that tracer recycling does not occur during the measurement period. Tracer recycling occurs if the labeled isotope disappears in tissue or cells and then reappears in the measured protein pool (123,

183). Recycling of the label would results in an underestimation in *total* R_a as measured with IV infusion or an overestimation of *Exo* R_a as measured with labeled food proteins. Proteins that rapidly turnover become highly labeled during an infusion protocol and consequently provide the greatest potential source for isotopic re-entry during the infusion period. As mentioned, splanchnic and renal tissue turnover at rapid rates and the half-life of many gut-, kidney- and liver- proteins range from a few h to multiple days (22, 142, 199, 215). Moreover, the turnover of these proteins are highly responsive to the fasting-feeding cycle (22, 101). Splanchnic protein breakdown, for instance, is greatly suppressed with feeding and rapidly rises at the onset of fasting (22, 101). Thus with prolonged infusions, involving one or more fasting-feeding cycles, there is potential of re-appearance of isotopic tracer from breakdown of highly labeled tissue proteins.

Surprisingly little work has been performed investigating the occurrence of tracer recycling during these types of studies and its potential for incorrect measures of protein turnover remains relatively unknown. The work by Schwenk et al. (183) in 1985 was one of the first to address the concern of tracer recycling as they suspected label re-entry had confounded findings of one of their previous papers (82). Subsequently, the authors observed tracer decay to be much slower in a group of subjects infused for 24h compared to a group infused for 4h, suggesting re-entry of the label from protein breakdown with prolonged infusion periods (183). Estimates from the literature indicate that tracer recycling occurs ~ 1-1.5 $\%/h^{-1}$ (220), a value that may be higher or lower depending on the amino acid tracer and/or the length on infusion (3, 30, 123, 151, 172, 183, 200). It can be concluded from these studies that measures of whole-body protein turnover become unreliable after > 10-12 h of isotope infusion (3, 30, 123, 151, 172, 183, 200). While such long infusions were common practice in the 1980s and 1990s, nowadays protein turnover is generally studied during shorter infusion protocols (lasting 8-10h) involving a single fasting-feeding cycle.

The steady decay of dietary-derived isotope after labeled food ingestion provides further evidence that tracer re-entry is not occurring to any great extent with shorter protocols. Results from our lab (209) (**Figure 3**) and others (18, 42, 43, 71, 72, 77, 104, 106, 152-154, 157, 205, 207) indicate that once dietary-derived isotopic enrichment has peaked there is a steady isotopic decay in the plasma. However, critics can argue a slight plateau in dietary-derived tracer enrichment observed in the late postprandial phase in our work (209) and that of others (18, 42, 43, 71, 72, 77, 104, 106, 152-154, 157, 205, 207). Even if this observation was due to label re-entry rather than continued release of dietary-derived label from the gut, any minor recycling occurring will have a negligible influence on the overall postprandial measurement of whole body protein turnover. A clear indication of label re-entry would be a rise in dietary-derived label enrichment in the late postprandial phase, however this has never been observed (18, 42, 43, 71, 72, 77, 104, 106, 152-154, 157, 205, 207, 209). Taken together these data can be taken as a suggestion that little to no tracer recycling occurs during a 5h measurement period.

A further point relates to the terminology used to describe the outcomes obtained from labeled food protein ingestion, namely protein digestion and absorption kinetics (18, 42, 43, 157, 207). As the modeling of protein digestion and absorption is based of isotopic enrichments in the plasma, the use of labeled food proteins does not provide a true measurement of ileal protein digestibility (58). Instead it provides quantitative insight in the amount of dietary-derived amino acids that become effectively digested and make it past first-pass splanchnic uptake. It could be argued that dietary-protein derived amino acid availability in circulation would be a more accurate description of the outcomes gained with labeled protein ingestion.

1.6 Measurement of muscle protein synthesis

While it is clear that that whole-body measures provide important information on the collective *in vivo* turnover of body proteins, such measures do not allow for insight in tissue specific turnover (88). Individual tissues and organs turnover at different rates depending on their role and function and differently contribute to whole-body protein turnover (67, 199). Knowledge of their turnover rates requires sampling of the tissue site during the stable isotope infusion protocols. These types of studies have revealed that intestinal- (~ 50 $\%/d^{-1}$) (22, 142), renal- (~ 42 $\%/d^{-1}$) (199), and hepatic tissue protein turnover (~ 12%/d^{-1}) (215) occurs more rapidly than skeletal muscle tissue (~ 1.5 $\%/d^{-1}$) (199). Nonetheless, the relative tissue contribution of skeletal muscle to whole-body protein turnover is highest of these tissues as it contributes to roughly half of total rates of whole-body protein synthesis (199), which is explained by the high contribution of skeletal muscle to total body protein mass.

Furthermore it is well established that skeletal muscle is *1)* the primary organ system for locomotion and physical function (24) *2)* plays a central role in long-term metabolic health (231) through the regulation of glycemia (85), lipidemia (113) and its substantial contribution to the basal metabolic rate (BMR) (99) *3)* its metabolic derangements to exercise/food stimuli are amongst the earliest of defects observed in various disease states including chronic kidney disease (9, 119, 139) and *4)* is highly regulated by dietary- (14, 178, 196, 201, 213, 214) and/or exercise stimuli (14, 159, 186) and thus highly modifiable by lifestyle interventions (81, 125, 189). For these reasons, measurements of muscle protein turnover are generally made during stable isotope studies.

It is common that only measures of muscle protein synthesis (and not breakdown) are made in these types of studies. The reasons for this are two-fold; *1*) alterations in synthesis rather than

breakdown generally serve as the primary variable dictating the change in postprandial muscle net protein balance (13, 159, 213, 214, 230) and *2*) the measurement of muscle protein breakdown is methodologically challenging (238, 239). While it is a clear shortcoming that muscle protein breakdown is not measured in the studies related to this thesis, and the field in general, measurement of one equation of the net balance (e.g. protein synthesis) is generally accurate in predicting the long-term phenotypic outcome; at least in healthy individuals (59, 81, 89, 131, 189, 223). The methodology pertinent to measuring muscle protein synthesis will have focus in the next sections.

Approximately 1 h into the primed-continuous infusion protocol **(Figure 1),** an initial skeletal muscle sample (~30-100 mg of tissue) is obtained (11, 50). This initial 1h lead-in is necessary to ensure isotopic steady-state is achieved in the free amino acid pool, although some workers have omitted the first biopsy and have used a background plasma mixed protein enrichments (i.e. albumin) as a proxy for initial muscle protein-bound enrichment (28). Roughly 3 h into the infusion protocol the skeletal muscle is sampled again. This time is needed to ensure that enough time has passed for reliable determination of changes in muscle protein-bound tracer enrichment between the two collected biopsy samples (229). Subsequently, additional biopsy samples are collected during a postprandial and/or post-exercise phase to study the influence of an acute nutritional and/or exercise manipulations on changes in the stimulation of muscle protein synthesis rates.

When calculating a rate of muscle protein synthesis, the measured change in protein-bound tracer enrichment between two sampling points is normalized to the enrichment of the precursor pool (i.e. the plasma free, muscle intracellular free or aminoacyl-tRNA) using the standard precursor-product equation (see below). Solving for this equation will provide a fractional synthesis rate (FSR) of muscle protein in % per unit of time (e.g. $\%/h^{-1}$):

$$FSR = \frac{\Delta E_p}{E_{precursor} \cdot t} \cdot 100\%$$

where ΔE_p is the increment in muscle protein-bound tracer enrichment after an incorporation period, $E_{precursor}$ is the weighted mean precursor pool tracer enrichment during that incorporation period, and t is the incorporation period (h).

1.7 Measurement of muscle anabolic signaling

On a molecular level, protein synthesis is regulated through coordinated changes in the translational machinery (111). This processes of mRNA translation (i.e. protein synthesis) involves coordinated changes in the phosphorylated state of eukaryotic initiation, elongation and termination factors (i.e. eIFs, eEFs and eRFs). The mechanistic target of rapamycin complex 1 (mTORC1), an evolutionary conserved serine/threonine protein kinase, plays a central role in the phosphorylation of several components of the translational machinery (218). For instance, mTORC1 can phosphorylate downstream targets such as p70S6K and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1) (218). mTOR and is downstream targets P70S6K and 4E-BP1 have been shown to be responsive to the stimuli of amino acids (65, 80). In particular, dietary protein-derived amino acids form the main precursors for protein synthesis (20, 77) and their flux across the sarcolemma is facilitated by amino acid transporters (such as LAT1, SNAT2 and CD98) resulting in subsequent activation of the mTORC1 pathway (94). Besides its nutrient sensitivity, mTORC1 and targets of P70S6K and 4E-BP1 are also responsive to exercise (52), with further potentiation of their phosphorylated-state when the stimuli of nutrients and exercise are combined (132). Another pathway by which exercise increases mRNA translation involves phosphorylation of proteins within the mitogen-activated protein kinase (MAPK) pathway such as

extracellular signal–regulated kinases (ERK) 1/2 and its downstream target of eukaryotic translation elongation factor 2 (eEF2) (132), which can be activated independent of mTORC1 (61).

To understand the molecular mechanism underpinning mRNA translation (i.e. protein synthesis) in response to the nutritional and/or exercise stimuli provided within this thesis, we used the Western Blotting technique to provide readouts on the phosphorylated state of proteins and total protein content of amino acid transporters that are acknowledged to play in important role in protein synthesis. **Figure 4** highlights the signaling proteins and amino acid transporters studied within the mTORC1 and MAPK pathway as part of this thesis.

The goal of this thesis was to combine the use of labeled food proteins with primed-continuous tracer infusions and the Western Blot technique to provide comprehensive insight in potential differences in postprandial whole-body and muscle protein metabolism after various nutritional and/or physical manipulations in both health and disease. The question that remains is: why go through the efforts of studying protein metabolism in particular as it relates to skeletal muscle mass?

1.8 The importance of skeletal muscle mass

Historically studied for its contractile abilities, the preservation of skeletal muscle mass across the lifespan is important for maintaining physical function (97). Adequate maintenance of muscle size and strength with advancing age will allow for active involvement in family-, community-, and work life. Perhaps less recognized is the central role of skeletal muscle tissue in whole-body metabolism (231). Skeletal muscle plays in important part in the regulation of 24h glycemia, lipidemia, and is a major determinate of basal metabolic rate and thus total daily energy expenditure (231). Unsurprisingly, the maintenance of skeletal muscle mass with advancing age is associated with a lower risk for development of metabolic diseases such as type II diabetes (150), obesity (195), and cardiovascular disease (194). A greater amount of skeletal muscle mass is also an important predictor of survival rate in various cancers (66, 110, 120, 163, 182) and chronic kidney disease (234) and can facilitate healing from physical trauma (44, 161). As skeletal muscle is the largest protein reservoir in the body, the breakdown of muscle proteins will be the main supply of amino acids to vital organs and tissues to maintain the increased rate of protein turnover typical to clinical states (231), including chronic renal failure (166). This loss of muscle proteins to supply amino acids to other organs likely underpins the losses of muscle mass that is observed in disease states such as chronic renal failure (115).

On a day-to-day basis, skeletal muscle mass is regulated by coordinated changes in protein synthesis and breakdown, which reflect dynamic protein turnover (129). Over a prolonged period over time the cyclic nature of turnover results in maintenance (synthesis = breakdown), loss (synthesis < breakdown) or gain (synthesis > breakdown) in skeletal muscle mass. For most individuals, physical activity and diet (in particular dietary protein) will be the main factors that

influence skeletal muscle protein turnover and ultimately determine phenotypic outcome (i.e. changes in skeletal muscle mass and function).

Performing an acute bout of resistance (14, 159) and/or endurance exercise (186) upregulates skeletal muscle protein turnover with muscle protein synthesis being suggested as the major variable (as opposed to breakdown) to alter protein turnover (159). The other main anabolic stimulus to skeletal muscle is food intake (in particular dietary protein) and much like physical activity, alterations in synthesis rather than breakdown is considered to be the major variable to improve net protein balance (13, 213, 214, 230). When combined; these two main anabolic stimuli act synergistically as the exercise-mediated enhancement of muscle protein synthesis to feeding persists for at least 24h after performing an acute bout of exercise (27). Thus the development of strategies that involve both physical activity and diet have the greatest potential for maintaining skeletal muscle health with advancing age.

1.9 Physical activity

Skeletal muscle tissue has a tremendous capacity to adapt to physical demands by altering its contractile, neuronal and/or metabolic capacity (60). The high degree of malleability of skeletal muscle is especially apparent in athletes, where specific training modalities result in very distinct phenotypic outcomes (i.e. endurance vs. hypertrophy/strength athletes). However, the clear alterations in skeletal muscle size and capacity are not limited to athletic populations as aging, inactivity and disease can result in substantial deconditioning of skeletal muscle tissue.

A reduction in skeletal muscle mass, strength and function are one of the earliest manifestations of the aging process and are commonly referred to as sarcopenia (55). The loss of muscle with aging becoming apparent during the 4th decade of life with potential losses of 1-2 % per year (92,

184). These losses in skeletal muscle mass are troublesome as it results in a greater fall risk, loss of independence, and increased risk for the development of chronic diseases (such as obesity, diabetes, CVD etc.) (150, 194, 195, 231). Once manifested, recovery from these conditions is proven difficult for individuals with low muscle mass. Amongst hospitalized patients, sarcopenia is associated with an increased risk of postoperative complications (114, 174, 206), a greater chance of readmission (34) and higher rates of mortality (33, 212).

In 2000, the estimated healthcare cost attributable to sarcopenia alone in the United States was already estimated to be $18.5 billion per year and it is likely that this amount has further increased in recent years (192). In the future sarcopenia is likely to become an even greater problem to public health as the population aged 65 and older is projected to have nearly doubled by the year 2050 (147). Decreased physical activity levels is one of the most important underlying causes of sarcopenia (39). Thus strategies involving physical activity that can prevent (or slow down) the age-related loss of muscle mass are likely to become of increasing importance to public health.

Irrespective of age, a growing number of adults also develop diseases such as type II diabetes (1), cardiovascular disease (69), cancer (187) and obesity (8), and chronic kidney disease (83) of which its development can be in part attributed to physical inactivity (112). Similar to aging, it would appear that negative alterations in skeletal muscle protein metabolism are one of the earliest manifestations of obesity (9), heart disease (119), cancer (139) and chronic kidney disease (4).

Given the strong stimulatory effects of physical activity on skeletal muscle protein metabolism, strategies involving regular performance of physical activity hold great promise to prevent or improve various disease states. In particular, performing a single bout of resistance and/or endurance exercise can substantially upregulate skeletal muscle protein synthesis rates for up to

48-72 h (126, 159), thereby stimulating replacement of dysfunctional proteins with newly synthesized functional proteins.

The specific skeletal muscle adaptation that occurs depends on the type of exercise (e.g. resistance versus endurance exercise) that is habitually performed (226). The adaptation after endurance based activities are generally directed towards an increase in skeletal muscle oxidative capacity; (e.g., mitochondrial-related proteins, angiogenic proteins, glucose transporters, etc.) (226), while regular performance of resistance exercise will lead to an increase in muscle hypertrophy and strength (51, 116). It should be noted, however, that adaptations in skeletal muscle are not completely divergent. In untrained adults, high intensity resistance exercise stimulates increases in the myofibrillar (contractile) and mitochondrial (energy-producing) sub-fractions in skeletal muscle (226). A stimulation of all the major protein sub-fractions in skeletal muscle is also observed after an acute bout of endurance exercise (23, 48, 226), especially when the performance of endurance exercise involves substantial mechanical loading (i.e. cycling) (48). However, with chronic training the acute muscle protein synthetic response (i.e. an increase in myofibrillar or mitochondrial) to a bout of physical activity will be more reflective of the phenotypic outcome (hypertrophy/strength-based vs. endurance) (226). For one of the studies in this thesis, resistance-based exercise was used as a model of physical activity to study the effects of divergent whole food manipulations on myofibrillar protein synthesis during post-exercise recovery.

1.10 Protein ingestion

The other main anabolic stimulus to skeletal muscle tissue is food ingestion; particularly dietary protein. It has been shown that the amount (135, 153, 180, 228, 235), timing (5, 118), and source (animal vs. plant based) (210) of are all factors that can potentially modulate the muscle anabolic response after protein feeding.

It has been suggested that ~20 g of dietary protein per meal will result in an optimal stimulation of myofibrillar protein synthesis in healthy young adults both in during post-exercise recovery and rested states (134). With aging, however, the muscle can become less sensitive to the anabolic properties of protein ingestion and greater intakes of dietary protein appear necessary to maximize muscle protein synthesis (134, 153). The reduced anabolic sensitivity to protein feeding, often termed 'anabolic resistance of aging' (25) may be caused by impairments in protein digestion and amino acid absorption (19), amino acid uptake in muscle (49), or a reduced amount or activation status of key anabolic signaling proteins (41, 64, 108). However, ingestion of ~ 35-40 g of protein per meal in older adults (> 40-50 y) can overcome the 'anabolic resistance' of aging and restore rates of muscle protein synthesis to a level that is similar to young adults (134). However, the consumption of such high amount of protein in a single meal is not realistic for the majority of the population, which generally consumed 15-30 g of dietary protein per meal (2). Furthermore, it is important to note that most of this work is done after providing protein in isolation, without the addition of other macronutrients. A focus of this thesis was to study postprandial muscle protein synthesis in aging individuals after mixed meal consumption containing moderate amounts of protein (20 g) in the form of a whole food source (**Chapter 5**). Indeed previous suggestions have been made that consumption of a whole food protein source may potentiate the subsequent muscle protein synthetic response to moderate protein feeding when compared to more isolated sources

of protein (55). This data may suggest that when protein is consumed as part of a whole food source, containing multiple macronutrients as part of its natural food matrix, this may improve the postprandial muscle anabolic response to moderate protein amounts and as such provides a realistic feeding strategy to overcome the 'anabolic resistance' to protein feeding in aging individuals. This notion was further investigated in **Chapter 5** of this thesis.

Another factor that may influence postprandial muscle protein synthesis is the timing (or distribution) of ingestion throughout the day. Specifically, it has been demonstrated that the consumption of an optimal dose of 20-35 g of protein every 3-5 h will result in greater stimulation of muscle protein synthesis throughout the day when compared to an uneven distribution where lower amounts (~10-15 g) are consumed more frequently (e.g. every 1.5h) (5, 118) or higher amounts (~ 40-60) are consumed infrequently (every 6-8 h) (118).

The protein source ingested is another main factor that may influence postprandial protein synthesis. In multiple studies, the ingestion of whey has been shown to result in higher rates of protein synthesis when compared to the ingestion of isonitrogenous amounts casein (152, 197) and the plant-based protein soy (197, 236). This observation is likely explained by the more rapid digestibility and absorption of whey and its higher essential amino acid content (152). In general, the ingestion of animal-based sources result in greater postprandial stimulation of muscle protein synthesis when compared to the ingestion of isonitrogenous amounts of plant proteins (73, 158, 197, 227, 236). These observations may be explained by the lower digestibility of plant-based sources and their relative low abundance in specific essential amino acids (210); a 'disadvantage' that may be overcome by consuming greater amounts of plant proteins (73) or by consuming a variety of sources to make for a complete amino acid profile (210).

Interestingly enough, most of our knowledge on postprandial protein metabolism comes from work studying the ingestion of isolated protein fractions (e.g., dairy-based whey and casein and plant-based soy) in liquid beverages (17, 26, 27, 36, 37, 42, 43, 71, 72, 91, 104, 106, 107, 152, 155, 173, 178, 197, 201, 216, 235, 236) or ingestion of free form, crystalline amino acids (15, 40, 127, 141, 168, 169, 202, 203, 213, 214).

The consumption of protein or amino acid containing beverages is not common practice as the majority of the population obtains dietary protein as part of nutrient-dense, whole foods that contain a variety of macro- and micronutrients. However, far less is known about how the ingestion of nutrient-dense whole foods impacts postprandial protein metabolism. The major focus of this thesis was to study protein metabolism after the ingestion of more generalizable whole foods. Therefore, the literature on whole food ingestion and protein metabolism will be discussed in the next part of this thesis.

Whole foods

One of the earliest observations on whole food ingestion and postprandial protein metabolism was made by Elia et al. (53) in 1989. After having participants consume a mixed, whole foods meal the authors noted that the postprandial rise in circulating amino acids was attenuated when compared to the consumption of a meal predominantly consisting of protein (53). However, the lack of labeled food proteins did not allow these workers to truly assess whether postprandial dietary-derived amino acid availability was attenuated with mixed meal ingestion, nor was its potential influence on muscle protein synthesis assessed.

Nearly two decades later, Elliot and colleagues (54) were one of the first to study muscle protein metabolism in response to whole food ingestion. The authors found that the ingestion of whole milk resulted in a greater net muscle protein balance when compared to the ingestion of

isonitrogenous amounts of skim milk (containing negligible amounts of fat) (54). While the authors were unable to provide a plausible explanation for the observation that co-ingestion of fat with protein improved rates of muscle protein synthesis, these findings are not exclusive. A few years prior Fouillet et al. (62) had shown that the addition of fat to milk protein resulted in greater dietary N retention in peripheral (skeletal muscle) tissue when compared to the addition of carbohydrates to milk protein, which resulted in greater dietary N retention in the splanchnic bed (62). These findings were assumed to be explained by 1) a reduced rate of gastric emptying with carbohydrate intake thereby delaying systemic appearance of dietary-derived amino acids and 2) the insulinemic response to carbohydrate co-ingestion which is shown to upregulate splanchnic protein synthesis (45). In recent years, this hypothesis was confirmed by work from van Loon's research group. Using the novel technique of labeled food proteins, the authors revealed that the consumption of carbohydrates with casein protein resulted in attenuated appearance of dietary-derived amino acids in circulation (37, 71), while the ingestion of fat with casein protein did not impact systemic deliverance of dietary-derived amino acids (72). In their work, the addition of fat or carbohydrates did not modulate the muscle anabolic response when compared to the ingestion of only casein protein (37, 71, 72). The differential findings of Elliot et al. (32) and the van Loon group (72) may relate to the mode of ingestion. Specifically, in the work by Elliot et al. (32) protein was ingested as part of the natural food matrix (e.g. whole milk), whereas Gorissen et al. (72) provided macronutrients as isolated food sources (e.g. milk fat was mixed with isolated casein protein). It is tempting to speculate that greater retention of intrinsic factors (such as micronutrients, miRNAs, growth factors, bioactive peptides etc.) in the whole food source compared to the ingestion of processed, isolated constituents (75, 76, 171, 181) could have influenced these findings.

Only a handful of other studies have assessed postprandial (muscle) protein metabolism after whole food ingestion and have focused on beef ingestion. It was demonstrated that the ingestion of 30 g of protein in the form of lean ground beef (90/10 %) doubled rates of muscle protein synthesis above baseline in both young and older adults, an effect not further enhanced with the ingestion of 90 g of beef protein (196). Similar results have been made by others who found that postprandial rates of protein synthesis were upregulated after ingestion of a beef patty containing 36 g (180) and 40 g of protein (103). We have recently compared the muscle anabolic response to isonitrogenous lean beef vs. skim milk ingestion (26). Despite divergent responses in the early (0-2h) postprandial phase, no differences were found in total dietary-derived amino acid availability and stimulation muscle protein synthesis when assessed over a more representative 5h postprandial phase.

Taken together these data suggests that whole food ingestion is an effective strategy to stimulate postprandial muscle protein synthesis in healthy adults (26, 54, 103, 180, 196), with some evidence suggesting that consumption of whole milk may have greater muscle anabolic stimulatory effects when compared to the ingestion of more isolated sources of protein (e.g. skim milk) (54). However, it is clear that the influence of whole food ingestion on postprandial protein metabolism remains largely understudied and a main focus of this thesis was to fill this research gap. To this end we have performed a number of studies:

1.11 Studied and hypothesis tested

The primary objective of the studies in this thesis was to apply innovative methodology to assess the regulation of whole body and muscle metabolic responses in a detailed manner after food ingestion in both healthy and diseased adults. The Chapters that follow describe the studies that were used to gain a better understanding of the regulation of postprandial metabolic responses *in vivo* in humans in both health and disease-states.

Chapter 2: Sustained postprandial muscle protein synthesis rates after protein ingestion in healthy young males

The temporal relationship between the postprandial release of dietary protein derived amino acids in circulation and their subsequent use for the stimulation of muscle protein synthesis has not been clearly established. Study 1 (**Chapter 2**) was designed to directly define the relationship between protein derived amino acid availability in circulation, anabolic signaling phosphorylation, and the subsequent stimulation of postprandial muscle protein synthesis rates in healthy adults. To accomplish this, we combined specifically produced labeled milk proteins with primed continuous IV stable isotope infusions. We hypothesized that the temporal response of dietary protein-derived amino acid availability in circulation coincides with increased muscle anabolic signaling and subsequent stimulation of postprandial protein synthesis after protein ingestion. Performing this work was important to provide a clear understanding of the relationship between postprandial dietary-derived amino acid availability and the subsequent stimulation of muscle protein synthesis. The goal of this study was to set a benchmark for future work studying protein metabolism to divergent nutritional stimuli in health and disease.

Chapter 3*:* Development of intrinsically-labeled eggs and poultry meat for use in human metabolic research.

Intrinsically-labeled food proteins are not commercially available and thus have to be produced by the researcher. **Chapter 3** details study 2 of this thesis and our efforts in producing intrinsically labeled proteins and aimed to provide the research field with an easy and cost-effective approach to producing intrinsically labeled food proteins to measure postprandial protein handling. Previously, researchers have primarily focused on manufacturing intrinsically-labeled cow derived food products (i.e. milk and beef) for use in human metabolic research (6-8). The labeled milk and beef were produced by prolonged intravenous (IV) infusions of stable isotope tracer into lactating dairy cows. The purpose of this study was to extend on previous efforts in cows (18, 175, 207) and chickens (57, 63, 122) to demonstrate the feasibility of producing nutritionally complete and intrinsically L-[5,5,5-$^{2}H_{3}$]leucine labeled dietary proteins (i.e. egg and poultry meat) at a modest production price point by the use of a non-invasive oral tracer administration method in egg laying hens. In addition, we wished to apply these labeled foods in humans as a proof-of-principle to assess *in vivo* dietary protein digestion and absorption kinetics and subsequent postprandial muscle protein accretion. Our rationale behind this approach was that chickens are naturally small animals, which makes their management and resource cost ideal for the researcher. We hypothesized that ~ 1/10th of the amount of tracer (45g) would be required to produce labeled eggs sufficiently enriched to model postprandial whole-body and muscle protein metabolism in the consumer as compared to cow experiments (400 g) (207).

Chapter 4*:* Consumption of whole eggs promotes greater stimulation of postexercise muscle protein synthesis than consumption of isonitrogenous amount of egg whites in young men

Co-ingestion of protein with other macronutrients has been shown to modulate dietary protein digestion and amino acid absorption kinetics when consumed as a liquid beverage (37, 71, 72). However, the effects of co-ingestion of fat and protein as part of the natural food matrix on dietary protein digestion and absorption kinetics and the subsequent postprandial muscle protein synthetic response during recovery from resistance exercise remains to be established. To examine the influence of protein co-ingestion with fat within a natural food matrix, **Chapter 4** compared the consumption of three whole eggs (18 g protein and 17 g fat) to an equivalent amount of protein from egg whites (18 g protein and 0 g fat) by combining primed constant infusion methods with the ingestion of specifically produced L-[5,5,5-$^{2}H_{3}$]leucine labeled eggs in healthy young adults.

We hypothesized that the consumption of whole eggs will result in a delayed appearance of dietary protein derived amino acid in systemic circulation. However, we expect that the total amount of dietary protein derived amino acid availability throughout the 5 h postprandial phase to be similar between the two conditions with no subsequent difference in the stimulation of the postprandial muscle protein synthetic response.

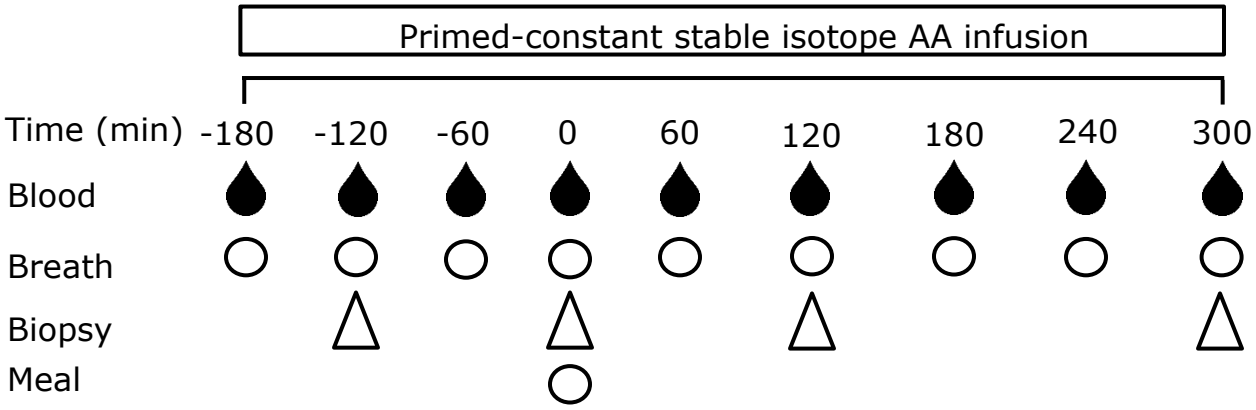
Chapter 5: Differential whole body and skeletal muscle protein metabolic responses to meal ingestion in maintenance hemodialysis patients.

Maintenance hemodialysis (MHD) is the most common method used to treat end-stage renal disease. Muscle wasting is a common feature in MHD patients, and forms a key factor responsible for the decline in muscle strength and loss of functional capacity in this patient population (117). The mechanisms responsible for the loss of muscle mass in MHD patients are not well defined, but likely relate to disturbances in protein metabolism from several co-morbid conditions (e.g., insulin resistance, chronic inflammation, metabolic acidosis, and hormonal abnormalities) (117). As a result MHD patients experience substantial whole body and muscle protein proteolysis that is maintained well beyond the dialysis period. However, it is currently unknown if protein-rich meal ingestion can improve whole body net balance and stimulate muscle protein synthesis in MHD patients and can thus mitigate some of the losses in body protein mass.

In **Chapter 5** (study 4)**,** we sought to fill this research gap and studied postprandial protein handling in MHD patients after the consumption of a mixed meal and compared these outcomes to healthy controls. We hypothesized that the postprandial protein handling in MHD patients will be impaired versus healthy controls.

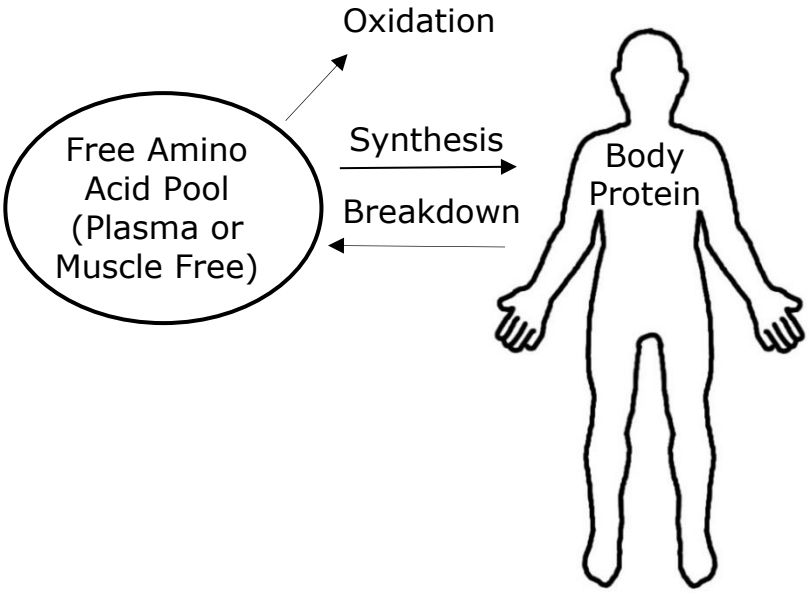
1.12 Figures

Figure 1.1



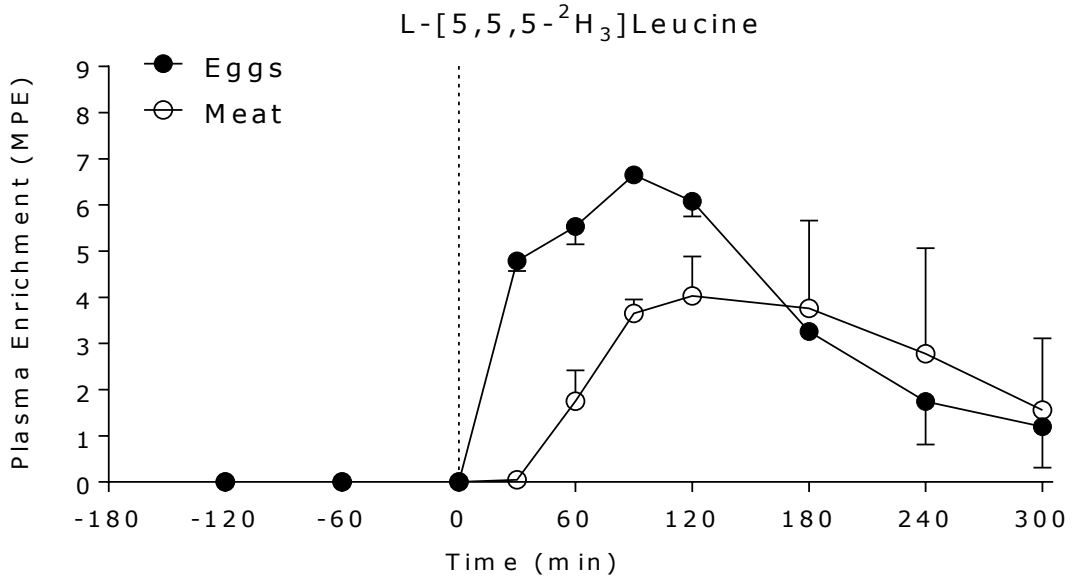
Example of primed-continuous stable isotope infusion protocol to assess protein metabolism in humans.

Figure 1.2



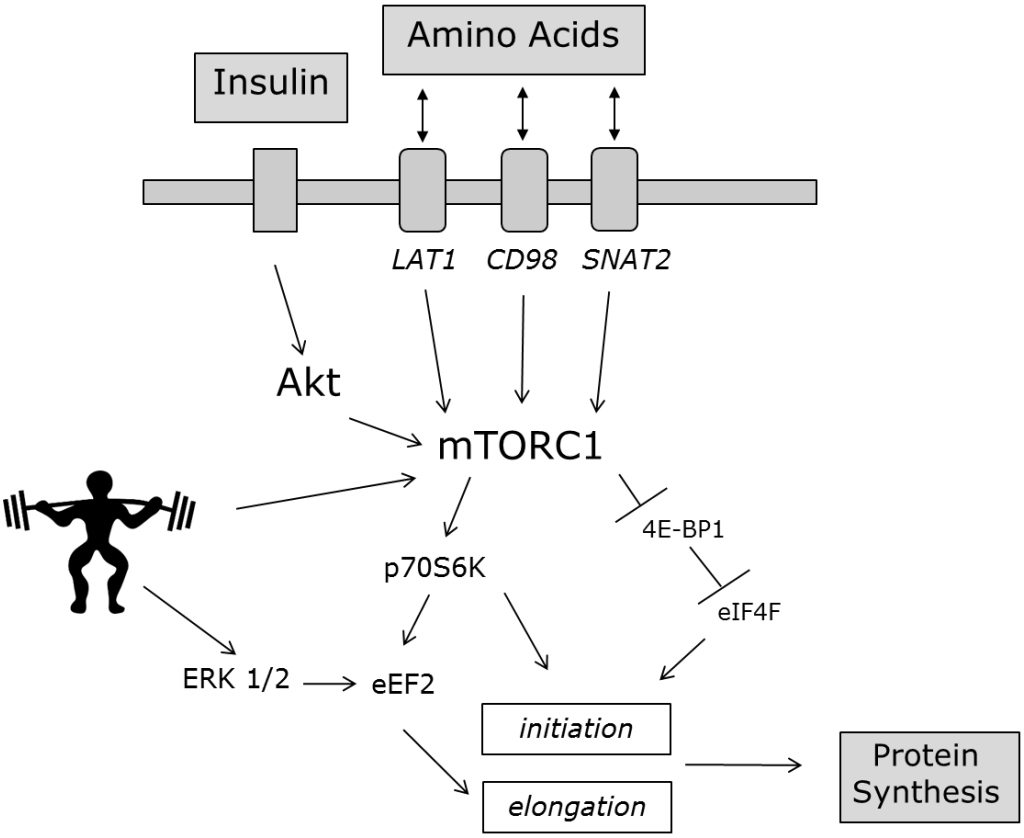
Simplified two-pool model to assess protein metabolism in humans.

Figure 1.3



Time course of isotopic enrichment after ingestion of L-[5,5,5-$^2H_3$]leucine-labeled eggs and meat.

Figure 1.4



Intramuscular signaling pathways and amino acid transporters involved in the regulation of protein synthesis (mRNA translation). An arrow indicates a stimulatory effect; a blunted end line indicates a repressive effect.

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CHAPTER 2: SUSTAINED POSTPRANDIAL MUSCLE PROTEIN SYNTHETIC RESPONSE AFTER PROTEIN INGESTION IN HEALTHY YOUNG MALES[1]

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Key words: amino acids, diet quality, time course, protein quality.

Abbreviations used: AA, amino acid; AKT, protein kinase B; FSR, fractional synthetic rate; LAT1, Large neutral amino acids transporter small subunit 1; LC/MS/MS, liquid chromatography-tandem mass spectrometry; GC/MS, gas chromatography-mass spectrometry; R_a, rate of appearance; R_d, rate of appearance; mTORC1, mammalian target of rapamycin complex 1, p70S6K, 70 kDa S6 protein kinase.

[1]All co-authors agreed to their contributions being included in the dissertation.

2.1 Abstract

We aimed to determine the effect of ingesting a single meal-like amount of protein on digestion and absorption kinetics and the subsequent time-dependent regulation of postprandial muscle protein synthesis rates *in vivo* in humans. Seven healthy young males (age: 22±1 y, BMI: 24.8±1.2 kg/m^2) received primed continuous L-[ring-$^2H_5$]phenylalanine and L-[ring-3,5-2H2]tyrosine infusions and ingested intrinsically L-[1-^{13}C]phenylalanine-labeled milk protein concentrate (38 g protein, 4.17 g carbohydrate, 1.4 g fat). Biopsies were collected from the *vastus lateralis* in the basal-state and 60, 120, 180 and 300 min after protein ingestion. Exogenous phenylalanine appearance in circulation rapidly increased after protein ingestion (P<0.001). Dietary protein-derived phenylalanine released in the circulation was 13±2% of the total intake within the first 60 min, followed by 14±1%, 12±1% 10±0%, and 9±1% within the subsequent 60-120, 120-180 min, 180-240 and 240-300 min of the postprandial period, respectively (total 56±4% over the 300 min period). mTORC1 phosphorylation increased at 120 min (22±8% above basal) after protein ingestion and remained elevated throughout the remainder of the postprandial period (all, P<0.05). Myofibrillar protein synthesis rates tended to increase (absolute change from basal: 0.010±0.008 %/h) immediately following protein ingestion between 0-60 min (P=0.10). Myofibrillar protein synthesis rates were significantly increased between 60-120 min (0.035±0.012 %/h) and myofibrillar protein synthesis remained elevated between 120-180 min (0.019±0.005 %/h) and 180-300 min (0.016±0.004 %/h) after protein ingestion when compared to basal rates (all, P<0.05). Our results show that muscle protein synthesis rates increased 60 min after protein ingestion and remain elevated above baseline values for at least 5 hours during the postprandial period in healthy young males.

2.2 Introduction

Skeletal muscle mass undergoes constant protein remodeling through coordinated changes in breakdown and synthesis. The increase in postprandial muscle protein synthesis rates after each meal is important for skeletal muscle mass maintenance , most notably through the stimulation of synthesis in the myofibrillar protein fraction (18). It is generally believed that dietary protein-derived amino acid availability in circulation is an independent and key regulator of the postprandial muscle protein synthetic response in humans (9, 16). Suggestions have been made that once enough dietary amino acids become available in circulation to signal muscle nutrient/anabolic signaling mechanisms, the stimulation of muscle protein synthesis is quite transient in nature and is subsequently inhibited despite prolonged elevated plasma amino acid concentrations after protein feeding (1, 2). This phenomenon has been referred to as the muscle-full effect (1). However, the direct measurement of the temporal relationship between the postprandial release of dietary protein-derived amino acids in circulation, phosphorylated-state of anabolic signaling pathways, and the subsequent stimulation of muscle protein synthesis rates after protein ingestion has not been clearly established.

Combining the consumption of intrinsically-labeled dietary proteins with primed continuous intravenous (I.V.) stable isotope amin acid infusions and muscle biopsy collections allows for direct insight into the effect of dietary amino acids on the time course of the phosphorylation of anabolic signaling proteins involved in translational control and the stimulation of postprandial muscle protein synthesis rates (5, 22). Therefore, the purpose of our current work was to apply primed continous I.V. infusions of L-[*ring*-$^{2}H_{5}$]phenylalanine and L-[*ring*-3,5-$^{2}H_{2}$]tyrosine with the ingestion of specifically produced intrinsically L-[1-^{13}C]phenylalanine labeled milk protein concentrate to assess dietary protein-derived amino acid availability in circulation and the time

course of the phosphorylation of mTORC1 and the stimulation of muscle protein synthesis rates in healthy young males. We hypothesized that the temporal release of dietary protein-derived amino acids into circulation coincided with increased phosphorylation of mTORC1 and the stimulation of postprandial muscle protein synthesis rates after protein ingestion in healthy young males.

2.3 Methods

Subjects

Seven healthy, young men (age: 22±1 y) were examined in the current study. Participant characteristics are presented in **Table 1**. All participants were informed about the experimental procedures to be used, the purpose of the study, and all potential risks before giving written consent. The study conformed to all standards for the use of human participants in research as outlined in the Helsinki Declaration and was approved by the local Institutional Review Board at the University of Illinois at Urbana-Champaign.

Experimental protocol

Participants reported to the laboratory at 0700 h after an overnight fast and having refrained from strenuous physical activity for at least 3 d prior to the experimental trial. A Teflon catheter was inserted into a heated dorsal hand vein for repeated arterialized blood sampling and remained patent by a 0.9% saline drip. After taking a baseline blood sample (t=-180), the plasma phenylalanine and tyrosine pools were primed with a single dose of L-[*ring*-$^{2}H_{5}$]phenylalanine (2.0 μmol·kg^{-1}), L-[*ring*-3,5-$^{2}H_{2}$]tyrosine (0.615 μmol·kg^{-1}) after which a continuous L-[*ring*-$^{2}H_{5}$]phenylalanine (0.05 μmol·kg^{-1}·min^{-1}) and L-[*ring*-3,5-$^{2}H_{2}$]tyrosine (0.015 μmol·kg^{-1}·min^{-1} intravenous infusion was initiated (t=-180 min) and maintained over the experimental infusion

trial. Participants received biopsies of the *vastus lateralis* before (*t*=-120 and 0 min) and after (*t*=60, 120, 180 and 300 min) the ingestion of 38 g of dietary protein (3.46 g leucine). Dietary protein was provided as intrinsically L-[1-^{13}C]phenylalanine milk protein concentrate dissolved in 300 ml of water (see below for details). Biopsies were collected in the middle region of the *vastus lateralis* (approximately 15 cm above the patella) with a Bergström needle that was custom modified for manual suction under local anesthesia (18). Muscle samples were freed from any blood, fat, and visible connective tissue and immediately frozen in liquid nitrogen prior to storage at -80°C until further analysis. Multiple blood samples were collected in EDTA containing tubes before (*t*=-180, -120, -60, -0 min) and after protein ingestion (t=30, 60, 90, 120, 180, 240 and 300 min). The blood samples were immediately analyzed for whole blood glucose concentrations (2300 Stat Plus, YSI Life Sciences, Springs, OH) and centrifuged at 3000 × g for 10 min at 4°C. The plasma samples were subsequently stored at -20°C for future analysis.

Intrinsically labeled milk protein

Intrinsically L-[1-^{13}C]phenylalanine labeled milk protein concentrate (MPC 80) was obtained by infusing L-[1-^{13}C]phenylalanine into a lactating Holstein cow, collecting the milk, and purifying the milk protein concentrate as previously described (6, 17, 21). The L-[1-^{13}C]phenylalanine enrichment in the milk protein concentrate was measured by GC-MS (Agilent 6890N GC coupled with a 5973 inert MDS; Little Falls, DE) and averaged 38.3 MPE. The MPC 80 met all chemical and bacteriologic specifications for human consumption. The beverage provided to participants contained 38 g protein (3.46 g leucine), 4.17 g carbohydrate and 1.4 g fat.

Plasma analyses

Plasma insulin concentrations were determined using a commercially available enzyme-linked immunosorbent assays (Alpco Diagnostics; Salem, NH). Plasma amino acid concentrations and enrichments were determined by GC-MS (Agilent 7890A GC/5975C; MSD, Little Falls, DE). Specifically, internal standards of [U-$^{13}C_6$]leucine, [U-$^{13}C_9{}^{15}N$]phenylalanine, and [U-$^{13}C_9{}^{15}N$]tyrosine were added to the plasma samples. The plasma was deproteinized on ice with 5-sulfosalicylic acid. Free amino acids were purified using cation exchange AG 50W-X8 resin (mesh size: 100-200, ionic form: hydrogen) (Bio-Rad Laboratories, Hercules, CA) columns. The free amino acids were converted to their tert-butyl dimethylsilyl (TBDMS) derivative before analysis by GC-MS. The amino acid concentrations were determined using electron impact ionization by monitoring ions at mass-to-charge (*m/z*) ratio 302 and 308 for unlabeled and [U-$^{13}C_6$]-leucine respectively, 336 and 346 for unlabeled and [U-$^{13}C_9{}^{15}N$]-phenylalanine respectively, and 466 and 476 for unlabeled and [U-$^{13}C_9{}^{15}N$]-tyrosine respectively. The plasma phenylalanine and tyrosine ^{13}C and ^{2}H enrichments were determined using selective ion monitoring at *m/z* 336, 337, and 341 for unlabeled and labeled (1-^{13}C and *ring*-$^{2}H_5$)phenylalanine respectively, *m/z* 466, 468, and 470 for unlabeled and labeled (*ring*-3,5-$^{2}H_2$, and *ring*-$^{2}H_4$)tyrosine respectively. Standard regression curves were applied from a series of known standard enrichment values against the measured values to assess the linearity of the mass spectrometer and to account for any isotope fractionation which may have occurred during the analysis. Phenylalanine and tyrosine enrichments were corrected for the presence of both the ^{13}C and ^{2}H isotopes.

Muscle analyses

Myofibrillar proteins were extracted from ~50 mg of wet muscle by hand-homogenizing in ice-cold homogenization buffer (10 μL·mg^{-1}) using a Teflon pestle. Homogenates were centrifuged at 700 × *g* for 5 min at 4ºC. The resultant supernatant was removed and the pellet was washed with 500 μL distilled water. Subsequently, the samples were centrifuged at 700 × *g* for 10 min at 4ºC. The remaining myofibrillar proteins pellet was solubilized by adding 1 mL of 0.3 M NaOH and heating at 50ºC for 30 min with vortex mixing every 10 min. Samples were centrifuged at 700 × *g* for 5 min at 4ºC. The supernatant containing the myofibrillar enriched protein fraction was collected and the collagen pellet was discarded. Myofibrillar proteins were precipitated by adding 1 mL of 1 M PCA and centrifuging at 2500 × *g* for 10 min at 4ºC. The myofibrillar protein pellet was washed twice with 70% ethanol. The amino acids were liberated by adding 2 mL of 6 M HCL and heating overnight at 110°C. Free amino acids were purified using cation exchange AG 50W-X8 resin (mesh size: 100-200, ionic form: hydrogen) (Bio-Rad Laboratories, Hercules, CA) columns before analysis of L-[*ring*-$^{2}H_{5}$]phenylalanine enrichments by GC/MS.

To reduce the signal-to-noise ratio during GC-MS analysis at low tracer enrichments, the phenylalanine from the myofibrillar protein hydrolysates were enzymatically decarboxylated to β-phenylethylamine (7) prior to tBDMS derivatization (19, 20). Enrichments of the protein bound samples were determined by selected ion monitoring for β-phenylethylamine-tBDMS mass to charge ratio at 183 (*m*+5) to 180 (*m*+2) and a single linear standard curve (to avoid slope influences on the measured TTR) from mixtures of known *m*+5 to *m*+2 ratios. To avoid saturation of the MS and eliminate bias due to any potential concentration dependencies (14), the split ratio was adjusted prior to the injection of each sample so that nearly equal amounts of phenylalanine were injected for all samples and standards.

Muscle intracellular free amino acids were extracted from a separate piece of wet muscle (~30 mg) using a Teflon-coated pestle and ice-cold 2% PCA and then centrifuged at 10000 × g at 4˚C for 10 min. The supernatant was then collected and this process was repeated 2 more times. The supernatant was taken as the muscle intracellular free amino acids and subsequently purified using cation exchange chromatography (Dowex 50W-X8-200 resin; Acros Organics, Geel, Belgium) before analysis by LC/MS/MS. The phenylalanine ^{13}C and ^{2}H enrichments were determined by multiple reaction monitoring (MRM) at *m/z* 166.0 → 103.0, 167.0 → 104.0 and 171.0 → 106.0 for unlabeled and labeled (L-[1-^{13}C] and *ring*-$^{2}H_5$)phenylalanine, respectively. Software Analyst 1.6.2 was used for data acquisition and analysis. Phenylalanine enrichments were corrected for the presence of both ^{13}C and ^{2}H isotopes.

Western blotting

A portion of whole muscle homogenates isolated during the myofibrillar protein extractions was used for Western blotting analysis. Protein content of the homogenates was determined by Bradford Assay (Bio-Rad Laboratories, Hercules, CA) and then equal amounts of protein were separated by SDS-PAGE before being transferred to polyvinyl difluoride membranes for blotting. After blocking, membranes were incubated in primary antibodies overnight at 4^{o}C to determine the phosphorylation status and total protein content mammalian target of rapamycin complex 1 (mTORC1) at Ser^{2448} and total protein content of large neutral amino acid transporter (LAT1/ SLC7A5) (Bioss Antibodies, Woburn, MA). Membranes from the respective proteins were then incubated with appropriate secondary antibodies and protein content was detected using West Femto Maximum Sensitivity substrate (SuperSignal, Thermo Scientific, Waltham, MA) and the ChemiDoc-It^{2} Imaging System (UVP, Upland, CA). After detection of phosphorylated proteins,

membranes were stripped with western blot stripping buffer (Restore, Thermo Scientific, USA) and re-incubated with antibodies against total protein (Cell Signaling Technology, Danvers, MA). Western blot data were normalized to an internal control (α-tubulin). Bands were quantified using ImageJ software (NIH), normalized to a control sample run on each blot to account for inter-blot variability, and expressed as fold change from basal.

Calculations

Ingestion of L-[1-^{13}C]-phenylalanine-labeled protein, intravenous infusion of L-[*ring*-$^{2}H_5$]-phenylalanine and L-[*ring*-3,5-$^{2}H_2$]-tyrosine, and arterialized blood sampling were used to assess whole-body amino acid kinetics in non-steady state conditions. Total, exogenous, and endogenous rate of appearance (R_a) and plasma availability of dietary protein-derived phenylalanine (i.e., the fraction of dietary protein-derived phenylalanine that appeared in the systemic circulation), were calculated using modified Steele's equations (4, 10) as described previously (12). Furthermore, total rate of phenylalanine disappearance (R_d), utilization of phenylalanine for protein synthesis, and phenylalanine hydroxylation (first step of phenylalanine conversion to tyrosine) were calculated (12).

Myofibrillar protein fractional synthetic rates (FSR) were calculated using the standard precursor-product equation:

$$\mathrm{FSR} = \frac{\Delta \mathrm{E_p}}{\mathrm{E_{precursor} \cdot t}} \cdot 100\% \qquad (9)$$

where ΔE_p is the increment in myofibrillar protein-bound L-[ring-$^{2}H_5$]-phenylalanine enrichment after an incorporation period (tracer-to-tracee ratio, TTR), $E_{precursor}$ is the weighted mean plasma

L-[ring-$^{2}H_5$]-phenylalanine enrichment or the muscle free pool during that incorporation period (TTR), and *t* is the incorporation period (h). Muscle biopsies at t=-120 and 0 min were used for basal FSR andbiopsies at t=60, 120, 180 and 300 min were used for postprandial FSR.

Statistics

Differences in time-dependent blood and muscle measurements were tested one-way repeated-measures ANOVA (time). When statistically significant time effects were observed LSD post hoc tests were performed to locate differences. Differences were considered statistically significant at $P<0.05$. All calculations were performed using IBM SPSS Statistics (version 24, Chicago, IL) unless otherwise designated. All data are expressed as mean ± SEMs.

2.4 Results

Plasma metabolites

Plasma glucose concentrations were increased above fasted values (t=0 min) at 30 min of the postprandial period reaching a peak concentration of 81 ± 1 mg·dL^{-1} **(***P*=0.03; **Figure 1A)**. Similarly, plasma insulin concentrations were increased above fasted values at 30 min of the postprandial period reaching a peak concentration of 19 ± 4 µIU·mL^{-1} at 30 **(***P*=0.02; **Figure 1B)**. Plasma phenylalanine- (**Figure 2A**), tyrosine- (**Figure 2B**) and leucine (**Figure 2C**) concentrations increased rapidly after protein ingestion and remained elevated above fasted values during the entire post-prandial phase (all $P<0.05$). Peak plasma concentrations for all three amino acids were observed at 30 min after protein ingestion with values of 97 ± 5, 116 ± 10, and 335 ± 32 µmol·L^{-1} for phenylalanine, tyrosine, and leucine, respectively.

Plasma amino acid enrichments

Plasma L-[1-^{13}C]-phenylalanine enrichment increased rapidly after protein ingestion and remained elevated above fasted values during the entire post-prandial phase (all, $P<0.05$; **Figure 3A**). Peak plasma L-[1-^{13}C]-phenylalanine enrichment was observed at 30 min after protein ingestion with a value of 14 ± 2 MPE. Plasma L-[*ring*-$^{2}H_5$]-phenylalanine enrichment (**Figure 3B**) decreased after protein ingestion (time effect: $P<0.01$) and remained reduced below fasted values (t=0 min) until 60 min after protein ingestion (all, $P<0.05$). Plasma L-[*ring*-3,5-$^{2}H_2$]tyrosine (**Figure 3C**) decreased after protein ingestion (time effect: $P<0.01$) and remained reduced below fasted values (t=0 min) until 120 min after protein ingestion (all, $P<0.05$).

Whole body amino acid kinetics

Exogenous phenylalanine rates of appearance (representing the appearance of dietary protein-derived phenylalanine in circulation) increased after protein ingestion and remained elevated above fasted values during the entire post-prandial phase (all, $P<0.01$; **Figure 4A**). Peak plasma exogenous phenylalanine rates of appearance were observed at 30 min after protein ingestion and reached a value of 313 ± 48 nmol phenylalanine$\cdot kg^{-1}\cdot min^{-1}$. The amount of dietary protein-derived phenylalanine that appeared in circulation over the 300 min postprandial period was calculated to be 58±4%. The amount of dietary protein-derived phenylalanine that appeared in circulation during min 0-60, 60-120, 120-180, and 180-240 and 240-300 min was 13±2, 14±1, 12±1, 10±0 and 9±1%, respectively (**Figure 4B)**. While dietary-protein-derived amino acid availibity remained elevated above fasted values (t=-60-0 min) throughout the entire postprandial period (all $P<0.05$), dietary derived amino acid availibity was significantly higher between 60-120 min when compared to later time points (120-180, 180-240 and 240-300 min) ($P<0.05$).

Whole body protein breakdown rates (**Figure 5A**) decreased after protein ingestion (time effect: P<0.01) with the lowest value of 572 ± 37 nmol phenylalanine·kg^{-1}·min^{-1} observed at 90 min after ingestion. Whole body protein oxidation rates (**Figure 5B**) increased after protein ingestion (time effect: P<0.01) and remained elevated above fasted values during the entire post-prandial phase (all *P*<0.05) with a peak value of 136 ± 14 nmol phenylalanine·kg^{-1}·min^{-1} observed at 60 min after ingestion. Whole body protein synthesis rates (**Figure 5C**) increased after protein ingestion (time effect: P<0.01) and remained elevated above fasted values (t=0 min) until 180 min after ingestion (all *P*<0.05) with a peak value of 920 ± 17 nmol phenylalanine·kg^{-1}·min^{-1} observed at 60 min after ingestion. Consequently, whole body net protein balance increased after protein ingestion (time effect: P<0.01) (**Figure 5D**) and remained elevated above fasted values during the entire post-prandial phase (all *P*<0.01) with a peak value of 258 ± 22 nmol phenylalanine·kg^{-1}·min^{-1} observed at 60 min after protein ingestion.

Muscle intracellular free amino acid enrichments

Muscle intracellular free L-[1-^{13}C]-phenylalanine enrichments increased after protein ingestion (time effect: *P*<0.01) and remained elevated above fasted values (t=0 min) during the entire post-prandial phase (*P*<0.05; **Figure 6A**). Peak muscle intracellular free L-[1-^{13}C]-phenylalanine enrichment was observed at 120 min after protein ingestion with a value of 4.0 ± 0.6 MPE. Muscle intracellular free L-[ring-$^{2}H_5$]-phenylalanine enrichments decreased after protein ingestion (time effect: P<0.01) and were suppressed below fasted values at 60 min after protein ingestion (*P*=0.03; **Figure 6B**).

LAT1 amino acid transport and mTORC1 phosphoryalation

Protein ingestion did not modulate LAT1 protein content throughout the entire postprandial period (time effect: P=0.53; **Figure 7A**). mTORC1 phosphorylation increased after protein ingestion above baseline between 180-300 min of the postprandial period (all P<0.01; **Figure 7B**).

Myofibrillar protein synthesis

Using plasma phenylalanine enrichments as the precursor, postprandial myofibrillar protein synthesis rates increased at 60-120 min after protein ingestion and remained elevated above fasted values (t=-120-0 min) throughout the remaining postprandial period (all, P<0.05; **Figure 8**). In the first 60 minutes after protein ingestion, myofibrillar protein synthesis showed a trend towards an increase above fasted values (absolute change from fasted: 0.010±0.005 %/h^{-1}; P=0.11). The postprandial increase in myofibrillar protein synthesis was greatest at 60-120 min after protein ingestion (0.037±0.01 %/h^{-1}) and remained elevated above fasted values at 120-180 min (0.022±0.005 %/h^{-1}) and 180-300 min (0.019±0.004 %/h^{-1}).

Using intracellular free phenylalanine enrichments as the precursor, postprandial myofibrillar protein synthesis rates increased at 60-120 min after protein ingestion and remained elevated above fasted values (t=-120-0 min) throughout the remaining postprandial period (all, P<0.05; data not shown). In the first 60 minutes after protein ingestion, myofibrillar protein synthesis showed a trend towards an increase above fasted values (absolute change from fasted: 0.025±0.014 %/h^{-1}; P=0.13). The postprandial increase in myofibrillar protein synthesis was greatest at 60-120 min after protein ingestion (0.114±0.04 %/h^{-1}) and remained elevated above fasted values at 120-180 min (0.057±0.020 %/h^{-1}) and 180-300 min (0.045±0.018 %/h^{-1}).

2.5 Discussion

Our repeated blood and muscle biopsy approach allowed us to directly compare the time course of the effect of dietary amino acids on the stimulation of the postprandial muscle protein synthetic response in healthy adults. We show that protein-derived amino acids appear rapidly in circulation immediately after protein ingestion. In particular, 13 ± 2 % (~5 grams) of dietary-protein derived amino acids became available in circulation within the first 60 min of the postprandial period. However, there is a ~60 min delay period after protein ingestion prior to a significant and sustained increase in both anabolic signaling mechanisms and the stimulation of muscle protein synthesis rates throughout a 300 min postprandial period. The anabolic effects of dietary amino acids on skeletal muscle tissue appear to be the most robust between 60-120 min after protein ingestion. Interestingly, protein oxidation was also highest between 60-120 min of the postprandial period and shows the amino acids were also being shifted to other metabolic processes beyond protein synthesis.

Defining the role of insulin and amino acids to regulate muscle anabolic responses to protein nutrition has made considerable progress. For example, it has been shown muscle protein synthesis rates respond in a dose-dependent manner to elevated amino acid availability despite insulin being clamped at postabsorptive concentrations (8). As such, insulin is considered to be permissive rather than modulatory for the stimulation of the postprandial muscle protein synthetic response. Here, we show that plasma insulin concentrations peaked at 30 min after protein ingestion (**Figure 1B**). However, both mTORC1 phosphorylation and muscle protein synthesis rates were not significantly increased until 60-120 min after protein ingestion. These anabolic responses occurred without changes in skeletal muscle LAT1 protein content throughout the postprandial period. In addition to the role of LAT1 in amino acid transport, it is also believed to

play a role in amino acid sensing to activate mTORC1 signaling events (13). Our data suggests that perhaps the total amount of muscle LAT1 protein is not rate limiting for amino acid transport/sensing in response to protein ingestion, but does not discount the potential of nutrient stimuli to augment recruitment of amino acid transporters to the sarcolemma (11).

Importantly, our study allowed us to directly compare the relationship between postprandial dietary protein derived amino acid availability and the subsequent increase in mTORC1 phosphorylation and muscle protein synthesis rates. Our findings are in contrast to the previously demonstrated 'muscle-full' effect (1). Specifically, Atherton et al. (1) showed a brief stimulation of postprandial muscle protein synthesis between 45-90 min after protein ingestion followed by subsequent rapid decline to basal values. These findings were made despite another 90 min of continued elevated postprandial plasma and intramuscular amino acid concentrations and elevated anabolic signaling events. In the present work, we show a sustained increase in the muscle protein synthetic response throughout the late postprandial period. The discrepancy between our data and Atherton et al. (1) may relate to the digestion rate of the ingested protein source. For instance, we fed milk protein (80 % casein/20 % whey) whereas the previous researchers used whey protein (1). Thus, the 'muscle full effect' may only occur when plasma and intramuscular amino acid pools experience an amino acid delivery pattern that is initially very rapid but is not sustained during the later postprandial phase (i.e. after ~ 120-180 min). This effect is common after the ingestion of a rapidly digested source such as whey (3, 15). In contrast, we provided a combination of a fast (i.e. whey) and slow (i.e. casein) protein fractions (3), as contained within the natural food matrix of milk protein, and observed a more sustained release of dietary protein derived amino acids and subsequently maintained postprandial stimulation of muscle protein synthesis (**Figure 9**).

In conclusion, our findings show the rise in dietary amino acid appearance precedes the increase in the phosphorylated-state of mTORC1 and muscle protein synthesis rates. In addition, we show a sustained postprandial stimulation of muscle protein synthesis rates that coincides with sustained plasma amino acid availability and elevated mTORC1 signaling after protein ingestion. For optimal clinical nutrition strategies, our finding provide insight into the design of optimal pulse feeding strategies (i.e., 3-4 meals daily). Specifically, consumption of protein sources that provide a sustained delivery of dietary amino acids will result in the maintenance of the postprandial muscle protein synthetic response throughout a prolonged postprandial period.

Acknowledgements

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Author contributions

The authors' responsibilities were as follows—SvV, LvL and NAB: contributed to the conception and the design of the experiment; all authors: contributed to collection, analysis, and interpretation of data; SvV, LvL and NAB: contributed to drafting or revising intellectual content of the manuscript and had primary responsibility for the final content; all authors: read, edited, and approved the final version of the manuscript. The authors declared no conflicts of interest related to this study.

2.6 Tables

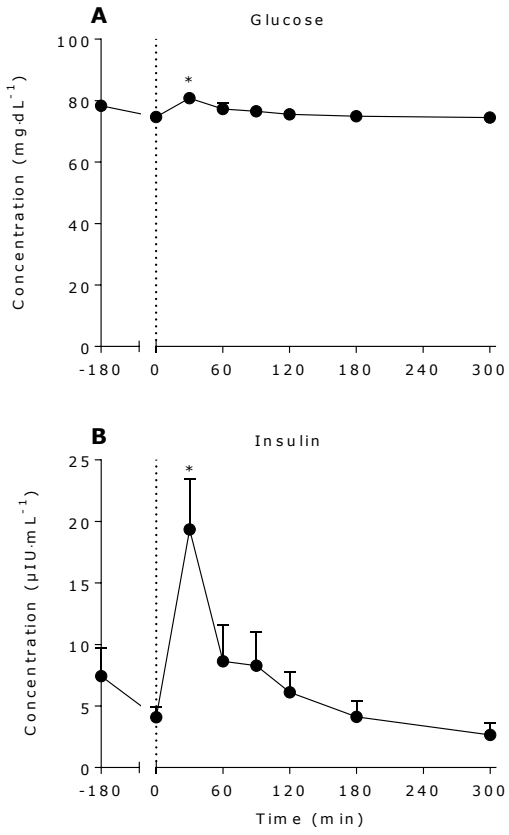
Table 2.1 Participant characteristics (*n*=10)

Variable			
Age (y)	22	±	1.3
Weight (kg)	79.2	±	4.9
BMI (kg/m^2)	24.8	±	1.2
Systolic BP (mmHg)	124.7	±	3.9
Diastolic BP (mmHg)	72.4	±	3.9
Fat (%)	16.6	±	1.4
Lean Body Mass (kg)	64.0	±	3.7
Fasting Glucose (mg/dL^{-1})	78.3	±	1.0
Energy Intake (J/d^{-1})	8740.4	±	423.0
Protein Intake (g/d^{-1})	113.0	±	4.8
Carbohydrate Intake (g/d^{-1})	228.7	±	21.1
Fat Intake (g/d^{-1})	83.0	±	1.17

Data are mean ± SEM.

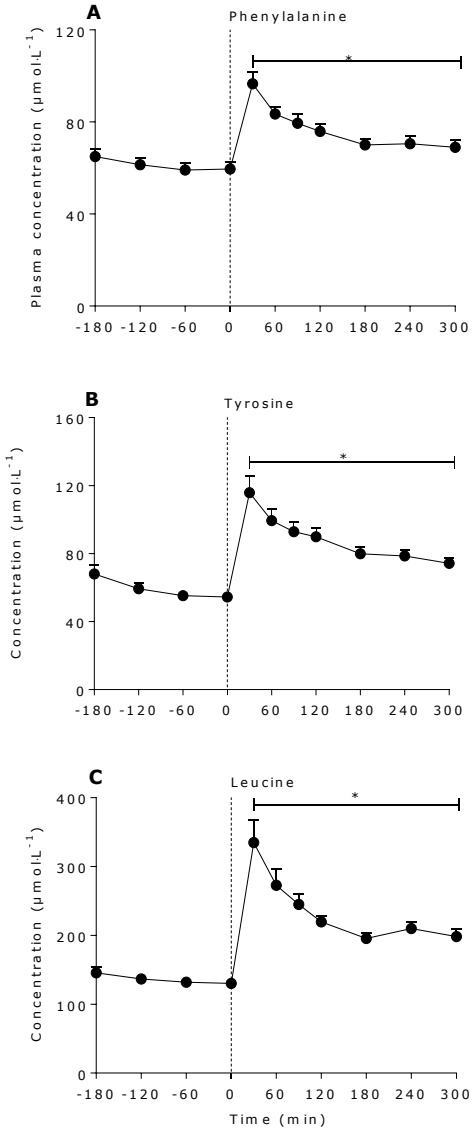
2.7 Figures

Figure 2.1



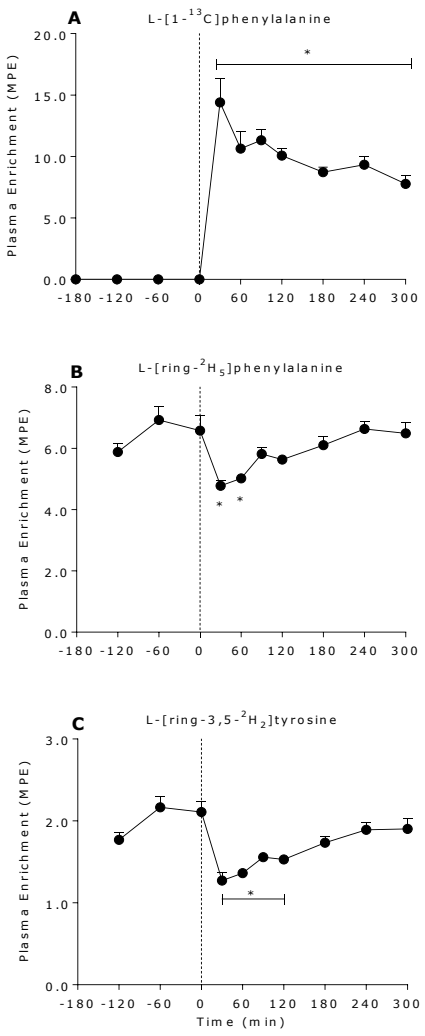
Blood glucose- (A) and plasma insulin (B) concentrations (mg·dL^{-1} and µIU·mL^{-1}, respectively) in the fasted state and after ingestion of milk protein (38 g) in healthy young men (n=7). Dashed line refers to protein ingestion. Data were analyzed with one-way repeated-measures ANOVA (time). A LSD post hoc test was used to locate differences between means when significance was observed. Glucose (A); Insulin (B): All time effect, P<0.01. *Significantly different from fasted (t=0 min) (P<0.05). Data are mean ± SEM.

Figure 2.2



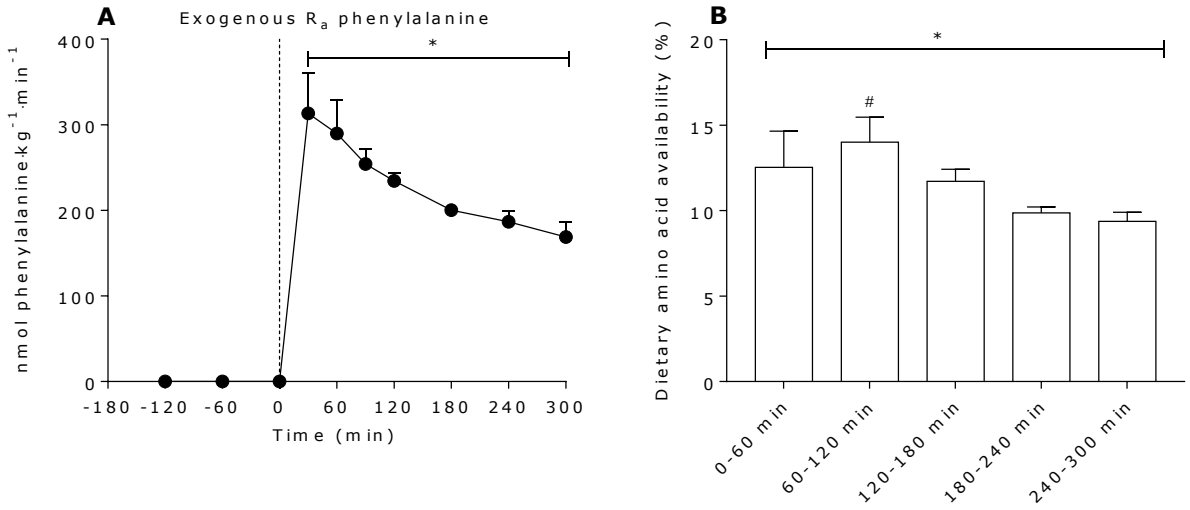
Plasma phenylalanine- (A), tyrosine- (B), and leucine (C) concentrations concentrations (µmol·L^{-1}) in the fasted state and after ingestion of milk protein (38 g) in healthy young men (n=7). Dashed line refers to protein ingestion. Data were analyzed with one-way repeated-measures ANOVA (time). A LSD post hoc test was used to locate differences between means when significance was observed. Phenylalanine (A); Tyrosine (B); Leucine (C): All time effect, P<0.01. *Significantly different from fasted (t=0 min) (P<0.05). Data are mean ± SEM.

Figure 2.3



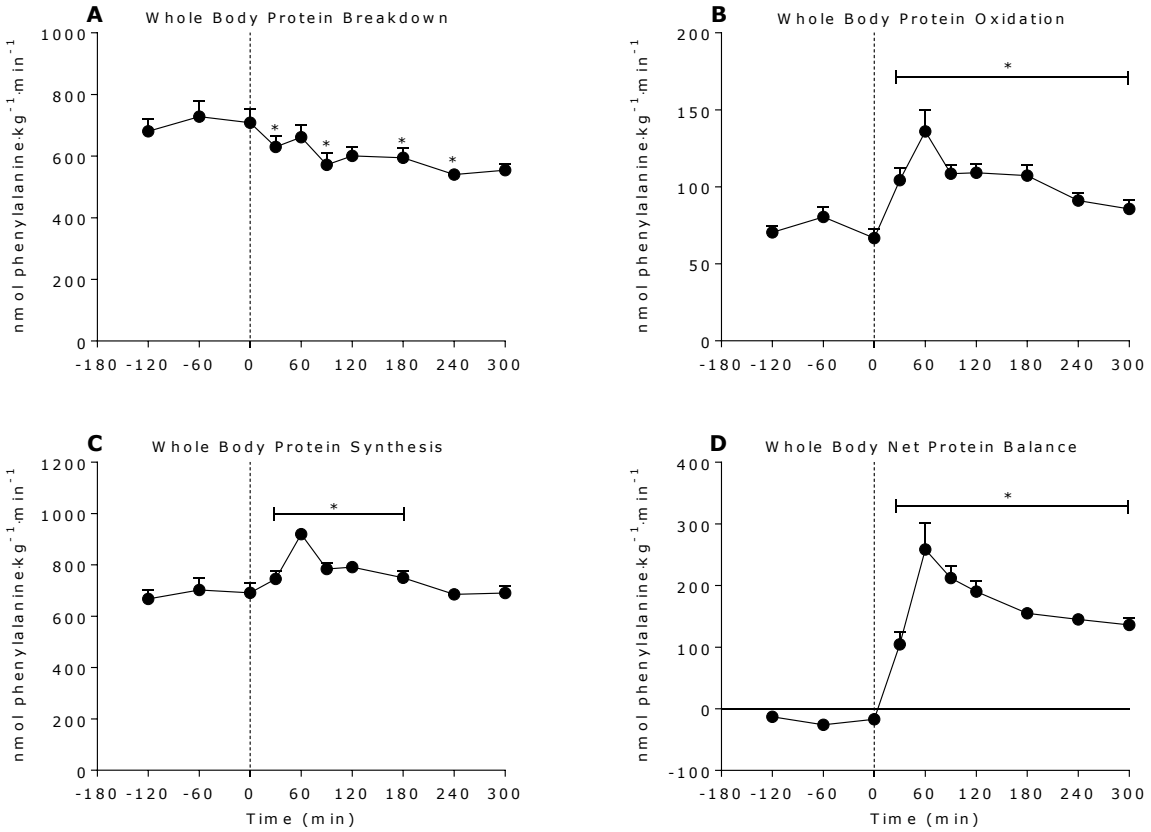
Plasma L-[1-^{13}C]phenylalanine- (A), L-[*ring*-$^{2}H_5$]phenylalanine- (B), and L-[*ring*-3,5-$^{2}H_2$]tyrosine (C) enrichments (MPE) in the fasted state and after ingestion of milk protein (38 g) in healthy young men (n=7). Dashed line refers to protein ingestion. Data were analyzed with one-way repeated-measures ANOVA (time). A LSD post hoc test was used to locate differences between means when significance was observed. L-[1-^{13}C]phenylalanine (A); L-[*ring*-$^{2}H_5$]phenylalanine (B L-[*ring*-3,5-$^{2}H_2$]tyrosine (C): All time effect, P<0.01. *Significantly different from fasted (t=0 min) (P<0.05). Data are mean ± SEM. MPE, mole percent excess.

Figure 2.4



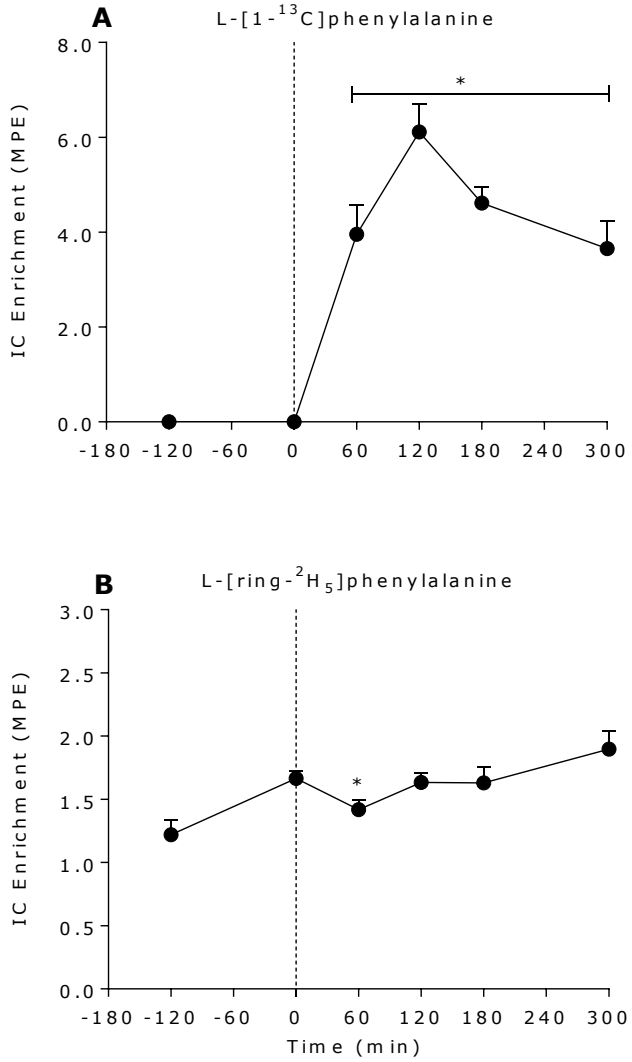
Exogenous phenylalanine R_a (A) (nmol phenylalanine·kg^{-1}·min^{-1}) and dietary phenylalanine availability (%) in the fasted state (not shown for Phe_{plasma}) and after ingestion of milk protein (38 g) in healthy young men (*n*=7). Dashed line refers to protein ingestion. Data were analyzed with one-way repeated-measures ANOVA (time). A LSD post hoc test was used to locate differences between means when significance was observed. Exogenous R_a (A); Total R_a (B); Total R_d (C): All time effect, $P<0.01$. *Significantly different from fasted (t=0 min) ($P<0.05$). $^{\#}$Significantly different from 120-180, 180-240, and 240-300 min. Data are Mean ± SEM. R_a, rate of appearance.

Figure 2.5



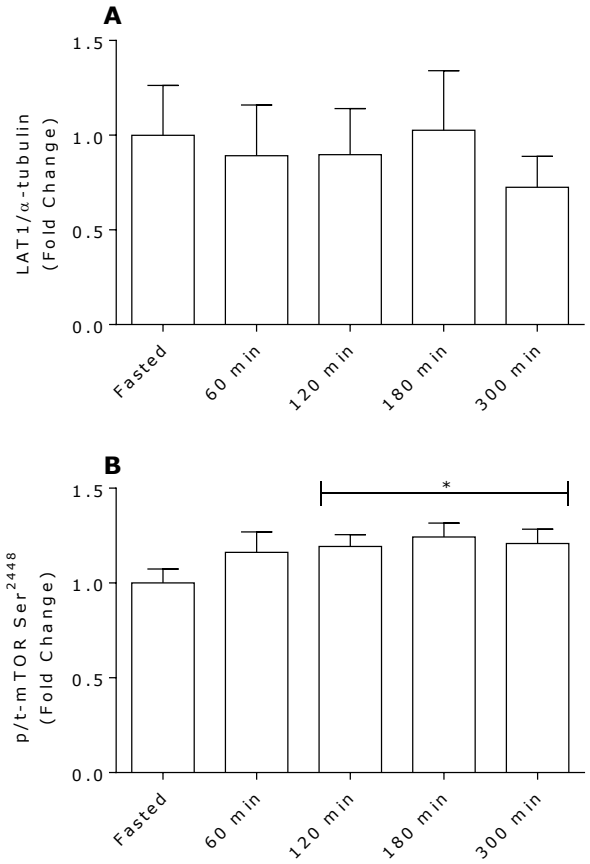
Whole body protein breakdown- (A), oxidation- (B), synthesis- (C) and net balance (D) rates (nmol phenylalanine·kg^{-1}·min^{-1}) in the fasted state and after ingestion of milk protein (38 g) in healthy young men (n=7). Dashed line refers to protein ingestion. Data were analyzed with one-way repeated-measures ANOVA (time). A LSD post hoc test was used to locate differences between means when significance was observed. Whole body protein breakdown- (A), oxidation- (B), synthesis- (C) and net balance (D): All time effect, P<0.01. *Significantly different from fasted (t=0 min) (P<0.05). Data are Mean ± SEM.

Figure 2.6



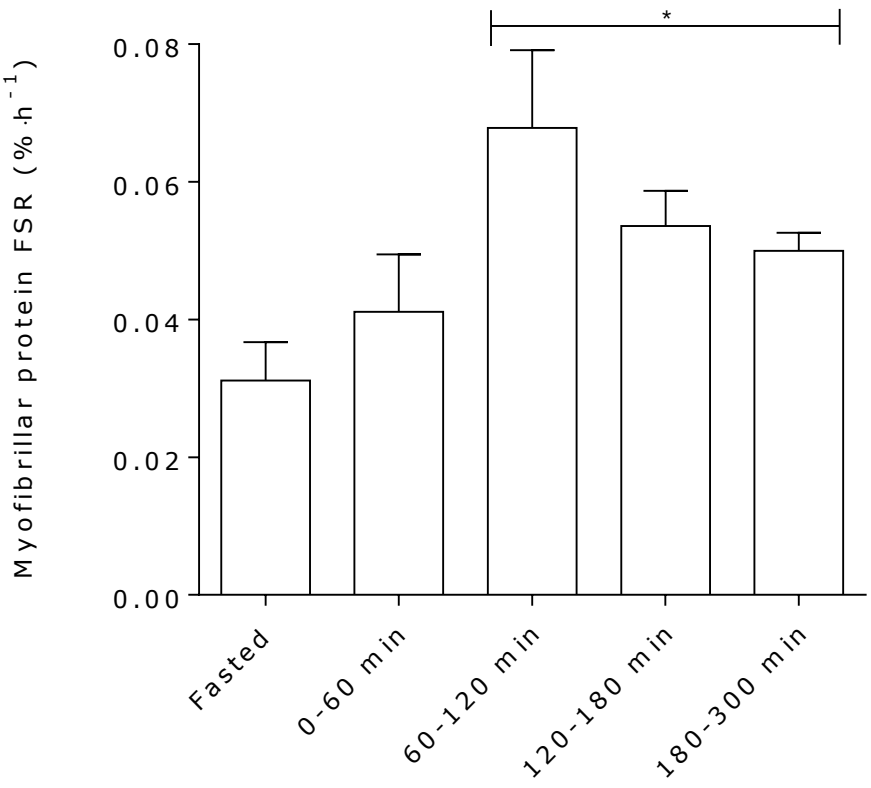
Muscle free L-[1-^{13}C]phenylalanine- (A) and L-[*ring*-$^{2}H_5$]phenylalanine- enrichments (MPE) in the fasted state and after ingestion of milk protein (38 g) in healthy young men (n=7). Dashed line refers to protein ingestion. Data were analyzed with one-way repeated-measures ANOVA (time). A LSD post hoc test was used to locate differences between means when significance was observed. L-[1-^{13}C]phenylalanine (A); L-[*ring*-$^{2}H_5$]phenylalanine: All time effect, P<0.01. *Significantly different from fasted (t=0 min) (P<0.05). Data are mean ± SEM.

Figure 2.7



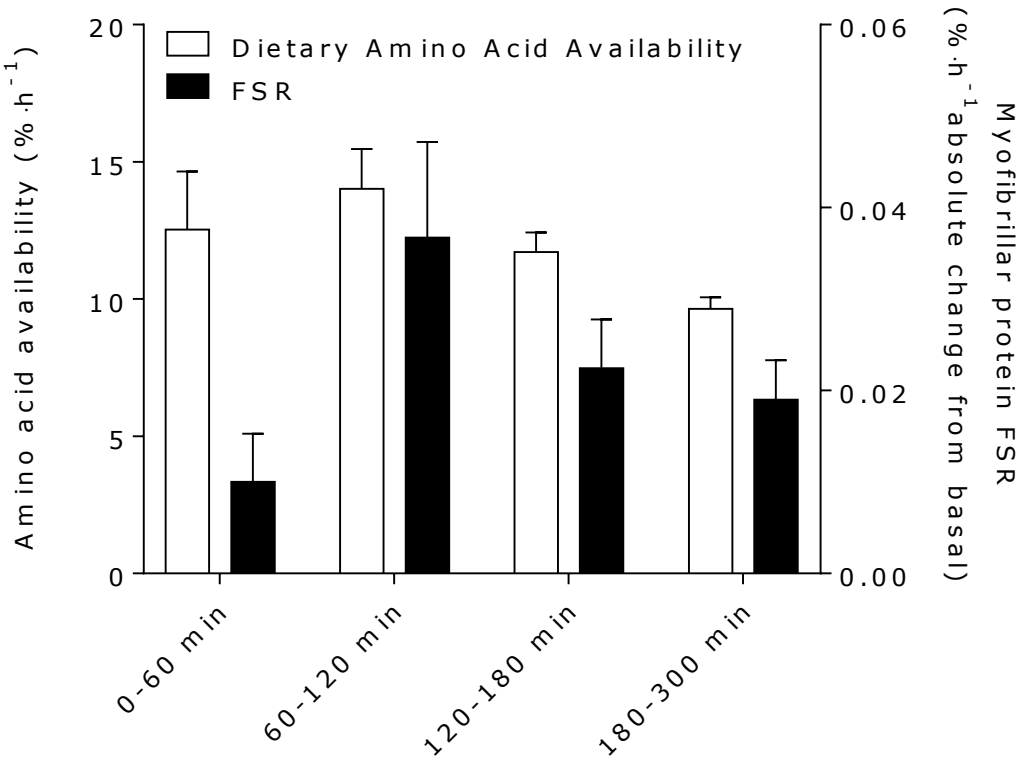
Total protein content of LAT1 (A) and phosphorylated state of mTOR^{Ser2448} (B) in the fasted state (t=0 min) and after ingestion of milk protein (38 g) in healthy young men (n=7). Data were analyzed with one-way repeated-measures ANOVA (time). A LSD post hoc test was used to locate differences between means when significance was observed. LAT1 (A): Time effect, P=0.53. mTOR^{Ser2448} (B): Time effect, P=0.02. *Significantly different from fasted (t=0 min) (P<0.05). Data are mean ± SEM. LAT1, Large neutral amino acid transporter small subunit 1; mTORC1, mammalian target of rapamycin complex 1.

Figure 2.8



Myofibrillar protein synthesis expressed as FSR (%·h^{-1}) in the fasted state and after ingestion of milk protein (38 g) in healthy young men (*n*=7). Data were analyzed with one-way repeated-measures ANOVA (time). A LSD post hoc test was used to locate differences between means when significance was observed. Time effect, *P*=0.02. Data are mean ± SEM. FSR, fractional synthesis rates.

Figure 2.9



Time-dependent comparison between responses of myofibrillar protein FSR (%·h^{-1} absolute change from basal) and dietary phenylalanine availability (%) after ingestion of milk protein (38 g) in healthy young men (*n*=7). Data are mean ± SEM. FSR, fractional synthesis rates.

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CHAPTER 3: DEVELOPMENT OF INTRINSICALLY-LABELED EGGS AND POULTRY MEAT FOR USE IN HUMAN METABOLIC RESEARCH[1]

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Keywords: muscle mass, muscle protein synthesis, nutrition, protein digestion, stable isotopes

Abbreviations used: AA, Amino Acid; GC/MS, Gas Chromatography Mass Spectrometry; HCL, Hydrochloric Acid; IACUC, Institutional Animal Care and Use Committee; IV, Intravenous; LC/MS/MS, Liquid Chromatography Tandem Mass Spectrometry; NaOH, Sodium Hydroxide; MPE, Mole Percent Excess; MPS, Muscle Protein Synthesis; NRC, National Research Council; PCA, Perchloric Acid; R_a, rate of appearance.

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3.1 Abstract

Background: Stable isotope amino acids are regularly used as tracers to examine whole body and muscle protein metabolism in humans. To accurately assess *in vivo* dietary protein digestion and absorption kinetics, it is required that the amino acid tracer is incorporated within the dietary protein food source (i.e., intrinsically-labeled protein). **Objective:** We assessed the practicality of producing intrinsically L-[5,5,5-$^{2}H_3$]leucine-labeled eggs and poultry meat through non-invasive oral tracer administration. **Methods:** A specifically formulated diet (containing 0.52% leucine) was supplemented with 0.3% L-[5,5,5-$^{2}H_3$]leucine and subsequently fed to 3 laying hens (Lohmann LSL Whites) for 55 d. On d 55, the hens were slaughtered and their meat, bones, and organs were harvested to determine tissue labeling. In experiment 1, two healthy young men (age: 22 ± 1.5 y; BMI: 23.7 ± 0.5 kg/m^2) ingested 18 g of L-[5,5,5-$^{2}H_3$]leucine-labeled egg protein. In experiment 2, two healthy young men (age: 20.0±0.0 y; BMI: 26.4±3.1 kg/m^2) ingested 28 g of L-[5,5,5-$^{2}H_3$]leucine-labeled poultry meat protein. Plasma samples (experiment 1 and 2) and muscle biopsies (experiment 1) were collected before and after labeled-food ingestion. **Results:** High tracer labeling (>20 mole percent excess, MPE) in the eggs was obtained after 7 d and maintained throughout the feeding protocol (P<0.05). Over a 55 d period, ~850 g of egg protein (145 eggs) was produced with a mean tracer enrichment of 22.0±0.8 MPE. Mean L-[5,5,5-$^{2}H_3$]leucine enrichment in the meat was 9.6±0.1 MPE. In experiment 1 and 2, the consumption of labeled eggs and poultry meat protein increased plasma L-[5,5,5-$^{2}H_3$]leucine enrichment with peak values of 6.7±0.1 and 4.0±0.9 MPE, respectively. The 5 h postprandial increase in myofibrillar L-[5,5,5-$^{2}H_3$]leucine enrichment after egg ingestion in healthy young men was 0.051±0.008 MPE (experiment 1). **Conclusion:** We have demonstrated the feasibility of producing intrinsically-labeled eggs and poultry meat for use in human metabolic research.

3.2 Introduction

Stable isotope labeled amino acids are regularly used as tracers to examine whole body and muscle protein metabolism in humans. The whole body and muscle protein synthetic response to food ingestion has been shown to be modulated by the pattern of postprandial (essential) amino acid availability (5, 10, 19, 28), which is mainly determined by the digestion and absorption kinetics of the ingested food source (9, 19). Thus, researchers have sought to develop novel approaches to assess dietary protein digestion and absorption kinetics and, more importantly from a biological regulatory standpoint, the subsequent dietary protein-derived plasma amino acid availability.

Intrinsic labeling of a dietary protein source with a stable isotope tracer is a novel approach used in human metabolic research to circumvent the differential amino acid intestinal absorption patterns between free amino acid tracer versus amino acids derived from the ingested food source (7). Thus, intrinsically labeled food proteins provide a tool to reliably model postprandial whole body protein metabolism *in vivo* in humans. In addition to whole body applications, the use of highly labeled food proteins (>30 mole percent excess; MPE) have allowed researchers to directly 'trace' the use of dietary protein-derived amino acids for postprandial muscle protein accretion (29).

To date, researchers have primarily focused on demonstrating the effectiveness of manufacturing intrinsically-labeled cow derived food products (i.e. milk and beef) by prolonged intravenous (I.V.) infusions of stable isotope tracer into lactating dairy cows (6, 25, 26). The purpose of this study was to extend on previous efforts in cows (6, 26, 29) and chickens (11, 13, 21) to demonstrate the feasibility of producing nutritionally complete and intrinsically labeled dietary proteins (i.e. egg and poultry meat) at a modest production price point by the use of a non-invasive oral tracer administration method in chickens. In addition, we wished to apply these

labeled foods in humans as a proof-of-principle to assess *in vivo* dietary protein digestion and absorption kinetics and subsequent postprandial muscle protein accretion. Our rationale behind this approach was: *1)* chickens are naturally small animals, which makes their management and resource cost ideal for the researcher. We hypothesized that ~ $1/10^{th}$ of the amount of tracer (45g) would be required to produce labeled proteins sufficiently enriched to 'trace' the dietary protein-derived into human skeletal muscle tissue after its ingestion when compared to cow experiments (400 g) (29); *2)* previous research applications with intrinsically labeled eggs have not assessed their suitability to model postprandial whole body and muscle protein synthesis in humans (11, 13, 21) and; *3*) egg protein is the current World Health Organization/Food and Agricultural Organization gold standard protein source, which will provide invaluable nutritional insight into the metabolism of this high quality protein (32). Therefore, egg protein is ideally suited to apply in human intervention trials with the endpoint of developing optimal guidelines for the retention and/or enhancement of protein mass and/or muscle health in various populations.

3.3 Methods

Development of intrinsically labeled eggs and poultry meat

Tracer administration

The diet of 3 egg laying hens (Lohmann LSL Whites, age: 29 wks; weight: 1.7 kg) was supplemented with 0.3% L-[5,5,5-$^{2}H_3$]leucine for 55 d with assistance from the Poultry Research Farm, Animal Science Laboratory, University of Illinois at Urbana-Champaign. Specifically, 45 g L-[5,5,5-$^{2}H_3$]leucine was mixed with 14,955 g of a specially formulated diet. In contrast to previous approaches that provided tracer on top of a normal mixed diet (13, 21), we used the approach by Evenepoel et al. (11) and formulated a diet to be deficient in (unlabeled) leucine in

order to minimize tracer oxidation and irreversible loss and to maximize recovery into protein (**Supplemental Table 1**). The diet was calculated to contain 0.52% leucine from the intact protein in the feed ingredients; the National Research Council (NRC) requirement for leucine is 0.82% (1). Thereafter, the diet was supplemented with 0.3% of L-[5,5,5-$^{2}H_3$]leucine to meet the requirement for leucine. All other amino acids were present at levels that were equal to or greater than the NRC requirements (1).

The hens were adapted to the modified diet for 7 d prior to the administration of the tracer feeding protocol to confirm proper consumption of the diet. The diet fed on those 7 d mirrored the diet fed during the 55 d feeding trial with the exception that the diet was supplemented with unlabeled free leucine and not tracer. The average daily food intake of the hens over the 55 d period was ~86 $g \cdot d^{-1}$. Residual feed was recycled and fed on the subsequent days. The experiment and animal handling procedures were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Illinois.

Egg and organ collection

Eggs were collected daily and stored at 4°C. Subsequently, the eggs were processed under sterile conditions and an aliquot of both the white and yolk of each egg was collected for future analysis of L-[5,5,5-$^{2}H_3$]leucine enrichment by GC/MS (see below for details) prior to storage at -20°C. On d 55 of the feeding trial, the hens were killed with CO_2. Immediately afterwards, the tissues were harvested and processed by an abattoir (Meat Science Laboratory, University of Illinois at Urbana-Champaign). Meat cuts (corresponding to the breast, drumstick, tenderloin, thigh, and wing), organs (the brain, crop, gizzard, heart, kidney, large intestine, liver, lungs, ovary, oviduct, small intestine and spleen), and bones (the femur, ilium, tibia, radius, and the femur's marrow)

were individually packaged, labeled, and stored at -20°C until further analysis of L-[5,5,5-$^{2}H_3$]leucine enrichment by GC/MS (see below for details).

Egg protein and lipid analysis

One egg, representative of the average weight of the eggs (i.e. ~52 g) collected during the 55 d feeding trial, was used for proximate analysis as described previously (23). In brief, the yolk and white were separated and homogenized in a food processor. Samples were dried at 110°C for 24 h and extracted in an azeotropic mixture of warm chloroform and methanol. Protein concentrations were determined by measuring nitrogen content using the combustion method (method 990.03; AOAC International, 2000; TruMac; LECO Corp., St. Joseph, MI). Moisture, protein, and extractible lipid analyses were performed in duplicate.

Egg and tissue enrichment analysis

The egg, meat and bone protein bound L-[5,5,5-$^{2}H_3$]leucine enrichments were determined by GC/MS analysis (Agilent 7890A GC/5975C; MSD, Palo Alto, CA, USA). L-[5,5,5-$^{2}H_3$]leucine enrichment of the samples was determined by selective ion monitoring for leucine mass-to-charge (*m/z*) ratio at 302 (*m*+0) and 305 (*m*+3). For complete analysis details see **Supplemental Methods**.

Human intervention trials

All participants were informed about the experimental procedures to be used, the purpose of the study, and all potential risks before giving written consent. Both experiments conformed to all standards for the use of human subjects in research as outlined in the Helsinki Declaration and

were approved by the local Institutional Review Board at the University of Illinois at Urbana-Champaign.

Experimental protocol

In **experiment 1**, 2 healthy young men (age: 22 ± 1.5 y; weight: 70.73 ± 4.04 kg; body mass index: 23.7 ± 0.5 kg/m^2) were studied during primed continuous intravenous infusion of L-[1-^{13}C]leucine to assess postprandial handling after the ingestion of 18 g of intrinsically L-[5,5,5-$^2H_3$]leucine labeled eggs. In **experiment 2**, 2 healthy young men (age: 20.0 ± 0.0 y; weight: 94.6 ± 12.4 kg; body mass index: 26.4 ± 3.1 kg/m^2) were studied to assess the feasibility of measuring postprandial release of dietary protein derived L-[5,5,5-$^2H_3$]leucine into circulation after the ingestion of 28 g intrinsically L-[5,5,5-$^2H_3$]leucine labeled protein contained in poultry meat. Arterialized blood samples were collected at *t*=-180, -120, -60 min (**experiment 1**), and -0 min (**experiment 2**) of the postabsorptive state and at *t*=30, 60, 90, 120, 180, 240 and 300 min (**experiment 1 and 2**) of the postprandial phase. Biopsies of the *vastus lateralis* were collected before (*t*=0 min) and after (*t*=300 min) the ingestion of 18 g of intrinsically L-[5,5,5-$^2H_3$]leucine labeled egg protein to assess the use of dietary protein derived amino acids for postprandial muscle protein accretion (**experiment 1**). No muscle biopsies were collected in **experiment 2**.

Preparation of intrinsically labeled protein and meal composition

In **experiment 1**, the intrinsically-labeled L-[5,5,5-$^2H_3$]leucine eggs were prepared by boiling the eggs for 12 min prior to peeling. Subsequently, the whole eggs were freeze-dried and finely ground using a hand mortar and pestle. The eggs were mixed to achieve a homogeneous mixture and stored at -20°C. Participants received a beverage containing 18 g protein, 17 g fat, and 60 g carbohydrate

(50% sucrose: 50% maltodextrin blend) in a total volume of 400 mL water. The beverages were uniformly flavored by adding 1 mL of vanilla extract. The L-[5,5,5-$^{2}H_3$]leucine enrichment of these eggs fed to the participants was 21.2 MPE. In **experiment 2**, the intrinsically labeled L-[5,5,5-$^{2}H_3$]leucine poultry meat was deboned and subsequently ground using a commercial meat grinder and stored at -20°C. The L-[5,5,5-$^{2}H_3$]leucine enrichment of the meat was 9.6 MPE. The ground poultry meat was thawed overnight in a refrigerator at 4° C. On the morning of the experiment, the 161 g ground chicken patty was prepared in a non-stick skillet until the inner temperature reached 65°C. Both participants ingested the meal within 10 min and were provided with 400 mL of water. Salt and pepper were added ad libitum. The protein content of the meal was 28 g protein and 13 g fat. All isotopic enrichments of the intrinsically labeled food sources were determined by GC/MS analysis.

Plasma analyses and muscle analyses

Plasma amino acid concentrations and enrichments were determined by GC/MS analysis (Agilent 7890A GC/5975C; MSD, Palo Alto, CA, USA). Briefly, plasma leucine enrichments were determined by ion monitoring at *m/z* 302 and 303 for unlabeled and labeled [1-^{13}C]leucine, respectively; and *m/z* 302 and 305 for unlabeled and labeled L-[5,5,5-$^{2}H_3$]leucine, respectively. Plasma amino acid concentrations were determined by integrating amino acid peak areas in comparison to an internal standard (DL-p-chlorophenylalanine) using the AMDIS software package (v. 2.71, NIST^{TM}) (17). Myofibrillar protein-enriched fractions were extracted from ~50 mg of wet muscle. Myofibrillar protein enrichments were determined by 5500 QTRAP LC/MS/MS (Sciex, Framingham, MA). The L-[5,5,5-$^{2}H_3$]leucine enrichments of the myofibrillar protein bound samples were determined by multiple reaction monitoring (MRM) at *m/z* 132.0 → 86.0 and

135.0 → 89.0 for unlabeled and labeled L-[5,5,5-$^{2}H_3$]leucine, respectively. Software Analyst 1.6.2 was used for data acquisition and analysis. For complete analysis details see **Supplemental Methods**.

Calculations

Whole-body amino acid kinetics were assessed in non-steady conditions by the ingestion of L-[5,5,5-$^{2}H_3$]leucine eggs combined with IV infusion of L-[1-^{13}C]leucine. Total, exogenous, and endogenous rate of leucine appearance (R_a), and postprandial protein-derived leucine availability in the circulation (Leu_{plasma}) were calculated using modified Steele's equations (7, 10) (see **Supplemental Methods**).

Statistics

Differences in tracer enrichment between the egg yolk and white were tested by a two-factor (time × treatment) analysis of variance (ANOVA) with repeated measures on the time factor. Differences between different meat cuts, organs, and bone tissue enrichments and concentrations were analyzed using a one-factor (condition) ANOVA. Time dependent differences in leucine Total R_a, Exo R_a, and Endo R_a as well as plasma enrichment were analyzed using a one-way repeated measures (time) ANOVA. When statistically significant interaction effects were observed, Bonferroni post hoc tests were performed to locate these differences. Statistical significance was set at $P<0.05$. Linear regression lines were fitted to the plasma L-[1-^{13}C]leucine enrichments to assess the existence of any deviation in enrichment indicated by lines with a significant positive or negative slope (GraphPad Prism, version 6, La Jolla, CA). All calculations were performed using IBM SPSS Statistics (version 23, Chicago, IL, USA) unless otherwise designated. All data are expressed as mean ± SEMs.

3.4 Results

Egg and poultry meat yield

The hen-day egg production performance of the 3 hens was ~88 % over the 55 d feeding trial. As such, a total of 145 labeled eggs (850 g of total protein) were produced over the 55 d protocol. A total of 322 g of poultry meat (absent of bone) was collected from the three hens resulting in 56 g of total meat protein.

Egg and poultry organ tissue enrichments

Consumption of the 0.3% L-[5,5,5-$^2H_3$]leucine enriched diet increased the L-[5,5,5-$^2H_3$]leucine enrichment in both the egg yolk and white during the 55 d protocol (**Figure 1**; $P<0.001$). The L-[5,5,5-$^2H_3$]leucine enrichments did not differ ($P=0.30$) between the egg yolk (22.8±1.0 MPE) and white (21.3±0.9 MPE). The rate of increase in L-[5,5,5-$^2H_3$]leucine enrichment over time did not differ between the egg yolk or white ($P=0.42$). Mean enrichment of the whole eggs was 22.0±0.9 MPE. Based on the protein yield of the eggs (~850 g) and a measured leucine content of 8.9%, it was calculated 17 g of the 45 g of L-[5,5,5-$^2H_3$]leucine that was included in the diet was recovered in the eggs. This resulted in a tracer recovery of ~37 %. The L-[5,5,5-$^2H_3$]leucine enrichment of the various cuts of poultry meat are shown in **Figure 2**. The L-[5,5,5-$^2H_3$]leucine enrichment of the collected poultry meat was 9.6 ± 0.1 MPE. Based on the protein yield of the meat (56 g) and an assumed leucine content of 7.8 % (2), the recovery of L-[5,5,5-$^2H_3$]leucine in the meat was calculated as ~1 %. The L-[5,5,5-$^2H_3$]leucine enrichment did not differ between the various meat cuts ($P=0.57$). However, as shown in **Figure 3**, the L-[5,5,5-$^2H_3$]leucine enrichments were different between the various collected organs. Specifically, the ovary was significantly different from the brain, large intestine, and gizzard ($p<0.05$); the oviduct was significantly different from

brain, and gizzard ($p<0.05$); the kidney was significantly different from gizzard ($p<0.05$); and the liver was significantly different from gizzard ($p<0.05$). The L-[5,5,5-$^{2}H_3$]leucine enrichments were different between the various collected bones with the highest enrichment observed in the femur's marrow ($P<0.001$).

Human plasma and muscle analysis

Experiment 1: Plasma leucine concentrations increased after egg ingestion ($P<0.05$) (data not shown). Linear regression analysis indicated that the slopes of the plasma L-[1-^{13}C]leucine enrichment over time curve were not different from zero ($P=0.88$) (data not shown). Plasma L-[5,5,5-$^{2}H_3$]leucine enrichments trended ($P=0.08$) towards a gradual increase after egg ingestion with values peaking at 90 min (6.7±0.1 MPE) and thereafter enrichments declined during the late postprandial period (**Figure 4A**). Exogenous (dietary) leucine R_a increased rapidly after intrinsically-labeled egg ingestion with peak values obtained at 90 min and declined during the remaining postprandial phase (**Figure 5**). The total amount of egg protein-derived leucine that became available in systemic circulation after first-pass splanchnic extraction during the 5 h postprandial period was 71±4%. The endogenous leucine R_a in plasma decreased during the postprandial phase (data not shown). The incremental change in myofibrillar protein-bound L-[5,5,5-$^{2}H_3$]leucine enrichment during the 0-300 min postprandial period after egg ingestion was 0.051±0.008 MPE. **Experiment 2:** Plasma leucine concentrations increased after meat ingestion ($P<0.05$) (data not shown). Plasma L-[5,5,5-$^{2}H_3$]leucine enrichment gradually increased after the consumption of meat, however the data did not reach statistical significance ($P=0.38$). Peak enrichment values were obtained at 120 min (4.0±0.9 MPE) after which values declined during the late postprandial phase **(Figure 4B).**

3.5 Discussion

Our work shows that intrinsically L-[5,5,5-$^{2}H_3$]leucine labeled eggs and meat can be produced by adding L-[5,5,5-$^{2}H_3$]leucine to the feed of laying hens. Moreover, egg proteins with L-[5,5,5-$^{2}H_3$]leucine enrichments of >20 MPE were produced and allowed for the assessment of postprandial release of dietary protein derived leucine and subsequent postprandial muscle protein accretion from the ingested food source

It is important to note that the rate of tracer recovery in the eggs was calculated to be ~37%, which is a 0.5 fold greater tracer recovery than previously reported by workers producing intrinsically labeled milk proteins using either L-[1-^{13}C]phenylalanine (25, 29) or L-[1-^{13}C]leucine (6). We suspect that the use of leucine, which is relatively more abundant in egg (2) and tissue protein mass compared to phenylalanine (3), could have increased the incorporation of the tracer into the *in vivo* proteins. Moreover, our calculated tracer recovery of ~37% was similar to others who provided a diet low in unlabeled leucine to egg laying hens (i.e. ~40%) (11), but is greater than those who provided free leucine tracer on top of the normal diet (i.e. ~20%) (21). Since excess dietary amino acids are preferentially oxidized (15), we suspect substituting the tracer for unlabeled leucine resulted in decreased rates of oxidation and irreversible loss of tracer. In addition, oral consumption of tracer (as opposed to IV administration) presumably allowed us to increase recovery of L-[5,5,5-$^{2}H_3$]leucine into the eggs. Specifically, oral consumption of tracer results in first pass uptake of tracer by hepatic tissue when compared to I.V. tracer administration. Since certain components of the egg are partly produced in the liver prior to transport to the ovary (4), this could have further contributed to the high(er) recovery of tracer in the egg protein compared to milk proteins (10, 12, 23). Importantly, the modified diet did not impair egg production performance (90%) or the mean weight of the eggs (~52 g), which is considered a

typical egg weight for the Lohmann LSL White strain (27). Therefore, meeting the dietary amino acid requirements of laying hens by adding tracer to the diet represents an efficient means to maximize tracer recovery into protein and thus allowed for the production of highly enriched and nutritionally complete dietary egg proteins.

In this study, we only recovered ~1% of tracer in the meat, which is lower than previous reports for the production of labeled beef via IV administration of tracer (i.e. ~6% recovery rate) (25). Indeed, the researchers used larger quantities and IV administration of tracer in the cows, which collectively would have allowed for greater relative amounts of tracer to be delivered to and subsequently taken up by skeletal muscle tissue given the ability to circumvent first pass uptake of the tracer by splanchnic tissue. In addition, the Lohmann LSL White strain are not birds bred for their meat, and thus would generally have a lower overall skeletal muscle mass and presumably lower rates of protein turnover compared to other chicken strains (Cornish and White Rock cross broilers) (18). Nevertheless, we still observed a muscle tissue enrichment of 9.6±0.1 MPE, which would be suitable for use in human metabolic research.

As shown in **Figure 3**, there were differences in L-[5,5,5-$^{2}H_3$]leucine enrichment between the sampled organs of the laying hens. The tracer enrichments in the ovary and oviduct were amongst the highest of the sampled organs with L-[5,5,5-$^{2}H_3$]leucine enrichments of 24.0±0.2 and 24.0±0.5 MPE, respectively. This is not surprising considering that the egg is fully matured in these tissues (4). L-[5,5,5-$^{2}H_3$]leucine enrichment was also found to be particularly high in the kidneys. This is representative of increased renal metabolic activity during the egg laying cycle as a mechanism to meet the increased calcium demand of egg-shell formation (12). In **Table 1,** we showed enrichment of L-[5,5,5-$^{2}H_3$]leucine in the bones of the laying hens. Although bone turnover is elevated during the laying cycle of the hens to meet the calcium demand of the egg shell (22), it is

well documented that avian bone turns over at a slower rate when compared to skeletal muscle tissue and the various cardiovascular (heart and lungs), digestive (intestines, liver and pancreas), excretory (liver and kidney) and reproductive (ovary and oviduct) organs (16). Caging of the hens likely further contributed to the low L-[5,5,5-$^{2}H_3$]leucine enrichment in the bones as limiting the hen's activity would result in reduced loading of the bone (31) and consequently a reduced stimulus for bone remodeling. Not surprisingly, we observed high L-[5,5,5-$^{2}H_3$]leucine enrichment in the marrow of the femur (22.6±0.7 MPE) **(Table 1)**. This is indicative of its hemopoietic activity, which involves the rapid synthesis of various blood proteins (20).

In human proof-of-principle **experiment 1**, we showed that the ingestion of intrinsically L-[5,5,5-$^{2}H_3$]leucine labeled eggs (21.2 MPE) resulted in rapid increase in postprandial L-[5,5,5-$^{2}H_3$]leucine enrichment in the plasma (**Figure 4A**). Combining intrinsically labeled egg ingestion with primed continuous IV infusion of L-[1-^{13}C]leucine allowed us to determine the postprandial release of dietary protein derived leucine in the circulation, which provides insight into the dietary protein digestion and absorption kinetics of the ingested food source. The percentage of the total amount of egg protein-derived leucine that makes it past first-pass splanchnic extraction and appeared in circulation throughout the 0-5 h postprandial period was 71±4%. This amount is similar to ingestion of other high quality protein sources such as whey and casein (10). In addition, we demonstrated that the ingestion of highly labeled eggs (21.2 MPE) would allow for the assessment of postprandial muscle protein accretion from the ingested food source independent of IV tracer infusions. Previously, this approach has only been performed with highly labeled (>30 MPE) milk-derived proteins (8, 14, 19, 24, 29, 30). Such an approach would be advantageous in populations or settings where continuous IV infusions may not be easily applied (e.g. elderly, clinical populations, nutrition field studies).

In **experiment 2**, we showed that the ingestion of intrinsically L-[5,5,5-$^{2}H_3$]leucine labeled meat (9.6 MPE) resulted in an increase in postprandial L-[5,5,5-$^{2}H_3$]leucine enrichment in the plasma (**Figure 4B**). No attempt was made to 'trace' meat protein-derived L-[5,5,5-$^{2}H_3$]leucine into skeletal muscle as the level of enrichment in the meat (9.6 MPE) would presumably be too low to enable its precise detection into skeletal muscle proteins. From a practical perspective, we have demonstrated the possibility producing labeled poultry meat to be used in human nutritional research to assess whole body protein kinetics. However, the meat protein yield per chicken is low (~19 g protein) and, as such, requires larger scale protocol consisting of multiple chickens to produce a larger meat protein yield for use in a larger scale human trial.

In conclusion, we demonstrated the feasibility of producing intrinsically labeled eggs and meat for use in human metabolic research. A potential strong point of intrinsically labeled egg production is the relatively high tracer recovery rate, which allows for the production of highly enriched food proteins that can be produced at reasonable price point. This metabolic tracer "tool" of a gold standard reference protein (i.e. egg) could be applied to a variety of populations to advance our knowledge of the optimal nutritional factors that enhance whole body and muscle protein metabolism in humans.

3.6 Tables

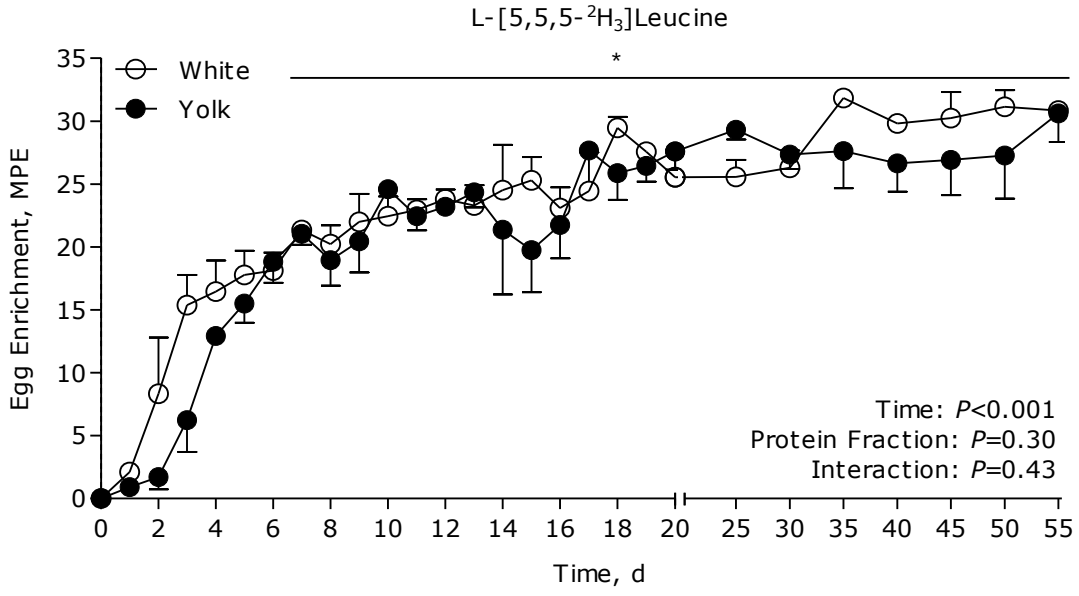
Table 3.1 Bone L-[5,5,5-$^{2}H_3$]leucine enrichment of Lohmann LSL White laying hens[1]

Bone	Enrichment (MPE)
Femur	3.0±0.6[a]
Femur Marrow	22.6±0.7[b]
Ilium	2.3±0.2[a]
Tibia	2.9±0.7[a]
Radius	2.9±0.7[a]

[1]Values are means ± SEMs, *n*=3. Labeled means in a column without a common letter differ (P<0.001). MPE; mole percent excess.

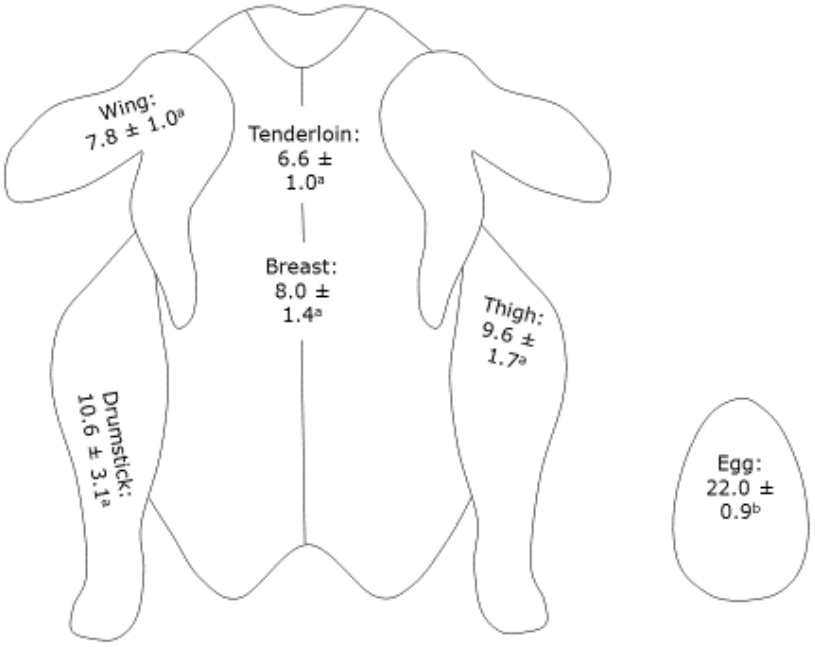
3.7 Figures

Figure 3.1



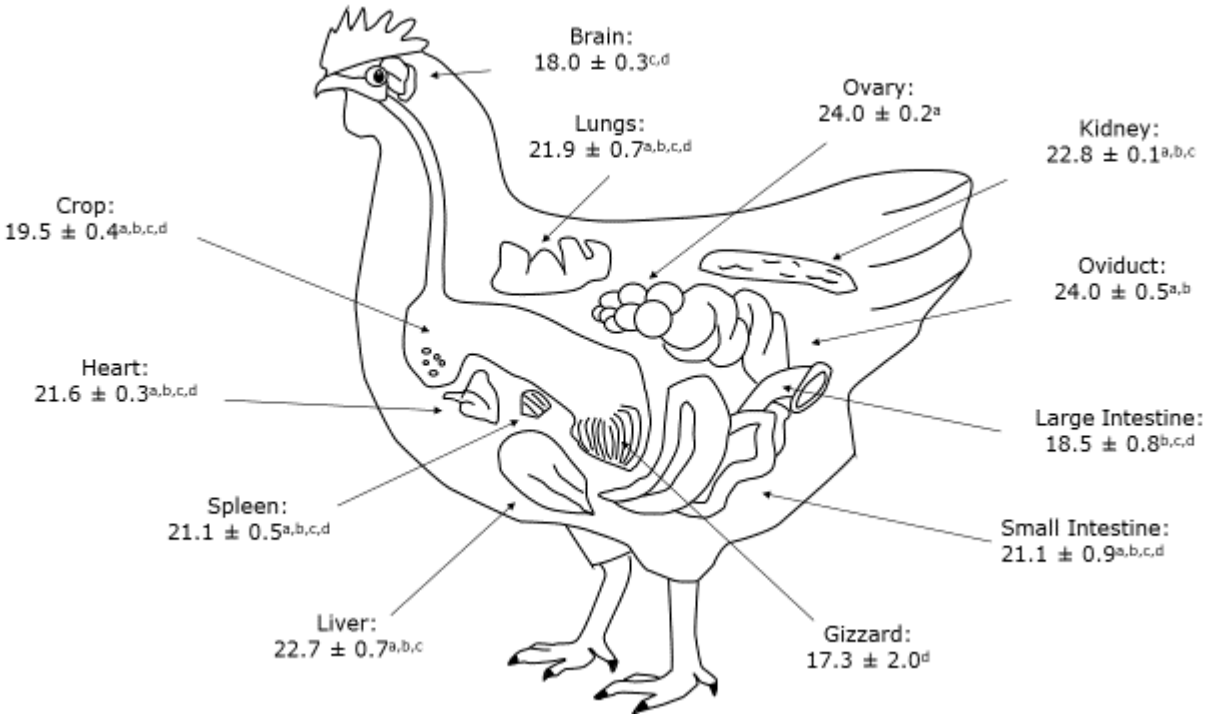
L-[5,5,5-$^2H_3$]leucine incorporation into the yolk and white protein fraction of laying hens (Lohmann LSL Whites) consuming a diet supplemented with 0.3% L-[5,5,5-$^2H_3$]leucine for 55 d. Values are means ± SEMs, *n*=3. *Different from d 0 (*P*<0.05). MPE, mole percent excess.

Figure 3.2



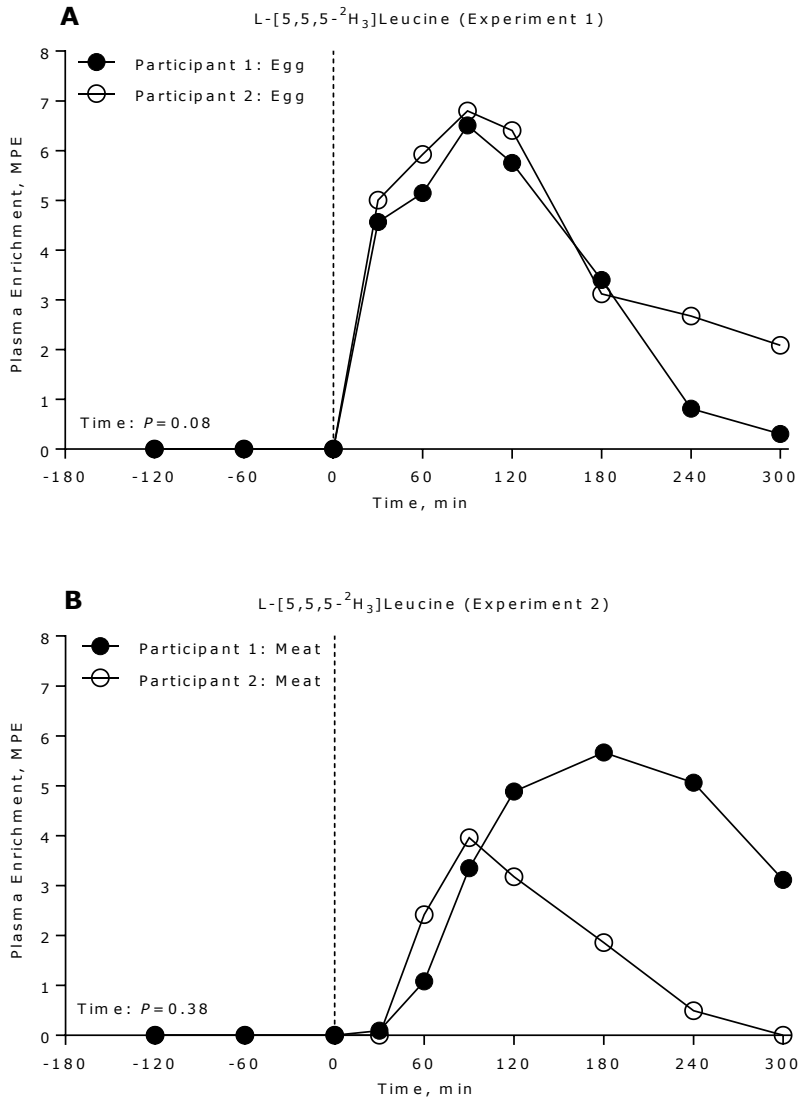
L-[5,5,5-$^{2}H_3$]leucine enrichment of the various meat cuts and whole egg enrichment of laying hens(Lohmann LSL Whites) consuming a diet supplemented with 0.3% L-[5,5,5-$^{2}H_3$]leucine for 55 d. Values are means ± SEMs, *n*=3. Labeled means without a common letter differ significantly (*P*<0.01). MPE, mole percent excess.

Figure 3.3



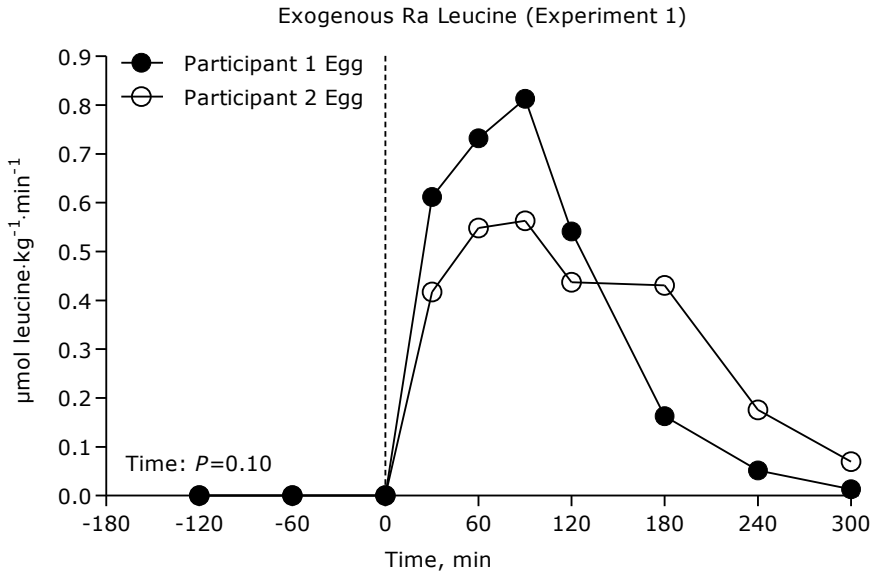
L-[5,5,5-$^{2}H_3$]leucine enrichment of various organ tissue of laying hens (Lohmann LSL Whites) consuming a diet supplemented with 0.3% L-[5,5,5-$^{2}H_3$]leucine for 55 d. Values are means ± SEMs, *n*=3. Labeled means without a common letter differ significantly (P<0.05). MPE, mole percent excess.

Figure 3.4



Plasma L-[5,5,5-$^2H_3$]leucine enrichment in the postabsorptive state and after consumption of intrinsically-labeled eggs (A; Experiment 1) or meat (B; Experiment 2) in healthy young men. Values are means ± SEMs, n=2 per experiment. The dashed line refers to food ingestion. A (Experiment 1): P=0.08. B (Experiment 2): P=0.38. MPE, mole percent excess.

Figure 3.5



Exogenous leucine R_a in the postabsorptive state and after consumption of intrinsically-labeled eggs in healthy young men. Values are means ± SEMs, *n*=2. The dashed line refers to food ingestion. Data were analyzed using a one-way repeated measures (time) ANOVA. *P*=0.08. R_a, rate of appearance.

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3.9 Supplemental methods

Egg and tissue enrichment analysis

The egg white and yolk protein bound L-[5,5,5-$^{2}H_3$]leucine enrichments were determined by adding a portion of the egg white or yolk to 500 µL of 0.6 M of ice-cold perchloric acid (PCA). The mixture was vortexed, centrifuged, and the supernatant was removed. The egg white and yolk protein pellets were washed with additional 0.6 M PCA. Subsequently, the egg protein pellets were washed with 500 µL of ethanol and lyophilized to dryness. The tissue protein bound L-[5,5,5-$^{2}H_3$]leucine enrichments were determined from ~30 mg of wet weight meat, organ and bone tissue by homogenizing on ice using a Teflon pestle with 500 µL of 0.6 M PCA. The 30 mg wet tissue was randomly selected from the overall meat, organ, and bone mass. Samples were centrifuged and the supernatant was removed. The meat, organ, and bone protein pellets were washed, centrifuged, and lyophilized. The resulting freeze-dried protein pellets were hydrolyzed overnight at 110°C with 1.5 mL of 6 M hydrochloric acid (to liberate the amino acids. The free amino acids were purified using a selective filter, dried under vacuum, and re-suspended in 100 µL of pyridine to separate from inorganics. The obtained solution was dried under vacuum and derivatized with 50µL of neat MTBSTFA+1%TBDMCS (Campbell Sci., Rockford, IL, USA) and 50 µL of acetonitrile (Fisher Sci., Fair Lawn, NJ, USA) for 4 h at 80°C. 3 µL of sample was injected in split mode (10:1) into the GC/MS system consisting of an Agilent 7890 (Agilent Inc., Palo Alto, CA, USA) gas chromatograph, an Agilent 5975 mass selective detector and Agilent 7683B autosampler in Metabolomics Lab of Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. Protein bound L-[5,5,5-$^{2}H_3$]leucine enrichment of the samples were determined by selective ion monitoring for leucine mass-to-charge (*m/z*) ratio at 302 (*m*+0) and 305 (*m*+3).

Plasma analyses

Plasma amino acid concentrations and enrichments were determined by GC/MS (Agilent 7890A GC/5975C; MSD, Palo Alto, CA, USA) in Metabolomics Lab of Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. Plasma (200 µL) was extracted using 1 mL of a single phase mixture of isopropanol:acetonitrile:water (3:3:2, v/v) and was centrifuged for 10 min at 4ºC. Subsequently, the supernatant was dried and the amino acids were converted into TBDMCS derivatives before analysis by GC/MS.

Muscle analyses

Myofibrillar proteins were extracted from ~50 mg of wet muscle by hand-homogenizing in ice-cold homogenization buffer (10 µL·mg^{-1}) using a Teflon pestle. Homogenates were centrifuged at 2000 × *g* for 10 min at 4ºC. The resultant supernatant was removed and the pellet was washed with 500 µL distilled water. Subsequently, the samples were centrifuged at 700 × *g* for 10 min at 4ºC. The remaining myofibrillar proteins pellet was solubilized by adding 1 mL of 0.3 M NaOH and heating at 50ºC for 30 min with vortex mixing every 10 min. Samples were centrifuged at 10000 × *g* for 5 min at 4ºC. The supernatant containing the myofibrillar enriched protein fraction was collected and the collagen pellet was discarded. Myofibrillar proteins were precipitated by adding 1 mL of 1 M PCA and centrifuging at 2500 × *g* for 10 min at 4ºC. The myofibrillar protein pellet was washed twice with 70% ethanol. The amino acids were liberated by adding 2 mL of 6 M HCL and heating overnight at 110°C. The free amino acids were purified using cation exchange chromatography (Dowex 50W-X8-200 resin; Acros Organics, Geel, Belgium) before analysis by 5500 QTRAP LC/MS/MS (Sciex, Framingham, MA) in Metabolomics Lab of Roy J. Carver Biotechnology Center, University of Illinois at Urbana-Champaign. The samples were dried and

re-suspended in 100 µL 60% methanol and centrifuged. Subsequently, samples were injected (5 µL) and the LC separation was performed on a Thermo Hypercarb column (4.6 × 100 mm, 5 µm) with mobile phase A (0.1% formic acid in water) and mobile phase B (0.1% formic acid in acetontrile). The flow rate was 0.4 mL/min. Mass spectra were acquired under positive electrospray ionization (ESI) with the ion spray voltage at +5500 V. The source temperature was 450 °C. The curtain gas, ion source gas 1, and ion source gas 2 were 35, 65, and 55 psi, respectively. The L-[5,5,5-$^{2}H_3$]leucine enrichments of the myofibrillar protein bound samples were determined by multiple reaction monitoring (MRM) at *m/z* 132.0 → 86.0 and 135.0 → 89.0 for unlabeled and labeled L-[5,5,5-$^{2}H_3$]leucine, respectively. Software Analyst 1.6.2 was used for data acquisition and analysis.

Calculations

Total, exogenous, and endogenous rate of leucine appearance (R_a), and postprandial protein-derived leucine availability in the circulation (Leu_{plasma}) were calculated using modified Steele's equations (1, 2). The specific equations used to calculate leucine fluxes were as follows:

$$\text{Total } R_a = \frac{F_{iv} - \left[pV \times C(t)leu \times \frac{dE_{iv}}{dt}\right]}{E_{iv}(t)} \quad (1)$$

$$\text{Exo } R_a = \frac{\text{Total } R_a \times E_{po}(t) + \left[pV \times C(t)leu \times \frac{dE_{po}}{dt}\right]}{E_{prot}} \quad (2)$$

$$\text{Endo } R_a = \text{Total } R_a - \text{Exo } R_a - F_{iv} \quad (3)$$

$$Leu_{plasma} = \left(\frac{AUC_{Exo\ Ra}}{Leu_{prot}}\right) \times BW \times 100 \quad (4)$$

Total R_a is the appearance rate of total leucine calculated with the IV tracer (μmol · kg^{-1} · min^{-1}); where F_{iv} is the IV tracer infusion rate (0.13 μmol · kg^{-1} · min^{-1}); *pV* (0.125 L/kg^{-1}) is the distribution volume (1); *C(t)* is the mean plasma leucine concentration between two consecutive time points (μmol · kg^{-1}); dE_{iv}/dt represents the time-dependent variations in plasma leucine enrichment (mole percent excess) derived from the IV tracer; and $E_{iv}(t)$ is the mean plasma leucine enrichment (mole percent excess) derived from the IV tracer between two consecutive time points. *Exo R_a* is the plasma entry rate (μmol · kg^{-1} · min^{-1}) of dietary protein-derived leucine after first-pass splanchnic extraction; $E_{po}(t)$ is the mean plasma leucine enrichment based on the oral tracer between two consecutive time points (mole percent excess); dE_{po}/dt is the time-dependent variations of plasma leucine enrichment derived from the oral tracer (mole percent excess) ; and E_{prot} is the L-[5,5,5-$^2H_3$]leucine enrichment of the dietary protein (21.2 mole percent excess). *Endo R_a* is the entry rate of endogenous leucine from whole body protein breakdown (μmol · kg^{-1} · min^{-1}); Leu_{plasma} represents the fraction of dietary leucine that appeared in circulation (%); where AUC_{ExoRa} represents the area under the time curve (AUC) of *Exo R_a*, which indicates the amount of dietary protein-derived leucine that appeared in the circulation during the 5 h postprandial period; Leu_{prot} is the amount of dietary leucine (11992 μmol) ingested and *BW* is the participants' body weight (kg).

3.10 Supplemental tables

Supplemental table 3.1. Diet of laying hens[1].

Ingredient	**g·kg^{-1}**
Wheat	310.4
Barley	269.7
Sucrose	120.4
Limestone	90.0
Sunflower Meal	67.2
Glutamic Acid	50.0
Soybean Oil	40.0
Dicalcium Phosphate	14.0
L-Lysine-HCl	5.50
Sodium Chloride	5.00
Glycine	4.00
L-Isoleucine	3.60
DL-Methionine	3.50
L-Valine	3.20
L-[5,5,5-$^{2}H_3$]leucine	3.00
L-Arginine	2.10
Vitamin Mix[2]	2.00
L-Phenylalanine	1.50
Mineral Mix[3]	1.50
L-Tryptophan	0.70

[1]Diet provided to Lohmann LSL White laying hens during 55d feeding period, N=3.[2] Provided per kg of diet: retinyl acetate, 4400 IU; cholecalciferol, 25 μg; d,l-∝-tocopheryl acetate, 11 IU; vitamin B_{12}, 0.01 mg; riboflavin, 4.41 mg; D-Ca-pantothenate, 10.00 mg; niacin, 22.00 mg; menadione sodium bisulfite, 2.33 mg.[3] Provided as mg per kg of diet: Mn, 75.00 from MnO; Fe, 75.00 from $FeSO_4 \cdot 7H_2O$; Zn, 75.00 from ZnO; Cu, 5.00 from $CuSO_4 \cdot 5H_2O$; I, 0.75 from ethylene diamine dihydroiodide; Se, 0.10 from Na_2SeO_3

3.11 Supplemental references

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CHAPTER 4: CONSUMPTION OF WHOLE EGGS PROMOTES GREATER STIMULATION OF POSTEXERCISE MUSCLE PROTEIN SYNTHESIS THAN CONSUMPTION OF ISONITROGENOUS AMOUNTS OF EGG WHITES IN YOUNG MEN[1]

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Key words: eggs, whole foods, muscle protein synthesis, protein quality, and exercise.

Abbreviations used: 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; AMPKα, adenosine monophosphate-activated protein kinase alpha; eEF2, eukaryotic translation elongation factor 2; ERK, extracellular signal–regulated kinases; FSR, fractional synthetic rate: LAT1, large neutral amino acids transporter small subunit 1; NOLD, non-oxidative leucine disposal; R_a, rate of appearance; R_d, rate of appearance; RM, repetition maximum; SNAT2, sodium-coupled neutral amino acid transporter 2; TTR, tracer-to-tracee ratio, mTORC1, mammalian target of rapamycin complex 1, p70S6K, 70 kDa S6 protein kinase.

[1]All co-authors agreed to their contributions being included in the dissertation.

4.1 Abstract

Background: Protein in the diet is commonly obtained from whole foods that contain various macro- and micro-nutrients. However, the effect of consuming protein within its nature food matrix on postprandial protein metabolism remains understudied in humans.

Objective: We aimed to compare the whole body and muscle protein metabolic responses after consumption of whole eggs versus egg whites during exercise recovery in young males.

Design: In crossover trials, 10 resistance-trained males (21±1 y; 88±3 kg; body fat: 16±1%) received primed continuous L-[*ring*-$^{2}H_5$]phenylalanine and L-[1-^{13}C]leucine infusions and performed a single bout of resistance exercise. After exercise, participants consumed intrinsically L-[5,5,5-$^{2}H_3$]leucine-labeled whole eggs (18 g protein, 17 g fat) or egg whites (18 g protein, 0 g fat). Repeated blood and muscle biopsies were collected to assess whole body leucine kinetics, intramuscular signaling, and myofibrillar protein synthesis.

Results: Plasma appearance rates of protein-derived leucine was more rapid after consumption of egg whites than whole eggs (P=0.01). Total plasma availability of leucine over the 300 min postprandial period was similar (P=0.75) between ingestion of whole eggs (68±1%) and egg whites (66±2%) with no difference in whole body net leucine balance (P=0.87). Both whole egg and egg white conditions increased the phosphorylation of mammalian target of rapamycin complex 1, ribosomal protein S6 kinase 1, and eukaryotic translation initiation factor 4E-binding protein 1 during postexercise recovery (all P<0.05). However, whole egg ingestion increased the postexercise myofibrillar protein synthetic response to a greater extent than ingestion of egg whites (P=0.04).

Conclusions: We show that the ingestion of whole eggs immediately after resistance exercise stimulates a greater stimulation of myofibrillar protein synthesis than the ingestion of egg whites

despite being matched for protein content in young males. Our data indicate that the ingestion of nutrient and protein dense foods differentially stimulate muscle anabolism versus protein centric foods.

4.2 Introduction

Dietary patterns that include the regular ingestion of high quality protein-dense foods are important to optimize the stimulation of postprandial muscle protein synthesis rates to augment skeletal muscle remodeling. Protein quality in human nutrition can be, at least partly, based on the bioavailability of dietary protein-derived amino acids in circulation to support whole body and muscle protein metabolic need. For example, studies have shown that various meal characteristics, such as protein source (9, 26) and ingested protein quantity (32), can influence the amount of dietary protein-derived amino acids available in circulation to stimulate postprandial muscle protein synthesis rates. However, most work has focused on the effects of consuming isolated protein fractions (e.g., dairy-based whey and casein and plant-based soy) dissolved in liquid beverages (7, 9, 12, 18, 19, 26-28, 34, 35, 41) with far less known about how the ingestion of nutrient- and protein-dense whole foods impact postprandial protein metabolism. This is significant as the ingestion of nutrient-dense whole foods, as compared to supplemental protein sources, is far a more common approach to achieve daily protein recommendations while concomitantly improving diet quality.

To better define whole food protein quality in human nutrition, we developed intrinsically L-[5,5,5-$^{2}H_{3}$]leucine eggs to allow for the detailed assessment of postprandial whole body and skeletal muscle protein metabolism *in vivo* in humans (44). Eggs are a nutritionally complete food source and commonly consumed at breakfast by U.S. adults (2). However, the removal of the yolk

is often promoted for improved health when multiple eggs are consumed. This an unsubstantiated belief related to the cholesterol and fat content of an egg yolk (25). The yolk is nutrient dense and contains nearly half of the total protein found in the egg and, as such, its removal is counterintuitive. Since nutrient-dense protein foods are often recommended to achieve protein recommendations (33), it is important to define how the ingestion of nutritionally complete and protein-dense foods modulates protein metabolism under a setting that includes other components of a healthy lifestyle (i.e., incorporation of regular exercise).

Therefore, the purpose of this study was to compare whole body leucine kinetics and postprandial myofibrillar protein synthesis rates after the ingestion of whole eggs and isonitrogenous amounts of egg whites during recovery from resistance exercise in young males. In addition, we examined skeletal muscle amino acid transporter protein content and the phosphorylated-state of protein signaling molecules that may regulate changes in myofibrillar protein synthesis rates (15). We hypothesized that whole egg ingestion will delay the appearance rates of postprandial protein-derived amino acids in circulation, but will not modulate plasma amino acid availability or myofibrillar protein synthesis rates throughout a 0-300 min recovery period when compared to egg white ingestion in healthy young males.

4.3 Methods

Participants and ethical approval

Ten healthy young males (mean ± SEM: age: 21±1 y) who were regularly engaged in structured resistance exercise training (mean ± SEM: training years: 5±1 y) volunteered for the study. Participant characteristics are presented in **Table 1**. All participants were deemed healthy based on responses to a routine medical screening questionnaire and had no prior history of participating

in stable isotope amino acid tracer experiments. All participants were informed about the experimental procedures to be used, the purpose of the study, and all potential risks before giving written consent. The study was approved by the Institutional Review Board at University of Illinois at Urbana-Champaign and conformed to standards for the use of human participants in research as outlined in the seventh revision of the Declaration of Helsinki.

Experimental design

A within-subject crossover design was used for this study. At least one week before the first infusion trial, participants reported to the laboratory for familiarization with the exercise equipment and for maximum strength testing as determined by their ten-repetition maximum (10-RM) for leg press and leg extension. In addition, body weight and height were measured as well as body composition by dual-energy X-ray absorptiometry (Hologic QDR 4500A, Bedford, MA, USA). On a separate occasion, at least 3 d after initial maximum strength testing and at least 3 d before the first infusion trial, participants re-visited the lab to confirm their 10-RM for leg press and leg extension. The 10-RMs for leg press and leg extension (233±32 kg and 112±10 kg, respectively; means ± SEM) were used to set the workload for the infusion trials. Participants were instructed to refrain from vigorous physical activity for 3 d prior to each trial and to record their dietary intake using online food tracker (MyFitnessPal™) for 2 d before each trial. Participants were subsequently instructed to follow their food dairy as closely as possible during the 2 d leading into the second infusion trial while recording their dietary intake again. Average 2 d macronutrient intake was similar between the whole egg and egg whites trials (P=0.16) (**Supplemental Table 1**). Participants were counterbalanced in random fashion to consume either whole eggs or egg whites for their first infusion trial. The time between crossover trials was 7-14 d (8±1 d).

Infusion protocol

On both infusion trials, participants reported to the laboratory at 0700 h after an overnight fast. After collecting a baseline breath sample, a Teflon catheter was inserted into an antecubital vein for baseline blood sample collection (*t*=-210 min) and participants received priming doses of $NaH^{13}CO_2$ (2.35 μmol·kg^{-1}), L-[1-^{13}C]leucine (7.6 μmol·kg^{-1}), and L-[*ring*-$^{2}H_5$]phenylalanine (2.0 μmol·kg^{-1}). Subsequently, a continuous intravenous infusion of L-[1-^{13}C]leucine (0.10 μmol·kg^{-1}·min^{-1}) and L-[ring-$^{2}H_5$]phenylalanine (0.05 μmol·kg^{-1}·min^{-1}) was initiated (*t*=-210 min) and maintained over the infusion trials. A second Teflon catheter was inserted into a heated dorsal hand vein for repeated arterialized blood sampling and remained patent by a 0.9% saline drip. In the postabsorptive state of infusion trial 1, muscle biopsies were collected at *t*=-150 and -30 min of infusion to determine fasted myofibrillar protein synthesis rates, relative skeletal muscle amino acid transporter content, and anabolic-related signaling. In the subsequent cross-over trial only 1 muscle biopsy was collected at *t*=-30 for anabolic-related signaling analysis and postabsorptive myofibrillar protein-bound tracer enrichment. After collection of the resting muscle biopsy at *t*=-30 for both trials, the participants performed resistance exercise that consisted of 4 sets of 10 repetitions at 80% of 10-RM for both leg press and leg extension exercise. The exercise external work (repetitions × load) was matched between the whole egg (9896 ± 52 kg) and egg whites trials (9893 ± 52 kg) (*P*=0.34). Immediately after completion of the exercise bout, participants consumed 3 whole eggs or an equivalent amount of protein from egg whites (*t*=0 min). Participants were also provided 300 mL of water to consume with the meal. Completion of the meal marked the start of the postprandial phase (*t*=0 min) and additional muscle biopsies were collected at *t*=120 and 300 min. Biopsies were collected from the middle region of the *vastus lateralis* (15 cm above the patella) with a Bergström needle modified for suction under local anesthesia (2% Lidocaine). The

postabsorptive muscle biopsies on trial 1 were collected from the same incision with the needle pointed to distal and proximal directions, respectively. The muscle biopsies obtained at t=120 and 300 min of post-exercise recovery were collected from the contralateral leg through separate incisions (2-3 cm apart). All muscle biopsy samples were freed from any visible blood, adipose, and connective tissue immediately frozen in liquid nitrogen, and stored at -80˚C until subsequent analysis. Breath samples and arterialized blood samples and were collected every 30 or 60 min during the postabsorptive- and postprandial states. Total CO_2 production rates were measured with a metabolic cart (TrueOne 2400, ParvoMedics, Sandy, UT) at regular intervals throughout the infusion trials. The blood samples were immediately analyzed for whole blood glucose concentrations (2300 Stat Plus, YSI Life Sciences, Springs, OH) and subsequently centrifuged at 3000 × g for 10 min at 4°C. Aliquots of plasma were frozen and stored at -80°C until subsequent analysis. The breath samples were collected in 10 mL Vacutainers and stored at 20°C until subsequent determination of $^{13}CO_2$ enrichment by isotope ratio mass spectrometry (IDmicro Breath, Compact Science Systems Ltd, Newcastle-Under-Lyme, UK).

Meal composition

The intrinsically L-[5,5,5-$^2H_3$]leucine labeled eggs were produced by supplementing the diet of laying hens (Lohmann LSL Whites) with 0.3% L-[5,5,5-$^2H_3$]leucine as described previously (44). A portion of the collected eggs had the yolks removed prior to storage at -20°C in aliquots of 18 g protein. The other portion of eggs had the yolks and whites completely mixed prior to storage in aliquots of isonitrogenous amounts. The L-[5,5,5-$^2H_3$]leucine enrichment of the whole egg and egg white aliquots were determined by gas chromatography mass spectrometry (GC-MS) and averaged 28.6 and 26.1 mole percent excess (MPE), respectively. Proximate analyses for protein,

lipid, and carbohydrate concentrations were determined by using the combustion method (method 990.03; AOAC International, 2000; TruMac; LECO Corp., USA) (44). Leucine content of the whole egg and egg white aliquots were determined by GC-MS with integration of amino acid peak areas compared to an internal standard (DL-p-chlorophenylalanine) using the AMDIS software package (v. 2.71; NIST) (23). Before the infusions, the egg aliquots were thawed overnight in a refrigerator at 4°C. On the morning of the experiment, the whole egg or whites were scrambled in a skillet until solid with no visible liquid remaining. The macronutrient composition and energy content was 18 g protein (1.57 g leucine), 17 g fat, and 226 kcals for the whole eggs and 18 g protein (1.60 g leucine), 0 g fat, and 73 kcals for the egg white treatments.

Plasma analyses

Plasma insulin concentrations were determined using a commercially available enzyme-linked immunosorbent assays (Alpco Diagnostics; Salem, NH). Plasma triglyceride concentrations were determined using a point-of-care chemistry analyzer (Piccolo Xpress Chemistry Analyzer, Abaxis, Union City, CA). Plasma amino acid concentrations and enrichments were determined by GC-MS analysis (Agilent 7890A GC/5975C; MSD, Little Falls, DE) as described in our previous work (44). Briefly, plasma samples were prepared for amino acid analysis using a mixture of isopropanol:acetonitrile:water (3:3:2, v/v) and centrifuged at 20000 × g for 10 min at 4ºC. Subsequently, the supernatant was dried and the amino acids converted into tert-butyldimethylsilyl (*t*-BDMS) derivatives prior to GC-MS analysis. Plasma L-[1-^{13}C]leucine and L-[5,5,5-$^{2}H_3$]leucine enrichments were determined by ion monitoring at mass/charge (*m/z*) ratios 302 (*m*+0), 303 (*m*+1), and 305 (*m*+3) with *m*+0 representing the lowest molecule weight of the ion or unlabeled leucine. For L-[*ring*-$^{2}H_5$]phenylalanine, *m/z* 336 (*m*+0) and 341 (*m*+5) were monitored for unlabeled and

labeled phenylalanine, respectively. Plasma enrichment of the *t*-BDMS derivative of α-[^{13}C]-ketoisocaproate (α-KIC) was measured by GC-MS analysis by ion monitoring at mass/charge (*m/z*) ratios *m/z* 232 and 233. The plasma leucine concentrations were determined by integrating amino acid peak areas in comparison to U-[$^{13}C_6$]leucine as an internal standard with the use of the AMDIS software package (v. 2.71; NIST) (23).

Muscle analysis

Myofibrillar protein-enriched fractions were isolated from ~50 mg of wet tissue as described previously (44). Myofibrillar-enriched protein pellets were hydrolyzed overnight in 6 M HCL at 110°C. The resultant free amino acids were purified using cation exchange chromatography (Dowex 50W-X8-200 resin; Acros Organics, Geel, Belgium) and dried under vacuum. Free amino acids were re-suspended in 60% methanol and centrifuged before the myofibrillar protein-bound enrichments were determined by 5500 QTRAP liquid chromatography-tandem mass spectrometry (LC/MS/MS; Sciex, Framingham, MA). The myofibrillar protein-bound L-[ring-$^2H_5$]phenylalanine enrichments were determined by multiple reaction monitoring (MRM) at *m/z* 166.0 → 103.0 and 171.0 → 106.0 for unlabeled and labeled L-[ring-$^2H_5$]phenylalanine, respectively. Muscle intracellular free amino acids were extracted from a separate piece of wet muscle (~30 mg) using a Teflon-coated pestle and ice-cold 2% PCA and then centrifuged at 10000 × *g* at 4˚C for 10 min. The supernatant was then collected and this process was repeated 2 more times. The supernatant was taken as the muscle intracellular free amino acids and subsequently purified using cation exchange chromatography (Dowex 50W-X8-200 resin; Acros Organics, Geel, Belgium) before analysis by LC/MS/MS. Muscle intracellular free L-[1-^{13}C]leucine and L-[5,5,5-$^2H_3$]leucine enrichments were measured by MRM at *m/z* 132.0 → 86.0, 133.0 → 87.0 and

135.0 → 89.0 for unlabeled and labeled leucine. For L-[*ring*-$^{2}H_5$]phenylalanine enrichments, MRM at171.0 → 106.0 for unlabeled and labeled phenylalanine were measured. Sciex Software Analyst 1.6.2 was used for data acquisition and analysis.

Western Blotting

A portion of whole muscle homogenates isolated during the myofibrillar protein extractions was used for Western Blotting analysis. The protein concentrations of the homogenates were determined by BCA protein assay (Thermo Fisher Scientific, Rockford, IL) and then used to prepare working samples of equal concentrations in Laemmli buffer and heated to 95°C for 5 min. Equal amounts of protein (10μg) were separated by SDS-PAGE and transferred to nitrocellulose membrane (wet transfer, 100V for 60 min) for antibody incubation. Membranes were blocked in 5% fat-free milk at room temperature for 60 min, washed in Tris-buffered saline with 0.1% Tween 20 (TBST) and incubated in primary antibody (Cell Signaling Technology; 1:1000) overnight at 4°C to determine phosphorylation status of AMPKα at Thr172 (cat. 2535), mTOR at Ser2448 (cat. 2971), ERK at Thr202/Tyr204 (cat. 4377), 4E-BP1 at Thr37/46 (cat. 9459), and eEF2 at Thr56 (cat. 2331). The antibodies (Abcam; 1:1000) used to determine the relative protein content of skeletal muscle amino acid transporters were large neutral amino acid transporter small sub unit 1 (SLC7A5/LAT1) (cat. ab85226), SLC3A/CD98 (cat. ab96721), and sodium-coupled neutral amino acid transporter 2 (SLC38A2/SNAT2) (cat. ab90677). Following overnight incubation of the primary antibody (4°C), membranes were then washed (3 x 5 min TBST), incubated in secondary antibody for 60 min at room temperature (1:20000 in 3% fat-free milk), and washed (3 x 5 min TBST), prior to the detection with chemiluminescence (Millipore; cat. WBKLS0500). To determine the phosphorylation status of p70S6K1 at Thr389 (cat. 9205), 25ug of protein was

loaded followed by incubation in primary (overnight, 1:1000 in 5% bovine serum albumin) and secondary antibody (1:5000 in 5% fat-free milk). Bands were imaged using FluorChem E Imaging System (Protein Simple; Alpha Innotech, Santa Clara, CA). Bands were quantified using AlphaView SA software (Protein Simple) and normalized to Ponceau S staining; this approach has been validated as an alternative loading control (e.g., α-actin) for Western Blot analysis (36, 37).

Calculations

Whole-body leucine kinetics were assessed under non-steady conditions by the ingestion of L-[5,5,5-$^{2}H_3$]leucine eggs combined with intravenous infusion of L-[1-^{13}C]leucine.Leucine oxidation was calculated from the appearance of the ^{13}C-label in the expired CO_2 using the α-KIC reciprocal pool model with fractional bicarbonate retention factors of 0.7 and 0.83 for postabsorptive and postprandial states, respectively (22). For the other leucine fluxes, calculations were performed using the plasma L-[5,5,5-$^{2}H_3$]leucine and L-[1-^{13}C]leucine enrichments and leucine concentrations. Total, exogenous, and endogenous leucine rates of appearance (R_a) and total leucine rates of disappearance (R_d) were calculated with the use of modified Steele equations (8, 14). Furthermore, plasma protein-derived leucine availability, total leucine oxidation, non-oxidative leucine disposal (NOLD), whole body leucine net balance, and whole body leucine retention were calculated (see **Supplemental Methods** for more details on calculations) (8, 14). Myofibrillar protein fractional synthesis rates (FSR) were calculated using the standard precursor-product equation by dividing the increment in L-[*ring*-$^{2}H_5$]-phenylalanine enrichment in the myofibrillar protein pool by the weighted average of L-[*ring*-$^{2}H_5$]-phenylalanine enrichment in the plasma precursor pool over time.

Statistics

A within-subject crossover design was used for this study. A power analysis based on previous research (9, 41) showed that an n=8 per condition was sufficient to detect differences in postprandial muscle protein synthesis between conditions when using a two sided statistical test (P<0.05, 80% power, f=1.2; G*power version 3.1.9.2, Kiel, Germany). Considering a potential dropout rate of 20% during the protocol, the final number of participants recruited was 10 per condition. Differences in time-dependent blood and muscle measurements were tested by 2-factor (time × condition) repeated-measures ANOVA. Differences in self-reported dietary intakes, exercise external loads, and the fraction of dietary protein derived leucine that appeared into plasma were tested by paired t-test. When statistically significant interaction effects were identified in the ANOVA, Bonferroni post hoc tests were performed to determine the differences between means for all significant main effects and interactions. For all analysis, differences were considered statistically significant at P<0.05. All calculations were performed using IBM SPSS Statistics (version 24, Chicago, IL) unless otherwise designated. All data are expressed as mean ± SEMs.

4.4 Results

Plasma metabolites and enrichments

Plasma leucine concentrations increased after egg ingestion (time: P<0.001) with no differences between conditions (time × condition: P=0.76). Peak plasma leucine concentrations were observed at 150 min (210 ± 14 μmol·L^{-1}) and 240 min (200 ± 11 μmol·L^{-1}) after ingestion of egg whites and whole eggs, respectively **(Figure 1A).** Plasma glucose concentrations tended to decrease transiently at 60 min of the postprandial period (P=0.06) with no differences between conditions (time × condition: P=0.73) **(Figure 1B).** Plasma insulin concentrations rapidly

increased after egg ingestion (time: $P<0.001$) with no differences between conditions (time × condition: $P=0.48$) **(Figure 1C)**. Plasma triglyceride concentrations were increased at 180 min after ingestion of whole eggs but not egg whites (time: $P=0.007$; time × condition: $P=0.03$) **(Figure 1D)**.

Plasma L-[5,5,5-$^{2}H_3$]leucine enrichment rapidly increased after egg ingestion (time: $P<0.001$) with no differences between conditions (time × condition: $P=0.37$) (**Figure 2A**). Plasma L-[1-^{13}C]leucine enrichments decreased after whole egg and egg white ingestion (time effect: $P<0.001$) with no differences between conditions (time × condition: $P=0.86$) (**Figure 2B**). Plasma α-[^{13}C] ketoisocaproate enrichments increased after egg ingestion (time effect: $P<0.001$) with no differences between conditions (time × condition: $P=0.68$) (**Figure 2C**). Plasma L-[*ring*-$^{2}H_5$]phenylalanine enrichments declined after egg ingestion (time effect: $P<0.001$) with no differences between conditions (time × condition: $P=0.78$) (**Figure 2D**).

Whole body leucine kinetics

Exogenous leucine rates of appearance (representing the appearance of dietary protein-derived leucine into circulation) increased after whole egg and egg white ingestion (time effect: P<0.001; **Figure 3A**). Exogenous leucine appearance rates were higher between 15-75 min after ingestion of egg whites when compared to whole eggs , whereas exogenous leucine rates of appearance were higher between 150 and 300 min after whole egg ingestion min when compared to egg whites (time × condition: $P=0.001$). Thus, more rapid peak exogenous leucine appearance rates were observed at 75 min (529 ± 56 nmol·kg^{-1}·min^{-1}) versus 120 min (496 ± 38 nmol·kg^{-1}·min^{-1}) of the postprandial period after ingestion of egg whites and whole eggs, respectively. The fraction of dietary protein-derived leucine that appeared in circulation during the first 120 min of the

postprandial period was greater after egg white (34 ± 2%) than whole egg ingestion (25 ± 3 %) (P=0.02). However, the cumulative fraction of dietary protein-derived leucine that appeared in circulation throughout the 300 min postprandial period was similar between the egg white (68 ± 1%) and whole egg conditions (66 ± 2 %) (P=0.75). Endogenous leucine rates of appearance (representing the appearance of leucine derived from whole-body protein breakdown into circulation) decreased after egg ingestion (time effect: P<0.001) with no differences between conditions (time × condition: P=0.17) **Figure 3B**). Total leucine rates of appearance increased after egg ingestion (time effect: P<0.001) with no differences between conditions (time × condition: P=0.83) (**Figure 3C**). Total leucine rates of disappearance increased after egg ingestion (time effect: P<0.001) with no differences between conditions (time × condition: P=0.84) (**Figure 3D**).

Area under the curves of fasted and fed rates of whole-body protein breakdown (Endo R_a), synthesis (NOLD), oxidation (Total Ox), and net balance (NOLD - Endo R_a) are presented in **Figure 4**. Regardless of condition, egg ingestion decreased whole body protein breakdown rates (time effect: P<0.001; time × condition: P=0.31) and increased whole body synthesis rates (P=0.031; time × condition: P=0.87), thereby resulting in an improved net protein balance when compared to fasted conditions (time effect: P<0.001; time × condition: P=0.39). Whole body leucine oxidation rates decreased (P=0.008) after egg ingestion with no differences between conditions (time × condition: P=0.74).

Whole body net leucine retention improved after egg ingestion when compared to fasted values (time effect: P<0.001) with no difference between conditions (time × condition: P=0.70). Whole body leucine retention improved from negative fasted values of -354 ± 29 and -347 ± 20 to positive

fed values of 131 ± 33 and 123 ± 22 nmol leucine·kg^{-1}·min^{-1} for egg whites and whole eggs, respectively.

Intramuscular signaling and amino acid transporters

There was no change in the relative protein content of skeletal muscle amino acid transporters (LAT1, CD98, or SNAT2) throughout post-exercise recovery (all time effect: $P>0.05$) (**Figure 5**). AMPKα phosphorylation decreased during post-exercise recovery (time effect: $P<0.001$) with no differences between conditions (time × condition: $P=0.52$) (**Figure 6A**). Phosphorylation of mTORC1 increased by 0.9 ± 0.3 and 0.6 ± 0.3 fold above fasting values at 300 min of post-exercise recovery ($P=0.01$) after whole egg and egg ingestion, respectively, with no differences between egg conditions (time × condition: $P=0.58$) (**Figure 6B**). Erk1/2 phosphorylation decreased during post-exercise recovery (time effect: $P<0.001$) with no differences between conditions (time × condition: $P=0.64$) (**Figure 6C**). Phosphorylation of p70S6K1 was elevated above fasted throughout postexercise recovery (time effect: $P<0.001$) with no differences between conditions (time × condition: $P=0.49$) (**Figure 6D**). 4E-BP1 phosphorylation was elevated throughout postexercise recovery (time effect: $P<0.001$) with no differences between conditions (time × condition: $P=0.23$) (**Figure 6E**). There was no change in the phosphorylated-state of eEF2 phosphorylation, regardless of egg condition, during recovery from exercise (time effect: $P=0.13$) (**Figure 6F**). Representative blots are displayed in **Supplemental Figure 1** and **2**.

Muscle intracellular free enrichments

Muscle intracellular free enrichments are presented in **Table 2.** Muscle-free L-[5,5,5-$^{2}H_3$]leucine enrichment increased after egg ingestion (time effect: $P<0.001$) with no differences between conditions (time × condition: $P=0.61$). Muscle-free L-[1-^{13}C]leucine enrichments remained steady over time (time effect: $P=0.30$) with no differences between conditions (time × condition: $P=0.62$). Muscle intracellular free L-[ring-$^{2}H_5$]phenylalanine were lower at t=-120 when compared to time points t=0, 120, and 300 (all $P<0.05$) with no differences between conditions ($P=0.63$) or other time points (all $P>0.05$).

Myofibrillar protein synthesis rates.

Postexercise myofibrillar protein synthesis, based on the plasma L-[*ring*-$^{2}H_5$]phenylalanine precursor pool, increased above fasted values (0.015 ± 0.002 %·h^{-1}) after egg white and whole egg ingestion (time effect: $P=0.015$) with a greater cumulative response (0-300 min) after whole egg ingestion (0.034 ± 0.004 %·h^{-1}; 2.7 ± 0.5 fold above fasted) when compared to egg white ingestion (0.024 ± 0.002 %·h^{-1}; 1.9 ± 0.4 fold above fasted) (time × condition: $P=0.04$) (**Figure 7**). The temporal pattern of change in the early (0-120 min) and late (120-300) postexercise myofibrillar protein synthetic responses were not different between the whole egg and egg white conditions (time × condition: $P=0.54$); however, both conditions were elevated above rest during the late and early postprandial phase (all $P<0.05$) (**Figure 7 Inset**).

Similarly, the cumulative postexercise myofibrillar protein synthetic response, based on the muscle free L-[*ring*-$^{2}H_5$]phenylalanine precursor pool, tended to increase above fasted values (0.07 ± 0.011 %·h^{-1}) (time effect: $P=0.001$) to a greater extend after whole egg ingestion (0.131 ± 0.017 %·h^{-1}; 2.1 ± 0.3 fold above fasted) when compared with egg white ingestion (0.88 ± 0.007 %·h^{-1};

1.6 ± 0.3 fold above fasted) (time × condition: P=0.05). Resistance exercise and egg ingestion, based on the muscle free as the precursor, showed a trend towards increasing myofibrillar protein synthesis rates above fasted values at 0-120 min and 120-300 min during postexercise recovery (time effect: *P*=0.12) with no differences between egg conditions (time × condition: *P*=0.53). Using the muscle free enrichment as the precursor, postexercise myofibrillar protein synthesis rates between 0-120 min were 0.140 ± 0.035 %·h^{-1} (2.5 ± 0.8 fold above fasted) and 0.098 ± 0.017 %·h^{-1} (1.8 ± 0.6 fold above fasted) after whole egg and egg white ingestion, respectively. Postexercise myofibrillar protein synthesis rates, based on the muscle free precursor, between 120-300 min were 0.123 ± 0.027 %·h^{-1} (1.8 ± 0.3 fold above fasted) and 0.078 ± 0.013 %·h^{-1} (1.4 ± 0.4 fold above fasted) after whole egg and egg white ingestion, respectively.

4.5 Discussion

To our knowledge, we report for the first time the potentiation of exercise-mediated stimulation of postprandial myofibrillar protein synthesis rates in response to ingestion of whole eggs versus isonitrogenous amounts of egg whites in healthy young males. Our work points to the concept that dietary protein may display differential anabolic properties on skeletal muscle tissue when consumed within its natural food matrix.

Similar to isolated protein sources (e.g., whey and casein) (7, 14), we also showed that consumption of protein-dense foods in the form of whole eggs and egg whites improved whole body net protein balance (**Figure 4**). While insight into whole body protein kinetics can provide valuable information on whole body protein remodeling and net anabolism, which are prerequisites for lean mass maintenance or growth, it is not uncommon that whole body protein kinetics may 'mask' important changes within specific tissues, such a skeletal muscle (9). In particular, the

observed potentiation of postexercise myofibrillar protein synthesis rates in response to whole egg versus egg white ingestion was not observed on a whole body level in which there was an equivalent stimulation of whole body protein synthesis in both conditions.

In an attempt to underpin how the ingestion of whole eggs supported a greater postexercise myofibrillar protein synthetic response when compared with egg whites, we studied various upstream factors that are often assumed to be regulatory for postprandial muscle anabolism. We showed more rapid appearance rates of protein-derived leucine into circulation and higher peak leucinemia after egg whites versus whole eggs ingestion (**Figure 3**), the latter of which is a similar pattern to previous studies using either isolated egg protein (31) or whole eggs (10). However, there were no differences between the total amount of dietary protein-derived leucine that became available in circulation throughout the 5 h postprandial period between the egg whites (68 ± 1%) and whole egg conditions (66 ± 2%). Similarly, there were no differences in the relative protein content of skeletal muscle amino acid transporters (such as LAT1, CD98, and SNAT2) (**Figure 5**) or muscle free L-[5,5,5-$^{2}H_{3}$]leucine enrichments during recovery from exercise between the egg conditions (**Table 2**). Collectively, these data imply that muscle amino acid sensing and/or amino acid uptake likely did not differentially contribute to the stimulation of postexercise myofibrillar protein synthesis rates between the egg conditions.

In addition, the temporal assessment of various metabolic and molecular readouts often associated with the control of translation initiation and elongation, such as MAPK-related and mTORC1-related signaling pathways, did not reveal significant differences in phosphorylation between the egg conditions (**Figure 6**). The lack of differences in the phosphorylated-state of mTORC1-mediated signaling between the egg conditions could imply that this anabolic pathway was maximized from the prior performance of resistance exercise and food ingestion (9, 28, 31,

34), thereby overriding any subtle non-protein nutritive influences of whole egg consumption on mTORC-1 phosphorylation and its downstream targets. We also examined metabolic regulatory pathways and energy sensing protein phosphorylation. Similar to the other assessed molecular readouts there were no observed differences in the phosphorylated-state of AMPK or Erk1/2.

The essential amino acid composition, and leucine in particular, are nearly identical between the egg conditions (1) and likely did not have an influential role in the muscle anabolic response. In addition to this, it would seem that the greater overall energy content, and subsequent insulinemia, within the whole egg (256 kcal) versus egg white (73 kcal) conditions likely did not influence the differential stimulation of the postprandial myofibrillar protein synthetic response. For example, it has been shown that only small amounts of plasma insulin concentrations (5 $\mu U \cdot mL^{-1}$) are required to maximize the muscle anabolic potential of elevated plasma amino acid availability in humans (13, 20). Moreover, the additional substrate (i.e. fat) for energy production with whole egg ingestion did not spare the use of amino acids for oxidative 'fuel' (**Figure 4**). It could be hypothesized, however, that the caloric difference between the whole egg and egg white conditions was not sufficient to spare amino acid oxidation as both meals provided were much lower in caloric content than the habitual diets of most of the resistance-trained young males in this study. Notwithstanding, previous reports have shown that additional energy to a protein containing meal does not amplify the anabolic properties of dietary amino acids to stimulate postprandial muscle protein synthesis rates when compared to protein alone (12, 17-19, 40).

Previous research has shown that other food components, beyond dietary amino acids, may have a supporting role in modulating postprandial muscle anabolism during recovery from exercise. For example, Elliot *et al.* (16) previously showed that whole milk ingestion immediately after resistance exercise resulted in greater amino acid leg uptake when compared to consumption

of isonitrogenous or isoenergetic amounts of skim milk in healthy young adults. Here, we showed a greater early (0-120 min) postprandial dietary-derived leucine availability after ingestion of egg whites (34 ± 2%) versus whole eggs (25 ± 3%). The greater postprandial plasma leucine availability after egg white versus whole egg ingestion, however, did not result in a greater early stimulation of the postprandial (0-120 min) myofibrillar protein synthetic response after ingestion of egg whites when compared to whole eggs. Given all this, it seems that perhaps 'extra' nutritional food constituents, and not simply a rapid aminoacidemia/leucinemia (7, 26, 35, 41), may also have a role in modulating the postprandial muscle protein synthetic response in healthy adults. For example, as part of its natural food matrix, the egg yolk contains various non-protein food components that may have anabolic properties such miRNAs (5), vitamins (11, 21), minerals (30) and lipids (e.g., phosphatidic- (24), palmitic- (46), and docosahexaenoic- acid (38)) by modifying pathways related to transcriptional and/or protein translational control. However, more work is required to systemically assess the role of such food components on modulating postprandial muscle protein synthesis rates in vivo in humans.

It is important to note that despite recent modifications of dietary guidelines to reflect that total dietary cholesterol intake is often misrepresented as a risk factor for cardiovascular disease (39, 43), popular practice may still dictate the discarding of the yolk when multiple eggs are consumed in a meal. However, the yolk is nutrient dense and may contain a variety of important bioactive compounds such as lipids, micronutrients, antioxidant carotenoids, and miRNAs (6, 45). Therefore, the removal of the yolk and its associated vital nutrients from the eggs may limit their benefit to stimulate muscle protein synthesis rates as well as overall human health (3, 4, 6). Overall, our current work lends support for the recommendation that nutrient and protein dense foods are

cornerstones to meeting daily protein requirements to optimize muscle protein synthesis rates with exercise.

In conclusion, we demonstrate that postexercise myofibrillar protein synthesis rates are stimulated to a greater extent after consuming whole eggs versus egg whites in healthy young males, despite being matched for protein content. We observed no differences between egg conditions in the commonly assumed regulators of postexercise muscle protein synthesis rates such as total postprandial plasma leucine availability, whole body leucine oxidation rates, skeletal muscle amino acid transport protein content, or molecular readouts associated with metabolic and anabolic protein signaling. Given this, future work is required to identify the potential role of non-protein food components to contribute to the stimulation of postprandial muscle protein synthesis rates in humans. This information is important as other nutritional components may contribute to food protein requirements, particularly when dietary protein is consumed in moderate amounts (~15-20 g).

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Author Contributions

The authors' responsibilities were as follows—SvV and NAB: contributed to the conception and the design of the experiment; all authors: contributed to collection, analysis, and interpretation of data; SvV, and NAB: contributed to drafting or revising intellectual content of the manuscript

and had primary responsibility for the final content; all authors: read, edited, and approved the final version of the manuscript. The authors declared no conflicts of interest related to this study.

4.6 Tables

Table 4.1 Participant characteristics (*n*=10)

Variable		
Age (y)	21	± 1
Weight (kg)	88	± 3
Fat (%)	16	± 1
Lean Body Mass (kg)	72	± 2
Systolic BP (mmHg)	121	± 4
Diastolic BP (mmHg)	70	± 2
Fasting Glucose (mg/dL^{-1})	76	± 1
10-RM Leg Press (kg)	242	± 32
10-RM Leg Extension (kg)	112	± 10

Data are mean ± SEMs.

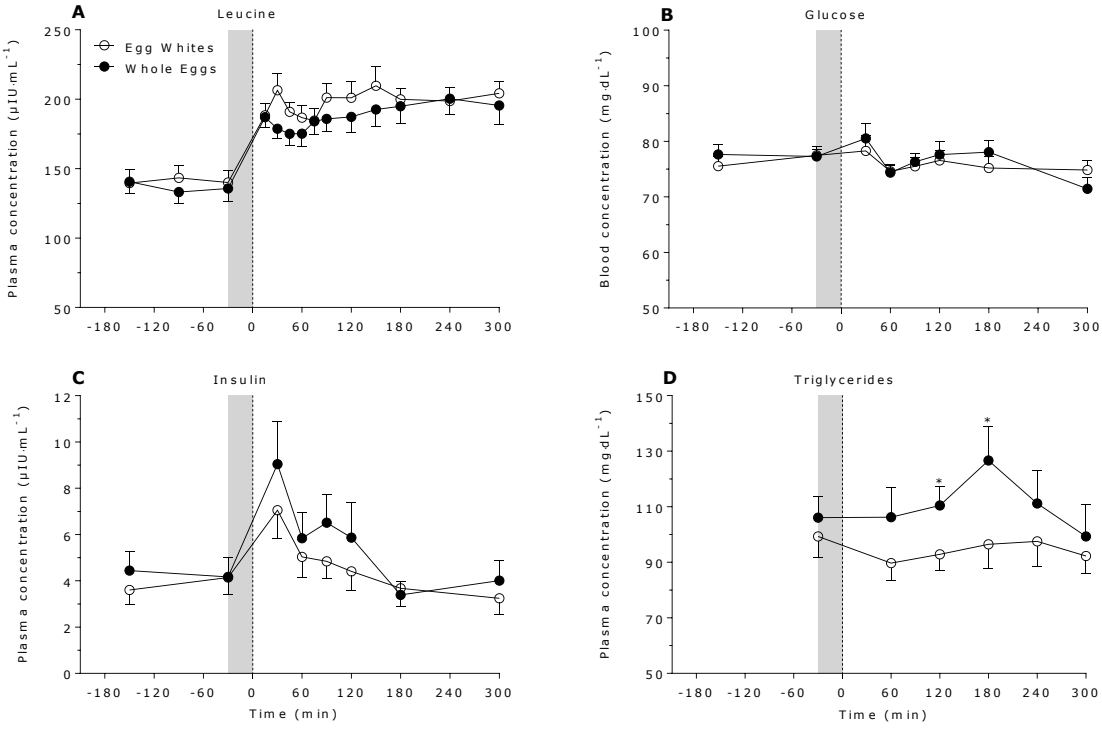
Table 4.2. Muscle intracellular free tracer enrichments before and after egg ingestion.

Tracer	-150 min (*n*=5)	-30 min (*n*=10)	120 min (*n*=10)	300 min (*n*=10)
L-[5,5,5-$^{2}H_3$]leucine				
Egg whites	0	0	3.5 ± 0.5$^{\#}$	1.4 ± 0.2$^{\#}$
Whole eggs	0	0	4.0 ± 0.4$^{\#}$	1.8 ± 0.1$^{\#}$
L-[1-^{13}C]leucine				
Egg whites	5.1 ± 0.6	5.1 ± 0.3	5.5 ± 0.5	4.8 ± 0.3
Whole eggs	4.1 ± 0.5	4.9 ± 0.3	5.1 ± 0.5	5.2 ± 0.4
L-[*ring*-$^{2}H_5$]phenylalanine				
Egg whites	0.8 ± 0.1	1.4 ± 0.1$^{\#}$	1.5 ± 0.2$^{\#}$	1.3 ± 0.1$^{\#}$
Whole eggs	0.7 ± 0.1	1.3 ± 0.1$^{\#}$	1.3 ± 0.0$^{\#}$	1.3 ± 0.1$^{\#}$

Data are mean ± SEMs. Muscle free L-[5,5,5-$^{2}H_3$]leucine-, L-[1-^{13}C]leucine-, and L-[*ring*-$^{2}H_5$]phenylalanine enrichments in MPE in the fasted state and after consumption of egg whites or whole eggs in young males. Muscle biopsies at *t*=-150 and -30 min represent the fasted-state and *t*=120 and 300 min represent the postprandial-state. Data were analyzed with a two-factor ANOVA with repeated measures on time. A Bonferroni post hoc test was used to locate differences between means for all significant interactions. L-[5,5,5-$^{2}H_3$]leucine: Time effect, $P<0.001$; Time × condition, $P=0.61$. L-[1-^{13}C]leucine: Time effect, $P=0.30$; Time × condition, $P=0.62$. L-[*ring*-$^{2}H_5$]phenylalanine (C): Time effect, $P<0.001$; Time × condition, $P=0.54$. $^{\#}$Significantly different from t=-150 min ($P<0.05$). MPE, mole percent excess.

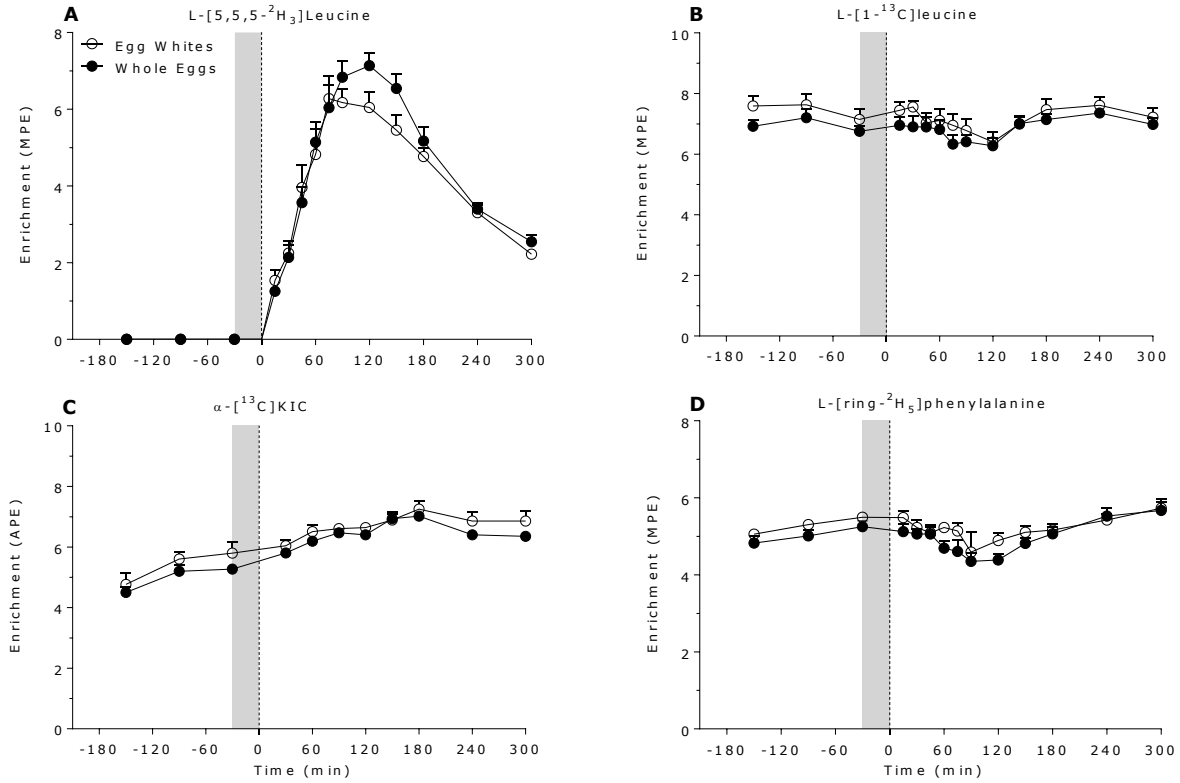
4.7 Figures

Figure 4.1



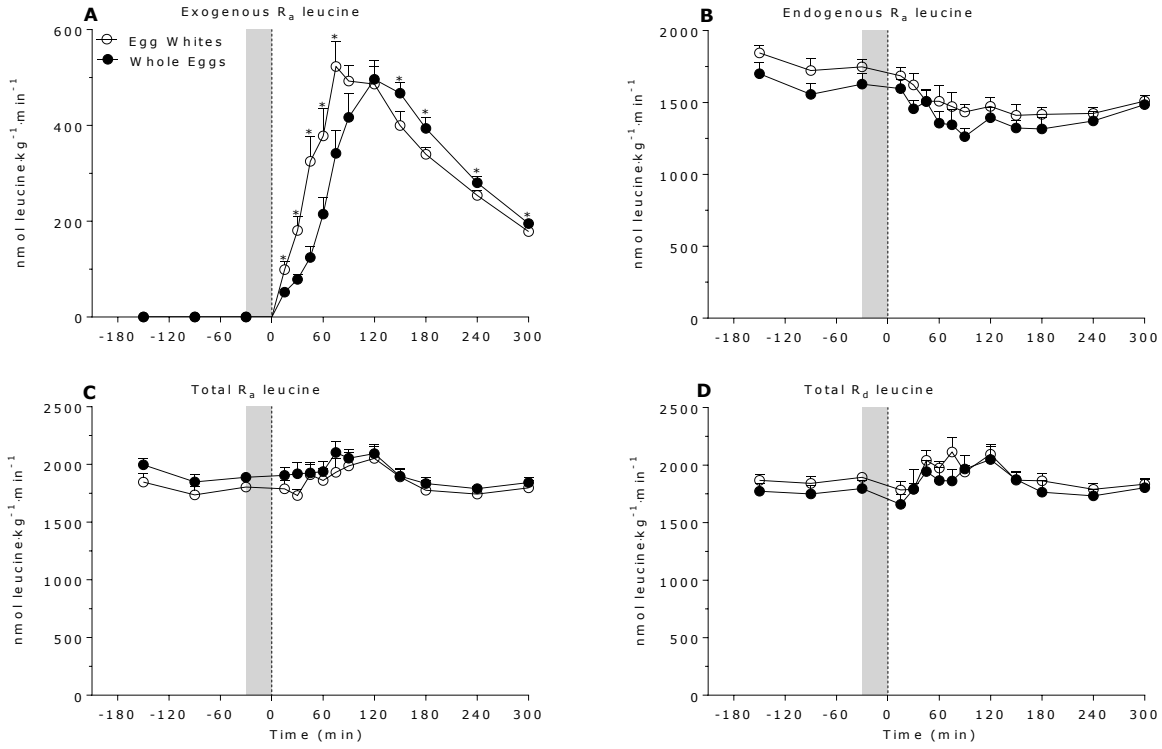
Data are mean ± SEMs. Plasma leucine (A), blood glucose (B), plasma insulin (C), and plasma triglycerides concentrations (D) in the fasted state and after consumption of egg whites or whole eggs in young males (n=10 per condition). Data are expressed as μmol·L^{-1} (A), mg·dL^{-1} (B), μIU·mL^{-1} (C), and μg·mL^{-1} (D), respectively. Gray area corresponds to the exercise bout; dashed line refers to egg ingestion. Data were analyzed with a two-factor ANOVA with repeated measures on time. A Bonferroni post hoc test was used to locate differences between means for all significant interactions. Leucine: Time effect, P<0.001; Time × condition, P=0.76. Glucose: Time effect, P=0.06; Time × condition, P=0.73. Insulin: Time effect, P<0.001; Time × condition P=0.484. Triglycerides: Time effect, P=0.007; Time × condition P=0.03. *Significantly different between egg conditions (P<0.05).

Figure 4.2



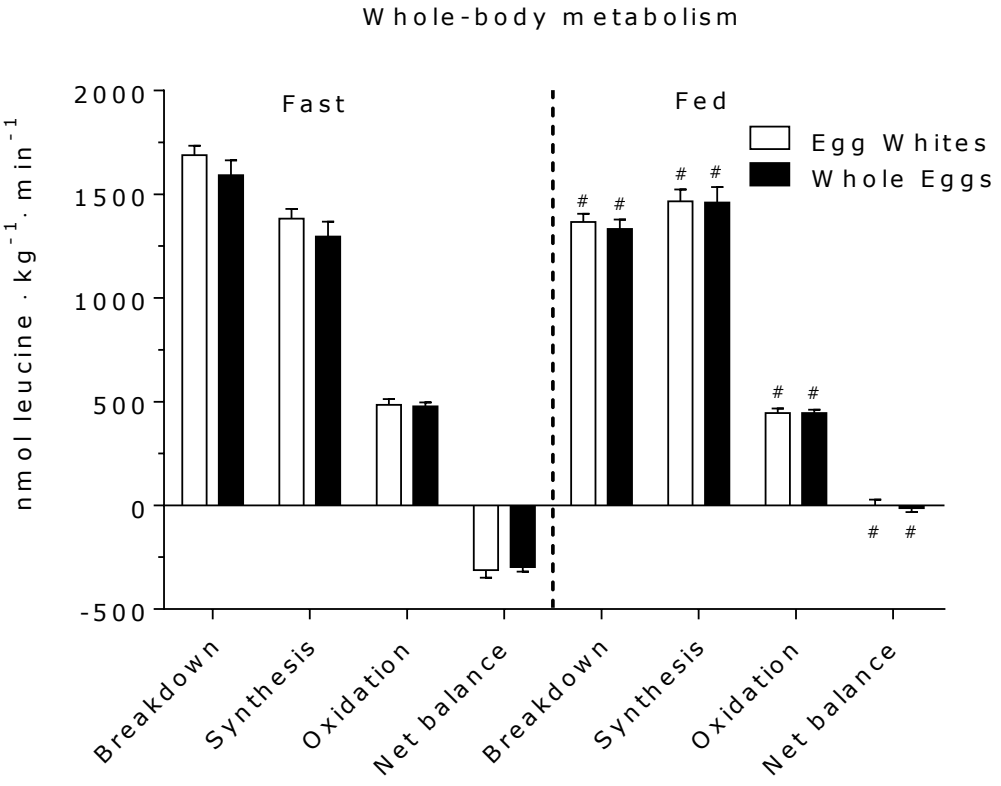
Plasma L-[5,5,5-$^{2}H_3$]leucine (A), L-[1-^{13}C]leucine (B), α-[^{13}C]-ketoisocaproate (C), and L-[*ring*-$^{2}H_5$]phenylalanine (D) enrichments in the fasted state and after consumption of egg whites or whole eggs in young males (n=10 per condition). Data are expressed as MPE (A, B, and C) and APE (D), respectively. Gray area corresponds to the exercise bout; dashed line refers to egg ingestion. Data were analyzed with a two-factor ANOVA with repeated measures on time. A Bonferroni post hoc test was used to locate differences between means for all significant interactions. L-[5,5,5-$^{2}H_3$]leucine: Time effect, P<0.001; Time × condition, P=0.37. L-[1-^{13}C]leucine : Time effect, P<0.001; Time × condition, P=0.86. α-[^{13}C]-ketoisocaproate: Time effect, P<0.001; Time × condition, P=0.78. L-[ring-$^{2}H_5$]phenylalanine: Time effect, P<0.001; Time × condition, P=0.78. MPE, mole percent excess; APE, atom percent excess.

Figure 4.3



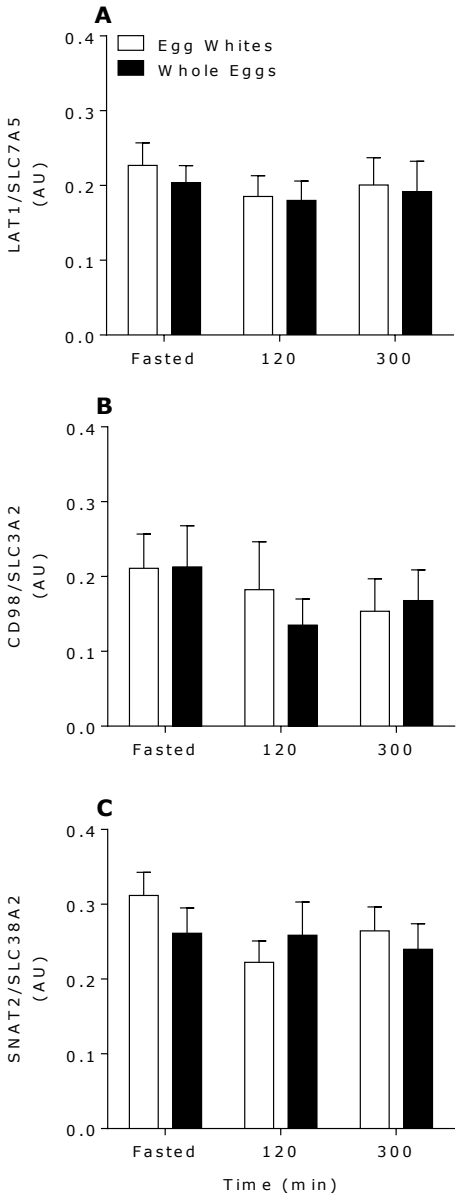
Data are mean ± SEMs. Whole body leucine kinetics over time in the fasted state and after consumption of egg whites or whole eggs in young males (n=10 per condition). Exogenous leucine R_a (A), endogenous leucine R_a (B), total leucine R_a (C), and total leucine R_d (D). Data are expressed as nmol leucine·kg^{-1}·min^{-1}. Gray area corresponds to the exercise bout; dashed line refers to egg ingestion. Data were analyzed with a two-factor ANOVA with repeated measures on time. A Bonferroni post hoc test was used to locate differences between means for all significant interactions. Exogenous R_a: Time effect, P<0.001; Time × condition, P=0.001. Endogenous R_a: Time effect, P<0.001; Time × condition, P=0.17. Total R_a: Time effect, P<0.001; Time × condition, P=0.83. Total R_d: Time effect, P<0.001; Time × condition, P=0.84. *Significantly different between egg conditions (P<0.05). R_a, rate of appearance; R_d, rate of disappearance.

Figure 4.4



Data are mean ± SEMs. Whole body leucine endogenous rate of appearance (R_a; marker of protein breakdown), non-oxidative leucine disposal (NOLD; marker of protein synthesis), leucine oxidation (oxidation) and net leucine balance expressed as the cumulative rates in the fasted state and after consumption of egg whites or whole eggs in young males (n=10 per condition). Data are expressed as nmol leucine·kg^{-1}·min^{-1}. Data were analyzed with a two-factor ANOVA with repeated measures on time. A Bonferroni post hoc test was used to locate differences between means for all significant interactions. Endogenous R_a: Time effect, P<0.001; Time × condition, P=0.31. NOLD: Time effect, P=0.031; Time × condition, P=0.87. Oxidation: Time effect, P<0.001; Time × condition, P=0.74. Net balance: Time effect, P<0.001; Time × condition, P=0.39. #Significantly different from fasted (P<0.05).

Figure 4.5

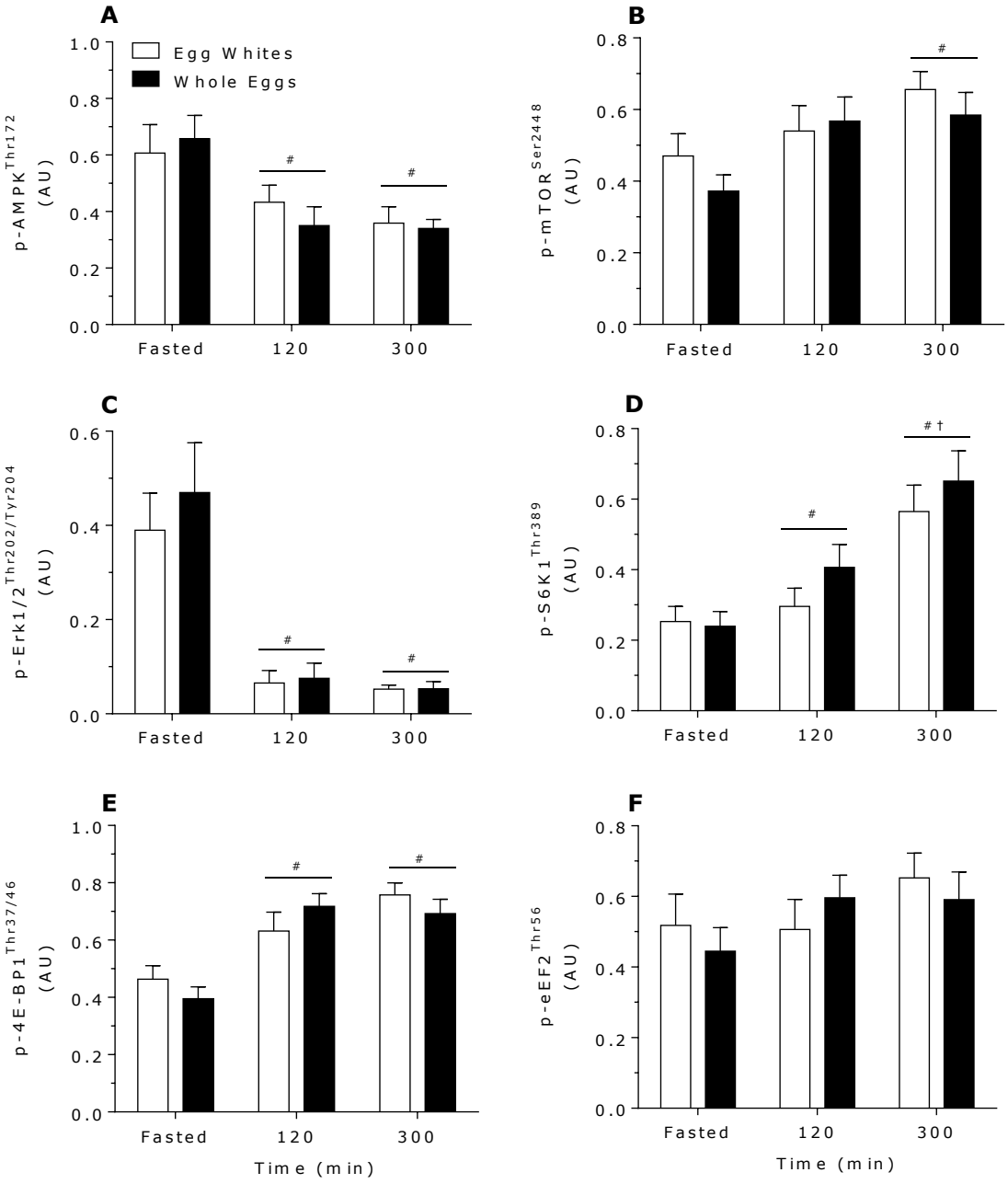


Data are mean ± SEMs. Skeletal muscle protein content of LAT1/SLC7A5 (A), CD98/SLC3A2 (B), SNAT2/ SLC38A2 (C) in the fasted state and after consumption of egg whites or whole eggs in young males (n=10 per condition). Data are expressed as arbitrary units. Data were analyzed with a two-factor ANOVA with repeated measures on time.

Figure 4.5 (cont.)

A Bonferroni post hoc test was used to locate differences between means for all significant interactions. LAT1/SLC7A5: Time effect, P=0.18; Time × condition, P=0.81. CD98/SLC3A2: Time effect, P=0.10; Time × condition, P=0.49. SNAT2/ SLC38A2: Time effect, P=0.12; Time × condition, P=0.16. LAT1, Large neutral amino acids transporter small subunit 1; SNAT2, sodium-coupled neutral amino acid transporter 2.

Figure 4.6

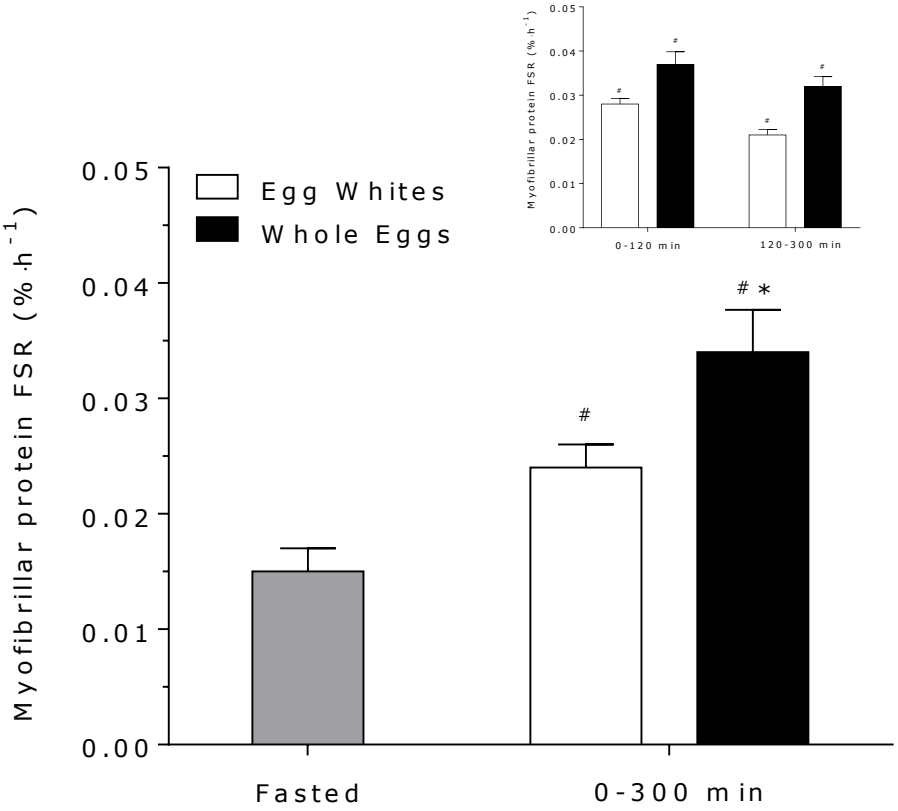


Data are mean ± SEMs. Phosphorylation status of AMPKα^{Thr172} (A), mTOR^{Ser2448} (B), ERK$^{Thr202/Tyr204}$ (C), p70S6K1^{Thr389} (D), 4E-BP1$^{Thr37/46}$ (E), and eEF2^{Thr56} (F) in the fasted state and after consumption of egg whites or whole eggs in young males (n=10 per condition).

Figure 4.6 (cont.)

Data are expressed as arbitrary units. Data were analyzed with a two-factor ANOVA with repeated measures on time. A Bonferroni post hoc test was used to locate differences between means for all significant interactions. p-AMPKα^{Thr172}: Time effect, P<0.001; Time × condition, P=0.52. mTOR^{Ser2448}: Time effect, P=0.010; Time × condition, P=0.577. ERK 1/2$^{Thr202/Tyr204}$: Time effect, P<0.001; Time × condition, P=0.64. p70S6K1^{Thr389}: Time effect, P<0.001; Time × condition, P=0.49. 4E-BP1$^{Thr37/46}$: Time effect, P<0.001; Time × condition, P=0.23. eEF2^{Thr56}: Time effect, P=0.13; Time × condition, P=0.41. $^{\#}$Significantly different from fasted (P<0.05). †Significantly different from 120 min. AMPKα, adenosine monophosphate-activated protein kinase alpha; mTORC1, mammalian target of rapamycin complex 1; ERK, extracellular signal–regulated kinases, p70S6K1, 70 kDa S6 protein kinase 1; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; eEF2, eukaryotic translation elongation factor 2.

Figure 4.7



Data are mean ± SEMs. Myofibrillar protein synthesis rates in the fasted state and after consumption of egg whites or whole eggs in young males (n=10 per condition). Data are expressed as FSR (%·h^{-1}). The inset shows the temporal responsiveness of myofibrillar protein synthesis rates during the early (0-120 min) and late (120-300 min) exercise recovery periods. Data were analyzed with a two-factor ANOVA with repeated measures on time. A Bonferroni post hoc test was used to locate differences between means for all significant interactions. FSR: Time effect, P=0.02; Time × condition, P=0.04. Inset FSR: Time effect, P=0.04; Time × condition, P=0.50. #Significantly different from fasted (P<0.05). *Significantly different between egg conditions (P<0.05). Data are Mean ± SEM. FSR, fractional synthesis rates.

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4.9 Supplemental methods

Calculations

Total, exogenous, and endogenous leucine rates of appearance (R_a), postprandial circulating protein-derived leucine availability, total leucine rate of disappearance (R_d), total leucine oxidation, non-oxidative leucine disposal (NOLD) and net balance were calculated with the use of modified Steele's equations (1, 2). The specific equations used to calculate leucine fluxes were as follows:

$$\text{Total } R_a = \frac{F_{iv} - \left[pV \times C(t)\text{leu} \times \frac{dE_{iv}}{dt}\right]}{E_{iv}(t)} \qquad (1)$$

Total R_a (Eq. 1) is the plasma appearance rate of total leucine calculated with the IV tracer (nmol · kg^{-1} · min^{-1}); where F_{iv} is the IV tracer infusion rate (e.g. 130 nmol · kg^{-1} · min^{-1} for leucine); pV (0.125 L/kg^{-1}) is the distribution volume (8); $C(t)$ is the mean plasma leucine concentration between two consecutive time points (nmol · kg^{-1}); dE_{iv}/dt represents the time-dependent variations in plasma leucine enrichment (mole percent excess) derived from the IV tracer; and $E_{iv}(t)$ is the mean plasma leucine enrichment (mole percent excess) derived from the IV tracer between two consecutive time points.

$$\text{Exo } R_a = \frac{\text{Total } R_a \times E_{po}(t) + \left[pV \times C(t)\text{leu} \times \frac{dE_{po}}{dt}\right]}{E_{prot}} \qquad (2)$$

Exo R_a (Eq. 2) is the plasma entry rate (nmol · kg^{-1} · min^{-1}) of dietary protein-derived leucine after first-pass splanchnic extraction; where $E_{po}(t)$ is the mean plasma leucine enrichment based on the

oral tracer between two consecutive time points (mole percent excess); dE_{po}/dt is the time-dependent variations of plasma leucine enrichment derived from the oral tracer (mole percent excess); and E_{prot} is the leucine tracer enrichment of the dietary protein (mole percent excess).

$$\text{Endo } R_a = \text{Total } R_a\text{-Exo } R_a\text{-}F_{iv} \quad (3)$$

Endo R_a (Eq. 3) is the plasma entry rate of endogenous leucine from whole-body protein breakdown (nmol · kg^{-1} · min^{-1}); where F_{iv} is the IV tracer infusion rate as in Eq. 1; and *Exo* R_a is the plasma entry rate of dietary protein-derived leucine as in Eq. 2.

$$\text{Leu}_{\text{plasma}} = \left(\frac{\text{AUC}_{\text{Exo Ra}}}{\text{Leu}_{\text{prot}}}\right) \times \text{BW} \times 100 \quad (4)$$

Leu_{plasma} (Eq. 4) represents the fraction of dietary leucine that appeared in the plasma (%); where AUC_{ExoRa} represents the area under the time curve (AUC) of *Exo* R_a, which indicates the amount of dietary protein-derived leucine that appeared in the circulation during the measured postprandial period; and Leu_{prot} is the amount of dietary leucine ingested (12209520 and 12005432 nmol for egg whites and whole eggs, respecitvely) and *BW* is the participants' body weight (kg).

$$\text{Total Ox} = \frac{\text{Vco}_2 \times \text{Eco}_2}{\text{E}_{\alpha\text{-KIC}}} \text{ x } \frac{1}{\text{k}} \quad (5)$$

Total Ox (*Eq. 5*) is the rate of total leucine oxidation calculated with the IV tracer (nmol · kg^{-1} · min^{-1}); where V_{CO2} is CO_2 production (nmol · kg^{-1} · min^{-1}); E_{CO2} is ^{13}C enrichment in expired CO_2; $E_{\alpha\text{-KIC}}$ is plasma ^{13}C-α-KIC enrichment, which best represents the precursor for irreversible

decarboxylation of leucine (29); and *k* is the fractional bicarbonate retention factor correcting for incomplete recovery of $^{13}CO^2$ in breath with chosen values of 0.7 and 0.83 for the fasted and fed state, respectively (22, 42).

$$R_d = \text{Total } R_a\text{-}(pV \text{ x } dC/dt) \quad (6)$$

Total R_d (Eq. 6) is the plasma leucine disappearance rate of calculated with the IV tracer (nmol · kg^{-1} · min^{-1}); where dC/d*t* is the difference in plasma leucine concentration between two consecutive time points; and *pV* is the distribution volume as in Eq. 1.

$$\text{NOLD} = \text{Total } R_d\text{-Total Ox} \quad (7)$$

NOLD (Eq. 7) is the plasma disappearance rate of total leucine used for protein synthesis (nmol · kg^{-1} · min^{-1}); where *Total R_d* is the plasma disappearance rate of total leucine as in Eq 6.; and T*otal Ox* is the rate of total leucine oxidation as in Eq. 5.

$$\text{Net Balance} = \text{NOLD -Endo } R_a \quad (8)$$

Net Balance (Eq. 8) is the difference between *NOLD* (i.e. protein synthesis) as in Eq 7. and *Endo R_a* (i.e. protein breakdown) as in Eq. 3.

$$\text{Leu}_{\text{retention}} = \text{Leu}_{\text{prot}} + F_{iv} \text{ - Total Ox} \quad (9)$$

$Leu_{retention}$ (Eq. 9) represents the leucine intake (meal and IV infusion) that is retained in the body ($nmol \cdot kg^{-1} \cdot min^{-1}$); where Leu_{prot} is the amount of dietary leucine ingested as in Eq. 4., under the assumption that all dietary leucine ingested is effectively absorbed; F_{iv} is the IV tracer infusion rate as in Eq. 1; and *Total Ox* is the rate of total leucine oxidation as in Eq. 5.

4.10 Supplemental tables

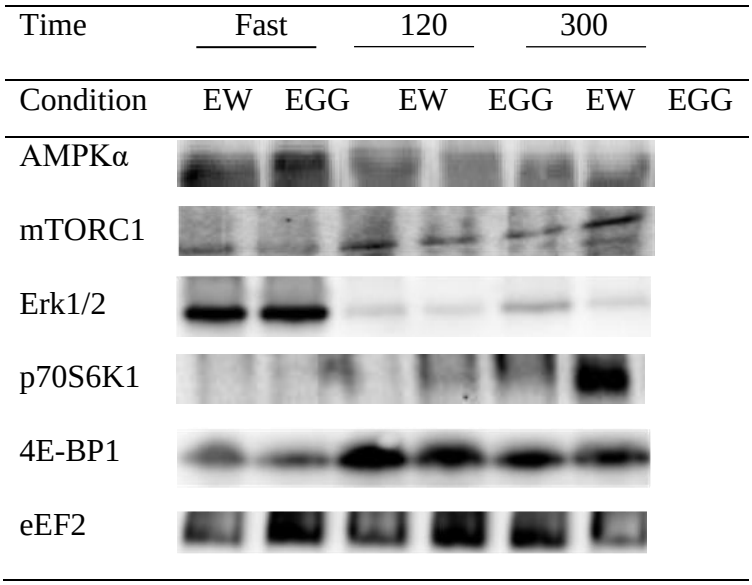
Supplemental table 4.1 Self-reported dietary intake during 2d prior to infusion trials.

	Whole eggs (*n*=10)			Egg whites (*n*=10)			P-value
Energy (kcal/d^{-1})	2705	±	163	2827	±	88	0.16
Protein (g/kg^{-1} body weight ·d^{-1})	1.9	±	0.2	2.0	±	0.2	0.38
Carbohydrate (g/kg^{-1} body weight ·d^{-1})	3.5	±	0.3	3.6	±	0.3	0.20
Fat (g/kg^{-1} body weight ·d^{-1})	1.0	±	0.2	1.1	±	0.2	0.22

Data are mean ± SEMs.

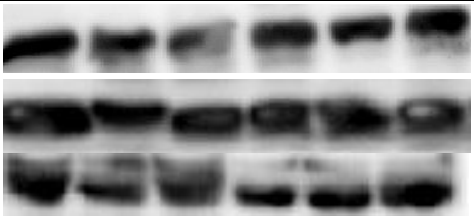
4.11 Supplemental figures

Supplemental figure 4.1



Representative blots for the phosphorylation status of AMPKα^{Thr172}, mTOR^{Ser2448}, ERK$^{Thr202/Tyr204}$, P70S6K1^{Thr389}, 4E-BP1$^{Thr37/46}$, eEF2^{Thr56} in the fasted state (t=-30 min) and after consumption (t=120 and 300 min) of egg whites or whole eggs during post-exercise recovery in young men. AMPKα, adenosine monophosphate-activated protein kinase alpha; mTORC1, mammalian target of rapamycin complex 1; ERK, extracellular signal–regulated kinases, p70S6K1, 70 kDa S6 protein kinase 1; 4E-BP1, eukaryotic translation initiation factor 4E-binding protein 1; eEF2, eukaryotic translation elongation factor 2.

Supplemental figure 4.2

Time	Fast		120		300	
Condition	EW	EGG	EW	EGG	EW	EGG
LAT1						
CD98						
SNAT2						

Representative blots for total protein content of LAT1, CD98, and SNAT2 in the fasted state (t=-30 min) and after consumption (t=120 and 300 min) of egg whites or whole eggs in young men. Large neutral amino acids transporter small subunit 1; SNAT2, sodium-coupled neutral amino acid transporter 2.

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CHAPTER 5: DIFFERENTIAL WHOLE BODY AND SKELETAL MUSCLE PROTEIN METABOLIC RESPONSES TO MIXED MEAL INGESTION IN MAINTENANCE HEMODIALYSIS PATIENTS[1]

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Key words: chronic kidney disease, aging, protein digestion, amino acids.

Abbreviations used: CKD, chronic kidney disease; ESRD, end stage renal disease, Endo, endogenous; Exo, exogenous; FFM, fat free mass; FSR, fractional synthetic rate; GC/MS, gas chromatography-mass spectrometry; LC/MS/MS, liquid chromatography-tandem mass spectrometry; MHD, maintenance hemodialysis R_a, rate of appearance; R_d, rate of appearance.

[1]All co-authors agreed to their contributions being included in the dissertation.

5.1 Abstract

Background: Maintenance hemodialysis (MHD) patients experience substantial skeletal muscle wasting. Thus, the attenuation of muscle mass loss is mandatory for the maintenance of physical performance and improved quality of life. The regular ingestion of protein-rich meals are recommended to offset this skeletal muscle protein loss. However, the protein metabolic responses before and after food ingestion remains undefined in MHD patients. **Objective:** To provide insight into whole body and muscle protein metabolism before and after food ingestion in MHD patients on a non-dialysis day. **Design:** Eight maintenance hemodialysis (MHD) patients (age: 56±5 y: BMI: 32±2) and eight controls (age: 50±2 y: BMI: 31±1) received primed continuous L-[*ring*-$^{2}H_{5}$]phenylalanine- and L-[1-^{13}C]leucine infusions. Participants consumed a mixed meal (546 kcal; 20 g protein, 59 g carbohydrates, 26 g fat) with protein provided as L-[5,5,5-$^{2}H_{3}$]leucine labeled eggs. Breath, blood, and muscle biopsies were collected to determine whole body amino acid kinetics and myofibrillar protein synthesis rates. **Results:** Postprandial dietary-amino acid availability in circulation tended to be reduced in MHD patients (43 ± 5%) vs. controls (56 ± 5%) (P=0.06). Breath $^{13}CO_2$ enrichments (indicative of leucine oxidation) were lower in MHD patients vs. controls (P=0.02). Basal myofibrillar protein synthesis rates were ~ 2.5-fold higher in MHD patients (0.032 ± 0.005 %·h^{-1}) vs. controls (0.014 ± 0.002 %·h^{-1}) (P=0.02). Meal ingestion failed to increase myofibrillar protein synthesis rates in MHD patients (-0.001 ± 0.008 %·h^{-1}) and controls (absolute change from basal: 0.006 ± 0.004 %·h^{-1}) (P=0.58). **Conclusion:** Basal-state muscle protein synthesis rates are elevated in MHD patients and protein-rich meal consumption did not elicit a postprandial rise in muscle protein synthesis rates in MHD patients, which may be attributed to reduced dietary amino acid availability after eating a meal.

5.2 Introduction

Chronic kidney disease (CKD) is estimated to be present in 11-13% of the overall global population (17) making it an important public health problem. Moreover, the prevalence of CKD is expected to further increase in the next decades (3) due to increasing incidences of lifestyle diseases such as hypertension, obesity and diabetes, which are leading causes for the development of renal failure (24). The most severe form of chronic renal failure is end-stage-renal-disease, with maintenance hemodialysis (MHD) as the most common treatment strategy. MHD patients experience a number of metabolic derangements including skeletal muscle wasting (48). MHD patients lose ~1-3 kg muscle mass annually (32), resulting in reduced physical function (21) and increased risk for morbidity and mortality (16). Thus, preventative strategies to attenuate muscle mass loss are mandatory to improve the quality of life in MHD patients. The underlying mechanisms of skeletal muscle wasting in MHD patients are likely multifactorial and include: chronic inflammation, reduced protein intake, metabolic acidosis, insulin resistance, hormonal abnormalities, increased substrate oxidation, and a loss of nutrients/amino acids during dialysis (47).

In healthy individuals, the maintenance of skeletal muscle mass is primarily determined by the postprandial stimulation of muscle protein synthesis after each meal. However, the effects of food ingestion on the stimulation of the postprandial muscle protein synthetic response in MHD patients remains to be firmly established. It has been demonstrated that dialysis increased basal-state muscle protein synthesis rates with an even greater elevation in protein breakdown rates (19, 34). As a result, this negative net protein balance induced by the dialysis procedure results in net skeletal muscle- and whole body catabolism (19). A troublesome issue for the maintenance of lean body mass (muscle) is that skeletal muscle- and whole body catabolism persists for several hours

after dialysis (19), suggesting prolonged post-dialysis disturbances in whole body- and skeletal muscle protein metabolism in MHD patients.

What is noteworthy is that the dialysis procedure itself results in ~20 % losses of circulating amino acids in the dialysate (14, 18), thus creating a need for replacement of amino acids by protein supplementation during and/or after dialysis. It is promising that intradialytic protein feeding can mitigate some of the amino acid losses on a whole body- (31, 45) and skeletal muscle level (31). However, due to concerns regarding patient safety and staff burden, meal consumption during dialysis is not common practice in the US (22). Therefore, consumption of protein-dense meals in between dialysis treatments represents an alternative dietary strategy to limit skeletal muscle protein loss in MHD patients. Besides the dialysis procedure itself, impaired protein metabolism resulting from a chronic inflammatory state (40), acidosis (26), increased substrate oxidation (4) and/or chronic illnesses (e.g., insulin resistance (35), and obesity (5)) may pose further challenges for the improvement of skeletal muscle health in this patient population. To limit losses in skeletal muscle mass, protein intakes of ≥ 1.2 g/kg^{-1} bodyweight/d^{-1} are often recommended (1). However, the effectiveness of protein-rich meal consumption on skeletal muscle and whole body protein turnover in MHD patients outside of the dialysis period is not well-defined.

Therefore, the purpose of this study was to characterize the whole body and muscle protein metabolic responses after mixed meal ingestion on a non-dialysis day in MHD patients and compare these responses to control subjects. To accomplish this aim, we combined contemporary stable isotope tracer methodology with the ingestion of intrinsically L-[5,5,5-$^{2}H_3$]leucine-labeled protein (44) to allow for the detailed assessment of postprandial protein handling in MHD patients and controls.

5.3 Methods

Participants and ethical approval

Eight maintenance hemodialysis (MHD) patients (mean ± SEM: age: 56±5 y: BMI: 32±2) and eight controls volunteered for this study (mean ± SEM: age: 50±2 y: BMI: 31±1). The participants were matched for age, sex, BMI and insulin resistance (as defined by HOMA IR). Full participant characteristics are presented in **Table 1**. MHD patients and controls subjects were deemed eligible based on responses to a routine medical screening questionnaire, routine blood panels and an in-person interview by the researchers. All participants were informed about the experimental procedures to be used, the purpose of the study, and all potential risks before giving written consent and had no prior of participating in stable isotope amino acid tracer experiments. For MHD patients, we requested physician clearance from their nephrologist to further ensure it was safe for the patient to participate in this study. The study was approved by the Institutional Review Board at University of Illinois at Urbana-Champaign and conformed to standards for the use of human participants in research as outlined in the seventh revision of the Declaration of Helsinki.

Screening protocol

Body weight and height were measured as well as body composition by dual-energy X-ray absorptiometry (Hologic QDR 4500A, Bedford, MA, USA). Participants were instructed to refrain from vigorous physical activity for 3 d prior to each trial and maintain their habitual dietary pattern. Furthermore, MHD patients were instructed to maintain daily medication as prescribed by their health-care provider. A single exception one diabetic MHD patient, who refrained from insulin injection during the infusion trial. The classes of medication were as follows: phosphatase binders (number of patients: 8), calcimimetics (8), ACE inhibitors (6), NSAIDs (6), beta-blockers (4),

calcium channel blockers (4), diuretics (4), statins (4), proton pump inhibitors (3), xanthine oxidase inhibitors (anti-gout) (3), opioids (3), vasodilators (2), P2Y12 (platelet) inhibitors (2), serotonin-reuptake inhibitor (anti-depressant) (2), anti-histamine (1), prokinetic (1), immunosuppressant (1).

All participants consumed a standardized meal of the same composition (320 kcal; 22 g protein, 43 g carbohydrates, 7 g fat) the evening prior to the infusion trial, after which participants were instructed to remain fasted. A 2-d dietary-recall was performed using dietary analysis software (Nutritionist Pro^{TM}, Version 2.1.13, Axxya Systems, Redmond, WA). Average 2-d protein intake tended to be lower in MHD patients (0.9 ± 0.14 g·kg^{-1} bodyweight·d^{-1}) when compared to control subjects (1.14 ± 0.05 g·kg^{-1} bodyweight·d^{-1}) (*P*=0.07) with no differences in other macronutrients (all, P>0.05) (**Supplemental Table 1**).

Infusion protocol

On the day of the infusion trial, participants reported to the laboratory at 0700 h after an overnight fast. MHD patients were studied during a day without dialysis, having dialyzed the day before. At the start of the trial, a Teflon catheter was inserted into an antecubital vein for baseline blood sample collection (*t*=-180 min) after which participants received priming doses of $NaH^{13}CO_2$ (2.35 μmol·kg^{-1}), L-[1-^{13}C]leucine (7.6 μmol·kg^{-1} FFM), and L-[*ring*-$^{2}H_5$]phenylalanine (2.0 μmol·kg^{-1} FFM). Subsequently, a continuous intravenous infusion of L-[1-^{13}C]leucine (0.10 μmol·kg^{-1} FFM ·min^{-1}) and L-[ring-$^{2}H_5$]phenylalanine (0.05 μmol·kg^{-1} FFM·min^{-1}) was initiated (*t*=-180 min) and maintained over the infusion trial. A second Teflon catheter was inserted into a heated dorsal hand vein of the same arm for repeated arterialized blood sampling and remained patent by a 0.9% saline drip. In the postabsorptive state of the infusion trial, muscle biopsies were collected at *t*=-120 and -0 min of infusion to determine fasting

myofibrillar protein synthesis rates. After collection of the basal muscle biopsy at *t*=-0 min, participants consumed a mixed macronutrient meal containing 20 g of dietary protein (see section below for further details). Completion of the meal marked the start of the postprandial phase (*t*=0 min) and an additional muscle biopsy was collected 300 min to determine postprandial myofibrillar protein synthesis rates. Biopsies were collected from the middle region of the *vastus lateralis* (15 cm above the patella) with a Bergström needle modified for suction under local anesthesia (2% Lidocaine). The postabsorptive muscle biopsies were collected from the same incision with the needle pointed to distal and proximal directions, respectively. The postprandial biopsy was collected through a separate incision 2-3 cm above the postabsorptive incision. All biopsy samples were freed from any visible blood, adipose, and connective tissue immediately frozen in liquid nitrogen, and stored at -80˚C until subsequent analysis. Arterialized blood samples and were collected every 30 or 60 min during the postabsorptive- and postprandial states. The blood samples were immediately analyzed for whole blood glucose concentrations (2300 Stat Plus, YSI Life Sciences, Springs, OH) and subsequently centrifuged at 3000 × g for 10 min at 4°C. Aliquots of plasma were frozen and stored at -80°C until subsequent analysis.

Meal composition

Participants ingested a meal consisting of three scrambled L-[5,5,5-$^{2}H_3$]leucine labeled eggs, one slice of toasted white bread, 300 ml of apple juice, and 10 grams of cow butter (546 kcal; 20 g protein, 59 g carbohydrates, 26 g fat). We chose this meal as this is a typical breakfast that is recommended for this patient population (2). The intrinsically L-[5,5,5-$^{2}H_3$]leucine labeled eggs were produced by supplementing the diet of laying hens (Lohmann LSL Whites) with 0.3% L-[5,5,5-$^{2}H_3$]leucine as described previously (44). The L-[5,5,5-$^{2}H_3$]leucine enrichment of the eggs

determined by gas chromatography mass spectrometry (GC-MS) and averaged 13.1 mole percent excess (MPE). Proximate analyses for protein, lipid, and carbohydrate concentrations of the eggs were determined by using the combustion method (method 990.03; AOAC International, 2000; TruMac; LECO Corp., USA) (44). For the other foods, macronutrient composition was determined from their respective food labels. Leucine content of the whole eggs (1.6 g) were determined by GC-MS with integration of amino acid peak areas compared to an internal standard (DL-p-chlorophenylalanine) using the AMDIS software package (v. 2.71; NIST) (20).

Blood analyses

Blood metabolites were determined using a point-of-care chemistry analyzer (Piccolo Xpress Chemistry Analyzer, Abaxis, Union City, CA). Plasma insulin concentrations were determined using a commercially available enzyme-linked immunosorbent assays (Alpco Diagnostics; Salem, NH). Plasma amino acid concentrations and enrichments were determined by GC-MS analysis (Agilent 7890A GC/5975C; MSD, Little Falls, DE) as described in our previous work (44). Briefly, plasma samples were prepared for amino acid analysis using a mixture of isopropanol:acetonitrile:water (3:3:2, v/v) and centrifuged at 20000 × g for 10 min at 4ºC. Subsequently, the supernatant was dried and the amino acids converted into tert-butyldimethylsilyl (*t*-BDMS) derivatives prior to GC-MS analysis. Plasma L-[1-^{13}C]leucine and L-[5,5,5-$^{2}H_3$]leucine enrichments were determined by ion monitoring at mass/charge (*m/z*) ratios 302 (*m*+0), 303 (*m*+1), and 305 (*m*+3) with *m*+0 representing the lowest molecule weight of the ion or unlabeled leucine. For L-[*ring*-$^{2}H_5$]phenylalanine, *m/z* 336 (*m*+0) and 341 (*m*+5) were monitored for unlabeled and labeled phenylalanine, respectively. The plasma leucine concentrations were determined by

integrating amino acid peak areas in comparison to U-[$^{13}C_6$]leucine as an internal standard with the use of the AMDIS software package (v. 2.71; NIST) (20).

Muscle analysis

Myofibrillar protein-enriched fractions were isolated from ~50 mg of wet tissue as described previously (44). Myofibrillar-enriched protein pellets were hydrolyzed overnight in 6 M HCL at 110°C. The resultant free amino acids were purified using cation exchange chromatography (Dowex 50W-X8-200 resin; Acros Organics, Geel, Belgium) and dried under vacuum. Free amino acids were re-suspended in 60% methanol and centrifuged before the myofibrillar protein-bound enrichments were determined by 5500 QTRAP liquid chromatography-tandem mass spectrometry (LC/MS/MS; Sciex, Framingham, MA). The myofibrillar protein-bound L-[ring-$^2H_5$]phenylalanine enrichments were determined by multiple reaction monitoring (MRM) at *m/z* 166.0 → 103.0 and 171.0 → 106.0 for unlabeled and labeled L-[ring-$^2H_5$]phenylalanine, respectively. Sciex Software Analyst 1.6.2 was used for data acquisition and analysis.

Calculations

Whole-body leucine kinetics were assessed under non-steady conditions by the ingestion of L-[5,5,5-$^2H_3$]leucine eggs combined with intravenous infusion of L-[1-^{13}C]leucine. Total, exogenous, and endogenous leucine rates of appearance (R_a) were calculated with the use of modified Steele equations (7, 11). Myofibrillar protein fractional synthesis rates (FSR) were calculated using the standard precursor-product equation by dividing the increment in L-[*ring*-$^2H_5$]-phenylalanine enrichment in the myofibrillar protein pool by the weighted average of L-[*ring*-$^2H_5$]-phenylalanine enrichment in the plasma precursor pool over time.

Statistics

A parallel design was used for this study. Differences in time-dependent blood and muscle measurements were tested by 2-factor (time × group) repeated-measures ANOVA. Differences in participant characteristics, dietary intakes and the fraction of dietary protein derived leucine that appeared into plasma were tested by unpaired *t*-test. When statistically significant interaction effects were identified in the ANOVA, Bonferroni post hoc tests were performed to determine the differences between means for all significant main effects and interactions. For all analysis, differences were considered statistically significant at $P<0.05$. All calculations were performed using IBM SPSS Statistics (version 24, Chicago, IL). All data are expressed as mean ± SEMs.

5.4 Results

Metabolites and tracer enrichments

Plasma leucine concentrations after meal consumption increased (time effect: $P<0.001$) with no differences between MHD patients and controls (time × group: $P=0.59$) (**Figure 1A**). However, the area-under-the-curve (AUC) of postprandial leucine concentrations was higher in controls when compared to MHD patients ($P=0.05$). Peak postprandial plasma leucine concentrations were observed at 180 min (243 ± 15 $\mu mol \cdot L^{-1}$) and 240 min (275 ± 23 $\mu mol \cdot L^{-1}$) after meal consumption in MHD patients and controls, respectively **(Figure 1A).** Plasma glucose concentrations increased after meal consumption (time effect: $P<0.001$) to a greater extend in MHD patients when compared to controls (time × group: $P=0.05$) **(Figure 1B).** Plasma insulin concentrations increased after meal ingestion (time: $P<0.001$) with no differences between MHD patients and controls (time × group: $P=0.29$) **(Figure 1C)**.

Plasma L-[5,5,5-$^{2}H_3$]leucine enrichment increased after meal ingestion (time effect: P<0.001) with no differences between MHD patients and controls (time × group: P=0.38) (**Figure 2A**). Plasma L-[1-^{13}C]leucine enrichments remained steady throughout the trial (time effect: P=0.50) with no differences between MHD patients and controls (time × group: P=0.64) (**Figure 2B**). Breath $^{13}CO_2$ enrichments (indicating the use irreversible loss of leucine to oxidation) increased after meal ingestion (P<0.001) and with no differences between MHD patients and controls (time × group: P=0.39) (**Figure 2B**). However, the area-under-the-curve (AUC) of breath $^{13}CO_2$ enrichments was higher in controls when compared to MHD patients (P=0.02). Plasma L-[*ring*-$^{2}H_5$]phenylalanine enrichments decreased after meal consumption (P<0.01) with no differences between groups (time × group: P=0.30) (**Figure 2D**).

Whole body leucine kinetics

Exogenous leucine rates of appearance (representing the appearance of dietary protein-derived leucine into circulation) increased after meal consumption (time effect: P<0.001) and tended to be lower in MHD patients when compared to controls (time × group: P=0.10) (**Figure 3A**). Peak exogenous leucine appearance rates after meal ingestion were observed at 120 min (409 ± 62 nmol·kg^{-1}·min^{-1}) and 180 min (479 ± 54 nmol·kg^{-1}·min^{-1}) for MHD patients and controls, respectively. Cumulative dietary-derived leucine availability tended to be reduced in MHD patients (43 ± 5%) when compared to controls (56 ± 5 %) (P=0.06) (**Inset Figure 3A**). Endogenous leucine rates of appearance (representing the appearance of leucine derived from whole-body protein breakdown into circulation) remained unchanged after meal consumption (time effect: P=0.20) with no differences between MHD patients and controls (time × group: P=0.55) (**Figure**

3B). Total leucine rates of appearance remained unchanged after meal consumption (time effect: *P*=0.79) with no differences between conditions (time × group: *P*=0.95) (**Figure 3C**).

Myofibrillar protein synthesis rates

Basal rates of myofibrillar protein synthesis were ~ 2.5-fold higher in MHD patients (0.032 ± 0.005 %·h^{-1}) when compared to controls (0.014 ± 0.002 %·h^{-1}) (group effect: *P*=0.02) (**Figure 4**). However, meal ingestion did not increase myofibrillar protein synthesis rates in MHD patients (absolute change from basal: -0.001 ± 0.008 %·h^{-1}) or controls (absolute change from basal: 0.006 ± 0.004 %·h^{-1}) (time effect: *P*=0.58; time × group: *P*=0.33) (**Figure 4**).

5.5 Discussion

MHD patients suffer substantial muscle mass loss (32), which impairs physical performance and metabolic health. As such, preventative strategies are required to attenuate muscle mass loss in MHD patients to improve their quality of life. In healthy people, protein ingestion is a main anabolic stimuli to skeletal muscle tissue. However, the whole body and muscle protein metabolic responses before and after food ingestion remains undefined in MHD patients. In this study, we demonstrated that basal-state myofibrillar protein synthesis rates are ~2.5-fold elevated in MHD patients when compared with controls. In addition, we show impairments in postprandial protein handling after the consumption of a protein- and nutrient dense meal in MHD patients. Thus, both defects in post absorptive and postprandial protein metabolic responses are likely contributing to the muscle mass loss in MHD patients.

Previously, it was demonstrated that dialysis results in prolonged whole body and skeletal muscle catabolism that persists for several hours after dialysis (19). The elevated basal rates of myofibrillar protein synthesis observed in the current study are likely indicative of a prolonged

hypercatabolic-state that is carried over to non-dialysis days (12, 33). For example, elevated post absorptive muscle protein synthesis rates are commonly observed in hyper-catabolic situations such as burn patients (30). The excessive muscle proteolysis results in an increased intramuscular amino acid availability to support the elevated basal muscle protein synthesis rates in burn patients (6). Indeed, we did not directly measure muscle protein breakdown rates in this study. However, other reports suggested elevated basal-state skeletal muscle proteolysis in MHD patients (9). In particular, the presence of systemic low-grade chronic inflammation (15) and the use of statins have been demonstrated to increase skeletal muscle protein degradation rates (43) both of which were common in the studied MHD patients (**table 1**). Again, the intracellular amino acids derived from excessive proteolysis could provide a further explanation for the elevated muscle protein synthesis rates in the basal-state in MHD patients vs. controls (6).

As a result of renal failure, skeletal muscle protein degradation increases and becomes the primary endogenous source of amino acids (8) to support protein turnover in vital organs such as the liver, gut and heart (38). Therefore, increased protein intake ($\geq$ 1.2 g/kg^{-1} bodyweight/d^{-1}) is often recommended for MHD patients to offset these protein losses (1). Interestingly, we show a tendency for reduced dietary protein derived leucine release into circulation in MHD patients vs. controls. Moreover, the recovery of breath ^{13}C (indicative of postprandial leucine oxidation) was significantly lower in MHD patients vs. controls. The lower postprandial protein derived amino acid availability in circulation in MHD patients (43 ± 5%) vs controls (56 ± 5%) may relate to increased splanchnic sequestration of amino acids. Specifically, MHD patients are commonly characterized by several splanchnic abnormalities such as intestinal small bacterial overgrowth (42), gastrointestinal inflammation (39), gut motility disorders (41), hypergastrinemia (27), and increased hepatic protein synthesis (34). In any case, we show for the first time that dietary protein

digestion and absorption kinetics are impaired in this patient population, which is limiting amino acid availability to skeletal muscle tissue. This provides further support that high(er) protein intakes may be required to compensate for these impairments in protein digestion and absorption to maximize dietary amino acid availability in MHD patients.

In this study, we assessed whole-body protein proteolytic responses in the basal- and postprandial-states in MHD patients. Previously, Veenemans *et al.* (45) demonstrated that protein consumption on a non-dialysis day improved whole body protein turnover through reductions in protein breakdown. However, we did not observe a reduction in whole body protein breakdown in our study. Dissociation between measurements of whole body- and skeletal muscle protein metabolism in MHD patients is not uncommon (19, 34). In particular the proteolytic response is often aggravated on the level of the muscle, pertaining to the important role of this organ to supply amino acids to other vital organs with chronic renal failure (13).

It is noteworthy that in this study we provided a breakfast meal (containing 20 g of dietary protein) that is typically recommended for the MHD patient population (2). However, the lack of inhibition in postprandial whole body protein breakdown rates after meal ingestion in the control group suggests that the protein amount provided was not optimal. It has been shown that ingested protein amounts of >35 g are necessary to inhibit whole protein breakdown rates (25) and stimulate muscle protein synthesis (36) in healthy aging populations. Unfortunately, such high intakes of dietary protein are generally not achieved by MHD patients (37). Thus, there is a need for alternative strategies to improve the postprandial muscle protein synthetic response to the consumption of a protein containing meal. Given the ability of exercise to enhance sensitivity of muscle protein synthesis rates to the anabolic properties of amino acids (10), it seems that exercise prior to food ingestion represents a logical adjunct strategy to nutritional intake. In particular, the

exercise-induced enhancement in postprandial muscle protein synthesis rates (28) may result in an improved net protein balance and potentially mitigate some of the muscle protein losses in MHD patients.

From a study design standpoint, muscle protein synthesis rates have been shown to be influenced by a number of factors such as: age (46), obesity (5), insulin resistance (35), metabolic acidosis (26), and habitual physical activity level (29). To rule out the influence of several non-uremic factors on protein synthesis, we intentionally recruited sedentary controls that were matched for age, gender, and presence of obesity and insulin resistance. In addition, the MHD patients in this study were corrected for metabolic acidosis through routine bicarbonate supplementation.

In conclusion, we demonstrated that basal-state muscle protein synthesis rates are elevated in MHD patients when compared to controls. Given that these measurement were made on a non-dialysis day, this highlights there is a hyper-catabolic state that is maintained well beyond the dialysis period. Furthermore, we demonstrated that consumption of a protein-rich meal by MHD patients did not elicit a postprandial rise in muscle protein synthesis rates. This finding can be explained by impairments in protein digestion and absorption and subsequent reductions in dietary amino acid availability in circulation for the stimulation of muscle protein synthesis rates. Furthermore, the presence of several co-morbidities such as inflammation, and the use of medication such as statins could have further negatively impacted protein metabolism in MHD patients. Skeletal muscle maintenance is one of the most important predictors of survival in this population (23). Therefore, future strategies involving the consumption of greater amounts of dietary protein and/or increases in physical activity should become a focus of treatment strategies to improve the protein metabolic responses in MHD patients.

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5.7 Tables

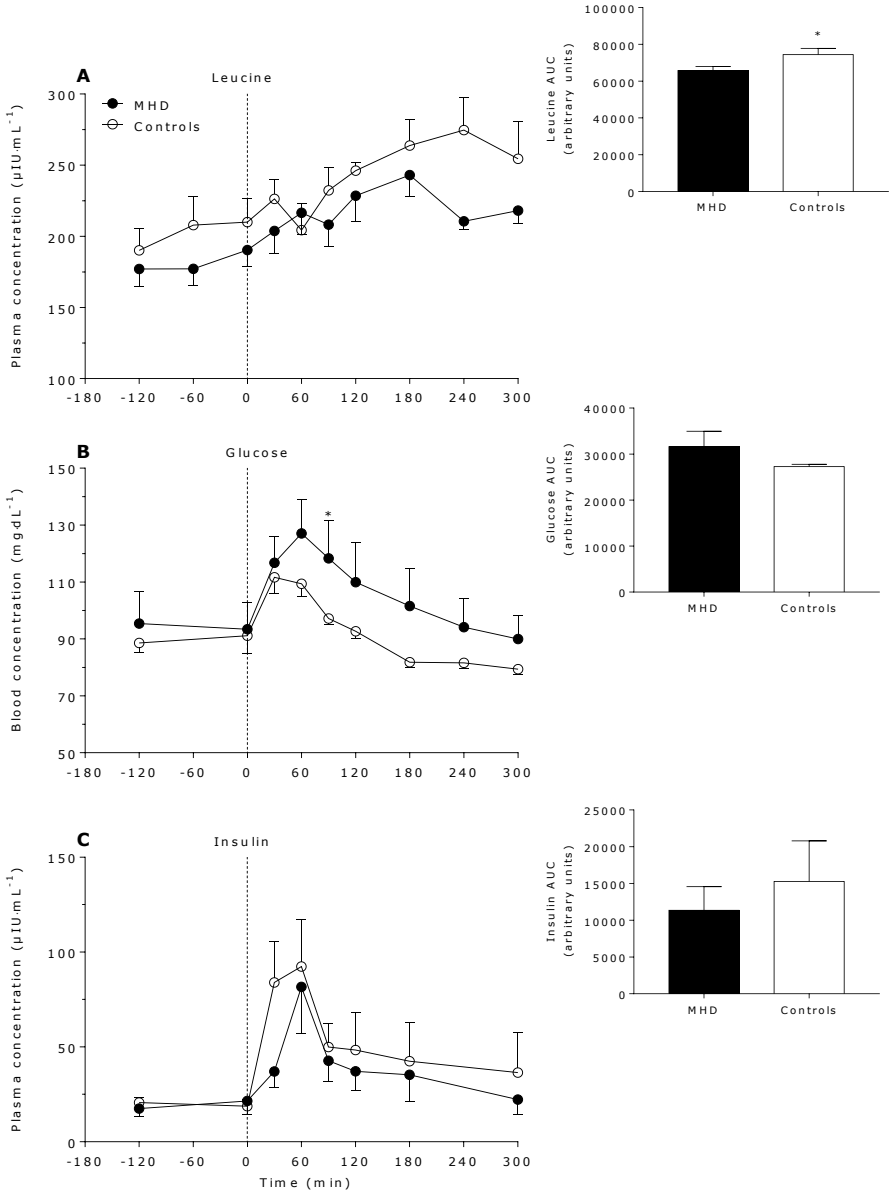
Table 5.1. Participant characteristics

	MHD patients (n = 8)	Controls (n = 8)	P-value
Dialysis period (y)	5 ± 1	-	-
Age, (y)	56 ± 5	50 ± 2	0.23
Male Sex, No. (%)	6 (75%)	6 (75%)	-
Weight (kg)	94 ± 9	94 ± 4	0.98
BMI (kg/m^2)	32 ± 2	31 ± 1	0.56
Fat (%)	31 ± 3	29 ± 2	0.75
Lean body mass (kg)	61 ± 5	65 ± 4	0.58
Systolic BP (mmHg)	144 ± 5*	129 ± 4	0.04
Diastolic BP (mmHg)	78 ± 4	83 ± 6	0.77
Statins, No %	4 (50 %)	0 (100 %)	-
Blood metabolites			
Fasting Glucose (mg/dL^{-1})	95 ± 11	89 ± 3	0.57
HOMA-IR	3.9 ± 0.9	4.0 ± 0.6	0.57
Blood urea nitrogen (mg/dL^{-1})	32 ± 3*	15 ± 1	<0.001
Creatinine (mg/dL^{-1})	7.4 ± 0.9*	1.1 ± 0.1	<0.001
eGFR ($mL/min^{-1}/1.73m^2$)	9 ± 1*	82 ± 6	<0.001
Total CO_2 (mEq/L^{-1})	23 ± 2	21 ± 1	0.40
C-reactive protein (mg/L^{-1})	16 ± 2	<5.0	-
Albumin (g/dL^{-1})	3.6 ± 0.1*	3.9 ± 0.1	0.04
Co-morbidities, No. (%)			
Hypertension	8 (100 %)	2 (25 %)	-
Diabetes mellitus	1 (12.5 %)	0 (0 %)	-
Obesity	4 (50 %)	4 (50 %)	-
Secondary hyperparathyroidism	8 (100 %)	0 (0 %)	-
Arthritis	8 (100 %)	0 (0 %)	-
Neuropathy	2 (25 %)	0 (0 %)	-
History of cardiovascular disease	2 (25 %)	1 (12.5 %)	-
Hematological disease	2 (25 %)	0 (0 %)	-
Gastroesophageal reflux disease	1 (12.5 %)	0 (0 %)	-
Liver disease	2 (25 %)	0 (0 %)	-

Data are mean ± SEMs, unless stated otherwise.

5.8 Figures

Figure 5.1

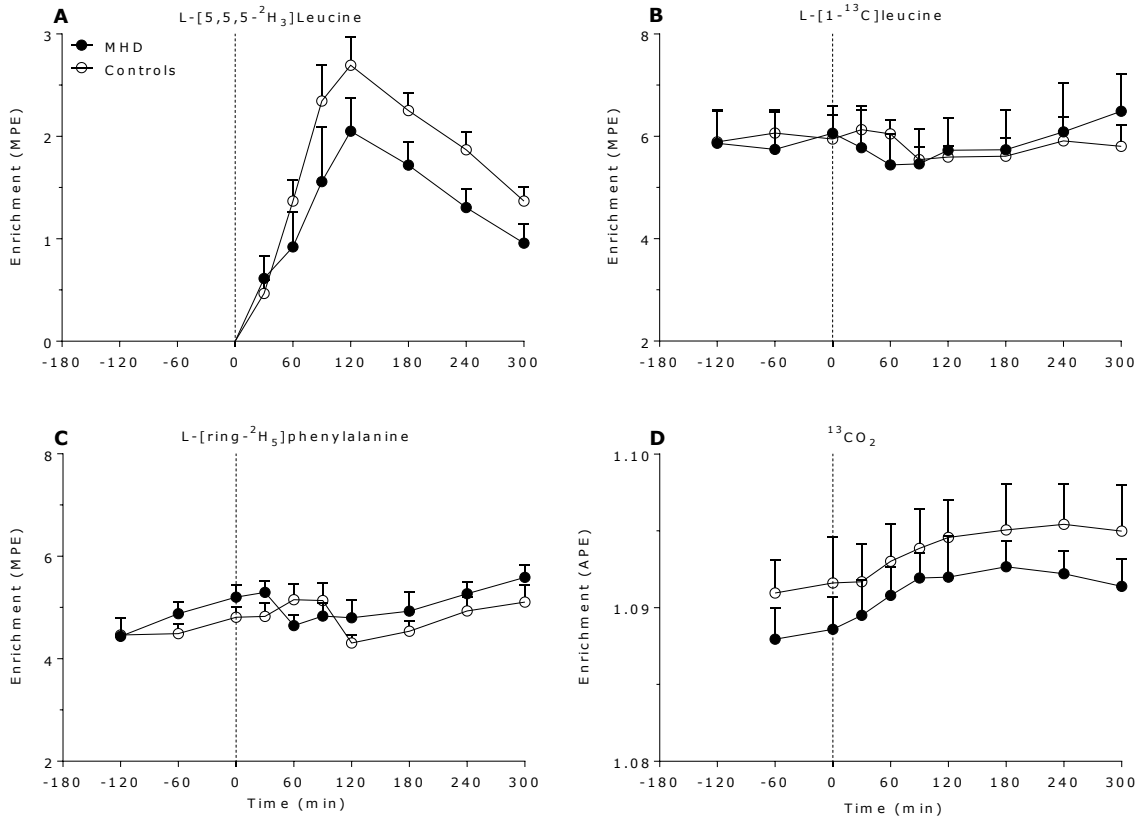


Data are mean ± SEMs. Plasma leucine (A), blood glucose (B), and plasma insulin concentrations (C) in the basal state and after meal consumption in MHD patients (*n*=8) and controls (*n*=8). Insets show the areas under the time curves for the fed-state. Dashed line refers to meal ingestion.

Figure 5.1 (cont.)

Data were analyzed with a two-factor (time × group) ANOVA. The areas under the time curves were analyzed using an independent samples t-test. Bonferroni post hoc test was used to locate differences between means for all significant interactions. Leucine: time effect, $P<0.001$; group effect, $P=0.33$; time × group: $P=0.59$. Glucose: time effect, $P<0.001$; group effect, $P=0.03$; time × group: $P=0.05$. Insulin: time effect, $P<0.001$; group effect, $P=0.51$: time × group: $P=0.29$. *Significantly different between conditions ($P<0.05$).

Figure 5.2



Plasma L-[5,5,5-$^{2}H_3$]leucine (A), L-[1-^{13}C]leucine (B), L-[*ring*-$^{2}H_5$]phenylalanine (C) and breath $^{13}CO_2$ (D) enrichments over time in the basal state and after meal consumption in MHD patients (n=8) and controls (n=6). Data are expressed as MPE (A, B, and C) and APE (D), respectively. Dashed line refers to meal ingestion. Data were analyzed with a two-factor (time × group) ANOVA. A Bonferroni post hoc test was used to locate differences between means for all significant interactions. L-[5,5,5-$^{2}H_3$]leucine: time effect, P<0.001; group effect, P=0.15; time × group: P=0.38. L-[1-^{13}C]leucine: time effect, P=0.50; group effect, P=0.99; time × group: P=0.64. L-[ring-$^{2}H_5$]phenylalanine: time effect, P<0.01; group effect, P=0.40: time × group: P=0.30. $^{13}CO_2$: Time effect, P<0.001; Group effect, P=0.02; time × group: P=0.39. APE, atom percent excess; MPE, mole percent excess.

Figure 5.3

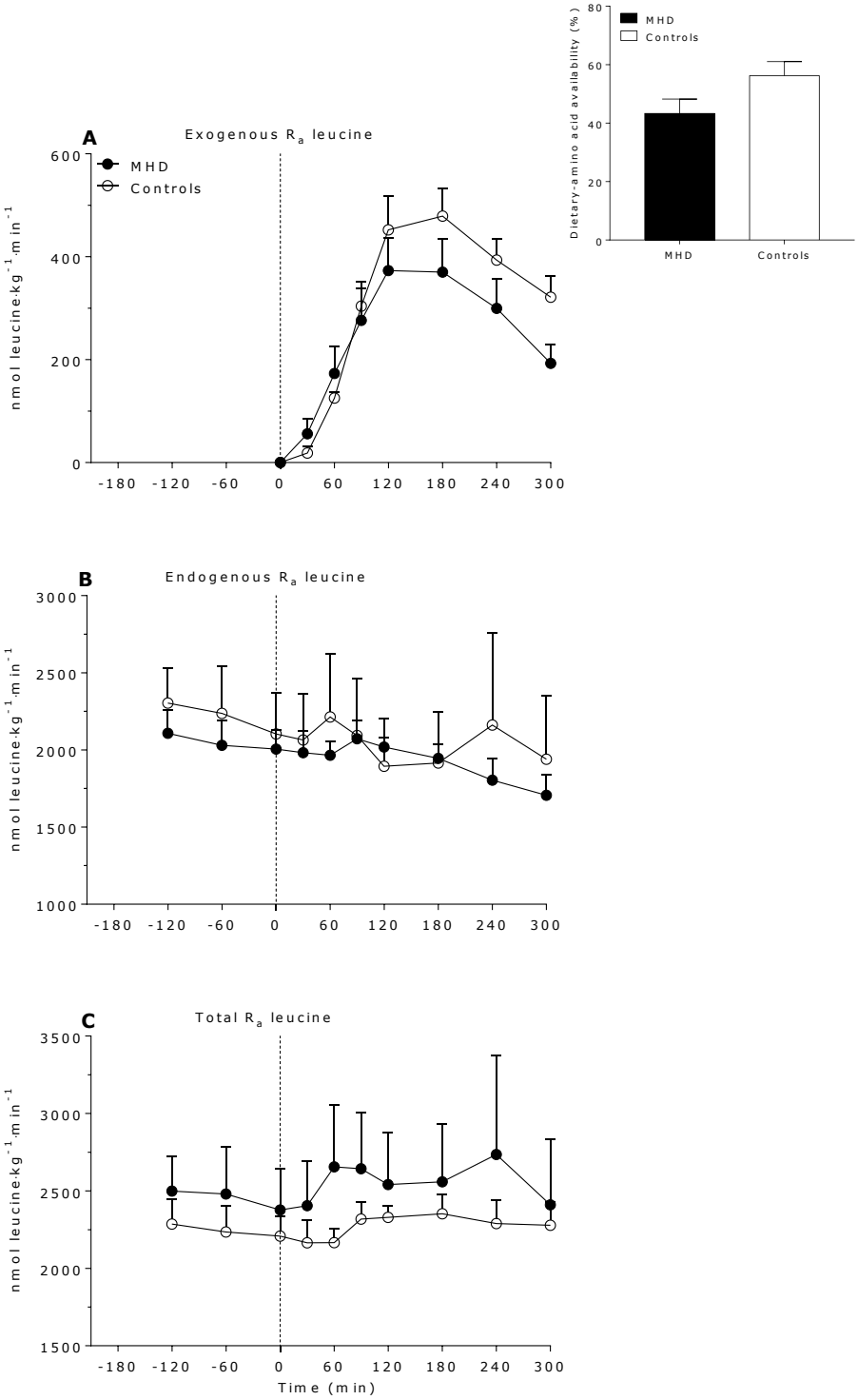
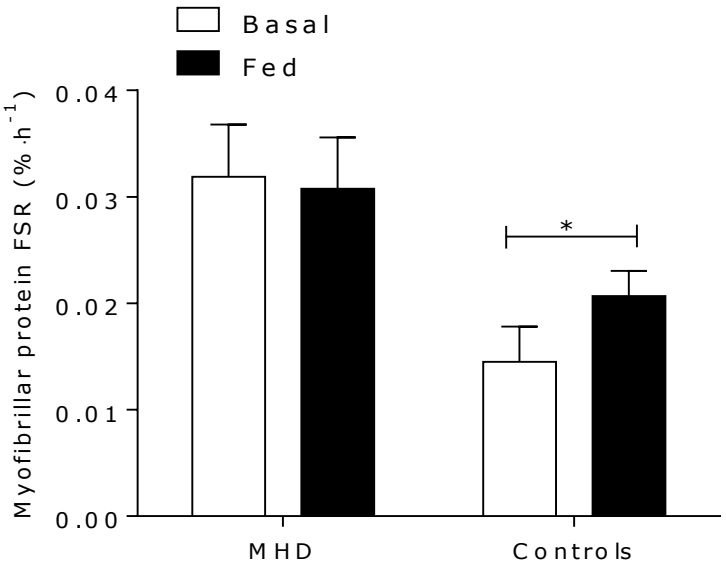

A
Exogenous R_a leucine
MHD
Controls
nmol leucine·kg^{-1}·min^{-1}
600
400
200
0
-180 -120 -60 0 60 120 180 240 300
Dietary-amino acid availability (%)
80
60
40
20
0
MHD
Controls
B
Endogenous R_a leucine
nmol leucine·kg^{-1}·min^{-1}
3000
2500
2000
1500
1000
-180 -120 -60 0 60 120 180 240 300
C
Total R_a leucine
nmol leucine·kg^{-1}·min^{-1}
3500
3000
2500
2000
1500
-180 -120 -60 0 60 120 180 240 300
Time (min)

Figure 5.3 (cont.)

Data are mean ± SEMs. Exogenous leucine R_a (A), endogenous leucine R_a (B), total leucine R_a (C) over time in the basal state and after meal consumption in MHD patients (n=8) and controls (n=6). Data are expressed as nmol leucine·kg^{-1}·min^{-1}. Dashed line refers to meal ingestion. Data were analyzed with a two-factor (time × group) ANOVA. The areas under the time curves were analyzed using an independent samples t-test. A Bonferroni post hoc test was used to locate differences between means for all significant interactions. Exogenous leucine R_a: time effect, $P<0.001$; group effect, $P=0.08$: time × group: $P=0.10$. Dietary amino acid availability: $P=0.06$. Endogenous leucine R_a: time effect, $P=0.20$; group effect, $P=0.76$: time × group: $P=0.55$. Total leucine R_a: time effect, $P=0.79$; group effect, $P=0.53$: time × group: $P=0.95$. Data are Mean ± SEM. R_a, rate of appearance;

Figure 5.4



Myofibrillar protein synthesis rates in the basal state and after meal consumption in MHD patients (n=8) and controls (n=6). Data are expressed as FSR (%·h^{-1}). Data were analyzed with a two-factor ANOVA with repeated measures on time. A Bonferroni post hoc test was used to locate differences between means for all significant interactions. FSR: time effect, P=0.58; group effect, P=0.02: time × group: P=0.33. *Different between groups (P<0.05). Data are Mean ± SEM. FSR, fractional synthesis rates.

CHAPTER 6: GENERAL DISCUSSION

The overarching aim of this dissertation was to improve the understanding of the nutritional modulation of postprandial protein metabolism after the consumption of protein-dense whole foods during resting and post-exercise recovery conditions in both healthy and diseased individuals. Previous work has focused on the regulation of postprandial protein metabolism after the consumption of isolated protein fractions (9, 11-14, 17, 18, 21, 22, 24, 26-28, 33, 35, 42, 44, 50, 51, 59, 62, 63) or ingestion of free form, crystalline amino acids (8, 16, 30, 32, 40, 41, 52, 53, 56, 57). However, this is not representative of normal human eating behavior. In this dissertation, we gained new information related to postprandial protein metabolism in response to whole food ingestion with health and disease. This Chapter will highlight the major findings from Chapters 2-5 and outline any potential limitations of the studies. Future areas for further research will also be discussed.

6.1 Chapter 2: Sustained postprandial muscle protein synthesis rates after protein ingestion in healthy young males

For the first study of this dissertation, we wished to establish a clear time course of the relationship between dietary-derived amino acid availability in circulation and the subsequent stimulation of muscle protein synthesis after protein consumption in healthy young adults. This information is important for the development of more effective clinical feeding strategies aimed at muscle mass maintenance.

It was previously demonstrated that rates of muscle protein synthesis after feeding are very short lived and rapidly return to basal values despite continued elevated postprandial amino acid concentrations and intramuscular anabolic signaling phosphorylation (5). However, these findings

were made after the consumption of ‘fast’ digested whey protein. It is not common practice for a a single meal or clinical feeding formulas to include only one isolated protein source. In **Chapter 2,** we demonstrated that protein blends consisting of ‘fast’ whey and ‘slow’ casein (20/80 blend) results in sustained muscle protein synthesis rates during a prolonged 300 min postprandial period.

These data illustrate that consumption of protein sources, which result in a gradual release of postprandial protein derived amino acids into circulation and ultimately a more sustained ‘anabolic drive’ to skeletal muscle tissue are preferred. To this end, nutritional supplements and/or clinical feeding formulas designed to support muscle mass maintenance will benefit from mixed protein blends (or mixed meal combinations) to maximize the postprandial stimulation of muscle protein synthesis rates under the time curve.

Study limitations and future directions:

An important limitation of the study is that we provided concentrated milk protein containing low amounts of the other macronutrients (4 g carbohydrates, 1 g of fat). Indeed, protein is generally consumed with other macronutrients which may slow down the rate at which dietary amino acids become available in circulation (14, 21, 22). Nevertheless, when measured over prolonged postprandial phase, total dietary amino acid availability and postprandial stimulation of muscle protein synthesis rates seems unaffected with macronutrient co-ingestion (14, 21, 22). Thus the sustained postprandial stimulation of muscle protein synthesis as observed in **Chapter 2** is perhaps expected with whole food sources as well.

6.2 Chapter 3: Development of intrinsically-labeled eggs and poultry meat for use in human metabolic research

In Chapter 2, we used an innovative tool referred to as intrinsically-labeled milk protein that allows the researcher to directly assess the rate of protein digestion and amino acid absorption and subsequent circulating dietary amino acid availability in humans. Plasma amino acid availability is an independent and important factor that regulates whole-body and muscle protein metabolism after the ingestion of a protein-containing meal (17, 26). Previously, researchers have focused on producing intrinsically-labeled cow derived food products (i.e. milk and beef) for use in human metabolic research (6-8). The labeled milk and beef were produced by prolonged intravenous (IV) infusions of stable isotope tracer into lactating dairy cows for ~7 days. However, producing labeled diary food protein in this manner requires large amounts of people-power and financial resources due to the infrastructure required to house and maintain lactating dairy cows. What is noteworthy is that Evenepoel et al. (20) suggested a simple and non-invasive method of producing intrinsically labeled protein foods by supplementing the feed of laying hens with an amino acid tracer.

We built on this work by supplementing a specifically formulated low-leucine diet (containing 0.52% leucine) with 0.3% L-[5,5,5-$^{2}H_{3}$]leucine and subsequently feeding this diet to 3 laying hens (Lohmann LSL Whites) for 55 d. We demonstrated that high tracer labeling (>20 mole percent excess, MPE) in the eggs was obtained after 7 d and maintained throughout the feeding protocol. Importantly, the modified diet did not impair egg production performance (90%) or the mean weight of the eggs (~52 g), which is considered a typical egg weight for the Lohmann LSL White strain (48). The rate of tracer recovery in the eggs was calculated to be ~37 %, which is a 0.5 fold greater tracer recovery than previously reported by workers producing intrinsically labeled milk proteins using either L-[1-^{13}C]phenylalanine (36, 55) or L-[1-^{13}C]leucine (10). Over a 55 d period,

a total of ~850 g of egg protein (145 eggs) was produced with a mean tracer enrichment of 22.0 ± 0.8 MPE. Subsequently, we confirmed in experimental human trials that the consumption of labeled eggs increased plasma L-[5,5,5-$^{2}H_3$]leucine enrichment to a peak values of 6.7 ± 0.1 MPE, which is more than enough to reliably study postprandial dietary amino acid availability (which requires ~ 0.5 MPE).

Overall, we demonstrated the feasibility of producing intrinsically labeled eggs for use in human metabolic research. In particular, we demonstrated the feasibility of producing nutritionally complete and intrinsically labeled dietary proteins at a modest production price point by the use of a non-invasive oral tracer administration method in chickens.

Study limitations and future directions:

Previously, researchers have demonstrated that the amino acid tracer from highly labeled (>30 MPE) milk-derived proteins can be 'traced' directly into myofibrillar protein (15, 23, 26, 34, 55, 60). Such an approach would be advantageous in populations or settings where continuous IV infusions may not be easily applied (e.g. elderly, clinical populations, nutrition field studies). An important limitation of our work was that direct measurement of egg-derived L-[5,5,5-$^{2}H_3$]leucine incorporation in myofibrillar proteins was only possible in physically smaller participants (<60 kg). The amino acid (L-[5,5,5-$^{2}H_3$]leucine) labeling in our eggs was 'only' 22 MPE and was presumably not high enough to reliably detect the incorporation of the tracer in skeletal muscle proteins. Enriching the diet (e.g., both the food and drinking water) with more L-[5,5,5-$^{2}H_3$]leucine or a 'heavier' tracer (i.e. $^{13}C_6$ or D_{10} leucine) could allow for more reliable detection of the tracer in human skeletal muscle tissue. However, this would also increase the price of production.

6.3 Chapter 4: Consumption of whole eggs promotes greater stimulation of postexercise muscle protein synthesis than consumption of isonitrogenous amount of egg whites in young men

The addition of other macronutrients (i.e. fat and carbohydrates) to isolated dairy protein drinks have previously been shown to modulate protein digestion and absorption kinetics, without modulating the cumulative postprandial stimulation of muscle protein synthesis rates when compared to the ingestion of only protein (14, 21, 22). However, little is known about how mixed meal combinations influence circulating postprandial dietary amino acid availability and the subsequent stimulation of muscle protein synthesis rates when fat is ingested as part of a natural food matrix.

It was previously demonstrated that consumption of whole milk resulted in greater net amino acid retention in the leg muscle when compared to skim milk ingestion that was matched for protein content (19). The workers were unable to find a clear explanation for their findings, however, it was believed that the addition of a non-protein nutrient such as fat somehow improved the muscle anabolic response. With consumption of multiple eggs, the yolk is often discarded due to the long held belief that the regular consumption of cholesterol contained in the yolk will increase risk of cardiovascular disease (49, 54). From a sports nutrition standpoint, the yolk is often discarded based on the belief that fat slows down protein digestion and absorption and subsequent delivery of amino acids to the muscle as well as the extra energy might influence body composition (fat mass gain).

In **Chapter 4**, we compared the consumption of three whole eggs (18 g protein and 17 g fat) to an equivalent amount of protein from egg whites (18 g protein and 0 g fat) on the stimulation of postexercise muscle protein synthesis rates. In contrast to our hypothesis (no differences in

postprandial stimulation of muscle protein synthesis between the ingestion of whole eggs and egg whites), we found that whole eggs resulted in greater postprandial rates of muscle protein synthesis when compared to egg whites. However, we did not observe differences on the whole-body level between the egg conditions. This underlines the importance of obtaining measurements of protein synthesis directly on the level of the muscle vs. the whole body when attempting to define optimal strategies for muscle mass maintenance.

A strong suit of this study was its randomized crossover design where each subject performed two trials (separated by ~ 1 week) and thus minimized inter-subject variability in the responses. The order on which they received the whole eggs and egg whites was randomized such that half the participants received whole eggs on their first trial, while the other half received egg whites and vice versa.

To elucidate why whole egg ingestion resulted in greater postprandial stimulation of muscle protein synthesis when compared with egg whites, we examined various factors that are acknowledged to dictate the postprandial muscle protein synthetic response. These factors include: *1.)* the amino acid composition of the ingested food source, *2.)* protein digestion and splanchnic release of dietary amino acids into circulation, *3.)* peak plasma leucine concentrations, *4.)* whole body leucine oxidation rates, *5.)* skeletal muscle amino acid transporter content, *5.)* anabolic signaling phosphorylation, and *6.*) metabolic sensors. However, none of these factors provided a clear indication for the greater anabolic property of whole egg ingestion vs. egg whites in healthy men in this study.

Overall, we showed that discarding the yolk from the egg limits the postprandial stimulation of muscle protein synthesis rates during post-exercise recovery in young males. The yolk is nutrient dense and may contain a variety of important bioactive compounds such as lipids,

micronutrients, antioxidant carotenoids, and miRNAs (7, 58). Therefore, besides limiting postprandial skeletal muscle anabolic, the removal of the yolk and its associated vital nutrients may also limit their benefit to improve overall human health (3, 4, 7). **Chapter 4** lends support for the recommendation that nutrient and protein dense foods should be the cornerstones to meeting daily protein requirements to optimize muscle protein synthesis rates.

Study limitations and future directions:

An obvious 'limitation' of the study was the lack of revealing a clear mechanism as to why whole egg vs. egg white ingestion resulted in greater postprandial stimulation of muscle protein synthesis rates. Within the current study we measured phoshorylation status of various molecular readouts related to mTORC1 and MAPK-pathways. The lack of differences in the phosphorylated-state of molecular readouts between the egg conditions could imply that this anabolic pathway was maximized from the prior performance of resistance exercise and food ingestion (11, 29, 31, 43), thereby overriding any subtle non-protein nutritive influences of whole egg consumption on mTORC-1 phosphorylation and its downstream targets. Moreover, the phosphorylated state of mTORC1 does not provide the dynamics of mTORC1. For increased translation (i.e. protein synthesis), mTORC1 needs to localize towards to the surface of the lysosome compartment where mTORC1 can bind with its well-known activator Ras homolog enriched in brain (Rheb) (47). Future analysis will be performed if mTORC1 resides in closer proximity to the lysosome after whole egg vs egg white ingestion.

6.4 Chapter 5: Differential whole body and skeletal muscle protein metabolic responses to mixed meal ingestion in maintenance hemodialysis patients.

After studying postprandial protein metabolism in healthy young individuals, we wished to extend our focus of the study of postprandial protein metabolism after whole food ingestion to more clinical population. Maintenance hemodialysis (MHD) patients experience severe skeletal muscle wasting for a number of reasons including: chronic inflammation, reduced protein intake, metabolic acidosis, insulin resistance, hormonal abnormalities, and a loss of nutrients/amino acids during dialysis (61). To offset the losses of skeletal muscle proteins, the consumption of protein-rich meals are recommended for this patient populations (1). However, little is known about the protein metabolic responses after food ingestion in MHD patients.

To fill this research gap, we recruited 8 MHD patients and 8 controls, matched for age, BMI and several co-morbidities known to influence protein metabolism (e.g. insulin resistance (39) and obesity (6)). We observed that basal rates of muscle protein synthesis were ~ 2.5 fold elevated in MHD patients when compared to controls, which suggests hypercatabolism in MHD patients (37, 38). Noteworthy is that measurements were made on a non-dialysis day; suggesting prolonged hypercatabolism that is maintained well beyond the dialysis period. In addition, we demonstrated that consumption of protein-rich meal (20 g protein, 560 kcal) typically recommended to dialysis patients did not elicit rise a postprandial rise in muscle protein synthesis rates (2). Using the research tool of L-[5,5,5-$^{2}H_{3}$]leucine labeled eggs, we were able to demonstrate reduced circulating dietary amino acid availability in MHD patients when compared to controls. The reduced amino acid availability to skeletal muscle tissue could have led to the inability to improve postprandial muscle protein synthesis rates in MHD patients after eating a meal. We attribute the reductions in dietary amino acid availability to splanchnic abnormalities, commonly witnessed in

MHD patients. In addition, the large number of co-morbidities (such as hyperthyroidism and inflammation) and high medicinal use (such as statins) could have further negatively impacted protein metabolism in this patient population.

Study limitations and future directions:

A possible limitation of the study was that the MHD patients experienced a number of non-uremic co-morbidities (e.g. gastroesophageal reflux disease, liver disease, hematological disease) that could have further impacted our findings. While this makes it difficult to untangle the effects of kidney failure on protein metabolism per se, the presence of a variety of co-morbidities is representative of a typical MHD patient. The goal of this research was to explore whether the consumption of a protein-rich mixed meal, commonly recommended for this population, can stimulate postprandial muscle protein synthesis rates and thus set a benchmark for future studies to explore more effective strategied to potentially offset some of the losses in skeletal muscle proteins. We were unable to elicit a feeding-induced stimulation in muscle protein synthesis rates in the controls, which may point towards the idea that we did not feed enough protein. In particular we fed 20 g of dietary protein, whereas it was previously suggest that ingested protein amounts of > 35 g are necessary to inhibit whole protein breakdown rates (25) and stimulate muscle protein synthesis (45) in aging populations. Unfortunately, such high intakes of dietary protein are generally not achieved by MHD patients (46), suggesting a need for alternative strategies improve postprandial protein metabolism in MHD patients. Given the ability of exercise to enhance sensitivity of muscle protein synthesis rates to the anabolic properties of amino acids (12), it seems that exercise prior to food ingestion represents a logical adjunct strategy to nutritional intake in this patient population.

6.5 Conclusions

The studies in this thesis lend support for the recommendation that the ingestion of whole foods should form the cornerstone for meeting daily protein requirements. Specifically, we show that postprandial muscle protein synthesis rates are strongly regulated after whole food ingestion and exercise. We also demonstrated that muscle protein rates are negatively altered in chronic renal failure, and could not be rescued with the ingestion of a protein dense meal alone. Clinical strategies to improve skeletal muscle health should involve a combination of increased physical activity and protein-dense whole food consumption.

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APPENDIX A: PROTOCOLS

A.1 Hen tissue and egg protein extraction

This section describes the extraction procedures utilized in Chapter 3 to determine the tracer enrichment of the main protein fractions in the egg (i.e. white and yolk) and the enrichments of the tissues of the egg laying hens.

SUPPLIES NEEDED:

1. Teflon Pestle
2. 1.5 ml Eppendorf Tube
3. 4.0 ml Glass Top Vial and Cap
4. Hydrion Ph Paper

REAGENTS NEEDED:

1. 0.6 M Perchloric Acid (PCA)
2. 70 % Ethanol (ETOH)
3. 6 M Hydrochloric Acid (HCL)
4. 6 M Sodium Hydroxide (NaOH)
5. 0.1 M HCL

PROTOCOL:

1. Place 20-25 mg of sample in 1.5 ml Eppendorf tube and add 500 µL of .6 M PCA
2. Homogenize with Teflon Pestle and vortex.
3. Spin at 3600 × *g* for 5 min (4°C).
4. Remove supernatant (1st shot) and discard.
5. Add 500 µL of .6 M PCA to pellet and vortex.
6. Spin at 3600 × *g* for 5 min (4°C).
7. Remove supernatant (2st shot) and discard.
8. Add 500 µL of 70% ETOH to pellet and vortex.
9. Spin at 3600 × *g* for 5 min (4°C).
10. Remove supernatant (3rd) shot and discard.
11. Poke hole in lid of Eppendorf and freeze dry.
12. Transfer pellet to 4 mL glass screw top vial and add 1.5 mL 6 M HCL
13. Hydrolyze overnight (> 12 h) at (110°C)
14. Add 6 M Sodium Hydroxide (1.45 mL) and confirm if neutral with pH paper (if not add increments of 0.1 mL).
15. Vortex glass vials and pipet out sample into Eppendorf tube (1.5 mL).

16. Pipet 1.5 mL out of the Eppendorf tube and run the sample through a .22 µm filter attached to a 4 mL glass screw top vial.
17. Continue until you have pipetted and ran the entire sample (3 mL total) through .22 µm filter.
18. Dry sample in SpeedVac concentrator, dissolve sample in 100 µL 0.1 M HCl.
19. Ready for derivatization and amino acid analysis on GC-MS.

A.2 Plasma amino acid extractions for amino acid quantifications

This section describes the extraction procedures and analysis performed in Chapter 3-5 to determine the amino acid concentrations in the plasma of participants.

SUPPLIES NEEDED:

1. 1.5 ml Eppendorf Tubes

REAGENTS NEEDED:

2. MIX A (3:3:2: Acetonitrile:Isopropanol:ddH_2O)
3. INTERNAL STANDARD ($^{13}C_9$-Phenylalanine and $^{13}C_6$-Leucine or p-chlorophenylalanine at 1 mg/mL)
4. 0.1 M HCL

EXTRACTION PROTOCOL:

4. Put 1 mL of MIX A (3:3:2: Acetonitrile:Isopropanol:ddH_2O) in an empty Eppendorf.
5. Put 10 ul of IS in the Eppendorf with MIX A.
6. Put 0.2 mL of Plasma in the Eppendorf with MIX A and IS and vortex.
7. Place the mixed sample in freezer (- 20°C) for 30 min.
8. Spin at 20800 × *g* for 10 min (4°C).
9. Transfer 1.0 mL of supernatant to new Eppendorf.
10. Ready for derivatization and amino acid analysis on GC-MS.

NOTE: Do not analyze IV-infused (i.e. [$^2H_5$]phenylalanine and/or [^{13}C]leucine) or dietary-derived tracers ([$^2H_3$]leucine) from the samples that have the [$^{13}C_9$]phenylalanine and [$^{13}C_6$]Leucine Internal Standard (IS) added! The IS will artificially 'enrich' the enrichments of the IV and dietary-derived tracer. In that case use 'Plasma Amino Acid Extraction for Tracer Enrichments' protocol to determine the IV and dietary-derived tracer enrichments.

QUANTIFICATIONS PROTOCOL:

1. Open Amdis_32 (chemdata.nist.gov
2. Go to FILE → BATCH JOB → CREATE AND RUN JOB)
3. Go to ADD and select all GC/MS output files to analyze (always labeled x.D; e.g., 1.D, 2.D etc).
4. Select SIMPLE as ANALYSIS TYPE.
5. Open TXT file with excel and quantify all individuals amino acids relative to IS (i.e. IS is always 1).
6. Plot this ratio to the generated standard curve to find the corresponding amino acid concentration in um/l.

A.3 Plasma amino acid extractions for tracer enrichments

This section describes the extraction procedures utilized in Chapters 2-5 to determine the amino acid tracer enrichment in the plasma of participants.

SUPPLIES NEEDED:

1. 1.5 ml Eppendorf Tubes

REAGENTS NEEDED:

1. MIX A (3:3:2: Acetonitrile:Isopropanol:ddH_2O)

EXTRACTION PROTOCOL:

1. Put 1 mL of MIX A (3:3:2: Acetonitrile:Isopropanol:ddH_2O) in an empty Eppendorf.
2. Put 0.2 mL of Plasma in the Eppendorf with MIX A and vortex.
3. Place the mixed sample in freezer (- 20°C) for 30 min.
4. Spin at 20800 × *g* for 10 min (4°C).
5. Transfer 1.0 mL of supernatant to new Eppendorf.
6. Ready for derivatization and amino acid analysis on GC-MS.

ENRICHMENT ANALYSIS PROTOCOL:

1. Open RTR data analysis (MSD ChemStation)
2. Go to CHROMATOGRAM → EXTRACT ION CHROMATOGRAM and select the IONS to monitor (e.g, 302, 303 and 305 for unlabeled and labeled L-[1-^{13}C]leucine; L-[5,5,5-$^{2}H_3$]leucine, respectively).
3. Click A/B tab next to mouse cursor icon and check MANUAL INTEGRATION box.
4. Select the entire area below the peak of interest (e.g., leucine) and go to CHROMATOGRAM → PERCENT REPORT
5. Find the value of corresponding corrected area as the integrated peak.
6. Express the peaks as a TTR (tracer-to-tracee) for each sample (with subtraction of the 'baseline' sample).
7. Convert to MPE (mole percent excess) using the following formula: MPE = TTR / (1 + TTR) x 100

A.4 Myofibrillar protein extraction protocol

This section describes the extraction procedures utilized in Chapters 2-5 to isolate myofibrillar proteins for tracer analysis and generate whole muscle homogenate to perform Western Blot analysis. Myofibrillar protein-bound tracer enrichments can reliably obtained from 5-10 mg of muscle, however since we also performed Western Blot analysis a larger piece of ~50 mg of muscle was used (to provide for enough whole muscle homogenate).

SUPPLIES NEEDED:

1. Teflon Pestles
2. 2 ml Eppendorf Tube
3. 4 ml Glass Top Vial

REAGENTS NEEDED:

1. 0.3 M Sodium Hydroxide (NaOH)
2. 1 M Perchloric Acid (PCA)
3. 70 % Ethanol (EtOH):
4. 6 M HCL

PREPARATION:

1. Label Eppendorfs: MYO, WEST and BRAD prior to protocol.
2. Make Homogenization Buffer (see below).

HOMOGENIZATION BUFFER RECIPE

SUPPLIES NEEDED:

1. 500 mL beker
2. 10 ml Falcon Tubes

REAGENTS NEEDED:

3. 50 mM Tris-HCL pH 7.4
4. 150 mM Sodium Chloride (NaCL)
5. 1 mM phenylmethylsulfonyl fluoride (PMSF)
6. 10 % nonyl phenoxypolyethoxylethanol-40 (NP-40)
7. 0.5 g Sodium Deoxycholate Powder
8. Sodium Dodecyl Sulfate (SDS) Powder

TO MAKE HOMOGENIZATION BUFFER:

1. To 50 mL ddH_2O add: 790 mg Tris-Base and 900 mg NaCL
2. pH to 7.4 with HCL (6 or 12 M)
3. Add 10 mL of 10 % NP-40
4. Add 0.5 g Sodium Deoxycholate Powder
5. Add 0.1 g SDS powder
6. Fill to 100 mL with ddH_2O (add however much ddH_2O until beker is at 100 mL line).
7. Store in 10 mL aliquots at -20°C.

MUSCLE HOMOGENIZATION PROCEDURE FOR MYOFIBRILLAR AND SARCOPLASMIC PROTEINS

8. Add 1 Roche Complete Mini with EDTA Protease Inhibitor and 1 Roche PhosStop to 10 mL of homogenization buffer prior to starting homogenization (vortex to fully dissolve).
9. Add 10 µL/mg of homogenization buffer to a 50 mg sample in an Eppendorf labeled MYO
10. Homogenize with Teflon pestle on ice.
11. Spin at 700 × *g* for 5 min (4°C).
12. Transfer supernatant to new Eppendorf labelled WEST, transfer 5 µL from the tube labeled WEST to BRAD (use small RNA tubes).
13. Freeze MYO, WEST and BRAD at -80°C to process later.

MYOFIBRILLAR ISOLATION

1. Add 500 µL of ddH_2O to Eppendorf labeled MYO and vortex.
2. Spin at 700 × *g* for 10 min at (4°C).
3. Discard supernatant.
4. Add 1 mL of 0.3 M NaOH to the pellet and vortex.
5. Put samples in heating block at 50°C for 30 min (vortex every 10 min).
6. Spin at 10000 × *g* for 5 min.
7. Transfer supernatant (this is your myofibrillar portion) to 4-ml screw top glass vials.
8. Add 1 mL of 0.3 M NaOH to the pellet that is left in Eppendorf and vortex.
9. Spin at 10000 × *g* for 5 min (4°C).
10. Again transfer supernatant (this is your myofibrillar portion) to 4-ml screw top glass vials that already have the supernatant from the previous step (you now have collected the myofibrillar proteins twice)
11. The pellet remaining in the Eppendorfs can now be frozen at -80°C as COLLAGEN.
12. Add 1 mL of 1 M PCA to 4-ml glass vials (should see snow globe effect).
13. Spin 15 min at 3000 × *g* (4°C). (this is the max the glass vials can be spun at or else they are at risk of breaking)
14. Remove supernatant and discard (use transfer pipets).
15. Add 1 mL of 70 % EtOH to sample (DO NOT VORTEX OR ADD ETOH DIRECTLY TO PELLET; RATHER PIPET IT DOWN SIDE OF GLASS).
16. Spin for 15 min at 3000 × *g* (4°C).

17. Remove supernatant (EtOH) and discard.
18. Add 2.0 ml of 6 м HCL to sample.
19. Hydrolyze overnight at (110°C).
20. Purify amino acids over Dowex Cation Exchange Column (See ‘Dowex Purification Protocol’ below).

A.5 Mixed muscle and intracellular muscle free extraction protocol

This section describes the extraction procedures utilized in Chapters 2-5 to isolate mixed muscle proteins and obtain the intracellular (IC) muscle free amino acids, which can subsequently be used to determine IC muscle free tracer enrichment. IC muscle free tracer enrichments can reliably obtained from ~10 mg of muscle.

SUPPLIES NEEDED:

1. Teflon Pestle
2. 2.0 ml Eppendorf Tube
3. 4.0 ml Glass Top Vial and Cap

REAGENTS NEEDED:

1. 0.6 M Perchloric Acid (PCA)
2. 70 % Ethanol (ETOH)
3. 6 M Hydrochloric Acid (HCL)
4. 0.1 M HCL

PROTOCOL:

1. Place 10-15 mg of sample in 2.0 mL Eppendorf tube and add 500 µL of .6 M PCA.
2. Homogenize with Teflon Pestle and vortex.
3. Spin at 3600 × *g* for 5 min (4°C).
4. Remove supernatant (1st shot) and place in 2.0ml Eppendorf labeled IC.
5. Add 500 µL of .6 M PCA to pellet and vortex.
6. Spin at 3600 × *g* for 5 min (4°C).
7. Remove supernatant (2st shot) and place in 2.0ml Eppendorf labeled IC.
8. Add 500 µL of 70% ETOH to pellet and vortex.
9. Spin at 3600 × *g* for 5 min (4°C).
10. Remove supernatant (3rd) shot and place in 2.0ml Eppendorf labeled IC.
11. Leave IC at room temp if going to column the next day (See 'Dowex Purification Protocol' below); otherwise freeze at -80°C.
12. Free mixed muscle protein pellet at -80°C for possible future analysis.

A.6 Mixed plasma protein extraction protocol

This section describes the extraction procedures utilized in Chapters 2 and 4 to extract mixed plasma proteins (i.e. albumin) from a baseline plasma sample (before administering tracer). This methods assumes that every protein in the body is similar in terms of 'background' tracer enrichment. Thus enrichment of the plasma protein is used as a surrogate for muscle tracer enrichment and is used to deduct 'background' tracer enrichment from the muscle free intracellular enrichments.

SUPPLIES NEEDED:

1. Teflon Pestle
2. 1.5 ml Eppendorf Tube
3. 4.0 ml Glass Top Vial and Cap

REAGENTS NEEDED:

1. 2 % Perchloric Acid (PCA)
2. 70 % Ethanol (ETOH)
3. 6 M Hydrochloric Acid (HCL)

PROTOCOL:

1. Add 500 µL of 2 % PCA to empty 2.0ml Eppendorf
2. Add 200 µL of plasma to Eppendorf with PCA and vortex.
3. Spin at 10000 × *g* for 5 min (4°C).
4. Discard supernatant.
5. Add 500 µL of ddH_20 to pellet and vortex.
6. Spin at 10000 × *g* for 5 min (4°C).
7. Discard supernatant.
8. Add 500 µL of 70% ETOH to pellet and vortex.
9. Spin at 10000 × *g* for 5 min (4°C).
10. Discard supernatant.
11. Poke hole in lid of Eppendorf and freeze dry.
12. Transfer pellet to 4 mL glass screw top vial and add 1.5 mL 6 M HCL
13. Hydrolyze overnight at (110°C).
14. Purify amino acids over Dowex Cation Exchange Column (See 'Dowex Purification Protocol' below).

A.7 Dowex purification protocol

This section describes the cation-exchanges procedures utilized in Chapters 3-6 to purify the plasma, myofibrillar- and intracellular muscle free amino acids prior to analysis by LC-MS/MS.

SUPPLIES NEEDED:

1. 1000 mL beker
2. Stir Bar
3. Hydrion Ph Paper

REAGENTS NEEDED:

1. Dowex™ 50WX8-200 ion-exchange resin
2. 4 M NH_4OH (FOR DOWEX PREPARATION)
3. 2 M NH_4OH (FOR COLUMN CLEAN UP)
4. 1 M HCL
5. 0.1 M HCL

DOWEX PREPARATION:

1. Weigh out 200 g of Dowex Resin in a 1000 mL beker.
2. Add 500 mL of water and stir for 5 min on stir plate.
3. Wash with ddH_2O (add ddH_2O let resin sink to the bottom and discard water).
4. Keep repeating until solution is neutral (check regularly with PH Paper).
5. Add 500 mL of 4 M NH_4OH and stir for 15 min on stir plate.
6. Let Resin settle to bottom and discard NH_4OH (check PH with PH Paper, should be very basic)
7. Wash with ddH_2O (add ddH_2O let resin sink to the bottom and discard water).
8. Keep repeating until solution is neutral (check regularly with PH Paper).
9. Store in fridge with a little layer of 0.1 m HCL on top of it (to ensure Resin does not dry out).

COLUMN PREPARATION:

1. Pull out plunger of 5 mL syringe.
2. Add small amount of glass wool to syringe (to fill up syringe to 1 mL mark). Push the wool down firmly with a pen.

COLUMN CLEAN UP:

1. Add 2 mL of ddH_2O to samples that have been hydrolyzed overnight.
2. Add 1 mL of ddH_2O to syringe.
3. Add 1 mL of Dowex Resin to each of the columns (keep it consistent across all columns).

4. Add 1.5 mL of 2 M NH_4OH to each of the columns to elute contaminants.
5. Test with PH Paper, repeat if not blue.
6. Add 3 mL of water to each column and repeat until neutral (keep track of the amount of 'shots' you add).
7. Add 1.5 mL of 1 M HCL to charge column.
8. Test with PH Paper, repeat if not red (keep track of the amount of 'shots' you add). 1 shot is generally enough.
9. Add samples to columns.
10. Add 3 mL of water to each column and repeat until neutral (keep track of the amount of 'shots' you add).
11. WORK WITH YOUR TEST COLUMN FOR THE NEXT STEPS TO DETERMINE THE AMOUNT OF NH_4OH YOU NEED (undershoot with 500 µL for your samples).
12. Add NH_4OH to your test column in 500 µL increments until pH is basic (pH paper is blue).
13. Record amount of 'shots' you added and substract by one 'shot' for your actual samples (thus you are erring on the safe side for eluting the amino acids). 1 shot is generally enough.
14. Place columns on top of labelled culture tubes in order to collect the eluted amino acids.
15. Add 4 ml of 2 M NH_4OH to elute all amino acids the column.
16. Dry down in Speedvac for 4 h (first 2h at 65 °C).
17. Label eppendorfs.
18. Reconstitute dried down sample in 0.1 M HCL (50 mg/ml sample) and transfer to labeled eppendorfs.
19. Store in Freezer (-80°C). When submitting for analysis by LC-MS/MS provide only 20 µL of total sample (which is 1000 µL for 50 mg muscle piece).