PROTEIN QUALITY AND MUSCLE PROTEIN TURNOVER IN AGING

THE ROLE OF PROTEIN QUALITY AND PHYSICAL ACTIVITY IN SKELETAL MUSCLE PROTEIN TURNOVER IN OLDER ADULTS

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LAY ABSTRACT

At the end of the 5th decade of life, adults will have lost an appreciable amount of muscle mass and strength versus what they had in their 3rd decade of life. This age-related loss of muscle mass and strength is known as sarcopenia. Additionally, as they age, adults will experience brief periods of reduced physical activity due to illness, injury, or recovery from surgery. Such periods are associated with a rapid loss of muscle and strength creating a brief period of 'accelerated sarcopenia'. Strategies to combat the loss of muscle and strength in these periods include increasing protein intake and even periodic exercise which may help to reduce the negative impact of physical inactivity. In particular, higher quality protein sources (protein derived from animal sources or soy) and weightlifting may better help muscles recover from inactivity. Our main findings were that consuming high quality protein (whey protein) stimulated the process of muscle building that is normally reduced with inactivity. Importantly, when combined with resistance exercise, we were able to increase the rate at which healthy older women built muscle with whey protein in comparison to a lower quality protein source (collagen peptides). These findings provide novel and insightful information for the recommendations of protein supplement types to older adults to increase daily protein intake to preserve muscle mass with age.

ABSTRACT

Recent recommendations are that older adults increase their dietary protein intake to intakes higher than are currently recommended to mitigate sarcopenia-induced muscle loss caused in part by anabolic resistance. Protein supplementation may serve as an effective strategy to meet protein intake goals; however, protein supplements vary in their quality, which may impact muscle protein turnover. Protein quality is determined by the digestibility and content of essential amino acids in a protein source and may play an important role in mitigating the loss of muscle mass and muscle protein synthesis (MPS) during energy restriction (ER), acute reductions in physical activity, which we modeled using enforced step reduction (SR), and during recovery from SR. We aimed to determine whether the quality of a protein supplement – whey protein (higher quality) versus collagen peptides (lower quality) – would impact the reduction in fat-free bone-free mass (FBFM) and MPS (Study 1), and also to compare differences in functional variables: strength loss in men and women, and single fibre function with SR in men (Study 2). In Studies 1 and 2 we compared supplementation with whey protein (WP) and collagen peptides (CP), higher and lower quality proteins respectively, as part of a higher protein diet provided to older adults during one week of ER (-500 kcal/d), two weeks of step reduction (< 750 steps/d) (ER+SR) and one week of recovery (RC). Two weeks of ER+SR significantly reduced FBFM in both the WP and CP groups with greater FBFM recovery with WP. MPS was significantly reduced following ER in both groups and did not decrease further during ER+SR. MPS was increased above ER+SR following 1 week of RC in the WP group only. ER+SR significantly reduced maximum voluntary

contraction (MVC) in both men and women; however, following RC men fully recovered their strength and women did not. In Study 3, we aimed to determine the impact of WP and CP supplementation combined with unilateral resistance exercise (RE) to augment the acute and longer term MPS response in healthy older women. Acutely, rates of MPS were elevated following WP+RE and with WP alone while MPS was elevated only in CP+RE. Six days of supplementation increased MPS in WP and WP+RE with no increase in MPS with CP or CP+RE. Collectively, these studies demonstrate that protein quality is an important variable to consider in selecting a protein supplement for older adults and for recovering from inactivity.

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LIST OF ABBREVIATIONS

Akt	Protein kinase B
APE	Atom percent excess
ANOVA	Analysis of variance
AUC	Area under the curve
BMI	Body mass index
CHO	Carbohydrate
CP	Collagen peptide supplement
CPS	Collagen protein synthesis
CRP	C-reactive protein
CSA	Cross sectional area
DIAAS	Digestible indispensible amino acid score
DXA	Dual-energy x-ray absorptiometry
D_2O	Deuterated water
EAA	Essential amino acid
EB	Energy balance
EER	Estimated energy requirement
ER	Energy restriction
ER+SR	Energy restriction and step reduction phase
FBFM	Fat and bone free mass
Fed	Feeding only leg
Fed Ex	Feeding and resistance exercise leg
FSR	Fractional synthetic rate
HP	High protein
LBM	Lean body mass
LLM	Leg lean mass
MPS	Muscle protein synthesis
mTORC1	Mechanistic target of rapamycin protein complex 1
MHCI	Myosin heavy chain I
MHCII	Myosin heavy chain II
MHCIIA	Myosin heavy chain IIA
MyoPS	Myofibrillar protein synthesis
MVC	Multiple voluntary contraction
NEAA	Non essential amino acids
PDCAAS	Protein digestibility corrected amino acid score
Pmax	Maximum power production
P70/S6K1	Protein of 70 kDa S6 kinase 1
RC	Recovery phase
RDA	Recommended daily allowance
RE	Resistance exercise
Rest	Rested leg
RT	Resistance training
SR	Step reduction

Tmax	Maximum isometric tension
TTPT	Time to peak torque
TUG	Timed up and go test
Vmax	Maximum shortening velocity
WP	Whey protein supplement
1RM	1-repetition maximum
4E-BP1	4E-binding protein-1
6MWT	6 minute walk test
30 CST	30 second chair stand test

PREFACE
DECLARATION OF ACADEMIC ACHIEVEMENT

FORMAT AND ORGANIZATION OF THESIS

This thesis is prepared in the "sandwich" format as outlined in the School of Graduate Studies Guide for the Preparation of Theses. It includes a general introduction, three original research papers prepared in journal article format, and a general discussion. The candidate is the first author on all of the manuscripts. At the time of thesis preparation Chapter 2 and 3 were published in peer-reviewed journals and Chapter 4 was in preparation for submission.

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Chapter 1 (sections of the literature review)

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S.Y. Oikawa drafted the manuscript and figures. All authors edited and approved the final version.

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S.Y. Oikawa, C. McGlory and S.M. Phillips designed the study. S.Y. Oikawa, C. McGlory, L.K. D'Souza, A.K. Morgan, S.K. Baker, and S.M. Phillips performed the data collection. S.Y. Oikawa and N.I. Saddler performed the laboratory analysis. S.Y. Oikawa and S.M. Phillips analyzed the data. S.Y. Oikawa drafted the manuscript. All authors edited and approved the final version.

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CHAPTER 1:

INTRODUCTION

1.1 Skeletal muscle

Skeletal muscle comprises approximately 40% of total body mass and is the principal reservoir of amino acids, comprising 50-65% of all proteins in the human body (2). Skeletal muscle is the principal end tissue of locomotion and is critically important to retain and maintain in a functional sense to allow independence in older age. Skeletal muscle also plays an important role in glucose disposal, storage of amino acids for the support of protein synthesis of organs and tissues, and is a substantial contributor to basal metabolic rate. As one of the most dynamic and plastic tissues in the human body, skeletal muscle can be influenced by a variety of factors including genetics, physical activity, nutrition, hormones, growth factors, injury, and disease, all of which contribute to muscle health in varying degrees throughout the lifespan.

1.2 Regulation of skeletal muscle

Muscle protein turnover describes the processes of muscle protein synthesis (MPS) and muscle protein breakdown (MPB), mechanisms that are energy costly and account for approximately 28% of resting metabolic rate (3). Muscle protein breakdown results in the release of protein-bound amino acids into the intracellular pool. In the postabsorptive state, rates of MPB exceed those of MPS resulting in negative net protein balance, which if sustained, would result in muscle protein loss. The process of MPS occurs with hypereaminoacidemia and culminates in the incorporation of free amino acids into muscle proteins (4). During hyperaminoacdidemia MPB is also suppressed due to increased intracellular aminoacidemia and feeding-induced hyperinsulinemia. When muscle

proteins are synthesized at a greater rate than they are removed the net result is positive protein balance resulting in muscle protein accretion. Typically, the ingestion of protein and habitual physical activity increase rates of MPS and results in a net positive protein balance that serves to balance periods when MPB exceeds MPS occurring in the postabsorptive state (5). The balance between MPS and MPB is true particularly in skeletal muscle of healthy younger adults where fluctuations in MPS and MPB are roughly equal resulting in the maintenance of skeletal muscle mass (5).

In addition to the provision of amino acids through protein ingestion, mechanical loading can also serve to stimulate rates of muscle protein synthesis. Indeed, both aerobic and resistance exercise have been shown to induce robust increases in MPS (6-9). However, although there is a rise in MPB with exercise in the postabsorptive state (10), the rise in MPS resulting from exercise is much greater than the rise in breakdown, resulting in an overall increase in protein balance. It is not, however, until there is a hyperaminoacidemia, which further stimulates MPS to exceed MPB and the result is net muscle protein accretion. Exercise, in particular resistance exercise (RE), is able to sensitize skeletal muscle to the anabolic effects of dietary protein ingestion (11, 12). Protein ingestion in combination with RE serves as potent stimuli to facilitate skeletal muscle protein remodeling and ultimately muscle hypertrophy. Indeed, previous literature from our laboratory has consistently shown that the consumption of dietary protein following resistance exercise results in a greater increase in MPS in comparison to protein ingestion alone (11, 12).

1.2.1 Mechanisms of MPS in skeletal muscle

The stimulation of MPS is mediated, in part, through the activation of the mechanistic target of rapamycin complex 1 (mTORC1) that when stimulated elicits a signaling cascade ultimately leading to the translation initiation, and translation elongation of muscle proteins. The mTORC1 signaling pathway can be activated by several factors including specific nutrients (amino acids - arginine and leucine (13)), hormones, and through mechanical loading (exercise). In brief, once active, mTORC1 phosphorylates 4E Binding Protein-1 (4EBP-1) and P70 ribosomal s6 kinase 1 (S6K1) which serve to activate parallel pathways to promote the binding of mRNA to the 43S preinitiation ribosomal complex resulting in the synthesis of new proteins (14). Previous work has shown that when rapamycin, a potent inhibitor of mTORC1 was administered to adults following essential amino acid (EAA) ingestion, it resulted in the attenuation (but not complete suppression indicating that there is mTORC1-independent protein synthesis) of the increase in MPS (15). Multiple investigations have shown the upregulation of mTOR and its downstream targets – S6K1, rpS6 and 4EBP-1 – in response to protein ingestion (11, 16, 17) and exercise (11, 18) confirming the requirement of this pathway in the up regulation of translation initiation in human skeletal muscle.

1.2.2 Measuring MPS with the use of stable isotope tracers

Studies conducted as parts of this thesis utilized stable isotope methodology to obtain measures of muscle protein synthesis acutely (4 hours) and integrated over time (up to 2 weeks). Stable isotopes are non-radioactive, but heavier atoms of a particular

element making them distinguishable from their more common elemental counterparts only through the measurement of their mass. Stable isotopes are chemically indistinguishable from the most common isotope and thus behave identically within the body (19). It is this characteristic that makes the use of stable isotope tracers particularly effective in the measurement of muscle protein metabolism as many tracers can be used simultaneously and even multiple labels can be used with any individual amino acid. When introduced into a system, stable isotopes can be monitored over time, providing a dynamic measurement of metabolism in a system (19). In non-diseased states, it is generally accepted that fluctuations in MPS play a relatively larger role than MPB in mediating changes in skeletal muscle mass (20). Indeed, research from our laboratory shows that rates of MPS in healthy humans even in a catabolic state (energy-restriction) were significantly suppressed from baseline and that rates of MPB were unchanged following 10 days of 40% caloric deficit (21). Though possible, the measurement of MPB is technically challenging *in vivo*, whereas measurements of MPS can be directly and reliably measured using a variety of techniques.

Two methods of administering stable isotopes were used for the studies conducted as part of this thesis. The first method involved the intravenous administration of stable isotopes through a primed constant infusion over a period of several hours to achieve a steady tracer to tracee ratio within the plasma (5-10% enrichments above background) and intracellular precursor pool (3-5% enrichments above background) (22). In this method, serial blood sampling usually serves to determine the steady state enrichment of the precursor pool coupled with muscle biopsy samples to determine the incorporation of

the tracer into components of skeletal muscle protein over time. The primed constant infusion technique provides a sensitive measurement of MPS for the assessment of the acute responses (2-18 hours) of skeletal muscle to specific stimuli such as feeding or exercise; however, a major limitation of this method is its restriction of measurement to a controlled-laboratory environment.

The second method for the measurement of MPS used in this thesis was the use of orally ingested deuterated water (D_2O). This method allows for greater freedom for the participant as they are not confined to the laboratory and allows for the determination of changes in MPS from hours, to days, to weeks depending on the interval between biopsies. Use of the D_2O method also allows for the incorporation of daily variations in MPS throughout the measurement, taking into account all phases of a particular intervention (postabsorptive, postprandial, activity, inactivity, sleep) occurring throughout the measurement period and may be more representative of alterations occurring *in vivo*. Methods for the administration pattern of oral D_2O to participants are varied, however, the method ultimately relies on the principals of the precursor-product labeling approach used in the primed constant infusion method (23). Once consumed, D_2O rapidly equilibrates to the total body water pool where amino acids with exchangeable hydrogen atoms become labeled with D (^2H) which occurs intracellularly and mostly in the liver through exchange reactions and transamination (24). Measurements of the incorporation of deuterium in the muscle are done with via use of an IRMS measuring incorporation of deuterated alanine, an amino acid that is in rapid isotopic equilibrium with the body water pool (25, 26). In theory, any amino acid could be used for the measurement of MPS with

the D_2O method, however, alanine is most often chosen as its equilibration with body water is rapid (10-20min) and the majority of its exchangeable hydrogens (4 in total) are exchanged with high efficiency allowing for higher measurement sensitivity (~3.7 of 4 exchange sites).

Fractional synthetic rate, the rate of muscle protein synthesis expressed as a percentage of the protein made in a given time, can be measured in several fractions of muscle tissue: myofibrillar, sarcoplasmic, collagen (perimysial), or as mixed proteins (all fractions). The measurement of myofibrillar MPS (MyoPS) is indicative of the remodeling of the myofibrils, which are responsible for the generation and transmission of contractile force. MyoPS has been shown to be very responsive to mechanical loading (7, 18) and to dietary amino acid ingestion (27, 28), in a dose dependent manner (4, 29). Sarcoplasmic protein synthesis (SarcPS) has also been shown to be responsive to mechanical loading and dietary amino acid provision, however, it appears to be less sensitive to dietary amino acids than myofibrillar proteins (29). Perimysial muscle collagen on the other hand, makes up approximately 15-20% of muscle proteins and is sensitive to mechanical loading (30, 31); however, rates of collagen protein synthesis (CPS) have not been shown to be responsive to nutritional provision alone (30, 31).

1.2.3 Role of protein quality and dose in MPS

Dietary protein quality is assessed via the protein digestibility corrected amino acid score (PDCAAS) and more recently, the digestible indispensible amino acid score (DIAAS), methods that take into account the essential amino acid content and

digestibility of the protein source. The PDCAAS derives its score from fecal digestibility of amino acids and is truncated at 1 (100%), meaning that high quality protein sources, such as isolated whey and soy proteins, are scored equally despite a differences in PDCAAS score of 0.17 (whey and soy protein have PDCAAS values of 1.21 and 1.04 respectively) (32). Alternatively, the DIAAS derives its score by examining ileal digestibility of indispensible amino acids and is considered to better reflect the amount of amino acids absorbed before colonic amino acid and nitrogen metabolism (33). Further, the DIAAS is not truncated at 1, allowing for differentiation of proteins of higher quality and the recognition that individual amino acids could play metabolic roles beyond their requirement intakes. Importantly, neither methods score protein sources that lack EAAs, resulting in any incomplete protein, which is a protein that lacks an essential amino acid, given a score of 0.

In skeletal muscle, EAAs have been shown to be potent regulators of the feeding induced rise in MPS (34). Indeed, in a seminal study by Tipton et al. (35), the authors showed that consumption of a carbohydrate beverage in combination with a small amount of EAAs was sufficient to stimulate muscle protein anabolism. In agreement, Volpi et al., showed that consuming either 18g of EAA or 40g of balanced amino acids (18 g EAA and 22 g non-essential amino acids (NEAA)) resulted in significant and similar increases in rates of muscle protein synthesis. Thus, the additional of NEAA did not have an effect on skeletal muscle metabolism (34). Taken together, these data highlight EAA as an essential component for the nutritional regulation of skeletal muscle protein synthesis.

Importantly, dietary protein sources and protein supplements do not contain equal

amounts of EAA (36) and it is clear that amino acid compositions of supplements can modulate the MPS response to feeding (11). Of the EAA, leucine, a branched-chain amino acid (BCAA), appears to be unique in its ability to stimulate MPS directly as a key regulator of translation initiation in the mTORC1 pathway (37, 38). In an elegant study by Churchward-Venne et al. (16), the authors compared the effects of mixed macronutrient whey protein supplements with varying amounts of leucine (up to 5 g) on rates of MPS with and without RE in healthy young men. Interestingly, MyoPS was similarly increased with the consumption of 25 g of whey protein and 6 grams of whey protein with 4.3 g of added leucine (5g leucine total) in both an exercised and non-exercised limb and both conditions were significantly greater than consumption of 6 g of whey protein (0.7g leucine total), 6 grams of whey protein with 2.25 g of added leucine (3g leucine total), and 6 g of whey protein with 6.3 g of added BCAA (5g leucine total) (16). Thus, the effect of ingesting 25g of whey (~2.5g of leucine) on MyoPS was recapitulated by an absolute protein dose 25% less, but contained ~5g of leucine.

1.3 Impact of aging on the regulation of MPS

The older population is growing in Canada (39) and around the globe (40). For the first time in 2017, the proportion of adults over the age of 65 in Canada was greater than the proportion of adolescents and children under the age of 14 and by 2030 it is estimated that one in every 4 Canadians will be over the age of 65 (41). This dramatic growth in the population of older adults is largely driven by an increase in lifespan that is not accompanied by a proportional reduction in morbidity meaning that though older adults

are living longer, it is not without incidence of chronic disease and mobility impairments (42). The increasing emphasis in geroscience is to focus on healthspan, as opposed to lifespan, which describes the proportion of one's life that one is healthy (43).

Aging is accompanied by the progressive loss of skeletal muscle mass and function, termed sarcopenia. It is uncertain when sarcopenia begins, but losses in muscle mass are measurable around the 5th decade of life compared to the 3rd decade of life. Population-based estimates suggest muscle loss occurs at a rate of ~1% per year past age 55 (44) with losses in muscle strength and power, more rapid at rates of ~3% and ~8% per year respectively (45, 46). Currently, there is no consensus on a definition for sarcopenia despite that it is considered to be a complex geriatric syndrome (47). The European Working Group on Sarcopenia in Older Persons (EWGSOP) and other special interest groups (48) have formed practical clinical definitions for the evaluation of sarcopenia using the presence of both low muscle mass and low physical function. In the EWGSOP definition, low muscle mass is defined as being greater than two standard deviations below the mean measured in a reference group (The National Health and Nutrition Examination Survey data is used in the EWGSOP) (44) and low physical function defined as gait speed < 0.8 m/second in a 4 m walking test (49). Using these criteria, a systematic review determined that close to 29% of older community dwelling adults are considered to be sarcopenic, with prevalence closer to 33% in populations in long term care (50); however, there is wide variance in prevalence that is highly dependent on which definition of sarcopenia is used (51). Importantly, sarcopenia is an independent risk factor for physical disability (52), falls (53), incidence of hospitalization

(54), length of hospital stay (54), reduced physical health related quality of life (55), and mortality (56). Therefore, reducing the incidence of sarcopenia or mitigating its progression would greatly impact the ability of older adults to age in good health.

The mechanisms driving the progressive loss of muscle mass with age are multifaceted, making the treatment of sarcopenia as a disease very difficult. Indeed muscle mass and strength loss are affected by a variety of factors (57) however for this thesis we will focus peripherally on skeletal muscle. Loss of muscle mass with aging is a combination of reductions in muscle fibre number (fibre denervation) (58), in particular, type II muscle fibers (59), and a decline in type II fiber cross sectional area (60) with losses appearing to affect men and women to a similar extent (61).

1.3.1 Nutrition to combat anabolic resistance

Basal rates of protein turnover have been shown to be similar in younger (18-30 years) and older (> 60 years) adults (62, 63) indicating that older adults should retain a similar capacity to maintain skeletal muscle over time. However with age, skeletal muscle exhibits a blunting of the muscle protein synthetic response to anabolic stimuli such as protein ingestion and mechanical loading despite no differences in postabsorptive rates of MPS with increasing age (61, 64). Thus strategies to overcome the diminished anabolic responses demonstrated by healthy aging populations are crucial for the maintenance of muscle health. Despite age related anabolic resistance, older adults maintain their ability to respond to nutritional stimuli to augment rates of MPS. Interestingly, there appears to be a saturable dose-response relationship between the quantity of protein ingestion and

the response of MPS in young and older healthy men following resistance exercise (65). Moore et al., retrospectively analyzed MPS data from 6 studies following the ingestion of high quality protein (5 with whey, 1 with egg) to determine the point at which the MPS response plateaued in men 18-37 years and men 65-80 years. The authors compared studies using the same tracer methodology examining MPS responses over a 3-4 postprandial window. Through breakpoint analysis, these authors found that younger adults reached a plateau in the rise in MPS following bolus protein ingestion greater than 0.24 g/kg while older adults would reach this plateau at 0.4 g/kg, a dose ~67% greater than for younger adults (65). These findings were extremely impactful in quantifying appropriate per-meal protein doses for younger and older healthy men however the authors speculated that factors such as disease status or lower protein quality supplements would result in a rightward shift of this breakpoint curve, thus increasing protein requirements (65). Given that the protein sources provided as part of the included studies were all considered to be high quality (DIAAS for whey and egg are 1.09 and 1.13 respectively), protein dose should still be acknowledged with the consideration of protein quality, as lower quality proteins may require a greater absolute dose of protein to maximally stimulate a muscle protein synthetic response.

Protein quality is a significant factor in determining the response of MPS with particular importance in older adults, who with age consume less protein overall than younger adults (66). Indeed, younger adults appear to be responsive to doses of leucine as low as 1 g (16) while it has been suggested that older adults require doses of leucine greater than 2.5 g in order to significantly increase rates of MPS above postabsorptive

levels (67). Thus, the selection of protein type and dose are crucial when prescribing supplemental protein to older adults as a lower quality protein, presumably with a lower leucine content, will require greater absolute consumption in order to stimulate MPS in older persons compared to younger persons. Recently, we showed that leucine content, and not total protein content, was a primary determinant of the rate of MPS in healthy older women (68). Devries et al., showed that the consumption of 25 g of whey protein stimulated rates of MPS similarly to the consumption of 10 g of milk protein when supplements were matched for total leucine (3 g) (68).

Supplementation with protein or amino acids may represent an effective strategy to combat anabolic resistance particularly if used to create a more even meal-to-meal pattern with protein intake at each meal and equivalent post-prandial aminoacidemia (69, 70). This is largely because older adults tend to consume the largest amounts of protein; amounts sufficient to induce maximal rates of MPS only at dinner meals (71) as shown in Figure 1 and therefore the addition of a protein supplement at meals throughout the day may serve to facilitate positive daily protein balance (70). Protein supplementation alone has demonstrated pronounced effects on strength and LBM in frail (72), pre-frail (72, 73), and healthy adults (74) and may influence the ability of older persons to attenuate muscle loss with age. However, a recent meta-analysis has shown that not all protein supplementation studies result in the augmentation of strength and LBM (75). Supplementation with, vitamin D (76), n-3 polyunsaturated fatty acids (n-3 PUFA) (77), and essential amino acid (EAA) (78) have also independently shown marked positive effects on LBM to augment LBM and strength.

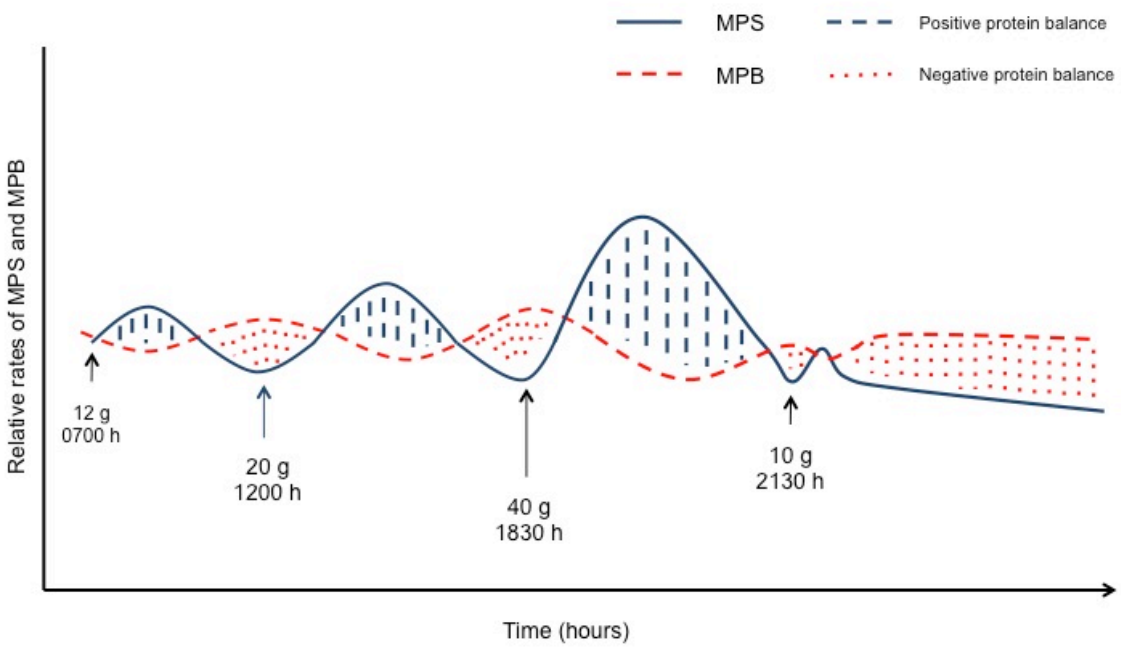

Figure 1. Muscle protein synthesis (MPS) and muscle protein breakdown (MPB) in responses to grams of protein per meal. Solid lines indicate MPS, dashed lines indicate MPB. Blue hashed areas indicate positive protein balance while the red dotted areas indicate negative protein balance. Blue hashed areas and red dotted areas equate to the same area under the curve indicating net protein balance.

1.3.2 Protein and exercise to combat anabolic resistance

While adults of advanced age have dampened response to respond acutely to anabolic stimuli they have been observed to undergo hypertrophy following prolonged protein ingestion and RE (79). Indeed work from our lab shows that resistance exercise sensitizes skeletal muscle to the anabolic effects of protein ingestion, augmenting the muscle protein synthetic response above feeding alone (12). Yang et al., showed that though a 40 g dose of whey protein was able to increase rates of MPS above basal levels, when combined with RE, this rate was augmented in comparison to the non-exercised limb indicating the older adults are still able to integrate the anabolic effects of protein ingestion paired with mechanical loading (12). Long term resistance training studies in older adults have shown substantial increases in muscle fibre cross sectional area (CSA)

(80), whole muscle CSA (80), and leg lean mass (81), when exercise is performed alone (82) and when combined with protein supplementation (83) over training periods from 12-16 weeks. Interestingly, Verdijk et al., showed that when protein intake is above the RDA (1.1 g/kg/day) the addition of a suboptimal protein dose (20 g) during resistance training did not further increase muscle fibre CSA or quadriceps CSA greater than the consumption of a placebo control (81). These data are in line with findings by Eliot et al., who showed similar increases in LBM following 14 weeks of resistance exercise training in older men consuming whey protein or a placebo control (84). In these findings, protein intake was only 0.2 g/kg/day above the RDA including the supplement and still well below the daily protein recommendations of 1.0-1.2 g/kg/day for older adults (67, 85). Optimal nutrition and regular resistance exercise training if tailored specifically for aging adults, may have the potential to drastically slow the loss of muscle with age; however, we know that the loss of skeletal muscle in habitual aging is not linear and therefore research should also examine strategies to augment muscle mass and strength during and in recovery from periods of inactivity during which the loss of muscle mass and strength is accelerated.

1.4 Impact of physical inactivity on aging skeletal muscle

In Canada, approximately 85% of individuals are not meeting physical activity guidelines (86). This highlights the potential for improvement that could be achieved with increased physical activity to reduce risks for a number of diseases and for all-cause mortality (87, 88). Older adults tend to engage in less physical activity in comparison to

younger adults (89) with a notable decline in levels of leisure time physical activity in older adults (90-92). Interestingly, social isolation may play a pivotal role in levels of physical activity in older persons and may result from numerous factors: inability to leave the house due to poor mobility, lack of transportation, or adverse weather conditions, illness of the individual or in their social circles, all of which highlight the complexity for the capacity of intervention in aging adults.

Exacerbating low levels of habitual physical activity in older adults are abrupt and acute reductions in activity resulting in lower levels of mechanical loading of skeletal muscle. These acute bouts of inactivity manifest due to a variety of circumstances (illness, injury, poor weather conditions) and are distinctly different from habitual sedentary behavior. Though these sudden but marked disruptions in activity may be seemingly benign, it is hypothesized that accumulated bouts of drastic inactivity superimposed on a physically inactive population is a major risk for negative physiological health outcomes and may accelerate sarcopenia and the development of chronic cardiometabolic conditions associated with aging.

Previous studies have employed various models to study physical inactivity in humans ranging from a brief reduction in habitual physical activity (90% reduction in daily steps for one day) (93) to spaceflight and microgravity (94). As shown in Figure 2,

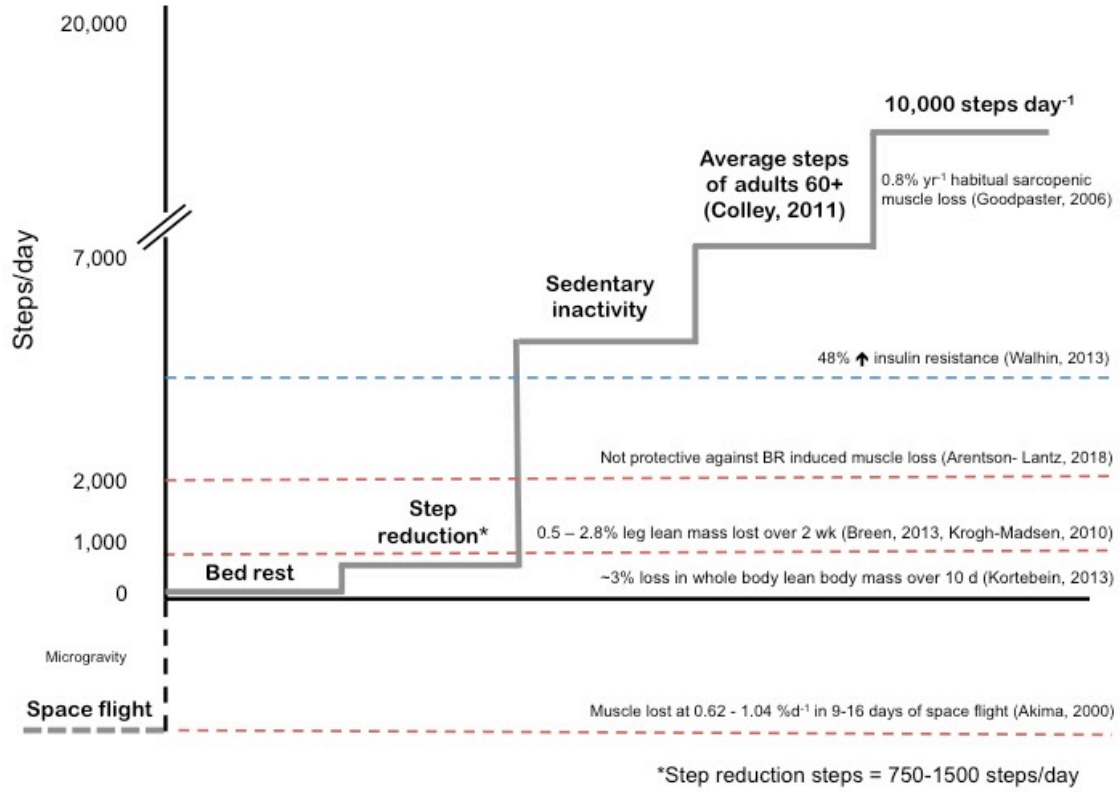

Figure 2. Physical inactivity models used in human skeletal muscle metabolism. Typical sarcopenic muscle loss based on population estimates (95). Sedentary behavior as categorized by Walhin et al., as < 4000 steps per day induced significant insulin resistance in healthy young adults (96). Interestingly, during one week of bed rest, participants walked for ~22 minutes per day (~2000 steps) in an effort to offset bed rest induced muscle atrophy however exercise was not able to mediate this effect and muscle loss was similar to controls (97). Step reduction, or abruptly reducing habitual daily steps to 750-1500 steps per day results in leg lean mass loss over two weeks in healthy young and older adults (98-100). Bed rest induces rapid muscle atrophy in healthy older adults (101) however the rate of muscle loss per day as a result of space flight or exposure to microgravity are staggering though affect only a small number of individuals (102).

each reduced activity model results in differences in daily activity levels, which are notably reduced from habitual physical activity levels in older adults. Inactivity during bed rest has provided researchers with a characteristic change in muscle phenotype in order to better understand the physiological consequences of disuse (103). Given that bed

rest requires inactivity of the whole body, it provides an excellent model to understand the systemic effect of disuse on multiple physiological systems and is clinically relevant (104). Conversely, single limb immobilization studies in older (105, 106) and younger (105, 107, 108) adults have emphasized the significant physiological consequences occurring with local muscle-level disuse. Immobilization-induced muscle loss is largely applicable to clinical scenarios of single-limb immobilization or elective orthopedic surgery, during which the recovery of the affected limb may be without loading for several weeks (105, 106, 109). More recently, the investigation of SR as a form of abrupt physical inactivity has been employed, to investigate the effects of abrupt reductions in activity but not complete disuse. During SR, participants are asked to reduce their daily steps (usually externally monitored by a pedometer or similar device) to a low maximal daily step count (750-5000 steps/d) (100, 110, 111). The lower end of daily step count (~750 steps/d) used in studies is in line with steps performed by patients in acute hospital stays (112). Alarmingly, the daily steps of patients in hospital (out of 708 days examined) exceeded 300 steps per day only 50% of the time; however, on average daily steps per patient were ~740 (112). Reductions of physical activity with SR to these low levels would not obviously constitute complete muscle disuse, but do have profound physiological consequences. Importantly, SR has similar whole body systemic effects, but obviously to a lesser degree as bed rest, in comparison to unilateral limb immobilization that largely targets the peripheral, affected tissues as shown in Figure 3.

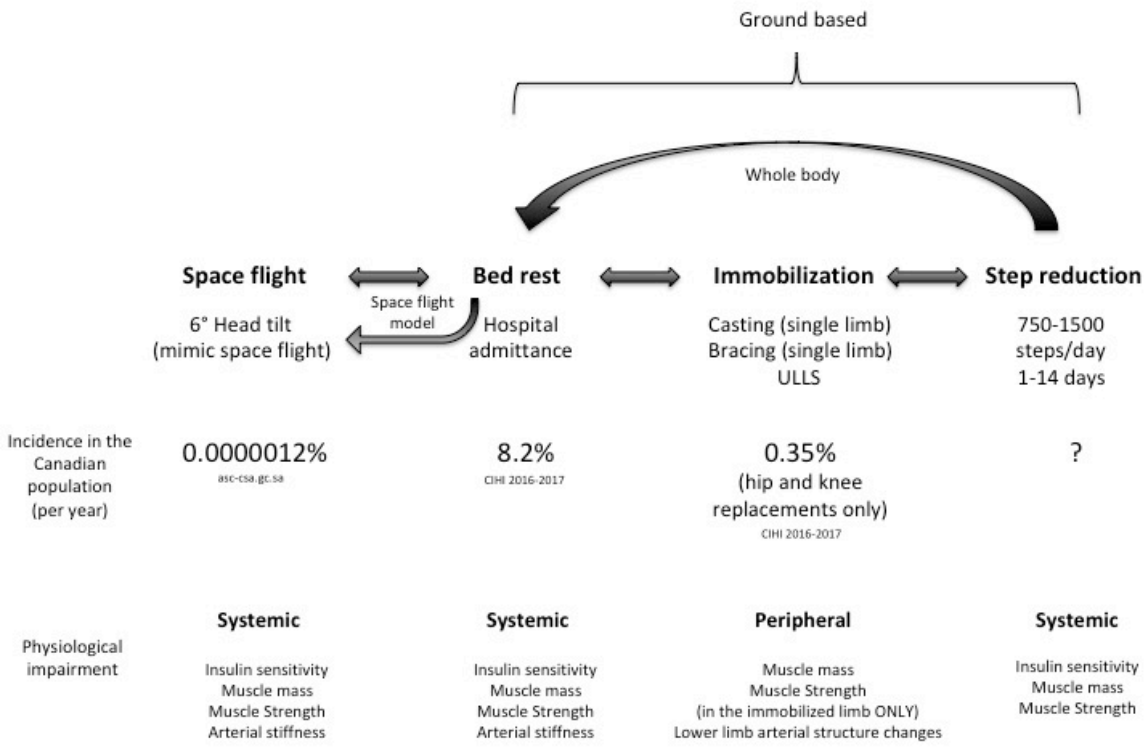

Figure 3. Comparison of disuse models used to study inactivity highlighting the whole body systemic nature of bed rest and step reduction compared to limb immobilization. Data regarding the prevalence of events with induced step reduction is currently unknown. ULLS, unilateral lower limb suspension.

Additionally, episodes of bed rest (typically due to hospital admittance) arguably occur less frequently than episodes of SR that occur periodically throughout the lifespan due to weather or illness such as influenza and likely affect a greater proportion of the population than is affected by complete bed rest.

1.4.1 Physiological consequences of SR in younger adults

The first study establishing the physiological consequences of SR was by Olsen and colleagues who elegantly demonstrated that reducing daily steps for as little as 3-wks had marked negative impacts on skeletal muscle (113). Participants (young healthy adults) reduced their step count by simply taking the elevator instead of stairs and utilizing cars

instead of walking or bicycling resulting in daily average steps totalling ~1300 steps/d (reduced from a habitual step count of ~10000 steps/d). Following 21 days of SR, participants had decreased insulin sensitivity, attenuation of postprandial lipid metabolism, and increased intra-abdominal fat mass (113). The negative alterations in glycemic control and impaired lipid metabolism (113) highlight the rapidity of how healthy and mobile individuals move to metabolic dysfunction through a period of SR and how easily, through alterations in use of personal and publication transportation that adults can reduce daily step count.

Knudsen and colleagues also demonstrated that reducing daily steps from 10,000 to 1500, in combination with over-feeding, increased visceral adiposity by 49% and decreased insulin sensitivity by 44% in healthy, active, young adults (114). Likewise, Krogh-Madsen et al., showed that reducing daily physical activity to levels similar to the previously described intervention but with maintaining energy balance (114), resulted in similar reductions in insulin sensitivity, a reduction in maximal aerobic capacity of 7 mL/kg/min, and a 0.5 kg decrease in leg lean mass (LLM) in healthy young men (99). Taken together, data from Knudsen et al. (114), and Krogh-Madsen et al. (99), demonstrate the potency of a SR model on multiple body systems and the susceptibility of healthy young adult populations to significant negative metabolic health outcomes following brief periods of inactivity.

Interestingly, Stephens and colleagues (93) showed that even one day of limited physical activity (~260 steps) was a sufficient stimulus to induce impairments in insulin action, as measured by whole body rate of glucose disappearance, in physically active,

young men and women by ~39%. Though the steps per day in that investigation (93) were low for a healthy adult, when viewed in the context of severe illness or hospital stay, the daily step counts are on par with those of hospitalized patients (112). Further, given that the impairments from reduced steps in young healthy adults are notably adverse, it is not unreasonable to hypothesize that the demonstrated adverse effects would be of greater magnitude in a compromised aging population (115). Nevertheless, measures to mitigate rapid declines in physical activity even in young adults should be further explored in order to offset the negative physiological adaptations to SR.

1.4.2 Physiological consequences of SR in older adults

Older adults, compared to their younger counterparts, may be at a greater risk for periods of inactivity in addition to declines in habitual physical activity (89). In addition to the frequency of periodic SR, the adverse consequences of such periods of inactivity in older persons are also likely much greater than they are in younger persons (105). Similarly, healthy older adults have shown marked susceptibility to acute reductions in daily stepping impacting glycemic control, markers of inflammation, and skeletal muscle, the recovery from which is incomplete following SR, underlining an obvious impaired regenerative capacity in aging skeletal muscle (109).

1.4.3 Glucose regulation and inflammation with SR in older adults

Similar to healthy young adults, SR invokes marked negative effects on glucoregulation in healthy older adults. McGlory et al. (109), examined how pre-diabetic

older adults responded metabolically to two weeks of SR and importantly whether participants were able to recover to pre-SR levels. In this study (109), participants reduced their daily steps to <1000 steps/d followed by a two-week recovery period in which they returned to habitual levels of activity. Fasted plasma glucose and insulin levels were significantly elevated following SR by 8% and 31% respectively and these values did not return to pre-SR levels following the two-week recovery (109). Comparably, Breen and colleagues had participants reduce their daily step count by ~75% or to ~1500 steps/day for two weeks and monitored changes in glycemic control. Though the authors found no significant elevation in fasted blood glucose following SR, they noted a significant elevation in fasted plasma insulin as well as an increase in glucose and insulin area under the curve as measured during an OGTT by 9% and 12% respectively confirming an impaired glycemic handling in response to SR (98). Interestingly, in both the aforementioned studies (98, 109), SR resulted in small but significant increases in systemic inflammatory cytokines. Breen et al., observed elevated levels of TNF- α and c-reactive (CRP) protein in response to inactivity in normoglycemic participants while McGlory et al., observed an increase in levels of TNF-α, IL-6 and CRP following SR in overweight and obese pre-diabetic participants. The concentrations of inflammatory markers were partially recovered following return to habitual activity in pre diabetic participants (109). Interestingly, the rise in inflammatory cytokines observed in older adults (98, 109) is something that was not seen in younger adults in response to SR (111, 114). While it is acknowledged that this is an observation, it is posited that this may be of

some significance in explaining why younger persons do and older persons do not recover from SR (116); however, this would require specific examination.

1.4.4 Changes in muscle protein turnover and skeletal muscle with SR

Previous work from our group (98, 109, 117) and others (118, 119), have attributed the loss of muscle mass with muscle disuse, for the most part, to a reduction in both fasted- and fed-state muscle protein synthesis (MPS) (120). Indeed, alterations in skeletal muscle protein synthesis are highly sensitive to modifications in physical activity and mechanical loading to a similar extent in both younger and older adults (101, 107). To date, no studies have examined the impact of SR on modifications in MPS in younger adults in response to SR. Nonetheless, Krogh Madsen et al. (111) did observe a loss of leg lean mass of 2.8% following two weeks of reduced daily stepping (<1500 steps per day), an observation that emphasizes the impact of SR in healthy young adults. Several studies have investigated the effects of SR on losses in LBM and modifications in rates of MPS in older adults (98, 100, 109, 117). Consistent with the concept of muscle disuse-induced 'anabolic resistance' (106, 107), work from our laboratory has shown reductions in MPS in response to two weeks of SR of varying degrees (750-1500 steps per day) (98, 100, 109) with rates reduced 13%- 26% from baseline. Given that MPS is a strong regulator of skeletal muscle mass in healthy populations (21), strategies to improve MPS in response to SR may prove to be promising in the maintenance of skeletal muscle during and in recovery from acute inactivity due to illness.

1.4.5 Changes in muscle function and physical capacity with SR

The association of skeletal muscle mass and skeletal muscle strength has been well established, where reductions in muscle mass/area are roughly correlated with reductions in muscle strength and power (95, 121). Low skeletal muscle strength (but not mass) is an independent risk factor for mortality in healthy individuals (122). Thus determining the impact of seemingly benign periods of reduced daily activity, via models such as SR, on skeletal muscle functional outcomes is imperative, specifically for older adults for whom losses in strength have a substantial effect on quality of life (123).

As mentioned previously, young adults exhibit notable decrements in maximal aerobic capacity (3-7% (111, 114)) following SR (<1500 steps per day) lasting only 14 days. Evidence substantiating alterations in maximum voluntary strength of the lower limbs in response to SR in older adults have been varied (109, 117, McGlory, 2017 #1710, 124). Recently, Reidy et al., found that knee extensor maximum voluntary contraction (MVC) was significantly reduced by ~8% in older adults during moderate SR (<3000 steps per day, two weeks) and was not recovered after 14 days of return to normal activity. Conversely, reductions in knee extensor MVC were not observed in previous investigations of SR despite marked metabolic and physiological perturbations in glucose regulation, inflammation and reductions in MPS (98, 109, 117); however, it is speculated that differences in familiarization procedures might underpin this heterogeneity of response. Substantial familiarization is required in order to obtain a true baseline strength measurement especially in older persons (125), along with the small changes in strength expected with SR (compared to complete disuse) may be responsible for the observed

heterogeneity in MVC as measured by dynamometry following SR. To date, no study has examined the impact of SR on strength or clinical functional parameters in healthy young adults though these outcomes appear to be preserved in older adults following SR, unlike the decrements observed in models of complete unloading (bed rest) (101, 126). Though the decrements in muscle strength reported by Reidy et al., is small, it should be acknowledged that a lack of strength recovery poses a significant threat in the progression of healthy aging. Without recovery of lost strength, each future perturbation in physical activity will reduce an individual's maximal strength output increasing risk for disability and mobility impairments (123). Thus, strategies to restore muscle strength and function following SR are imperative in order for maintenance of independence and quality of life throughout aging.

1.4.6 Mitigating the physiological consequences of disuse and SR with exercise

Muscular contraction is a potent stimulus to attenuate the negative effects of disuse on skeletal muscle loss (117, 127). Resistance training (RT) has been shown to increase skeletal muscle mass (128, 129), capillary density (130) and satellite cell activation in older adults (131) making it an obvious countermeasure to combat skeletal muscle atrophy. Previous literature employing resistance exercise to offset declines in LBM during bed rest has been successful (127, 132-134). Bamman et al., found significant decreases in Type I and II fibre CSA in the non-exercising group while myofibre CSA was maintained in the exercise group of young men during 14 days of bed rest (132). Similarly, Alkner et al., showed that following 90 days of bed rest that the RT group showed no decrease in total quadriceps muscle volume while the non-exercise control

group showed a decrease of ~18% (127). Interestingly, Oates et al also showed that even a very low volume of RT performed every other day was sufficient to mitigate declines in muscle CSA of the triceps surae and knee extensors in healthy young men (135). Thus, the use of RT to mitigate bed rest induced muscle atrophy is clearly an effective means to preserve skeletal muscle in healthy young adult populations.

To date, only one study has been conducted in which periodic low-level resistance exercise has been used to offset SR-induced muscle atrophy (117). Devries et al., utilized a unilateral model of resistance training during SR in which older participants were asked to reduce their daily step count to <1500 steps per day for two weeks (117) and performed unilateral low-load resistance exercise at 30% of their maximal strength (~20-25 repetitions) three times per week. Low load RT has significant promise to induce skeletal muscle hypertrophy and even strength (136, 137) and could possibly be useful in situations where high load exercises are not possible such as hospitalization or when home bound due to illness. Following 2-weeks of SR, the leg that performed RT was protected against the SR-induced reduction in postabsorptive and postprandial MPS seen in the non-exercised SR leg (117). Data from the same group of participants was analyzed for alterations in satellite cell activation in both the SR and SR plus RT limbs and found that RT was effective at preserving Type I and II fibre cross sectional area, similar to findings during bed rest (132), and in the preservation of $Pax7^{+}$ positive cells (satellite cells) in type I and II fibres (138), which were lower in the SR leg. Given the robust impact of resistance exercise on skeletal muscle anabolism in younger and older adults (131, 136) it is not wholly surprising that RT was able to preserve aged skeletal muscle

during SR to levels similar to healthy controls. Though the applicability of these data (117) is debatable since it is recognized that older adults who are taking <1500 steps per day may not be able to perform RT if the nature of the inactivity is caused by illness or injury. Nonetheless, it is proposed that the potentially favourable effects that even infrequent low load muscular contractions may have on the preservation of skeletal muscle health should be considered in an effort to reduce muscle mass and strength loss with disuse. Interestingly, a study by Arentson-Lantz et al. (97), aimed to determine whether taking 2000 steps/d (~22 minutes of walking per day) during one week of bed rest would be a sufficient stimulus to offset losses in skeletal muscle mass and physical function. Following one week, participants lost ~1 kg of LLM with no effect of the added daily stepping, findings that were confirmed by immunohistochemistry determining fiber CSA. Further, increased daily stepping did not attenuate the reduction in leg MVC, with participants exhibiting strength losses of ~12% (97). This study provides compelling data, highlighting the powerful effects that complete bed rest can have. Indeed, strategies to reduce the impact of bed rest and disuse are imperative to the conservation of skeletal muscle and functional outcomes in older adults.

1.4.7 The role for protein in attenuating LBM loss with inactivity

The role of nutrition to mitigate the negative physiological consequences of inactivity has been examined largely in the context of bed rest (119, 126, 139-141) and single leg immobilization (106, 142) with a majority of studies examining protein or amino acid based supplements (119, 141, 143, 144) however, to date, results have been

incongruent in bed rest models. Ferrando and colleagues found that supplementation with 15 g of essential amino acids (5.3 g of leucine) thrice daily, during 10 days of bed rest did not alleviate LBM loss in healthy older adults in comparison to a control group (126). Similarly, English et al., found that meal time supplementation with doses of 4.5 g of leucine only partially protected LBM loss after 7 days of bed rest but did not significantly protect LBM at 14 days of bed rest (119). Conversely Paddon-Jones et al., showed a protective effect of 16.5 g of EAA (3.1 g of leucine) provided three times daily, during 28 days of bed rest on total LBM in young adults (144). Given that the lack of agreement on the efficacy of amino acid supplementation on the sparing of LBM is in both young and older adults during bed rest, much more research is needed in order to definitively determine whether there is indeed a benefit of EAA and leucine supplementation during bed rest.

Interestingly, energy balance appears to play a significant role on LBM loss during bed rest. As might be expected, consumption of a hypocaloric diet results in an accelerated losses of LBM during bed rest, largely through suppression of MPS (21). Indeed, Biolo et al., showed that 14 days of bed rest in combination with a 20% caloric deficit led to the greater wasting of LBM compared to the same participants consuming a eucaloric diet in a cross-over study design (140). However, in a subsequent study, Biolo and colleagues also examined the effects of positive energy balance during bed rest in comparison to negative energy balance. These authors found that during 35 days of bed rest, participants in positive energy balance, lost 1.5 kg more LBM than participants in negative energy balance, a finding that the authors attributed to an activation of

inflammatory pathways associated with the increase in fat mass accompanying the positive energy balanced state. Thus, in addition to nutrient supplementation, a consideration should be made to encourage maintenance of energy balance during periods of disuse for optimal nutrition to attenuate skeletal muscle loss.

Protein and amino acid supplementation has not been widely examined in the literature to offset muscle loss during immobilization. Dirks et al, showed that following 5 days of cast immobilization, quadriceps CSA was reduced by 1.5% in controls and by 2% in healthy older men consuming a placebo or twice daily 20.7 g protein supplement respectively (106). Interestingly participants in this study were provided with a protein supplement that may have been below optimal thresholds (0.4 g/kg/dose (65)) as based on average mass of the participants, a protein dose closer to 30 g may have been more effective to attenuate the loss of LBM with cast immobilization of the knee (65). Given that to our knowledge, the study by Dirks et al., is the only to address protein supplementation during unilateral immobilization, further research is required in order to conclude the efficacy of protein to mitigate immobilization induced muscle atrophy.

1.5 Recovery and regenerative capacity in older adults

Previous studies have been unsuccessful in formulating a dietary plan to "out nutrition" muscle loss, meaning that regardless of dietary protein intake, older adults lose muscle mass during bed rest (126) and immobilization (106). Protein and resistance exercise however, may serve as potent and effective strategies to aid in the recovery of skeletal muscle loss following an abrupt disuse event.

Though it has been shown that older adults retain the capacity to induce skeletal muscle hypertrophy up until the 8th decade of life (81, 82), findings comparing hypertrophy in young and older adults with resistance training are mixed (82, 145, 146). However, following disuse atrophy and subsequent re-training or rehabilitation, older adults are able to increase skeletal muscle volume (105), CSA (147, 148) and muscle fibre area (149). Indeed previous work has shown that younger and older adults who were either cast immobilized or bed-ridden for 14 days, show significant muscle atrophy (105, 150) and that following four (105) and two (150) weeks of resistance exercise rehabilitation, older adults show an incomplete recovery in the ability to restore skeletal muscle mass to baseline levels in comparison to younger adults who experienced a full recovery. In addition to overcoming anabolic resistance in order to restore skeletal muscle with disuse, postprandial and post exercise rates of MPS in older adults must also be restored to increase periods of positive net protein balance and ultimately increase muscle mass. McGlory and colleagues showed in healthy older adults that in the absence of rehabilitative measures (i.e. additional physical activity/loading above baseline levels, nutritional intervention) rates of MPS were not recovered to baseline levels following two weeks of return to habitual activity (17). Importantly, older adults typically have less muscle mass in comparison to younger adults (151) when they become inactive. While the absolute loss of skeletal muscle in older adults in response to reduced activity models may be less (105) the relative loss is significantly greater than losses in younger adults in bed rest protocols (150). Nonetheless, the lack of recovery seen in older as opposed to younger adults is a troubling observation (105, 109).

1.6 Objectives

The aims of the studies that are part of this thesis were to examine the importance of protein quality on skeletal muscle recovery from inactivity in healthy older adults. To date, no study has examined the impact of protein supplementation to mitigate declines in skeletal muscle protein synthesis and potential atrophy during step reduction or to enhance recovery. Further, no studies have compared the effect of protein supplement quality during, and in recovery from inactivity. A further aim was to study the impact of protein quality in determining a stimulation of protein turnover in older women with and without an anabolic stimulus (resistance exercise) using short and long-term measures of muscle protein synthesis.

In study 1 the aim was to examine the effect of a hypocaloric diet and two weeks of step reduction in healthy older adults consuming a higher protein (1.6 g/kg/day) diet supplemented with either 30 g of whey protein or collagen peptides twice daily. Collagen peptides were used as a control protein as they are an isonitrogenous but biologically inactive protein, lacking tryptophan, with a PDCAAS and DIAAS score of 0. We used orally administered D_2O to assess the integrated myofibrillar protein synthesis response to 4 phases: energy balance (1 week), energy restriction (1 week), energy restriction and step reduction (2 weeks), and recovery (1 week), during which participants received their randomly allocated protein supplement twice daily in the last 3 phases. We hypothesized that energy restriction and step reduction would result in a loss of LBM and MPS but that whey protein supplementation would mitigate the loss in muscle mass and decline in MPS in comparison to collagen peptide supplementation.

In Study 2, we examined a subset of the male participants from Study 1 to determine the single muscle fibre response to SR. We also compared physical function outcomes during each phase of the trial in men and women. We hypothesized that combined energy restriction and step reduction would result in a reduction in whole muscle strength and whole body physical function. Additionally, we hypothesized that decrements in whole muscle strength would be associated with reductions in single muscle fibre contractile function.

Following the results of Study 2, in which we observed that female participants were noticeably compromised as a result of the step reduction intervention, Study 3 aimed to examine the effects of short term whey protein and collagen peptide supplementation with and without resistance exercise on the acute and longer-term MPS response in older women. Acute MPS was measured via the primed continuous infusion of L-[*ring*-$^{13}C_6$]-phenyalanine and integrated (6 day) MPS was measured through the oral administration of D_2O. Based on previous work using a similar model (68, 152), we hypothesized that both acute and longer-term MPS would be greater following the consumption of whey protein compared to collagen peptides and that resistance exercise would enhance the muscle protein synthetic response, particularly with whey protein.

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CHAPTER 2:

A randomized controlled trial of the impact of protein supplementation on leg lean mass and integrated muscle protein synthesis during inactivity and energy restriction in older persons. Published in *Am J Clin Nutr* 108(5): 1060-8, 2018

A randomized controlled trial of the impact of protein supplementation on leg lean mass and integrated muscle protein synthesis during inactivity and energy restriction in older persons

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ABSTRACT

Background: In older persons, muscle loss is accelerated during physical inactivity and hypoenergetic states, both of which are features of hospitalization. Protein supplementation may represent a strategy to offset the loss of muscle during inactivity, and enhance recovery on resumption of activity.

Objective: We aimed to determine if protein supplementation, with proteins of substantially different quality, would alleviate the loss of lean mass by augmenting muscle protein synthesis (MPS) while inactive during a hypoenergetic state.

Design: Participants (16 men, mean ± SD age: 69 ± 3 y; 15 women, mean ± SD age: 68 ± 4 y) consumed a diet containing 1.6 g protein · kg^{-1} · d^{-1}, with 55% ± 9% of protein from foods and 45% ± 9% from supplements, namely, whey protein (WP) or collagen peptides (CP): 30 g each, consumed 2 times/d. Participants were in energy balance (EB) for 1 wk, then began a period of energy restriction (ER; –500 kcal/d) for 1 wk, followed by ER with step reduction (ER + SR; <750 steps/d) for 2 wk, before a return to habitual activity in recovery (RC) for 1 wk.

Results: There were significant reductions in leg lean mass (LLM) from EB to ER, and from ER to ER + SR in both groups ($P < 0.001$) with no differences between WP and CP or when comparing the change from phase to phase. During RC, LLM increased from ER + SR, but in the WP group only. Rates of integrated muscle protein synthesis decreased during ER and ER + SR in both groups ($P < 0.01$), but increased during RC only in the WP group ($P = 0.05$).

Conclusions: Protein supplementation did not confer a benefit in protecting LLM, but only supplemental WP augmented LLM and muscle protein synthesis during recovery from inactivity and a hypoenergetic state. This trial was registered at clinicaltrials.gov as NCT03285737. *Am J Clin Nutr* 2018;108:1–9.

Keywords: muscle protein synthesis, older adults, whey protein, collagen peptides, step reduction

INTRODUCTION

Periods of inactivity and muscle disuse, such as during bed rest and hospitalization or protracted illness, are more common in older persons (1). The decline in muscle mass and function during hospitalization can transiently accelerate sarcopenic decline, resulting in incomplete recovery, particularly for older persons (2). We have shown that periods in which fewer steps are taken, as a model of inactivity but not outright muscle disuse, result in reductions in anabolic sensitivity to protein (3, 4) and declines in leg lean mass (3). Such periods of inactivity are, we suggest, more common than bed rest and complete disuse, and may be deleterious in older persons particularly if frequent and incomplete recovery occurs.

In addition to reduced ambulation, hospitalization or illness can be accompanied by a decrease in appetite and food intake, which can lead to an energy deficit and muscle loss (5). Typically, ∼25% of body mass lost in an energy deficit can be attributed to fat-free mass (6), some of which is likely muscle (7). Hospitalization is also associated with energy and protein underfeeding that may further exacerbate muscle catabolism (8).

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Abbreviations used: APE, atomic percentage excess; CP, collagen peptide; CRP, C-reactive protein; CSA, cross-sectional area; DXA, dual-energy X-ray absorptiometry; EAA, essential amino acid; EB, energy balance phase; ER, energy-restricted phase; ER + SR, energy-restricted and step-reduction phase; LBM, lean body mass; LLM, leg lean mass; MPS, muscle protein synthesis; PASE, physical activity scale for the elderly; RC, recovery phase; RDA, Recommended Dietary Allowance; SR, step reduction; WP, whey protein.

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An increase in dietary protein intake may alleviate inactivity-induced muscle loss (9, 10). Whey protein has a high essential amino acid (EAA) content, particularly leucine, and its ingestion stimulates muscle protein synthesis (MPS) (11). Supplementing the diet with protein sources rich in EAA and leucine is known to enhance rates of MPS (12, 13), and may serve to offset losses in muscle mass and strength during periods of physical inactivity (14). Although studies have examined the influence of increased protein intake on muscle atrophy during immobilization (15), no study has examined the efficacy of increased protein consumption to offset loss of muscle mass during inactivity while hypoenergetic and to promote recovery in older adults.

We investigated whether providing healthy older adults with twice the Recommended Dietary Allowance (RDA) of protein ($1.6\ g \cdot kg^{-1} \cdot d^{-1}$) would attenuate the inactivity-induced loss of leg lean mass (LLM) and integrated rates of MPS while energy restricted. We also examined whether supplementation with proteins of different quality would affect muscle outcomes. Supplements were high-quality whey protein (WP) or lower-quality collagen peptides (CP). We selected collagen as a comparator as it provides an isonitrogenous and isoenergetic comparison (as opposed to carbohydrate, which is often used) (16), and as it has shown considerable and impressive anabolic properties in older adults (17) [which have been questioned (18)]. To our knowledge, no other study had compared WP and CP for their effect on MPS in older adults. We hypothesized that energy restriction and step reduction would result in reductions in LLM and MPS as primary outcomes. Further, we hypothesized that WP, but not CP, would mitigate declines in LLM and maintenance of MPS. As secondary outcomes, we believed that ER + SR would induce an increase in levels of systemic inflammation independent of supplement type. We also hypothesized that ER + SR would result in impaired glucose handling congruent with previous findings from our laboratory (4).

METHODS

Ethical approval

The study was approved by the Hamilton Integrated Research Ethics Board, and conformed to the standards for the use of human subjects in research as outlined by the Canadian Tri-Council Policy on the ethical use of human subjects in research (http://www.pre.ethics.gc.ca/pdf/eng/tcps2/TCPS_2_FINAL_Web.pdf). Each participant was informed of the purpose of the study, experimental procedures, and potential risks before written consent was provided. The trial was registered at clinical trials.gov as NCT03285737.

Participants

Thirty-two older adults were recruited from the greater Hamilton area, in response to local advertisements, to participate in this study. Potential participants were screened first by telephone to ensure they were nonsmokers, nondiabetic, and between the ages of 65 and 80 y. Exclusion criteria included significant loss or gain of body mass in the past 6 mo (>2 kg); regular use of: nonsteroidal anti-inflammatory drugs (with the exception of daily low-dose aspirin); use of simvastatin or atorvastatin; use of anticoagulants; the use of a walker, cane, or assistive walking device; current or recently remised cancer; infectious disease; or gastrointestinal disease. **Figure 1** shows the Consolidated Standards of Reporting Trials (CONSORT) diagram for subject flow through the protocol.

Study overview

An overview of the study is shown in **Figure 2**. The study was a double-blind, parallel-group, randomized controlled trial. Eligible participants were allocated to consume 1 of 2 types of protein supplement: 30 g 2 times/d of WP or CP. Allocation was concealed from the participants and researchers for the duration of the study and until all analyses were complete. After baseline testing and familiarization with all study measures, participants commenced the 5-wk-long protocol during which they consumed a controlled diet provided by the study investigators. The protocol was divided into 4 distinct phases. The first phase was a week-long run-in phase in which subjects were in energy balance (EB) with protein intake equal to the RDA ($0.8\ g\ protein \cdot kg^{-1} \cdot d^{-1}$). Subjects were then placed in an energy restriction phase (ER) for 1 wk where they consumed an energy-restricted diet (–500 kcal/d) and protein intake was increased to twice the RDA ($1.6\ g \cdot kg^{-1} \cdot d^{-1}$) by consumption of a twice-daily supplement (30 g/dose) of either WP or CP. Inactivity, as step reduction (SR), was superimposed on ER (ER + SR) for 2 wk. During the ER + SR phase, participants were instructed to reduce their daily step count to ≤750 steps/d, which is a daily step count similar to what is observed in older hospitalized patients (19). Participants monitored their daily step counts with the use of a waist-mounted pedometer (PiezoX, Deep River, ON, Canada) and recorded their steps at the end of each day on a log sheet that was checked at each visit. Energy intake during the ER + SR phase was adjusted to account for subjects' inactivity (20). Finally, during recovery (RC, 1 wk), participants returned to their habitual levels of activity (matching their average daily step count seen in EB and ER). During RC, participants maintained their high protein intake ($1.6\ g \cdot kg^{-1} \cdot d^{-1}$) while consuming the same supplements and an energy intake matching their activity levels during EB. Before and after each dietary phase, participants had blood collected for fasting serum and plasma. Before and at the end of each phase participants underwent a dual-energy X-ray absorptiometry (DXA) scan (GE-Lunar iDXA; Aymes Medical, Newmarket, ON, Canada).

Baseline testing

Before study commencement, participants were asked to complete a physical activity and weighed food record (Nutribase version 11.5; Cybersoft Inc., Phoenix, AZ) for 3 d (2 weekdays and 1 weekend day) to assess habitual physical activity levels and dietary intakes.

Diets

Each participants' energy requirement was determined with the use of the Oxford prediction equations for basal metabolic rate (21) using height and body mass for men and women aged >60 y (20). Activity factors were determined for each participant on the basis of their baseline physical activity records, daily step

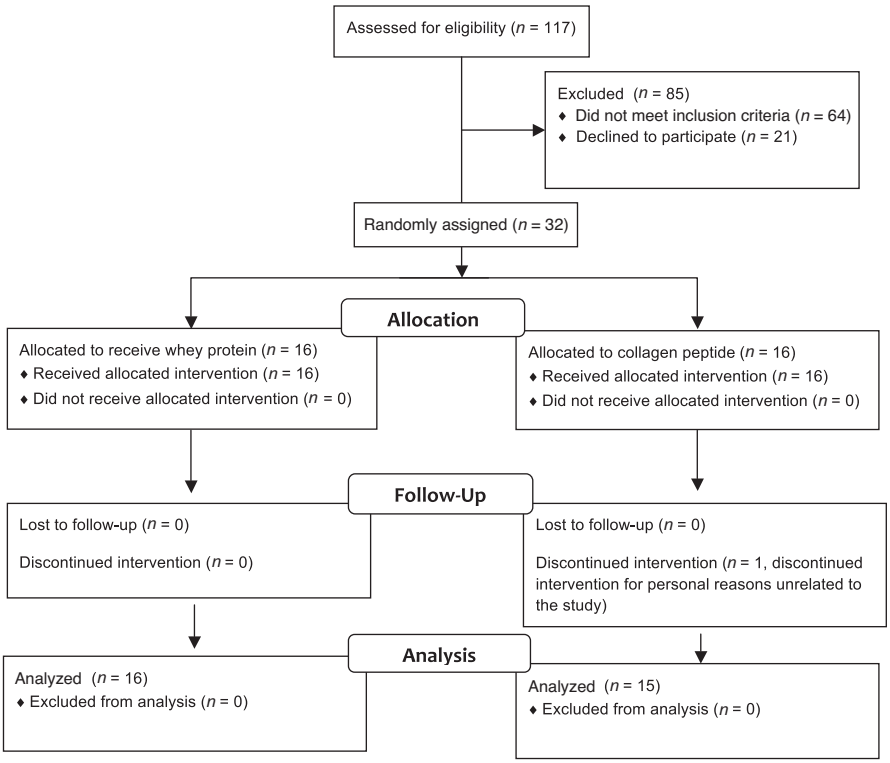

FIGURE 1 CONSORT flow diagram. CONSORT, Consolidated Standards of Reporting Trials.

counts, and Physical Activity Scale for the Elderly questionnaire (PASE) (22) for energy intake during the EB, ER, and RC phases. During the ER + SR phase, a reduced activity factor (of 1.3) was applied to the basal metabolic rate in order to match caloric intake to activity level. During the ER and ER + SR phases, a reduction in total energy intake of 500 kcal/d below the estimated energy requirement was applied to the diet to simulate a moderate energy restriction that is common during hospitalization (23). During the EB phase of the study, participants were provided with a protein intake of 0.8 g · kg^{-1} · d^{-1}, which reflects the current RDA for protein in adults ≥19 y (24). For the ER, ER + SR, and RC phases, participants were provided with a

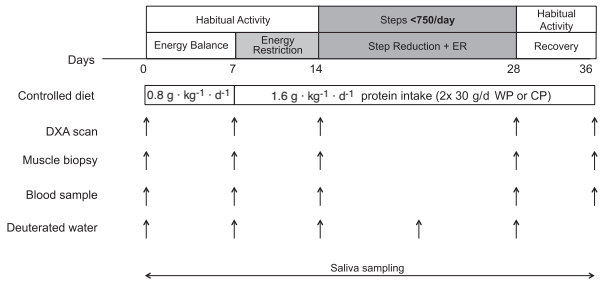

FIGURE 2 Schematic of study design. CP, collagen peptide supplement; DXA, dual-energy X-ray absorptiometry; WP, whey protein supplement.

protein intake of 1.6 g · kg^{-1} · d^{-1}, in line with recommendations from a number of expert committees for optimal protein intake for older adults who are hospitalized (25). Increasing protein intake during the ER, ER + SR, and RC phases of the study was achieved by reducing the proportion of food energy provided from carbohydrates, whereas the proportion of energy from fat was maintained at ∼25% of total energy across all phases. Dietary protein was derived via a combination of plant- and animal-based protein sources throughout the 5-wk trial. During phases ER, ER + SR, and RC, participants were provided with their prepackaged protein supplements (either WP or CP) to be consumed 2 times/d, once in the morning prior to breakfast and once in the evening ∼1–2 h before sleep.

Participants were prescribed a customized meal plan according to food preferences and food was supplied at the beginning of each week. Food consisted of prepackaged frozen meals (Heart to Home Frozen Meals, Brampton, ON) and items that required minimal preparation. Participants were provided with a dietary log where they were to indicate the percentage of the provided food consumed during the day and were strongly encouraged to consume only the study diet. If food outside of the provided diet was consumed, additions were recorded in the dietary log. Overall, compliance with the prescribed diets and supplements was excellent with subjects consuming 98% ± 2% of what was provided.

TABLE 1
Amino acid composition of protein supplements[1]

	WP supplement		CP supplement	
	g/100 g	g/30 g	g/100 g	g/30 g
Alanine	5.7	1.7	8.6	2.6
Arginine	3.0	0.9	7.3	2.2
Aspartic acid	12.5	3.8	5.8	1.7
Cystine	4.0	1.2	0	0
Glutamic acid	17.6	5.3	10.2	3
Glycine	1.8	0.5	22.2	6.7
Histidine	2.0	0.6	1.0	0.3
Proline	4.5	1.4	12.7	3.8
Serine	4.5	1.4	3.2	1.0
Tyrosine	4.2	1.3	0.8	0.2
Tryptophan	2.4	0.7	0	0
Isoleucine	6.3	1.9	1.4	0.4
Leucine	14.3	4.3	2.7	0.8
Lysine	11.2	3.4	3.6	1.1
Methionine	2.4	0.7	0.9	0.3
Phenylalanine	3.8	1.1	2.1	0.6
Threonine	5.3	1.6	1.8	0.5
Valine	5.6	1.7	2.4	0.7
ΣEAAs	51.3	15.4	14.9	4.5
ΣNEAAs	59.8	17.9	71.8	21.5

[1]CP, collagen peptide; EAA, essential amino acid; NEAA, nonessential amino acid; WP, whey protein.

Supplementation

Supplements contained WP isolate (Whey Protein Isolate 895, Nealanderes International Inc., Mississauga, ON, Canada), or hydrolyzed collagen peptide (Gelita, Eberbach, Germany). Individual servings were identically flavored and packaged by Infinit Nutrition (Windsor, ON, Canada) in powdered form. Participants were instructed to mix each package with 300 mL of water before ingestion, and were asked to consume the beverage within a 5-min period. Supplements were isonitrogenous and energy-matched; their contents appear in **Table 1**.

Isotope protocol

Oral consumption of ^2H_2O (70 at.%; Cambridge Isotope Laboratories) was used to label newly synthesized myofibrillar proteins as previously described (26). Participants reported to the laboratory in the fasted state on day 0, and after the collection of a saliva sample (26) and a muscle biopsy from the vastus lateralis, participants consumed a single 100-mL oral bolus of ^2H_2O. This process was repeated at the beginning of each dietary phase of the study. An additional 100 mL dose of ^2H_2O was provided to participants at the beginning of the second week of ER + SR. Total body water enrichment of ^2H was used as a surrogate of the precursor for plasma alanine labeling, which remains in a constant ratio of ∼3.7 with water. This has been confirmed in our laboratory (data not shown) and by others (26–28), and was determined from saliva swabs that were collected by participants between ∼0700 and 0900 each morning.

All muscle biopsies were obtained with the use of a 5-mm Bergström needle that was adapted for manual suction under 1% xylocaine local anesthesia. Muscle tissue samples were freed from any visible connective and adipose tissue, rapidly frozen in liquid nitrogen for measurement of MPS, and mounted in optimal cutting temperature medium for immunohistochemistry; samples were then stored at –80°C for further analysis.

Analytic methods

Myofibrillar proteins were isolated from the muscle biopsies as previously described (29). The incorporation of deuterium into protein-bound alanine was determined and rates of protein synthesis were calculated as detailed previously (30).

Saliva samples were analyzed for ^2H enrichment by cavity ring-down spectroscopy with the use of a liquid isotope analyzer (Picarro L2130-i analyzer, Picarro, Santa Clara, CA) with an automated injection system. The water phase of saliva was injected 6 times and the average of the last 3 measurements was used for data analysis (coefficient of variation ≤0.5%). Standards were measured before and after each participant run. The ^2H isotopic enrichments for muscle and saliva initially expressed as δ^2H were converted to atomic percentage excess (APE) using standard equations (27).

Body composition

Body composition was assessed following an overnight fast (∼12 h). DXA measurements were conducted using a GE Lunar iDXA total body scanner (GE Medical Systems Lunar, Madison, WI) and analyzed (Lunar enCORE version 14.1, GE Medical Systems) in medium-scan mode. The machine was calibrated each testing day with a 3-compartment Universal Whole Body DXA Phantom (Oscar, Jr; Orthometrix, Naples, FL). The analysis regions were standard regions where the head, torso, arms, and legs were subdivided by the software, but were subsequently

checked manually, in a blinded manner, by a single investigator (SYO).

Blood metabolites and hormones

Serum glucose concentrations were measured with the use of the glucose oxidase method (YSI 2300; YSI Life Sciences, Yellow Springs, OH). Plasma insulin concentrations were measured with the use of the dual-site chemiluminescent method (Immulite 2000 immuno-assay system; Siemens, Germany). High-sensitivity C-reactive protein (CRP) levels were measured with an Express Plus autoanalyzer (Chrion Diagnostics Co, Walpole, MA) and using a commercially available high-sensitivity CRP-Latex kit (Pulse Scientific, Burlington, ON). IL-6 and TNF-α levels were measured with a Bio-Plex reagent kit on a Bio-Plex 200 (Bio-Rad Laboratories, Hercules, CA). Intra-assay coefficients of variation were all <5% for all blood analyses.

Calculations

The fractional synthetic rate of myofibrillar protein was determined from the incorporation of deuterium-labeled alanine into protein with the use of enrichment of body water, corrected for the mean number of deuterium moieties incorporated per alanine (27), as the surrogate precursor labeling between subsequent biopsies. In brief, the following standard equation was used: $FSR(\%/d) = [(APE_{Ala})]/[(APE_p) \times t] \times 100$ where FSR is the fractional synthetic rate, APE_{Ala} is the deuterium enrichment of protein-bound alanine, APE_p is the mean precursor enrichment over the time period, and t is the time between biopsies.

The HOMA-IR was calculated with fasted glucose and insulin levels using the standard equation [(glucose × insulin)/22.5] (31).

Histologic staining

Muscle cross-sections, 7-μm thick, were prepared from optimal cutting temperature medium–embedded samples and allowed to air-dry for ∼30 min before being stored at –80°C. Tissue sections were thawed and fixed as previously described (32). Primary antibodies used were A4.951 (MHCI; slow isoform; neat; DSHB); myosin heavy-chain type II (MHCII; fast isoform; 1:1000; ab91506; Abcam, Cambridge, MA); laminin (1:500; ab11575; Abcam). Secondary antibodies used were MHCI (Alexa Fluor 488 anti-mouse, 1:500); MHCII (Alexa Fluor 647 anti-rabbit, 1:500), and laminin (Alexa Fluor anti-rabbit 488, 1:500). Slides were refixed with 4% PFA in between the MHCII and laminin staining steps to limit cross-reactivity. Nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI, 1:20,000; Sigma-Aldrich) before slides were coverslipped with fluorescent mounting media (DAKO, Burlington, ON, Canada). Images were observed with a Nikon Eclipse 90i microscope and captured with a Photometrics Cool SNAP HQ2 fluorescent camera (Nikon Instrument, Melville, NY). Analysis was completed per our previous work (32–35), fiber typing was conducted using ≥300 fibers, and cross-sectional area (CSA) was based on ≥50 fibers/fiber type. Muscle fibers on the periphery of muscle cross-sections were not used in the analysis.

TABLE 2
Participants' characteristics[1]

	WP supplement (n = 16, 8F)	CP supplement (n = 15, 7F)
Age, y	69 ± 4	68 ± 2
Height, m	1.71 ± 0.09	1.70 ± 0.10
Body mass, kg	92.4 ± 14.2	82.0 ± 17.9
BMI, kg/m^2	31.2 ± 5.2	28.0 ± 4.8
Body fat, %	41.1 ± 8.6	37.2 ± 8.6
LBM, kg	52.3 ± 9.7	49.3 ± 11.0
Steps/d	6237 ± 2890	8392 ± 4290
Knee extensor MVC, Nm	143 ± 62	146 ± 43
Chair stands, stands/30 s	15 ± 3	16 ± 3
TUG, s	7.8 ± 2.0	7.1 ± 1.2
6MWT distance, m	542 ± 99	562 ± 67
Gait speed, m/s	1.5 ± 0.3	1.6 ± 9.2

[1] Values are means ± SDs. CP, collagen peptide; LBM, lean body mass; MVC, maximum voluntary contraction; TUG, timed up and go test; WP, whey protein; 6MWT, 6-min walk test.

Statistics

Data were compared using a 2-way mixed-model ANOVA with between (group) and within (time, EB, ER, ER + SR, and RC) factors. The ANOVA revealed no interaction between group and sex, and thus groups were collapsed across sex for all measures. All significant interaction terms for the ANOVA were further tested with the use of Tukey's post hoc test. Significance was set at $P < 0.05$. All statistical analyses were completed using SPSS (IBM SPSS Statistics for Mac, version 21; IBM Corp., Armonk, NY). Data in tables are presented as means ± SDs. Graphical representation of data is in box and whisker plots with the box representing the IQR, the line indicating the median and the cross indicating the mean, and the whiskers indicate the maximum and minimum values.

RESULTS

Subjects' characteristics

Subjects' characteristics are presented in **Table 2**. There were no significant differences between groups for any variable. The baseline step counts of participants were 6237 ± 2890 and 8392 ± 4290 in the WP and CP groups, respectively and 2 wk of ER + SR resulted in a decrease in average daily steps by ∼84% and ∼90% ($P < 0.001$). Subjects returned to taking similar steps during RC (6336 ± 2348 and 8473 ± 3586) in both groups, showing no difference during RC compared with EB ($P > 0.05$).

Diet

There were no significant differences in any dietary variable between groups at any time points ($P > 0.05$) (**Table 3**). All required supplements were consumed by each participant and recorded in a dietary log. Supplemental protein accounted for 45% ± 9% of total protein intake in ER, ER + SR, and RC with the remaining 55% ± 9% derived from food sources.

TABLE 3
Nutrient intake during each dietary phase of the study[1]

		WP supplement			CP supplement			
	EB	ER	ER + SR	RC	EB	ER	ER + SR	RC
Intake, kcal	2535 ± 305	1986 ± 353	1406 ± 244	2337 ± 350	2442 ± 431	1989 ± 466	1394 ± 343	2193 ± 457
Intake, kcal/kg	30 ± 4	22 ± 4	16 ± 3	27 ± 4	29 ± 5	21 ± 5	15 ± 4	26 ± 5
Protein, g/kg	0.8 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	0.8 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1
Protein, g	75 ± 12	87 ± 20	86 ± 21	87 ± 20	67 ± 15	71 ± 29	71 ± 29	71 ± 29

[1]There were no significant differences in protein or calorie intake between WP and CP at any phase. Values are means ± SDs. CP, collagen peptide; EB, energy balance phase; ER, energy-restricted high-protein diet phase; ER + SR, energy-restricted high-protein diet and step-reduction phase; RC, habitual activity and caloric consumption, combined with high-protein intake recovery phase; WP, whey protein.

Body composition

Total lean body mass (LBM) was significantly reduced in ER + SR in comparison to EB ($P < 0.001$). In RC, there was an increase in LBM, but only in WP (**Figure 3A**). Losses in LLM mimicked losses in LBM with a significant reduction at ER + SR in comparison to ER that was increased at RC compared with other times in WP, but not in CP (Figure 3B).

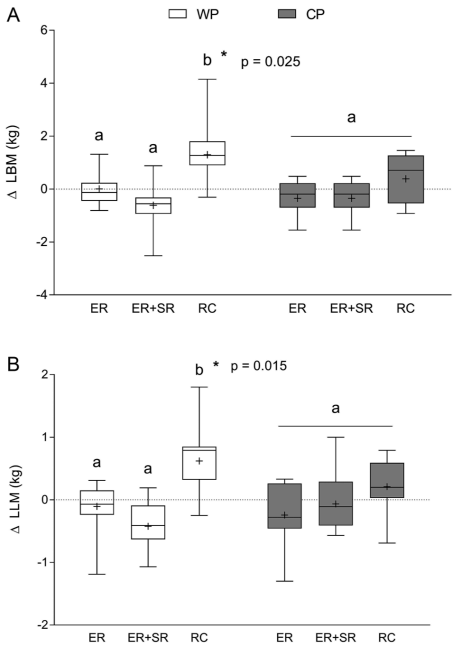

FIGURE 3 Changes in (A) LBM between sequential study phases, and (B) LLM between sequential study phases. The box plot shows the median (line) and mean (+), with the box representing the IQR, and the whiskers representing the maximum and minimum values. Data were analyzed with 2-factor ANOVA with group as a between factor, and repeated measures for phase. WP ($n = 16$), CP ($n = 15$). Means that do not share a letter are significantly different within the group, $P < 0.05$. ∗Differences between groups at that time point, $P < 0.05$. P value indicates the interaction of WP and CP at RC. CP, collagen peptide; ER, energy-restricted phase; ER + SR, energy-restricted and step-reduction phase; LBM, lean body mass; LLM, leg lean mass; RC, recovery phase; WP, whey protein.

Myofibrillar protein synthesis

There were no significant differences between basal rates of myofibrillar MPS between groups during EB ($P > 0.05$). ER resulted in a significant reduction in fractional synthetic rate in both groups ($P < 0.001$). MPS was significantly elevated from ER at RC in the WP group and in comparison to the CP group ($P = 0.05$). Rates of MPS remained suppressed at ER + SR ($P < 0.001$), and RC ($P < 0.001$) from EB in the CP group (**Figure 4**).

Glycemic control and inflammation

There was a significant increase in fasted blood glucose in ER + SR compared with EB and ER that remained elevated at RC ($P < 0.001$) (**Table 4**). There were no significant differences

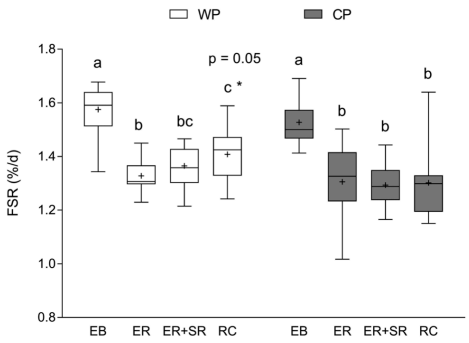

FIGURE 4 Rates of integrated myofibrillar muscle protein synthesis (%/d) during EB, ER, ER + SR, and RC. The box plot shows the median (line) and mean (+), with the box representing the IQR and the whiskers representing the maximum and minimum values. Data were analyzed with 2-factor ANOVA with group as a between factor and repeated measures for phase. WP ($n = 16$), CP ($n = 15$). Means that do not share a letter are significantly different within the group, $P < 0.05$. ∗Differences between groups at that time point, $P < 0.05$. P value indicates the interaction of WP and CP at RC. CP, collagen peptide; EB, energy balance; ER, energy restriction; ER + SR, energy restriction and step reduction; RC, recovery; WP, whey protein.

TABLE 4
Fasted glucose, insulin, and levels of systemic inflammation during each dietary phase of the study[1]

	WP supplement				CP supplement			
	EB	ER	ER + SR	RC	EB	ER	ER + SR	RC
Glucose, mM	4.9 ± 0.5[a]	5.0 ± 0.6[a]	6.2 ± 1.2[b]	5.6 ± 0.9[b]	4.6 ± 0.6[a]	5.1 ± 0.6[a]	6.2 ± 0.9[b]	5.7 ± 0.8[b]
Insulin, uIU/mL	9.9 ± 2.3[a]	6.9 ± 1.5[b]	9.2 ± 3.6[a,c]	8.0 ± 2.3[b,c]	10.0 ± 1.8[a]	6.5 ± 1.7[b]	7.9 ± 2.8[a,c]	8.0 ± 2.6[b,c]
HOMA-IR	2.2 ± 0.5[a]	1.5 ± 0.4[b]	2.5 ± 1.1[a]	2.0 ± 0.7[a]	2.1 ± 0.5[a]	1.6 ± 0.4[b]	2.2 ± 0.9[a]	2.1 ± 0.8[a]
TNF-α, pg/mL	15.1 ± 3.9[a]	16.8 ± 2.9[a]	23.5 ± 5.9[b]	16.17 ± 3.8[a]	16.3 ± 3.6[a]	16.9 ± 3.6[a]	22.8 ± 6.1[b]	17.3 ± 4.8[a]
IL-6, pg/mL	7.4 ± 1.6[a]	6.1 ± 1.7[b]	12.2 ± 4.8[c]	8.2 ± 3.3[a]	7.4 ± 1.7[a]	6.9 ± 1.2[b]	11.9 ± 5.7[c]	8.7 ± 3.4[a]
CRP, mg/L	9.0 ± 3.2[a]	10.2 ± 2.9[a,b]	12.7 ± 4.9[b,c]	13.9 ± 4.6[c]	9.1 ± 2.4[a]	11.3 ± 2.5[a,b]	12.4 ± 4.4[b,c]	11.4 ± 4.3[c]

[1]Data were analyzed with 2-factor ANOVA with repeated measures on time. There were no significant differences between the WP and CP groups at any time point. Values are mean ± SD. Means that do not share a letter are significantly different within the group, $P < 0.05$. CP, collagen peptide; CRP, c-reactive protein;EB, energy balance phase; ER, energy-restricted high-protein diet phase; ER + SR, energy-restricted high-protein diet and step-reduction phase; RC, habitual activity and caloric consumption, combined with high protein intake recovery phase; WP, whey protein.

between groups for any measures of insulin sensitivity or inflammation ($P > 0.05$).

Plasma insulin concentration decreased significantly from EB at ER ($P < 0.001$) but returned to levels similar to EB at ER + SR ($P = 0.03$) before decreasing again at RC ($P = 0.001$).

The calculation of HOMA-IR demonstrated a similar trend to fasted insulin levels where HOMA-IR was significantly decreased from EB at ER ($P < 0.001$), but then was significantly elevated from ER at ER + SR, similar to EB ($P = 0.02$) and then remained at levels no different to those of EB and ER + SR at RC ($P > 0.05$).

Concentrations of TNF-α at ER + SR were significantly elevated from all other phases. Levels of IL-6 were significantly decreased at ER from EB ($P = 0.049$) and elevated from all other time points at ER + SR (EB and ER $P < 0.0001$, ER + SR $P = 0.018$). Levels of CRP were significantly elevated at ER + SR from EB ($P = 0.034$), and remained elevated at RC from EB ($P = 0.003$) and ER ($P = 0.029$).

Type I and II fiber CSA

There were no significant changes in either type I or type II fiber CSA from EB to ER + SR ($P > 0.05$) with no significant differences between groups ($P > 0.05$; **Table 5**).

DISCUSSION

The novel finding of the present investigation was that 2 wk of physical inactivity (step reduction, <750 steps/d) in combination with a mild energy deficit (–500 kcal/d) resulted in a significant reduction in LLM in older men and women consuming a protein intake twice the RDA. Importantly, we observed that consuming a WP supplement, in comparison to the consumption of a CP supplement, resulted in an increase in integrated MPS with return to habitual levels of physical activity. To our knowledge, this study is the first to examine the impact of protein supplementation with different protein sources during simulated hospitalization and convalescence concurrent with a state of energy restriction in older men and women.

Consistent with previous reports (36, 37), we show that a reduction in energy intake induced a decline of ∼16% in integrated myofibrillar MPS, and that during ER + SR there was no further decline. The reduction in rates of MPS in the present investigation are similar to those from our previous study in which 2 wk of inactivity alone resulted in an ∼13% decline in integrated rates of myofibrillar MPS in older men and women (4). Thus, it appears that energy restriction and reduced activity do not synergistically lower rates of myofibrillar MPS, and that a lower limit exists to which MPS can decline in these scenarios, at least in healthy older adults. Importantly, in the present investigation, we report that twice-daily supplementation with WP was effective at increasing rates of MPS from ER + SR during RC in comparison to the consumption of a CP supplement. Interestingly, rates of MPS in the CP group remained suppressed following return to habitual activity. This finding is particularly relevant as our previous work (4) showed that a return to habitual activity in the absence of intervention was insufficient in restoring rates of MPS following 2 wk of return to habitual activity.

Another important finding of the present study was that the introduction of SR, in addition to a period of ER, did not result in a further decrease in LBM or LLM in comparison to ER alone when participants consumed twice the RDA for dietary protein intake.

TABLE 5
Fiber CSA of type I and type II fibers at EB and ER + SR[1]

	WP supplement		CP supplement	
	EB	ER + SR	EB	ER + SR
Type I CSA, μm^2	5570 ± 1987	6479 ± 2912	5501 ± 940	4533 ± 1699
Type II CSA, μm^2	4377 ± 1758	4334 ± 1897	4533 ± 1699	4375 ± 1588

[1]Data were analyzed with 2-factor ANOVA with repeated measures on time. There were no significant differences in type I or II CSA at EB or ER + SR or between WP and CP. Values are means ± SDs. CP, collagen peptide supplement; CSA, cross-sectional area; EB, energy balance phase; ER + SR, energy restricted high protein diet and step reduction phase; WP, whey protein.

We also show that supplementation with WP increased LLM and LBM from ER + SR during RC. However, supplementation with CP did not result in increases in LLM or LBM during RC. Previously, daily supplementation with WP, albeit at what we would consider a suboptimal dose for an older adult population (38), has been shown to be ineffective at reducing losses in skeletal muscle with immobilization (15). Congruent with these findings, our changes in LLM and LBM do not show a significant benefit of supplementation to offset muscle loss during a period of reduced activity; however, this is the first study, to our knowledge, to show an increase in LLM and LBM with WP, but not CP, supplementation during recovery. The pronounced increase in LLM and LBM with WP supplementation provides support for increasing protein intake in older adults in an effort to overcome the heighted anabolic resistance to protein feeding that occurs with age (38).

Consistent with our work from our laboratory (4), we showed that 2 wk of reduced daily activity resulted in a significant impairment in glycemic control following ER + SR that did not fully recover at RC in both groups. We report that ER alone is capable of inducing a favorable reduction in plasma insulin concentration in older adults; however, the addition of inactivity during ER + SR resulted in the elevation of plasma insulin and impairment of glucose handling. Mirroring changes in fasted blood glucose, we found that levels of TNF-α, IL-6, and CRP were significantly elevated following ER + SR; however, ER alone did not result in marked changes in systemic inflammation. These findings are congruent with existing literature using a bed-rest model in which the authors found a significant mediating effect of bed rest on increases in systemic inflammation following 35 d of inactivity (39).

The progression of dietary and activity phases in the present study was a strength of this protocol as it allowed us to determine the effects of ER alone, and in combination with ER + SR with high protein intake, on muscle metabolism in older adults. However, there are some limitations of the current investigation that we acknowledge. First, we did not directly measure rates of muscle protein breakdown, and therefore the relative contributions of MPS and muscle protein breakdown to changes in LBM and LLM are unknown. Second, all the participants in the current study were healthy and free of any chronic condition, thus limiting the applicability of the intervention to older persons with clinically prevalent chronic conditions. However, if the detrimental effects of ER and SR on skeletal muscle are significantly pronounced in a cohort of healthy older adults, we propose that losses in muscle mass and impairments in glycemic control would be worsened in a compromised older adult population as we have reported (4).

In conclusion, we show here that 2 wk of inactivity resulted in the loss of LLM and a decrement in MPS. Importantly, we show that WP was able to stimulate recovery of MPS and increase LLM in 1 wk of return to habitual activity that was not seen in men or women supplemented with CP. Congruent with previous literature, we show that protein supplementation alone was insufficient to offset the absolute loss of muscle mass with acute inactivity, but that supplementation with WP may be protective on LLM from a bout of inactivity combined with a hypocaloric diet and even enhance recovery following return to habitual activity.

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The authors' responsibilities were as follows—SYO, CM, and SMP: conceived and designed the study, and drafted the manuscript; and all authors: participated in some aspect of data collection and/or analysis, provided content and/or editorial corrections, and read and approved the final version of the manuscript. SMP has received competitive research funding, travel expenses, and honoraria for speaking from the US National Dairy Council. The remaining authors had no conflicts of interest to declare.

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CHAPTER 3:

Maintenance of skeletal muscle function following reduced daily physical activity in healthy older adults: a pilot trial.

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Maintenance of skeletal muscle function following reduced daily physical activity in healthy older adults: a pilot trial

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Running title: Muscle function following disuse

6MWT, six-minute walk test; 30 CST, 30 second chair stand test; CSA, muscle fibre cross sectional area; DXA, dual-energy x-ray absorptiometry; EAA, essential amino acid; EB, energy balance phase; EER, estimated energy requirement; ER, energy restricted phase; ER+SR, energy restricted and step reduction phase; LBM, lean body mass; LLM,

leg lean mass; MVC, maximum voluntary contraction; RC, recovery phase; RDA, recommended daily intake; TTPT, time to peak torque; TUG, timed up-and-go test.

Abstract

Introduction: Older adults can experience periods of inactivity related to disease or illness, which can hasten the development of physical disability, in part, through reductions in skeletal muscle strength and power. To date no study has characterized adaptations in skeletal muscle physical function in response to reduced daily physical activity. *Methods:* Participants (15 men, 69±2 years, 15 women, 68±4 years) restricted their daily steps (<750steps/d, SR) while being energy restricted (ER, -500kcal/d) for 2wks before returning to normal activity levels during recovery (RC, 1wk). Before and after each phase, measures of knee extensor isometric maximum voluntary contraction (MVC), time-to-peak torque (TTPT), and physical function were performed and muscle biopsies were taken from a subset of participants. *Results:* Following the energy restriction and step-reduction phase (ER+SR), MVC was reduced by 9.1 and 6.1 Nm in men and women respectively ($p = 0.02$), which returned to baseline after RC in men, but not women ($p = 0.046$). Tmax (maximum isometric tension) in MHC IIA fibres ($p<0.01$) and Pmax (maximum power production) in MHC I and IIA ($p = 0.05$) were increased by 14%, 25%, and 10% respectively following ER+SR. Reductions in muscle strength could not be explained by changes in single muscle fibre function in a sub-sample (n=9 men) of volunteers. *Discussion:* These data highlight the resilience of physical function in healthy older men in the face of an acute period of ER+SR and demonstrate sex-based differences in the ability to recover muscle strength upon resumption of physical activity.

Key words: skeletal muscle, muscle function, muscle strength, single fibre, older adults, physical inactivity

Introduction

Periods of absolute disuse (i.e., bed rest) (Kortebein et al. 2007; Coker et al. 2015), limb immobilization (Suetta et al. 2009; Dirks et al. 2014)) or a period of inactivity resulting in a relative muscle unloading (i.e., reduced daily steps) (Breen et al. 2013; McGlory et al. 2017; Oikawa et al. 2018) reduce muscle size and strength in older adults, which may accelerate habitual sarcopenic declines (Breen et al. 2013). Bed rest and limb immobilization are potent models of the most severe of disuse states and both promote rapid, profound loss of skeletal muscle mass and function (D'Antona et al. 2003; Kortebein et al. 2007; Glover et al. 2008; Suetta et al. 2009; Arentson-Lantz et al. 2016), which can be explained by atrophy and reduced contractility at the cellular level (Alkner et al. 2004); (Trappe et al. 2004) (D'Antona et al. 2003)

Although there is growing literature on the impact of complete muscle disuse on muscle mass and strength in older adults (D'Antona et al. 2003; Kortebein et al. 2007; Suetta et al. 2009), there is comparably less work examining the influence of seemingly benign periods of physical inactivity on changes in strength and functional capacity (Breen et al. 2013; Devries et al. 2015; McGlory et al. 2017; Oikawa et al. 2018; Reidy et al. 2018). Additionally, there are, so far as we are aware, no studies examining how inactivity impacts single muscle fibre function (isometric strength, tension, velocity). Previously, we have shown that reducing daily steps by 75-80% for two weeks induced a substantial negative impact on rates of muscle protein synthesis, a loss of muscle mass and a significant impairment in glucoregulation (Breen et al. 2013) (Devries et al. 2015). Further, during periods of illness or marked reduced activity, older adults tend to consume insufficient calories and specifically, insufficient protein (Drevet et al. 2014).

To make this investigation as applied to relevant clinical settings as possible, we designed the study to include both a reduction in physical activity via daily steps and a diet in energy deficit. We investigated the effects of two-weeks of reduced daily steps (<750/d) and hypocaloric diet on whole muscle and body function in healthy older men and women, and in a select group of older men, single fibre function. We hypothesized that combined step reduction (SR) and energy restriction (ER) would result in a reduction in whole muscle strength and whole body physical function. Furthermore, decrements in whole muscle strength would be associated with reductions in single muscle fibre contractile function.

Methods

Ethical Approval. The study was approved by the Hamilton Integrated Research Ethics Board and conformed to the standards for the ethical use of human subjects in research as outlined by the Canadian Tri-Council Policy (http://www.pre.ethics.gc.ca/pdf/eng/tcps2/TCPS_2_FINAL_Web.pdf). Informed consent was obtained before enrollment. Participants reported on in this paper were a part of another cohort (Oikawa et al. 2018) investigating the effects of protein supplementation on muscle protein synthesis and lean mass retention in older adults (registered as NCT03285737 on clinicaltrials.gov).

Participants. Thirty older adults were recruited from the greater Hamilton area to participate in this study. Potential participants were screened to ensure they were non-smokers, non-diabetic, and between the ages of 65-80 years. Detailed exclusion criteria

can be found elsewhere (Oikawa et al. 2018). Participant characteristics are presented in **Table 1**.

Protocol. The protocol for this trial has been described previously (Oikawa et al. 2018). Participants underwent 5-weeks of controlled dietary intake (**Supplementary Figure S1**). During week 1, participants were in energy balance (EB) with protein intake at the recommended daily allowance (RDA – 0.8 g protein/kg/d) and were instructed to maintain their habitual levels of physical activity. During week 2, participants consumed an energy-restricted diet (ER, -500 kcal/d) to simulate a reduction in energy-intake congruent with dietary patterns observed in institutionalized adults (Konturek et al. 2015) while consuming a high protein diet (1.6 g protein/kg/d),. During weeks 3 and 4, participants maintained the energy restricted and high protein diet from the previous week but reduced their daily step count to <750 steps/day (ER+SR) using a waist-mounted pedometer (PiezoX, Deep River, ON, Canada) with energy intake adjusted for the reduction in physical activity. During week 5, participants recovered (RC) and returned to habitual levels of activity and adequate caloric intake while maintaining their high protein intake (matching their daily step-count in EB and ER). In order to increase protein intake during weeks 2-5, participants were provided with a twice-daily supplement of either whey protein isolate, or collagen peptides (30 g/serve).

Physical function. Participants completed 4 assessments of physical function that were measured at baseline, and then at the end of each dietary phase (end of weeks 1, 2, 4, and 5). The 30s chair stand test (CST) required participants to rise from a chair without the use of their arms as many times as possible in 30 s (Jones et al. 1999). For the timed up-and-go (TUG), participants were instructed to rise from the same chair, walk to and from

a clearly marked point a distance of 3 m away, and sit back down in the shortest amount of time possible (Bell et al. 2017). Participants were given a practice trial before both tests, and the best of 3 trials (with 2 min rest allowed between trials) (Bell et al. 2017)as recorded for each outcome. The 6 min walk test (6MWT) was performed on a 200 m indoor track as described previously (Bell et al. 2017). Participants were instructed to attempt to cover as much distance as possible within 6 min while walking at their usual walking speed. Gait power was calculated as (body mass (kg)·9.8 ($m \cdot s^{-1}$)·average gait speed ($m \cdot s^{-1}$)), where 9.8 represents the acceleration of gravity. Participants were familiarized with the protocol for the physical function tests and the MVC on 3 occasions prior to beginning the study to increase stability in the measurement (Wallerstein et al. 2010). Participants were seated in a Biodex dynamometer (Shirley, NY) with set up as described previously (Devries et al. 2015). Participants performed three 5-s maximal isometric unilateral knee extensions at 70° of knee flexion (0° being full knee extension) separated by a 60-s rest to determine MVC on their dominant leg. MVC was taken as the greatest peak torque from the three contractions. Time-to peak torque (TTPT) was analyzed manually with use of the curve analysis program of the Biodex Dynamometer (Biodex Advantage Software 4.0). The peak torque was considered to be the highest point in each curve.

Muscle biopsy and single fibre function.

In the fasted state, on the first day of week 1, 2, 3 and 5 and on the final day of the study, a muscle biopsy from the *vastus lateralis* was performed using a 5-mm Bergström needle under 1% xylocaine local anesthesia. Though sex based differences may exist, muscle biopsy samples for the measurement of single fibre function were taken only from a

subset of male participants due to the cost of the analysis. Tissue was prepared for single fibre functional assessments, as described (Callahan et al. 2014b). Briefly, muscle fibre bundles were carefully dissected, tied to glass rods at slightly stretched lengths and ultimately stored at -20°C in storage solution (170 mM potassium proprionate,10 mM imidazole, 5 mM EGTA, 2.5 mM $MgCl_2$, 2.5 mMATP-Na_2H_2, and 1 mM sodium azide) at pH 7.0 (Miller et al. 2009) and 50% glycerol (v/v). Samples were then shipped on dry ice to the University of Vermont. Contractile function was measured on chemically-skinned single fibre under maximal Ca^{2+}-activated (pCa 4.5) conditions. Shortening velocity and power output were assessed during isotonic load clamps performed at 15ºC, as described (Callahan et al. 2014b). Single fibres were fibre typed by gel electrophoresis, as described (Toth et al. 2013). Biopsies were shipped within one week following collection and single fibre measurements were completed within 4 weeks following each biopsy.

Statistics. All variables were assessed for normality and statistical outliers. In our original paper (Oikawa et al. 2018), participants were randomized to a protein treatment, however that treatment did not have any main or interaction effects in any of our analysis and therefore groups were pooled for this study. Data were analyzed using a two-way mixed model ANOVA with between (sex) and within (time: EB, ER, ER+SR and RC) factors. Groups were also collapsed across sex with the exception of comparison of isometric MVC in which there was a significant time × sex interaction. All significant interaction terms for the ANOVA resulted in a follow-up post-hoc test to determine pairwise differences using Tukey's post hoc test. For single fibre function measures, a linear mixed model (SAS Version 9.3; SAS Institute, Cary, NC) was used, with time as a

within-subject factor and fibre number as a random effect to account for the fact that individual fibres are clustered within volunteers. Relationships between variables were determined using Pearson correlation coefficients. Analyses, except those on single fibre, were conducted with SPSS software version 16 (SPSS Inc.; Chicago, IL, USA).

Results

Participants. Thirty participants completed the study (15 men, 15 women; 69 ± 3 yrs, 87 ± 17 kg) of which 11 men had muscle fibres collected for single fibre analysis at EB and post ER+SR. Two samples were unable to be analyzed due to technical problems, which left 9 participants available for analysis.

Isometric torque and function. MVC was reduced at ER+SR from EB by 9.2 ± 18.4 and 6.1 ± 7.7 Nm in men and women respectively ($p = 0.02$). MVC was recovered in men ($p>0.05$) but not in women at RC ($p = 0.043$) (**Figure 1**). There were no significant differences in MVC reduction between men and women at ER+SR and no significant changes in time to peak torque (TTPT) between any phase ($p>0.05$).

Gait power was reduced from baseline following ER+SR by 26.3 ± 38.9 and 22.3 ± 17.8 W in men and women respectively ($p<0.05$), but no differences existed at any time point in any other functional task (30s CST, TUG, and 6MWT) ($p>0.05$) (**Table 2**). Reductions in MVC were not correlated to changes in any whole body functional measure (**Supplementary Table S1**).

Single fibre function (**Table 3**). An average of 19 ± 1 fibres were measured per participant at EB with 19 ± 2 fibers measured per participant at ER+SR. There was a trend for reduction in CSA in MHCI fibres following ER+SR ($p = 0.07$) with no change in CSA in MHCII fibres. There was an increase in maximum power production (Pmax) in MHCI ($p = 0.02$) and MHCIIA fibres ($p = 0.05$) and an increase in maximum isometric tension (Tmax) in MHC IIA fibres ($p < 0.01$). Maximum shortening velocity (Vmax) was inversely correlated with MVC ($p = 0.024$).

Discussion

The novel finding of the present investigation was that 2 weeks of combined SR+ER resulted in reduced muscle isometric strength in older men and women. However, no alterations in physical performance measures were observed and single muscle fibre function was unchanged or paradoxically increased when measured in men. Finally, women, as opposed to men, did not recover strength as measured by MVC that was lost during the SR+ER period during RC.

Following ER+SR, isometric knee extensor MVC was reduced by ~5%, confirming that 2 weeks of SR provides a sufficient stimulus to impair muscle function, albeit the magnitude is less than previous investigations in older adults who were on complete disuse of -15% (Kortebein et al. 2007; Hvid et al. 2010). Moreover, following one week of RC (recovery), muscle strength recovered to baseline. Though the decrements in strength in the present investigation are relatively small, periods of reduced physical activity occur at frequently in older populations (Milanovic et al. 2013), increasing the frequency of scenarios during which older adults have compromised

strength. Interestingly, when men and women were analyzed separately, women failed to recover strength losses, suggesting impaired resiliency in older women that may portend greater functional impairment over time (Janssen et al. 2002). Similar findings have been shown comparing the recovery of forearm flexor strength following 3 weeks of cast immobilization demonstrating that women had slower voluntary strength recovery (Clark et al. 2009). Data from the aforementioned work draws attention to a similar reduction in central activation in comparing men and women following casting suggesting that decrements at the level of the muscle may be the cause for a slower recovery in voluntary strength in women rather than differences in neural activation. How these contributions are altered with increasing age however, cannot be determined from the current investigation.

We found no alterations in functional performance outcomes following SR in our cohort of healthy older men and women, with the exception of a small reduction in gait power. Although muscle strength is a strong predictor of whole body functional measures (Bean et al. 2008), the decrement in strength observed in our volunteers may be insufficient to elicit reductions in physical function. Alternatively, there may be adequate reserve in other physiologic systems to maintain physical function. Reductions in physical performance may be expected in clinical populations with SR, where physiological reserve is compromised.

Interestingly, single fibre function, which we measured in only the male participants, remained unchanged or increased with SR+ER, in contrast with changes in whole muscle strength. Our results are not unprecedented, as a recent longitudinal study reported discordance in single fibre and whole muscle contractile performance in

mobility limited individuals (Reid et al. 2014). Considering that we observed these cellular level adaptations in response to acute disuse related to SR, we posit that they may reflect compensatory adaptations to mitigate decrements in whole muscle contractility. Our results are congruent with previous literature comparing single fibre function with a disuse model (knee osteoarthritis) highlighting a potential mitigation of muscle force decrements by the increase of single fibre velocity (Callahan et al. 2014a). Enhanced single fibre function may occur in response to deficits in neuromuscular activation or muscle cellular components of excitation-contraction coupling that develop with disuse, although we acknowledge that a larger cohort of volunteers with single fibre analysis will be needed confirm that SR potentiates myofilament function.

Our ability to compare men and women following a stimulus akin to acute illness was a significant strength of the current investigation. However there are limitations of the present study that we acknowledge. Firstly, our sample size, specifically for the single fibre analyses were small and therefore observations in single fibre function, notably increases in Pmax and Tmax parameters, may be at risk for type II errors. Further investigations into the effects of energy restriction, step reduction, on single fibre function are warranted. Secondly, we did not include women in our analyses of single fibre function due to cost of the analyses. As mentioned previously, women may be at greater risk for the recovery of voluntary strength due to alterations at the peripheral level (rather than central activation) and therefore changes in single fibre in women would provide valuable insight into sex based differences in single fibre function with disuse. Previous literature has attributed slowing of myosin actin-cross-bridge kinetics, increases in myofilament lattice stiffness, and increases in isometric tension associated with aging

to be primarily driven by women (Miller et al. 2013). Thus, including women in future analyses of single fibre function with disuse may provide valuable insight into the mechanisms underlying disparate responses between men and women.

Our data show the resiliency of physical function in healthy older men as a whole despite reduced whole muscle function. In women, the failure to remediate muscle function following resumption of activity levels may hasten disability however other measures of physical function evaluated were unchanged. The investigation of SR on muscle function may provide a more relevant model of real-world disuse in comparison to bed rest or immobilization.

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Conflicts of interest

The authors have no conflicts of interest to report.

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Figure 1. Isometric maximum voluntary contraction of the knee extensors at 70° of flexion in men (A) and women (B). MVC, maximum voluntary contraction; EB, energy balance phase; ER, energy restriction phase; ER+SR, energy restriction and step reduction phase; RC, recovery phase. Means that do not share a letter are significantly different within the group, $p<0.05$. Data were analyzed with 2-factor ANOVA with repeated measures.

Table 1. Participants' characteristics.

	EB	ER	ER+SR	RC
Body mass (kg)	86.4 ± 16.4[a]	85.9 ± 16.5[b]	84.7± 16.4[c]	85.2 ± 16.3[b]
LBM (kg)	50.2 ± 10.3[a]	50.0 ± 10.4[a]	49.5 ± 10.3[b]	50.4 ± 10.2[a]
Body fat (%)	39.2 ± 8.7[a]	39.2 ± 8.9[a]	38.9 ± 9.2[b]	38.2 ± 9.2[c]
Steps (day^{-1})	7315 ± 3757[a]	7883 ± 3814[a]	920 ± 370[b]	7919 ± 3371[a]

Values are means ± SD. LBM, lean body mass; EB, energy balance phase; ER, energy restriction phase; ER+SR, energy restriction and step reduction phase; RC, recovery phase. Means that do not share a letter are significantly different, $p<0.05$.

Table 2. Functional measures

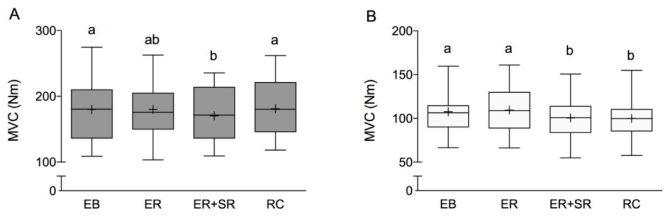
	EB		ER		ER+SR		RC	
Whole body								
30 CST	15.7 ± 3.4[a]		15.7 ± 3.3[a]		15.5 ± 3.1[a]		15.8 ± 3.5[a]	
TUG (sec)	7.3 ± 1.7[a]		7.2 ± 1.6[a]		7.3 ± 1.7[a]		7.2 ± 1.6[a]	
6MWT (m)	560.9 ± 93.2[a]		554.2 ± 91.0[a]		561.8 ± 94.8[a]		561.7 ± 89.8[a]	
Gait speed (m/s)	1.6 ± 0.3[a]		1.5 ± 0.3[a]		1.6 ± 0.3[a]		1.5 ± 0.4[a]	
Gait power (W)	1290.6 ± 424.7[a]		1267.0 ± 418.2[b]		1265.2 ± 415.0[b]		1258.9 ± 491.5[b]	
	Men	Women	Men	Women	Men	Women	Men	Women
Whole muscle								
MVC (Nm)	180.0 ± 49.7[a]	107.7 ± 24.2[a]	180.1 ± 42.4[a]	108.8 ± 26.2[a]	170.9 ± 41.0[b]	102.7 ± 23.1[b]	174.9 ± 44.8[a]	100.8 ± 22.9[b]
TTPT (sec)	2.9 ± 0.9[a]	2.5 ± 1.1[a]	3.0 ± 1.1[a]	2.6 ± 1.1[a]	3.2 ± 1.1[a]	2.5 ± 1.2[a]	3.2 ± 0.9[a]	2.6 ± 1.1[a]

Values are means ± SD. EB, baseline phase; ER+SR, step reduction phase; MVC, Maximum voluntary contraction; 30 CST, 30 second chair stand test; TUG, timed up and go test velocity; 6MWT, six minute walk test distance; TTPT, time to peak torque; EB, energy balance phase; ER, energy restriction phase; ER+SR, energy restriction and step reduction phase; RC, recovery phase. Data were analyzed a 2-way ANOVA (sex and time). Means that do not share a letter are significantly different, $p<0.05$.

Table 3. Single fiber contractile function in older men (n = 9)

	Fiber Type	EB	ER+SR	p value
Tmax (mN/mm^2)	MHC I	102.0 ± 8.5	107.8 ± 8.4	0.16
	MHC IIA	138.9 ± 14.1	167.3 ± 13.8	< 0.01
Vmax (ML/s)	MHC I	0.68 ± 0.12	0.72 ± 0.12	0.29
	MHC IIA	1.29 ± 0.11	1.34 ± 0.11	0.41
Pmax (W/L)	MHC I	0.03 ± 0.01	0.04 ± 0.01	0.02
	MHC IIA	0.09 ± 0.02	0.10 ± 0.02	0.05
CSA (μm^2)	MHC I	6089 ± 622	5602 ± 616	0.07
	MHC IIA	5596 ± 483	5382 ± 472	0.47

Values are least squared means ± SE derived from the mixed model analysis. EB, baseline phase; ER+SR, step reduction phase; Tmax, maximum isometric tension; Vmax, maximum shortening velocity; Pmax, maximum power production.



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198x64mm (300 x 300 DPI)

CHAPTER 4:

Whey protein but not collagen peptides stimulate acute and longer term muscle protein synthesis with and without resistance exercise in healthy older women: a randomized controlled trial. *Submitted to the Journal of Nutrition.*

Whey protein but not collagen peptides stimulate acute and longer-term muscle protein synthesis with and without resistance exercise in healthy older women: a randomized controlled trial

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Running title: Whey and collagen peptides on protein synthesis

Abbreviations

1RM, 1-repetition maximum; AKT, protein kinase B; AUC, area under the curve; C_{max}, concentration max; CP, collagen peptides; DXA, dual-energy x-ray absorptiometry; EAA, essential amino acid; Fed, feeding limb; Fed Ex, feeding plus exercise limb; LBM, lean body mass; MPS, muscle protein synthesis; mTOR, mammalian target of rapamycin; MCPS, muscle collagen protein synthesis; MyoPS, myofibrillar muscle protein synthesis; RE, resistance exercise; T_{max}, time to concentration maximum; WP, whey protein

Abstract

Skeletal muscle protein synthesis (MPS) is stimulated by the hyperaminoacidemia that follows ingestion of protein and is further stimulated with performance of resistance exercise (RE). Aging appears to attenuate the MPS response to anabolic stimuli such as hyperaminoacidemia, which could be affected by protein quality, and RE. The purpose of this study was to determine the effects of protein quality on feeding- and feeding plus RE-induced increases on acute and longer-term MPS following ingestion of whey protein (WP) and collagen protein (CP). In a double-blind parallel-group design, twenty-two healthy older women (69 ± 3 years, n=11/group) were randomly assigned to consume a 30 g supplement of either WP or CP twice daily for 6d. Participants performed unilateral RE twice during the 6d period to determine acute (via [$^{13}C_6$]-phenylalanine infusion) and longer-term (ingestion of deuterated water) MPS. Acutely, WP increased MPS by 0.017 ± 0.008%/h in the rested leg (Fed), and 0.032 ± 0.012%/hr in the feeding and exercise leg (Fed Ex) (both $p<0.01$) whereas CP increased MPS only in Fed Ex (0.012 ± 0.013%/hr) ($p <0.01$) and MPS was greater in WP than CP in both the Fed and Fed Ex legs ($p = 0.02$). Longer-term MPS increased by 0.063 ± 0.059%/day in Fed and 0.173 ± 0.104%/day in Fed Ex ($p<0.0001$) with WP however MPS was not significantly elevated above baseline in Fed (-0.011 ± 0.042%/day) or Fed Ex (0.020 ± 0.034%/day) with CP. Longer-term MPS was greater in WP than CP in both Fed and Fed Ex ($p<0.001$). Supplementation with WP elicited greater increases on both acute- and longer-term MPS than did CP supplementation, which is suggestive that WP would be a more effective supplement to support skeletal muscle retention in older women versus CP.

Introduction

Sarcopenia is the loss of muscle mass and muscle strength with age, progresses at rates of loss of ~0.8% and 1-3% per year respectively, that are measureable in the sixth decade of life (2). Contributing to sarcopenic muscle decline is a decreased response of muscle protein synthesis (MPS) to normally robust anabolic stimuli such as protein ingestion (and the subsequent hyperaminoacidemia) and resistance exercise (RE). This age-related attenuation of MPS has been termed anabolic resistance (3, 4). Previously, we have shown that older men required ~40% more protein per dose of isolated protein to stimulate comparable rates of muscle protein synthesis (MPS) in comparison to younger men (3). Given that many older adults, in particular older women (5), are not meeting protein intakes recommended at 1.0-1.3 g/kg/day (6) through their habitual diet, protein supplementation may be an effective strategy to augment total protein intake and combat anabolic resistance to maintain skeletal muscle health with aging (7).

Resistance exercise improves muscle strength (8), increases skeletal muscle mass (8), and improves functional outcomes (9) in older adults. Further, RE induces marked increases in rates of MPS in both young and older, men and women (10-12) and serves to sensitize skeletal muscle to the anabolic effects of protein ingestion (4, 13). However, to date, few studies have examined the effects of protein supplements of varying quality on the stimulation of MPS in older adults.

Recently, we showed that recovery from inactivity was more effective with consumption of whey protein (WP), which increased rates of MPS in healthy older men and women (14) versus an isonitrogenous collagen peptide (CP) placebo. Our findings

highlight the important role that protein quality can play in recovery and retention of muscle mass. Nonetheless, two studies have reported positive effects of collagen peptide supplementation on lean body mass gains with resistance training in sarcopenic older men (15) and pre-menopausal women (16). Interestingly, a CP supplement improved nitrogen balance in older women (17); however, to our knowledge no study has examined the muscle protein synthetic response that may be underpinning the ostensibly favourable CP supplement-induced changes in body composition (15, 16) or increased nitrogen balance (17). Thus, the aim of this study was to compare the acute and longer-term effects of WP or CP supplementation on MPS alone and when combined with RE. As an exploratory outcome, and given that CP supplementation has been theorized to support greater rates of connective tissue synthesis/renewal (18) we aimed to determine if CP supplementation would facilitate increases in muscle-derived collagen protein (perimysium) synthesis (MCPS). We hypothesized that both acute and longer-term MPS would be greater following consumption of WP compared to CP and that RE would enhance the MPS response but more so in WP.

Methods

Ethical Approval. The study was approved by the Hamilton Integrated Research Ethics Board (#3916) and conformed to the standards for the use of human subjects in research as outlined by the Canadian Tri-Council Policy on the ethical use of human subjects in research (http://www.pre.ethics.gc.ca/pdf/eng/tcps2/TCPS_2_FINAL_Web.pdf). Each participant was informed of the purpose of the study, experimental procedures, and

potential risks before written consent was obtained. The trial was registered at clinicaltrials.gov as NCT03281434.

Participants. Twenty-two healthy older women were recruited from the greater Hamilton area, in response to local advertisements, to participate in this study. Potential participants were screened first by telephone to ensure they were non-smokers, non-diabetic, and between the ages of 60-80 years. Exclusion criteria included: significant loss or gain of body mass in the past 6 months (>2kg); regular use of: non-steroidal anti-inflammatory drugs (with the exception of daily low-dose aspirin); taking either simvastatin or atorvastatin; use of anticoagulants; the use of a walker, cane, or assistive walking device; current or recently remised cancer; infectious disease; and/or gastrointestinal disease. See CONSORT diagram, in **Figure 1** for subject flow through the protocol.

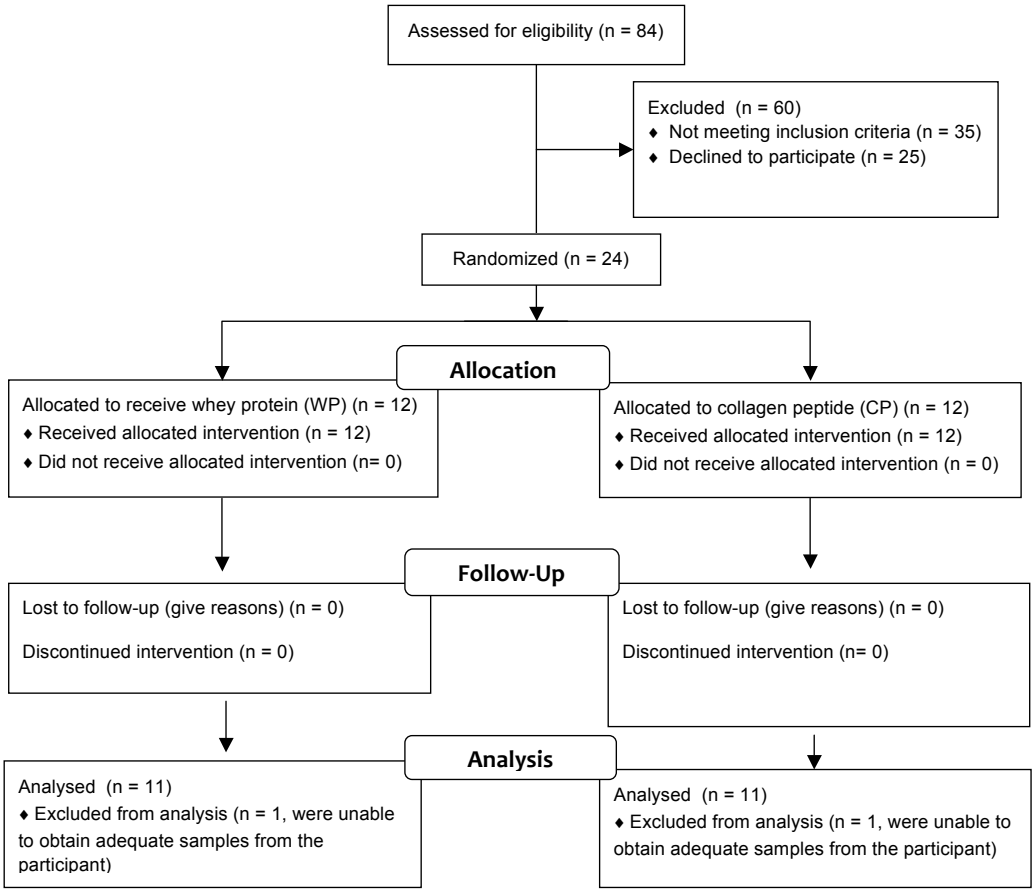

Figure 1. CONSORT flow diagram.

Study overview. An overview of the study is shown in **Figure 2.** The study was a double blind, parallel group, randomized controlled trial. Eligible participants were allocated to consume one of two types of protein supplement: 30g twice daily of WP or CP. Allocation was concealed from the participants and researchers for the duration of the study and until all analyses were complete. After baseline testing and familiarization with all study measures, participants commenced the 9-day protocol during which they consumed a controlled diet with all meals provided by the study investigators.

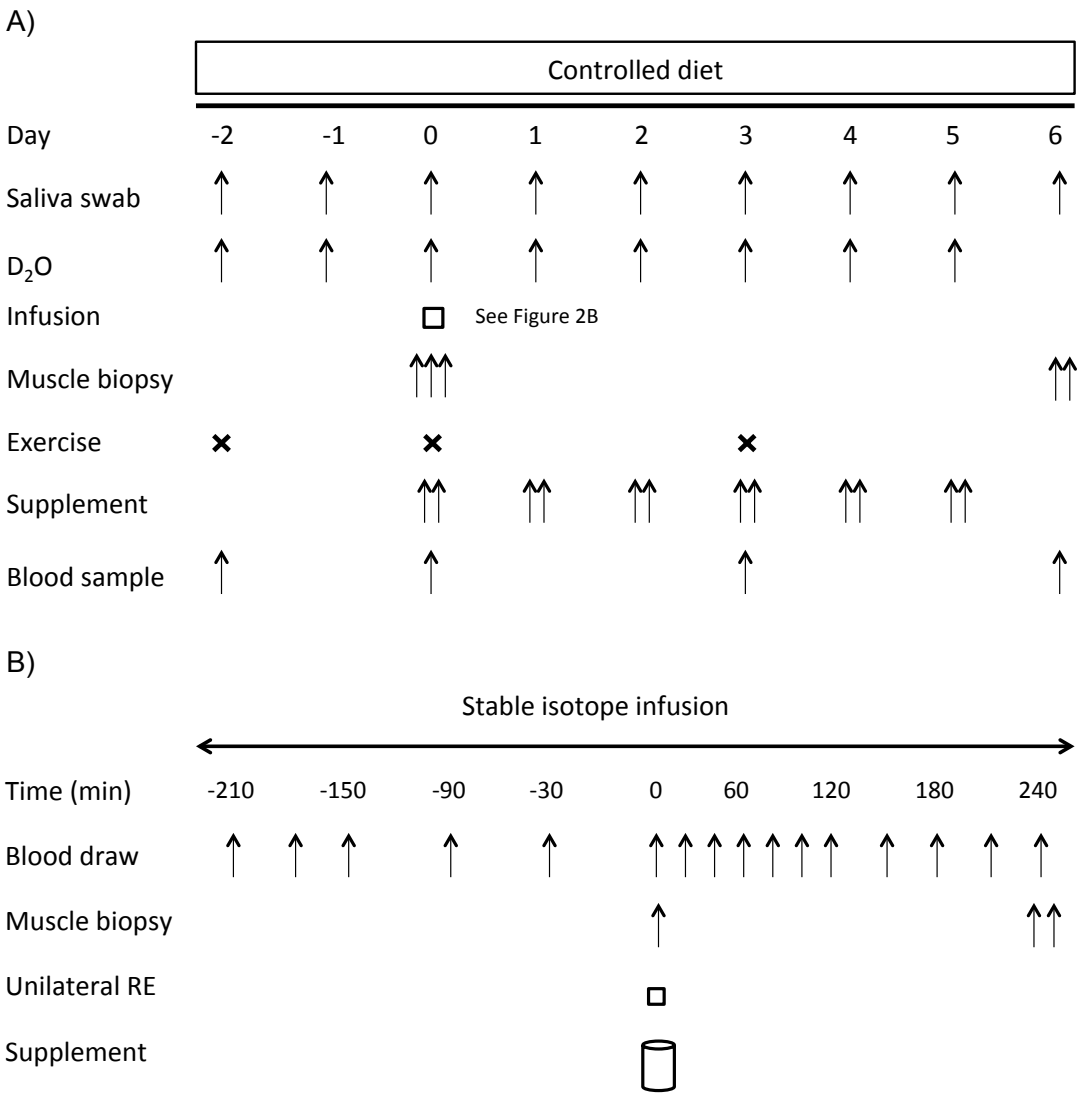

Figure 2. Study schematic over the 9-day protocol (A) and infusion protocol on day 0 (B). RE, resistance exercise.

Baseline testing. Prior to commencement of the protocol, participants were asked to complete a physical activity and weighed food record (Nutribase version 11.5, Cybersoft Inc., Phoenix, AZ, USA) for 3d (2 week days and 1 weekend day) to assess habitual physical activity levels and dietary intakes. Participants also underwent a dual-energy x-

ray absorptiometry (DXA) scan (GE-Lunar iDXA; Aymes Medical, Newmarket ON) for the determination of total fat- and bone-free (lean) body mass (LBM). Participants were assessed for single leg muscle strength (1RM) on a manually loaded leg extension machine (Precor, WA, USA). Following a 5-minute cycling warm up at a self-selected resistance, participants were familiarized with the knee extension machine by performing a single set of unloaded knee extensions for 10 repetitions. Participants rested for 2 minutes and then began testing for a 1 repetition maximum. 1RM values were used to calculate the load corresponding to ~60% to be used for the resistance exercise sessions throughout the study protocol.

Diets. Each participants' energy requirement was determined with the use of the Oxford prediction equations for basal metabolic rate (19) using height and body mass for women over the age of 60 years. Activity factors were determined for each participant on the basis of their baseline physical activity records and the Physical Activity Scale for the Elderly questionnaire (20) for the determination of total energy intake throughout the study protocol. Participants were provided with a protein intake from food sources of 1.0 g/kg/d, which reflects protein intakes consumed by older adults aged 51 and older in Canada (21) on days -2 and -1. Participants were then provided with a twice-daily protein supplement to increased total protein intake to 1.6 g/kg/day on days 0 to 6, achieved by reducing the proportion of food energy provided from carbohydrates, while the proportion of energy from fat was maintained at ~22-25% of total energy. Dietary protein came from a combination of plant- and animal-based protein sources. Participants were prescribed a

customized meal plan according to food preferences and food was supplied at the beginning of each week. Food consisted of pre-packaged frozen meals (Heart to Home Meals, Brampton, ON) and items that required minimal preparation. Participants were provided with a dietary log where they were to indicate the percentage of the provided food consumed during the day and were strongly encouraged to consume only the study diet. If food outside of the provided diet was consumed, additions were recorded in the dietary log. Overall, compliance with the prescribed diets and supplements was excellent with subjects consuming 97 ± 2% of what was provided.

Supplementation. Supplements contained whey protein isolate (Whey Protein Isolate 895, Fonterra, Auckland, NZ), or hydrolyzed collagen peptide (Gelita, Bodybalance®, Eberbach, Germany). Individual servings were identically flavoured and packaged by Infinit Nutrition (Windsor, ON, Canada) in powdered form. Participants were instructed to mix each package with 250 mL of water before ingestion and were asked to consume the beverage in a single sitting within a 5-minute period. Participants consumed the supplements twice daily, once in the morning prior to breakfast and once in the evening ~1-2 h before sleep. Supplements were isonitrogenous and energy-matched. Their contents appear in **Table 1**.

Table 1. Amino acid composition of protein supplements (g/100g)

	WP	CP
	g/30g	g/30g
Alanine	1.7	2.6
Arginine	0.9	2.2
Aspartic acid	3.8	1.7
Cystine	1.2	0
Glutamic acid	5.3	3
Glycine	0.5	6.7
Histidine	0.6	0.3
Proline	1.4	3.8
Serine	1.4	1.0
Tyrosine	1.3	0.2
Tyrptophan	0.7	0
Isoleucine	1.9	0.4
Leucine	4.3	0.8
Lysine	3.4	1.1
Methionine	0.7	0.3
Phenylalanine	1.1	0.6
Threonine	1.6	0.5
Valine	1.7	0.7
ΣEAA	15.4	4.5
ΣNEAA	17.9	21.5

EAA, essential amino acids; NEAA, non-essential amino acids; WP, whey protein supplement; CP, collagen peptide supplement.

Acute and integrated Muscle and Collagen Protein Synthesis. Consumption of $^{2}H_2O$ (70 atom%; Cambridge Isotope Laboratories) was used to label newly synthesized myofibrillar proteins as previously described (22). Participants reported to the laboratory in the fasted state on day -2, and after the collection of a saliva sample (22) consumed 8 doses (0.625 mL/kg of LBM) of $^{2}H_2O$ spread over 10.5 hours (one dose every 1.5 hours).

An additional dose (0.625 mL/kg LBM) of $^{2}H_2O$ was provided to participants to consume each morning following collection of a fasted saliva sample. Total body water deuterium enrichment was used as a surrogate of the precursor for plasma alanine labeling, (22-24). Body water enrichment was determined from saliva swabs that were collected by participants between ~0700-0900 each morning. Days -2 to 0 served for the determination of baseline acute muscle protein synthesis while day 0 to 6 served as the exercise and nutritional supplemented phases.

On day 0, participants reported to the laboratory in the fasted state, having restrained from strenuous exercise for 3 days, for the assessment of acute phase MPS (Figure 2B). Catheters were placed in an antecubital vein of each arm- 1 for the sample of venous blood, and 1 for the infusion of L-[*ring* $^{-13}C_6$] phenylalanine. Baseline blood samples were drawn and then participants received a priming dose of the stable isotope (2 μmol /kg) before initiating a constant tracer infusion (0.05 μmol • kg $^{-1}$ • min^{-1}); (Cambridge Isotopes, Andover, MA). Participants rested on a bed throughout the infusion and blood samples (8 mL) were taken every 20-30 minutes using evacuated heparinized tubes. After 210 minutes, a muscle biopsy was taken from the *vastus lateralis* of the non-exercising leg for the determination of fasted state MPS. Upon completion of the biopsy, participants completed 4 sets of unilateral resistance exercise (1 set at a non-fatiguing load, 3 sets at ~60% 1RM) on their exercise leg. Participants rested for 2 minutes between each set. Loads were adjusted in order to maintain a repetition range between 8-10 where participants exercised until volitional fatigue in the final set. Upon completion of the exercise protocol, a blood sample was drawn and the participants immediately consumed

their study beverage. Four hours after the consumption of the study beverage, participants had muscle biopsies taken from the *vastus lateralis* of both legs for the determination of feeding and feeding plus exercise effects.

Upon completion of the acute infusion, participants were provided with the remainder of their study beverage packages to be consumed for the next 5 days. Participants returned to the laboratory in the fasted state on day 3 to perform the resistance exercise protocol performed on day 0. On the morning of day 6, participants returned to the laboratory following an overnight fast for the collection of a blood sample and bilateral muscle biopsies for the determination of integrated rates of MPS and CPS.

All biopsies were taken following administration of 1% xylocaine local anesthesia with the use of a 5-mm Bergström needle that was adapted for manual suction under. Muscle tissue samples were freed from any visible connective and adipose tissue, rapidly frozen in liquid nitrogen for measurement of MPS and stored at -80°C for further analysis.

Analytic methods. Muscle samples (~30–50 mg) were homogenized to yield the myofibrillar and collagen fractions. Samples were homogenized on ice in buffer [10 μL/mg 25 mM Tris 0.5% v/v Triton X-100 and protease/phosphatase inhibitor cocktail tablets (Complete Protease inhibitor Mini-Tabs; Roche, and PhosSTOP; Roche Applied Science)] and centrifuged at 15000 *g* for 10 min at 4°C and the pellet retained. For the measurement of MPS, the myofibrillar protein pellet was solubilized and centrifuged as previously described (25) and the supernatant containing the myofibrillar proteins was collected leaving the collagen pellet. Myofibrillar proteins were precipitated in 1 mL of

1M perchloric acid, the supernatant discarded, and the fraction was washed with 70% ethanol. The myofibrillar-protein-enriched pellets were hydrolyzed in 1 M HCl at 110°C for 72 h to release their respective amino acids. The collagen-protein enriched pellet was washed with 0.5 M acetic acid and spun at 1600 *g* for 20 minutes. The collagen protein fraction was extracted overnight in 1 mL of 0.1% Pepsin using a vortex mixer at 4°C and collected by centrifugation. Protein bound amino acids were purified by ion exchange chromatography on Dowex H^+ resin. Myofibrillar [$^{13}C_6$]-phenylalanine enrichment was determined using GC pyrolysis-isotope ratio MS (25). Myofibrillar and collagen ^2H-alanine enrichment were determined using Thermo Finnigan Delta V isotope ratio MS coupled with Thermo Trace GC Ultra with GC pyrolysis interface III and Conflow IV as previously described (24).

Western blotting. Expression of intracellular signaling proteins was assessed by using Western blotting. Following homogenization for integrated MyoPS, total protein concentration of the sarcoplasmic fraction was determined by using a bicinchoninic acid assay (Thermo Fisher Scientifc,Waltham,MA,USA).Working samples of equal concentration were prepared in Laemmli buffer. Equal amounts of protein (10 mg) from each sample were run on 4–15% Criterion TGX Stain-Free protein gels (Bio-Rad, Hercules, CA, USA) at 200 V for 45 min. A protein ladder (Fermentas PageRuler Prestained Ladder; Thermo Fisher Scientific) and a calibration curve were run on every gel. Proteins were then transferred to nitrocellulose membranes and were blocked for 1 h in 5% bovine serum albumin. Transfer was visually checked with UV activation of the gel as well as the membrane pre- and post transfer (ChemiDoc MP Imaging System; Bio-

Rad). Membranes were then exposed for 12 h at 4°C to primary antibodies after which they were washed in Tris-buffered saline and Tween 20 (MilliporeSigma) and incubated in anti-rabbit/anti-mouse IgG conjugates with horseradish peroxidase secondary antibodies (GE Healthcare Life Sciences) for 1 h at room temperature. Signals were detected by using chemiluminescence Super-SignalWest Dura Extended Duration Substrate (Thermo Fisher Scientific), and bands were quantified by using Image Lab 6.0.1 (Image Lab Software for Mac Version 6.0.1, Hercules, CA, USA). Protein content was normalized using the calibration curve obtained from each gel (32, 33 The following antibodies used were purchased from Cell Signaling Technology (Danvers, MA, USA): p-4E-BP1$^{Thr37/46}$ (1:1000; 2855), mechanistic target of rapamycin (p-mTOR^{Ser2448}; 1:1000; 2972S), protein kinase B (p-AKT^{Ser473}; 1:1000; 9271), ribosomal protein S6 (p-S6$^{Ser235/236}$; 1:1000; 5364) and p70 S6 kinase 1 (p-p70S6K1^{Thr389}; 1:750; 9202L). Analyses of candidate protein expressions were conducted at rest, Fed, and Fed Ex on day 0.

Plasma and Saliva analysis. Saliva samples were analyzed for ^{2}H enrichment by cavity ring-down spectroscopy using a liquid isotope analyzer (Picarro L2130-*I* analyzer, Picarro, Santa Clara, CA) with an automated injection system. The water phase of saliva was injected six times and the average of the last three measurements were used for data analysis (coefficient of variation ≤0.5%). Standards were measured before and after each participant run. The ^{2}H isotopic enrichments for muscle and saliva initially expressed as $\delta^{2}H$ ‰ were converted to atom percent excess (APE) using standard equations (23).

Plasma [$^{13}C_6$]-phenylalanine enrichment was determined by gas chromatography (GC)-mass spectrometry (MS) (GC: 6890N, MS, 5973; Hewlett-Packard) as previously described (25).

Plasma AA concentrations were measured using the EZ:faastTM amino acid analysis kit for gas chromatography-mass spectrometry (Phenomenex; Torrance, CA). Samples were analyzed using an Agilent 5975C GC/MS (Source 240°C; Quad 180°C; MS Transfer Line 310°C). The instrument was configured to use electron impact ionization and was set to run in scan mode using the column supplied by Phenomenex (Phenom cgo-7169ZB-AAA; 325°C; 10 m x 250 μm x 0.25 μm). Aliquots of 1 μL of the derivatized sample/standard were injected into the MM inlet set to run a 15:1 split mode injection at 250°C. The GC was operated in constant pressure mode at approximately 2.9 psi producing and starting flow rate of 1.4 mL/min with an initial oven temperature of 110°C. The temperature ramp used was as follows: 110°C with no hold, followed by a ramp of 30°C/min to 320°C, followed by 1 min hold and 1 min post run at 320°C for a total run time of 8 min. Chromatographs were quantified using the enhanced data analysis software provided by Agilent in conjunction with the method instructions and EI ion database provided with the Phenomenex kit.

Calculations. The fractional synthetic rate of myofibrillar proteins, determined as %/h and %/d for [$^{13}C_6$]- phenylalanine and [^{2}H]-alanine, respectively, and for collagen proteins determined as %/d for [^{2}H]-alanine using the precursor- product equation as described (14, 26, 27). In brief, the following standard equations were used for acute MPS and longer term MPS: $FSR(\%/h) = [(E_{Phe2} - E_{Phe1})]/[(APE_p) \times t] \times 100$ where E_{Phe} is the

enrichment of the bound (myofibrillar) protein, APE_p is the average enrichment of the intracellular free amino acid precursor pool of two muscle biopsies, and *t* is the tracer incorporation time in hours. The use of tracer-naïve subjects allowed us to use a pre-infusion blood sample (i.e., a mixed plasma protein fraction) as the baseline enrichment (E_{Phe1}) for the calculation of the baseline (fasted) FSR, an approach that has been validated previously by our research group (28); $FSR(\%/d) = [(APE_{Ala})]/[(APE_p) \times t] \times 100$ where FSR is the fractional synthetic rate, APE_{Ala} is the deuterium enrichment of protein-bound alanine, APE_p is the mean precursor enrichment over the time period, and *t* is the time between biopsies. Enrichments of [$^{13}C_6$]- phenylalanine and [^{2}H]-alanine can be found in **Figure 3**.

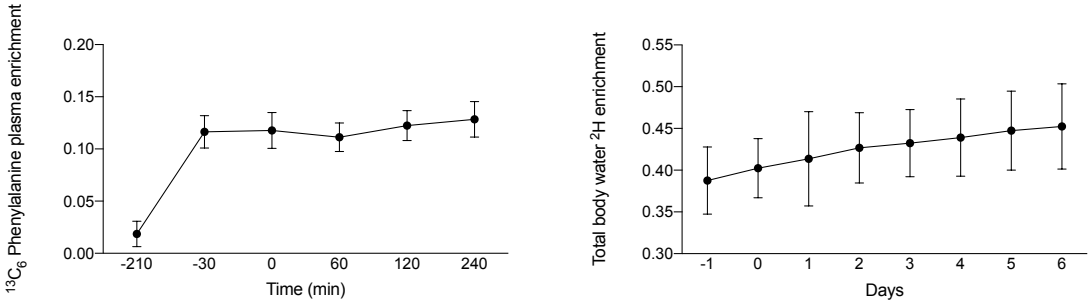

Figure 3. Enrichments of [$^{13}C_6$]- phenylalanine in plasma in minutes and [^{2}H]-alanine in total body water in days.

Statistics. Baseline participant characteristics and amino acid concentrations (maximum concentrations: peak concentration, C_{max} , area under the curve [AUC]) were compared using a non-paired *t*-test. MPS and western blotting analyses were compared using a 2-way mixed design ANOVA with between (group) and within [condition: baseline, feeding (Fed), feeding + exercise (Fed Ex)] factors. CPS was compared using a 2-way

mixed model ANOVA with between (group) and within (conditions Fed and Fed Ex). All significant interaction terms for the ANOVA were further tested by a post-hoc using Tukey's post hoc test. Significance was set at $p < 0.05$. All statistical analyses were completed using SPSS (IBM SPSS Statistics for Mac, version 21; IBM Corp., Armonk, NY). Data in tables are presented as means ± SD. Graphical representations of data are as box and whisker plots with the box representing the interquartile range, the line in each box indicating the median and the cross in each box indicating the mean, and the whiskers indicate the maximum and minimum values.

Results

Participants' characteristics. Participant characteristics are presented in **Table 2.** There were no significant differences between groups for any variable. Twenty-two healthy older women were recruited for this study and were randomly assigned to each group. During processing of the MPS samples, a malfunction in a piece of laboratory equipment resulted in the loss of samples from 3 participants from WP and 4 participants from CP, thus analyses for these data are $n = 8$ and $n = 7$ respectively.

Table 2. Participants' characteristics.

	WP (n = 11)	CP (n = 11)
Age (y)	67 ± 2	69 ± 4
Height (m)	1.61 ± 0.06	1.60 ± 0.03
Body mass (kg)	79.7 ± 13.2	70.6 ± 17.1
BMI (kg/m^2)	30.6 ± 4.3	27.6 ± 5.8
Body fat, (%)	45.2 ± 5.7	40.5 ± 6.3
LBM (kg)	41.71 ± 3.7	40.2 ± 6.1
Knee extensor 1RM (kg)	11.6 ± 3.3	9.4 ± 2.8
Steps/d	9630 ± 3122	9062 ± 2564
Daily PA > 3 METs, kcal/d	151 ± 67	149 ± 65
Average METs	1.5 ± 0.3	1.5 ± 0.2

Values are means ± SD. WP, whey protein supplement; CP, collagen peptide supplement; BMI, body mass index; LBM, lean body mass; 1RM, one-repetition maximum; MET, metabolic equivalent; PA, physical activity.

Dietary intake. There were no differences between groups in any dietary variable between groups ($p > 0.05$) (**Table 3**). Protein per kilogram of body mass and absolute protein intake were significantly greater during the supplementation phase than at baseline ($p < 0.001$).

Table 3. Diet nutrition composition over the baseline and supplemental periods.

	WP (n = 11)	CP (n = 11)	*p*
Energy (kcal)			
Baseline	2468 ± 179	2337 ± 259	0.18
Supplementation	2328 ± 179	2397 ± 259	0.18
Energy (kcal/kg)			
Baseline	31 ± 2	35 ± 4	0.15
Supplementation	31 ± 3	34 ± 5	0.13
Protein (g/kg)			
Baseline	1.00 ± 0.03	0.98 ± 0.04	0.74
Supplementation	1.76 ± 0.12*	1.87 ± 0.21*	0.16

Values are means ± SD. WP, whey protein supplement; CP, collagen peptide supplement. * Indicates significantly different from baseline.

Plasma amino acids. Summed total AA concentration was increased in response to supplementation during the infusion trial in both groups ($p = 0.006$, $p = 0.05$, WP and CP, respectively) and returned to baseline by 240 minutes (**Figure 4A**). There were no differences in total AA area under the curve (AUC) ($p > 0.05$), concentration max (C_{max}) ($p > 0.05$) or time of maximum concentration (T_{max}) ($p > 0.05$) between supplements (**Table 4**). Summed essential amino acid (EAA) concentration increased in response to supplement provision in WP ($p = 0.04$) and returned to baseline by 240 minutes but did not increased above baseline in CP ($p > 0.05$) (**Figure 4B**). Summed EAA AUC, and C_{max} were greater after WP ingestion than with CP ingestion ($p = 0.003$, $p = 0.003$, respectively). There was no difference in EAA T_{max} between supplement types ($p > 0.05$).

Plasma leucine concentrations increased above baseline in response to supplement provision in the WP group at 40 minutes by ($p = 0.001$) and remained elevated above baseline at 240 minutes ($p = 0.012$). Plasma leucine concentrations were not increased above baseline in CP ($p > 0.05$) (**Figure 4C**). Leucine AUC and C_{max} were greater after WP ingestion than with CP ingestion ($p = 0.011$, $p < 0.001$, respectively).

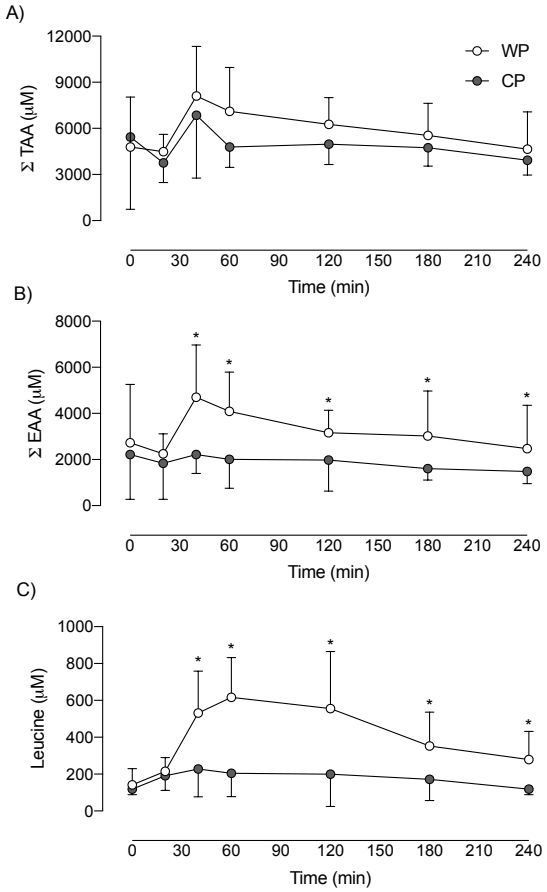

Figure 4. Concentrations of summed total amino acids (TAA) (A), summed total essential amino acids (EAA) (B), and leucine (C) over 4 hour post-ingestion of either WP or CP. * Different between supplement groups at that time, $p < 0.05$. Values are means ± SD. TAA, total amino acid; EAA, essential amino acids; WP, whey protein; CP, collagen peptides.

Table 4. Amino acid concentrations of healthy, older women participants during the acute infusion trial

	WP (n = 11)	CP (n = 11)	*P*
ΣTotal amino acids			
C_{max}, μM	10041 ± 4793	8808 ± 5276	0.15
T_{max}, min	64 ± 31	84 ± 37	0.10
AUC, μmol · min/L	1431636 ± 10908	1205330 ± 16858	> 0.05
ΣEAA			
C_{max}, μM	5733 ± 2368	2869 ± 1604	0.003
T_{max}, min	50 ± 10	46 ± 13	0.23
AUC, μmol · min/L	775083 ± 12823	442865 ± 10785	0.003
Leucine			
C_{max}, μM	645 ± 206	223 ± 117	< 0.001
T_{max}, min	54 ± 9	62 ± 31	0.22
AUC, μmol · min/L	103823 ± 17700	43600 ± 10078	0.012

Values are means ± SD. AUC was calculated over the 240 min following study beverage ingestion. Analysis by non-paired *t* test. Σ, sum of; C_{max}, maximum concentration; T_{max}, time of maximum concentration.

Acute MyoMPS. Acute MyoPS responses are shown in **Figure 5**. There were no differences in basal rates of myofibrillar MPS (MyoPS) between WP and CP. In response to WP supplementation, MyoPS was increased significantly by 0.017 ± 0.008%/h in the feeding only leg (Fed), and 0.032 ± 0.012%/hr in the feeding and exercise leg (Fed Ex) ($p < 0.001$). CP supplementation did not significantly increase Fed only MyoPS (0.009 ± 0.014%/hr) however Fed Ex MyoPS was increased by 0.012 ± 0.013%/hr from resting levels ($p < 0.001$). Postprandial MyoPS was significantly greater in the WP group than the CP group in both the Fed and Fed Ex legs ($p = 0.02$).

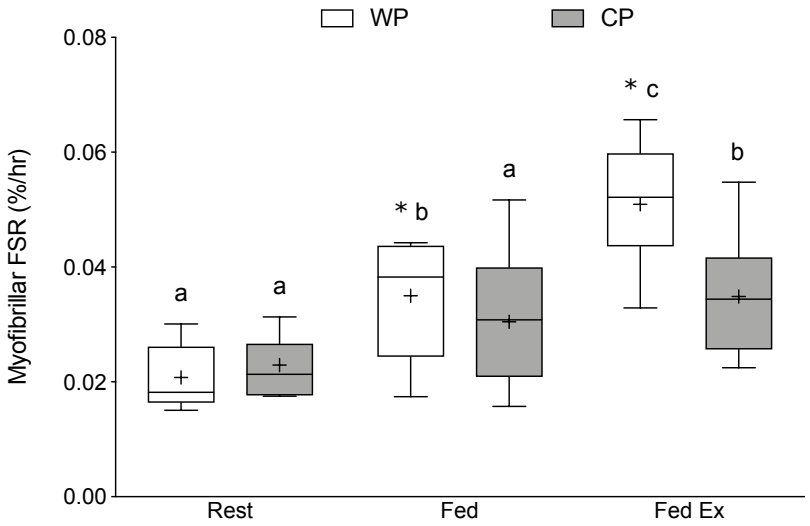

Figure 5. Acute myofibrillar muscle protein synthesis (%/hr) in the fasted state (Rest) and in response to feeding (Fed) and feeding with exercise (Fed Ex). The box plot shows the median (line) and mean (+), with the box representing the inter-quartile range and the whiskers representing the maximum and minimum values. Data were analyzed with a 2-factor ANOVA with group as a between factor and repeated measures for time. Means that do not share a letter are significantly different within the group, $p<0.05$. * Denotes differences between groups at that time point, $p<0.05$.

Integrated MPS and MCPS. Integrated MyoPS responses are shown in **Figure 6A**. There were no differences in baseline rates of MyoPS between WP and CP ($p > 0.05$) (days -2 to 0). In response to WP supplementation, MyoPS was increased by 0.063 ± 0.059%/day in the Fed leg and by 0.173 ± 0.104%/day in the Fed Ex leg ($p < 0.001$). With CP supplementation, rates of MyoPS were not significantly elevated above baseline in the Fed leg (-0.011 ± 0.042%/day) or the Fed Ex leg (0.020 ± 0.034%/day). Rates of integrated MyoPS were significantly greater in WP than CP in both the Fed and Fed Ex limbs ($p < 0.0001$). There were no differences in MCPS between groups ($p = 0.154$) (**Figure 6B**).

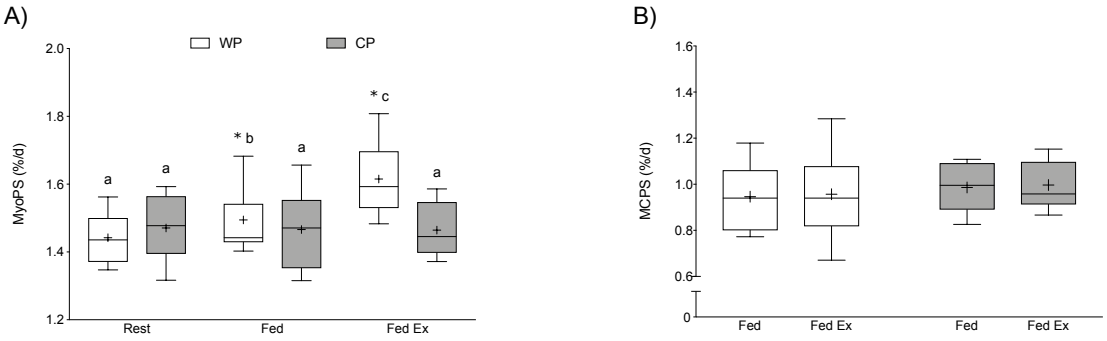

Figure 6. Integrated myofibrillar muscle protein synthesis (%/day) (5A) in the fasted state (Rest) and in response to feeding (Fed) and feeding with exercise (Fed Ex). Integrated intramuscular collagen synthesis (%/day) (5B) in response to Fed and Fed Ex in WP and CP groups. The box plot shows the median (line) and mean (+), with the box representing the inter-quartile range and the whiskers representing the maximum and minimum values. MPS and MCPS data were analyzed with a 2-factor ANOVA with group as a between factor and repeated measures for time. Means that do not share a letter are significantly different within the group, $p<0.05$. * Denotes differences between groups at that time point, $p<0.05$.

Muscle anabolic signaling. There were no significant differences between supplemental groups in any target measured for changes in phosphorylation status ($p > 0.05$). In response to feeding, phosphorylation of p-4EBP1$^{Thr37/46}$, p-Akt^{Ser473} and p-mTOR^{Ser2448} was significantly reduced from rest but not different from rest in the Fed Ex limb ($p = 0.027$, $p = 0.006$, $p = 0017$, respectively). Phosphorylation of p-p70^{Thr389} and p-s6$^{Ser235/236}$ were unchanged with feeding and exercise ($p > 0.05$). Anabolic signaling data can be found in **Table 5**.

Table 5. Protein signaling at rest and 4 hours following supplement ingestion.

Phospho- 4EBP1$^{The37/46}$	
Rest	0.91 ± 0.29^{a}
4 –h Fed	0.79 ± 0.21^{b}
4 –h Fed Ex	0.91 ± 0.35^{ab}
Phospho- Akt$^{Ser\ 473}$	
Rest	0.94 ± 0.23^{a}
4 –h Fed	0.77 ± 0.22^{b}
4 –h Fed Ex	0.91 ± 0.27^{a}
Phospho- mTOR$^{Ser\ 2448}$	
Rest	0.83 ± 0.51^{a}
4 –h Fed	0.69 ± 0.46^{b}
4 –h Fed Ex	0.95 ± 0.35^{a}
Phospho- p70^{Thr389}	
Rest	1.04 ± 0.28
4 –h Fed	1.01 ± 0.35
4 –h Fed Ex	1.05 ± 0.38
Phospho- s6$^{Ser235/236}$	
Rest	0.97 ± 0.49
4 –h Fed	1.03 ± 0.64
4 –h Fed Ex	1.03 ± 0.52

Values are means ± SD, n=22. WP, whey protein supplement; CP, collagen peptide supplement; Fed, feeding only leg; Fed Ex, feeding and exercise leg. Unshared letters within a protein are significantly difference from each other ($p < 0.05$).

Discussion

The novel finding of our investigation was that supplementation with WP enhanced the MyoPS response to feeding compared with the ingestion of an isonitrogenous and isoenergetic quantity of CP in healthy older women. We show here that ingestion of WP induced an acute increase in MyoPS above fasted levels that was further enhanced with resistance exercise; however, acute rates of MyoPS were increased only with exercise in the CP group and to a lesser extent than seen with WP. Importantly, we showed that supplementation with WP for 6 days, resulted in an increased rate of integrated MyoPS above baseline in a rested leg and that 2 bouts of resistance exercise within the supplementation period further enhanced rates of integrated MyoPS with no effect of supplementation or exercise in the CP group. These findings have important clinical implications and highlight the importance of protein quality for the maintenance of skeletal muscle mass in older women.

Increased availability of EAA, particularly leucine, has been shown to be a key stimulus to increase rates of MPS (13, 29, 30), particularly when combined with resistance exercise (4, 30). The contribution of EAA to the WP and CP in the present study were 46% and 17% respectively, with ~5.5 times (3.5 g) more leucine provided per supplement dose in WP compared to CP. Previous work from our laboratory, which used a similar experimental model to that employed in this study, compared a protein blend with a milk protein supplement containing ~3.2 times (2.9 g) greater leucine. Despite the beverages being isonitrogenous (~15g of protein per drink) consumption of the higher

leucine-containing beverage resulted in greater acute and chronic elevations in MyoPS, an effect that was also enhanced with resistance exercise (13).

Our findings are in accordance with previous work in which WP has been shown to be highly effective at stimulating MPS (30-32). A previous study from our laboratory showed no effect of CP supplementation on rates of MyoPS following two weeks of reduced daily activity and one week of recovery in healthy older adults (14). To date, three additional studies have looked at the efficacy of CP on body composition and showed that CP supplementation increased lean body mass (LBM) with resistance training in older men (15), pre-menopausal women (16) and in maintaining nitrogen balance in older women (17). It should be noted that the effects of CP supplementation on LBM gains with resistance exercise shown by Zdzieblik et al., were exceptional (~5kg) (15) and have been questioned (33). This is particularly relevant given that several recent meta-analyses have concluded that protein supplementation during resistance exercise did not augment gains in LBM in older persons (34, 35). Given that WP has a digestible indispensable amino acid score (DIAAS) of 1.09 and CP of 0 (as it lacks tryptophan and even with supplemental tryptophan is low in methionine and leucine), the lack of stimulation of postprandial MyoPS acutely and following 6 days of supplementation with CP is perhaps not surprising and provides no support for proposed amino acid or peptide-based mechanisms that ostensibly underpinned the marked increase in LBM reported previously (15). Interestingly, rates of MyoPS were not significantly elevated with CP in the Fed Ex condition after 6 days of supplementation as previous work from our laboratory has shown elevated MPS responses to exercise alone in male participants at 48

hours (36) and 72 hours (37) following a similar RE protocol. These findings may allude to a potential sex based difference anabolic response as Devries et al., have previously shown no elevation in rates of MPS 72 hours following RE also in older women who were consuming a control, lower protein quality containing beverage (13).

Provision of an amino acid mixture (containing all amino acids) has been shown to increase rates of MyoPS (38) largely through the activation of the mechanistic target of rapamycin complex-1 by the essential amino acid leucine (39) . Further, the administration of leucine has been repeatedly shown to independently stimulate MPS due to its interaction directly on the mTORC1 pathway (40, 41), the primary signaling pathway affecting translation and initiation. Our finding of increased plasma leucine concentration following ingestion of WP in combination with the postprandial increase in acute and integrated MyoPS aligns with seminal dose response data (42). Given that all essential amino acids are required in amounts sufficient to build muscle protein, it is not surprising that CP was unable to stimulate a robust longer-term MyoPS response either in the rested or in the exercised leg, particularly when observing the significantly lower levels of plasma leucine elicited by CP ingestion.

In order to determine the effects of protein quality on skeletal muscle protein kinetics, we examined the phosphorylation status of proteins involved in the mTOR pathway and its downstream effectors. Interestingly, we did not find significant increases in the phosphorylated targets of mTOR or its downstream targets in either group. Our data are in line with previous work that showed no changes in phosphorylated targets of the mTOR pathway 4 hours following feeding and exercise (43). We hypothesize that

since our muscle biopsy time points were chosen for measurements of MyoPS that the peak phosphorylation events for the proteins may have occurred earlier following protein ingestion and exercise.

Muscle-located (perimysium) collagen has been shown to be responsive to exercise stimuli but unresponsive to the nutritional provision of EAAs or a multi-nutrient, protein based supplement in humans (44, 45). The CP supplement had a high content of glycine, proline, and arginine, AA found in large quantities in collagen tissue (46). Thus, we hypothesized that supplementation with CP rather than EAA would result in an increase in MCPS with feeding alone. However, we saw no effect of feeding on rates of MCPS. These data are in line with work examining the distribution of ^{14}C labeled gelatin in which authors report no change in the distribution of radioactivity to skeletal muscle which was hypothesized to be due to a 90% removal of radioactivity from the gastrointestinal tract by excretion within the first 6 hours of administration (47). We were not able to obtain a baseline biopsy in order to determine baseline MCPS and therefore were only able to compare CPS in protein supplementation phases with and without exercise.

In the current investigation we chose to examine the effects of protein ingestion and exercise in healthy older women due in part to scant study of older women compared to men in these types of mechanistic studies. We also note that older women are at an increased risk for falls, fractures, and mobility impairments in comparison to men (48). Unique aspects of male physiology, such as higher testosterone concentrations compared to post-menopausal females, may interact with intracellular pathways related to MPS and

contribute to this sex-based difference in muscle growth and MyoPS responses. Determining strategies to augment MPS in older women is imperative in the ability to better tailor nutritional interventions specifically to women. Recently we showed that older women may not fully recovery strength losses following acute inactivity (49). Further, given the increased risk for falls (48), and greater life expectancy of women in comparison to men (50), strategies that promote retention of skeletal muscle may serve to prolong the health and independence of the aging female population.

In summary, our findings show that consumption of whey protein enhanced skeletal muscle protein anabolism both acutely and when measured over days. Corroborating previous work, we show that consumption of whey protein when consumed in conjunction with resistance exercise resulted in a further stimulation of MPS, reinforcing the importance of resistance exercise in the maintenance of skeletal muscle health. Importantly we also show that resistance exercise in combination with a low quality protein was not sufficient to elevate rates of MyoPS above baseline in healthy older women, a potentially discrepant finding in comparison to older men indicating that the selection of protein sources for older women may be of great significance. Older women should aim to select high quality dietary proteins and engage in regular resistance exercise in an effort to attenuate sarcopenic muscle declines.

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CHAPTER 5:

GENERAL DISCUSSION

5.1 Introduction

Aging is associated with the progressive loss of skeletal muscle mass and strength termed sarcopenia. Low skeletal muscle mass and strength are strong predictors of all-cause mortality (1), increased risks for falls, fractures (2), development of metabolic disorders (1), and a reduced quality of life (1, 3, 4). Dietary habits of older adults, particularly consumption of lower than recommended protein intake (5), and acute periods of reduced physical activity, act in confluence with sarcopenia to accelerate declines in skeletal muscle mass and strength. Importantly, recovery from such periods may be slow and is often likely incomplete. Increased dietary protein and participation in resistance exercise, or some form of muscle loading, may serve to attenuate sarcopenic losses as well as enhance the recovery from brief periods of disuse and convalescence. Increased dietary protein and loading work through the restoration of anabolic signaling and, over time, augment or maintain skeletal muscle mass (6, 7). With advancing age, the quality of dietary protein becomes increasingly important due to a heightened resistance of skeletal muscle to anabolic *stimuli* such as protein ingestion and exercise. Given that increasing protein intake in older adults is sometimes difficult to achieve (8), evidence in the support of high quality protein supplements is crucial in an effort to guide the selection of protein sources to benefit muscle health of older adults.

The aim of this thesis was to explore the relationship between protein quality and physical activity in aged skeletal muscle health and recovery. One novel aspect of this work was the use of both acute and longer-term assessments of muscle protein synthesis (MPS) to allow for the determination of MPS from periods as short as 4 hours to as long

as 2 weeks. Additionally, despite the growing evidence involving disuse models in humans, Chapter 2 (Study 1) was the first to examine the effect of a nutritional intervention with step reduction (SR) and recovery in older persons. Chapter 3 (Study 2) was the first study in which changes at the level of the single fibre following SR were examined in any population. Further, Chapter 4 (Study 3) added to the findings of Chapter 2 (Study 1) to allow a better understand the influence of resistance exercise and feeding responses on MPS to both whey protein (WP) and collagen peptides (CP) in healthy older women. Women, and in particular older women, are underrepresented in the literature regarding studies of skeletal muscle and protein turnover and thus highlight a further novelty of this work.

In Study 1 we hypothesized that the consumption of a high quality protein (WP), would attenuate the decline in skeletal muscle mass and rates of MPS. Study 1 demonstrated that despite the consumption of a diet containing twice the recommended daily allowance (RDA) for protein, older adults lost leg lean mass (LLM) and had reduced rates of MPS independent of protein supplement type following two weeks of SR while in an energy deficit. Importantly, following one-week of return to habitual activity (*i.e.,* without structured exercise rehabilitation) supplementation with WP facilitated an increase in MPS with no change in the CP supplemented group. WP supplementation also improved the restoration of LLM during recovery to a greater extent than CP supplementation. Given the few studies that have examined alterations in skeletal muscle health with step reduction, Study 2 aimed to examine the alteration in skeletal muscle function (men and women) and single fibre function (a subset of men only) to 2 weeks of

reduced daily stepping. For study 2, we hypothesized that whole body muscle function would decline in both men and women and that single fibre function would decrease in men. Interestingly, though maximum voluntary contraction (MVC) was significantly decreased in both men and women, there were no significant changes in any other clinical measure of physical function. Further, following resumption of habitual physical activity, older men were able to recover strength losses while older women remained at a small but significant strength deficit from baseline. Increases in single muscle fibre maximum power production (Pmax) in MCHI and MCHIIA fibres, and increases in maximum isometric tension (Tmax) in MCHIIA fibres were also demonstrated in a subset of male participants following the 2-weeks of SR. Given the beneficial effects of WP supplementation during recovery involving only habitual levels of physical activity (no structured exercise), Study 3 examined the MPS response to feeding and feeding combined with resistance exercise during supplementation with WP or CP in older women. It was hypothesized that WP would result in greater stimulation MPS with both feeding and when combined with exercise in comparison to CP supplementation. Acutely, ingestion of a single bolus of WP increased MPS in the feeding only limb (9) and the feeding plus exercise limb (Fed Ex) while CP ingestion induced an increase only in Fed Ex. Six days of twice daily supplementation with WP resulted in an increase in both Fed and Fed Ex rates of MPS and no increases in MPS with CP in either the Fed or Fed Ex conditions.

Taken together, this thesis provides insight into the importance of protein quality and physical activity on the impact of skeletal muscle health in aging adults. This chapter

integrates the findings from Study 1, 2, and 3 and highlights the collective contribution of this thesis to the current literature. This chapter will also discuss strengths, limitations, and areas for future direction.

5.2 Influence of protein quality on integrated myofibrillar protein synthesis, lean body mass, and glycemic response during energy restriction, step reduction, and recovery

Muscle protein synthesis (MPS), the primary regulator of skeletal muscle mass (10) (in the non-diseased state), is highly sensitive to caloric restriction (11), protein ingestion (12), exercise (13, 14) and energy status (15, 16). Previous work has shown that two weeks of SR induced physical inactivity reduced rates of MPS by 12% where healthy older adults took a maximum of 1000 steps per day (15). Additionally, our laboratory has also shown that mild energy restriction (ER) of just 300 kcal while maintaining habitual physical activity resulted in decreased rates of MPS in healthy older men (11). Augmenting dietary protein intake during weight loss mitigated the loss in LBM during ER (17) and the provision of an essential amino acid mix containing 3.1 g of leucine was shown to protect lean body mass (LBM) loss during 10 days of bed rest in young adults (18). Given that ER often accompanies instances of reduced daily steps, the aim of Study 1 was to examine the effects of a higher protein diet via supplementation, while consuming a hypocaloric diet to mitigate the declines in MPS and subsequent LBM in healthy older adults. In order to attain a protein intake of 1.6 g/kg/day, participants were supplemented twice daily with 30 g of either whey protein (WP) or collagen peptides

(CP) that served as an isonitrogenous but biologically inactive control supplement. MPS was significantly reduced (~ 16% decrease from baseline) following one week of ER alone in both supplemental groups (-500 kcal/day), a finding congruent with literature on ER (11, 19, 20). However, following 2 weeks of reduced daily stepping (< 750 steps/day) combined with ER, rates of MPS were not further decreased (~14% decrease from baseline), independent of supplement provision. Following 1 week of return to habitual activity while maintaining a high protein diet, WP facilitated a greater recovery of LLM in comparison to CP, and MPS was elevated above ER levels, while MPS remained attenuated in the CP group. In addition to alterations in skeletal muscle, ER + SR induced an impairment in glucoregulation and elevated levels of inflammatory cytokines in both groups. Following one week of return to habitual activity, fasted glucose and insulin levels did not return back to baseline however levels of inflammatory cytokines were restored back to levels in energy balance. This study was the first to examine the effects of a nutritional intervention during SR in older adults.

A major contribution of Chapter 2 to the literature was the demonstration of maintenance of MPS in response to SR following ER as it was hypothesized that the addition of SR would induce further declines in MPS from ER alone. Given that both ER (11, 19) and SR (21) act independently to decrease rates of MPS, Study 1 shows that they do not act synergistically. Though this may be a function of the population studied (healthy older adults, living independently), these findings highlight the resiliency in protein metabolism in healthy older adults to mitigate an accumulation of catabolic stimuli and the potential role for protein supplementation for use during rehabilitation.

We previously showed that consuming a high quality protein supplement (WP) during ER attenuated the decline in postprandial MPS in comparison to consuming soy protein (lower quality in comparison to whey) or a carbohydrate control (19). Additionally, English et al., showed that the consumption of a mealtime leucine supplement attenuated the decline in MPS following 14 days of bed rest in comparison to alanine (non-essential amino acid) supplementation in healthy young men (22). Thus, there does appear to be merit in the selection of high quality protein supplements to mitigate the declines in MPS and LBM with ER and disuse, yet these factors have not been examined in combination in healthy older adults. To date, no studies have examined the recovery of MPS following a disuse event involving use of a nutritional supplement. We recently showed returning to habitual activity after 2 weeks of step reduction did not restore rates of MPS following 2 weeks of SR in older pre-diabetic participants (21) who consumed their habitual diet *ad libitum*. Accordingly, our finding of augmented skeletal muscle mass recovery and MPS with WP in comparison to CP supplementation during recovery highlight the importance of nutrition during rehabilitation with particular attention to the selection of high quality protein sources in order to augment total protein intake. Since the protein digestibility corrected amino acid score (PDCAAS) and Digestible Indispensible Amino Acid Score (DIAAS) of CP are 0, as it is an incomplete protein source (lacking tryptophan), it can be hypothesized that augmenting dietary nitrogen intake during recovery is not sufficient to restore rates of MPS and rather that the quality of protein plays a role in the ability to recover skeletal muscle anabolism in older adults. The mechanisms underpinning our observation of enhanced recovery with WP supplementation following ER and SR cannot

be determined from Study 1. Due to its technically challenging nature, the assessment of MPB was not possible and therefore the impact of alterations in MPB and subsequent effects on LBM remain unclear. Though we have previously shown that MPB is not augmented with ER in young adults (20), the contributions of MPB to muscle loss in SR and when combined with ER are currently unknown.

Few studies have examined the effects of nutritional supplementation on recovery from muscle-disuse in humans. Hespel et al., provided young healthy adults with 5 g of creatine monohydrate four times a day during a 2 week lower limb immobilization period and during 10 weeks of subsequent resistance exercise rehabilitation (23). The authors showed that during immobilization, quadriceps muscle cross sectional area (CSA) was reduced by 10% with no differences between the supplement group and the placebo group, however following 3 and 10 weeks of rehabilitation, CSA was increased to a greater extent in the creatine-supplemented group (23). Brooks et al., showed that essential amino acid (EAA) supplementation (15 g) alone and with resistance training (RT) did not protect against the loss of total LBM following 28 days of bed rest and energy restriction (24). Further, in response to 14 weeks of active recovery, both groups regained muscle mass with no differences between groups (24). Given that EAAs were provided to all participants in the aforementioned study (24), it is difficult to infer whether consumption of a non-essential amino acid (NEAA) supplement would have limited the recovery in LBM following bed rest, however, considerations should be made to examine the provision of EAA to improve LBM recovery during active rehabilitation in both younger and older adults.

The observations in Study 1 of impaired glycemic handling following SR are congruent with findings from previous work in our lab utilizing SR as a model for whole body disuse (15, 25). McGlory et al., showed an adaptive response in glucose handling following 2 weeks of SR in pre-diabetic older adults (15), while Breen et al., (25) also showed similar alterations in rates of fasted insulin that were elevated following SR in a healthy older adults. Breen et al., (25) did not assess recovery from 2 weeks of SR (<1500 steps/day); however, pre-diabetic participants were assessed after 2 weeks of return to habitual activity following SR at < 1000 steps per day and the authors found only a partial improvement in fasted insulin and glucose levels (15). Interestingly, in Study 1, we also observed a failure of recovery in fasted glucose and insulin but in healthy older adults following one week of return to habitual activity, suggesting that perhaps existing pre-diabetic status may not influence glucose and insulin recovery from SR in older adults (15) given that glycemic response in both normoglycemic and pre-diabetic participants did not fully recover.

5.3. Physical function alterations at the whole muscle and single fibre level following step reduction

The loss of skeletal muscle strength is a strong predictor for poor mobility status (26). Specifically, older men with leg power less than 58% of average power, and older women with leg power less than 69% of average power, were found to be 9 and 3 times respectively, more likely to develop incident mobility disability over a 6 year period (26). Further, loss of muscle strength and the development of mobility impairments or

disability drastically decreases the quality of life of older adults largely due to a reduction in the ability to carry out activities of daily living (27, 28). It is estimated that the risk for reduction in independence to carry out ADLs during hospital admission is upwards of 30% in older persons (29). Of direct relevance to this point, in Study 2 we examined the effects of 2 weeks of SR at < 750 steps/day on changes in whole muscle functional outcomes and strength in healthy older adults consuming a high protein, hypocaloric diet. In a subset of participants (n = 9 men), changes at the single fibre level were examined following SR. The main finding from this study is the significant loss of isometric maximum voluntary contraction (MVC) strength in older men and women following SR; however, with one week of return to habitual activity, older men recovered strength losses while older women did not. Single muscle fibre analysis, from a subset of men, revealed somewhat paradoxical increases in maximum isometric tension (Tmax) in MHC IIA fibres and in maximum power production (Pmax) in both MHC I and IIa fibres following SR. Further, no changes were found in measures of whole body muscle function in men or women, and changes in single fibre outcomes were not correlated with changes at the whole muscle level.

Losses of muscle strength are of particular concern in older adults as Hvid et al., showed that just 4 days of unilateral leg immobilization induced a 9% decrease in isometric MVC that was not recovered within 7 days of active rehabilitation (30). Though the observed decrement in MVC in female participants in Study 3 was smaller than the aforementioned study (5.6%), losses in muscle strength over periods of muscle-disuse that are not recovered could result in accumulated strength deficits that may affect the

independence of older women. Indeed, Hughes et al., estimated that older adults require ~78% of their MVC in order to rise from a low chair height without the use of an aid (31). Though the required strength to stand from a chair is dependent on body mass, if each brief SR event induced a deficit of 5% that was not fully recovered, it is plausible that the minimum required strength suggested by Hughes et al., could be reached within 5 disuse events. Previous studies investigating changes in muscle strength and function following SR have been mixed (15, 32, 33), however, no studies to date have examined sex differences in strength with SR and recovery. Yasuda et al., (34) have previously shown that following 14 days of unilateral knee immobilization, women exhibited a greater decrease in isometric knee extensor MVC compared to men (3.1 Nm in men 17.1 Nm in women); however, changes in quadriceps CSA were similar between sexes (5.7 and 5.9% in men and women respectively). Additionally, these authors (34) showed similar atrophy in *vastus lateralis* fibre area of type I, IIa, and IIx between men and women indicating that perhaps CSA did not wholly dictate declines in force generating capacity in females. Similarly, we reported that although men and women had comparable relative losses and recovery of skeletal muscle following SR, recovery of MVC was not demonstrated in female participants indicating that perhaps alterations in neural recruitment may have contributed to attenuated increase in strength. Indeed, Deschenes et al., compared neuromuscular adaptations to unloading in young men and women and found alterations in the recruitment pattern in women in comparison to men after one week (35). Thus, it is possible there are sex-based differences in neuromuscular adaptations to affect muscle disuse and force generating capacity that negatively impacts females.

Alterations at the single fibre level in humans in response to immobilization (36, 37) and bed rest (38, 39) have been previously studied. In Study 2, we aimed to determine single muscle fibre-level changes in a subset of male participants in response to SR. This study was the first to examine the effects of reduced daily stepping on muscle fibre function. The findings of increases in single fibre function following SR are interesting yet, not wholly surprising. Callahan et al., compared single fibres from healthy active older adults with inactive older adults with advanced stage knee osteoarthritis and showed at reduced power output in shortening velocity in MHC IIa fibres in inactive females but an increase in power output and shortening velocity in inactive males (40). The increase in single fibre function is thought to be compensatory to offset the decline in muscle power output that occurs with muscle-disuse and can be attributed largely to differences in cross bridge kinetics in men (40). Specifically, inactive women had longer myosin attachment times than active women however there were no differences in cross bridge kinetics between active and inactive men (40). Reduced attachment of myosin to actin in men would be expected to increase the maximum shortening velocity and in turn maximum power that may account for the observed differences in single fibre function between men and women. Interestingly, D'Antona et al., examined single fibres from healthy men, younger adults, control older adults, and older adults who had one leg immobilized for 3.5 months (36). Similarly, these authors observed an increase in maximum shortening velocity of type I and IIa fibres in immobilized older men compared to younger men such that immobilization counteracted the age dependent decrease in shortening velocity as demonstrated by the control population of older men (36).

Therefore, it appears as though augmentation of single fibre contractile function may exist in older men as a compensatory mechanism to counteract losses in whole muscle function during marked disuse. Indeed, Trappe et al., examined single fibre from the *vastus lateralis* of young men following 84 days of bed rest and noted significant decreases in muscle fibre CSA in type I and IIa fibres (15% and 8% respectively) and notable decrements in single fibre contractile function. Specifically, they showed decreases of 46%and 25% in type I and IIa fibre maximum power respectively and slowed shortening velocities of 21% and 6% respectively (39). Interestingly, the observed augmentation of single fibre function in older men but not older women or younger adults with disuse was not demonstrated by Hvid et al., (41). However, the older adults in the study by Hvid et al., were younger (~65 years) in comparison to the participants in the studies by D'Antona (~73 years), Callahan (males ~70 years, females 72 years), and those studied in Study 2 (~70 years) and thus perhaps an age related effect may persist. Further, the study by Hvid et al., notes that there were no differences in physical activity levels between young and older adults as assessed by a physical activity questionnaire despite population estimates suggesting that older adults are typically less physically active than younger adults (42). Thus perhaps prior lower levels of activity (as shown by D'Antona et al.) may have mitigated the age related effects of single fibre function in this particular study (42).

To date, one study has examined the effect of protein supplementation on single fibre function. Trappe et al., (43) observed the effect of a leucine-enriched high protein diet on changes in single fibre with muscle-disuse in young healthy women and found no

difference in changes in single fibre function following 60 days of bed rest compared to participants in the control diet bed rest group. Though the aforementioned study was in young adults, no other study has examined the effects of nutritional countermeasures on single fibre function. As such, a major limitation of Study 2 was that we did not assess changes in single fibre function following 1 week of return to habitual activity and high protein diet due to monetary and time constraints. We also note that given the observations of attenuated recovery in older females and the potential for sex based differences in single fibre adaptation to disuse, another limitation to Study 2 is that we did not assess single fibre function in older women. Budget constraints restricted our observations to a limited number of participants for analysis and we therefore chose to examine only one sex to reduce variability.

5.4 Influence of protein quality and resistance exercise on acute and integrated myofibrillar protein synthesis

The two main anabolic *stimuli* influencing MPS are protein intake and alterations in physical activity. Specifically, foods or proteins that are high in essential amino acids (EAA) and that are fully digested are considered to be high quality protein sources. On a weight-to-weight basis, high quality protein sources increase rates of MPS to a greater extent than lower quality protein sources, a characteristic that may be largely attributable to total leucine content (44). The influence of protein quality on postprandial MPS has been examined with (45-47) and without (48, 49) exercise; however, only a small number of studies examining protein quality on MPS have been in women exclusively (6, 50).

Study 3 examined the feeding and exercise MPS response in healthy older women to whey protein (WP) or collagen peptides (CP) in an attempt to elucidate the mechanisms by which WP facilitated a greater recovery of MPS following SR from Study 1. Study 3 also aimed to determine the acute effects of CP supplementation with resistance exercise (RE) in an effort to provide insight to previous literature suggesting its potent anabolic nature (51, 52). The major contribution of Study 3 to the literature is the finding of augmented acute and longer term MPS with WP in comparison to CP supplementation in older women consuming a eucaloric-controlled diet. Importantly, these data show no benefit of CP supplementation on acute MPS without exercise and when consumed twice daily for 6 days, CP did not augment integrated MPS alone or when combined with RE. These findings are not wholly surprising given the low essential amino acid content of the CP supplement and 5.5 times lower (0.8 g) leucine content per dose in comparison to WP (4.3 g). Collagen peptides, also referred to as collagen hydrolysate, or hydrolyzed collagen, are derived from porcine or bovine bone, hide, or hide split, and sometimes from marine sources. In brief, collagen proteins are denatured into gelatin, and then enzymatically degraded into collagen peptides to enhance absorption and solubility. The use of collagen to improve tendon and cartilage health (53, 54), increase collagen synthesis (55, 56), and reduce joint pain (57) has been well studied, however it has been scarcely used as a supplement to improve skeletal muscle health (51, 52, 58). The lack of data for the use of CP in skeletal muscle anabolism can be attributed to the low ranking of collagen peptides via the PDCAAS and the DIAAS that score CP at 0 since it is an incomplete protein due to a lack of tryptophan, an essential amino acid (EAA). Thus, it

was expected that CP supplementation would result in a lower feeding and feeding + exercise responses compared to WP supplementation. Importantly, all EAAs are required in order to build muscle proteins and thus, ingestion of CP as a protein supplement to support muscle health requires serious consideration and future research, particularly in populations such as older adults wherein simply increasing dietary nitrogen intake may not suffice for the maintenance or increase in skeletal muscle. The lack of augmentation of integrated rates of MPS with CP + RE was surprising. Previous research from our laboratory using deuterated water for the measurement of integrated MPS showed an augmented MPS response following RE after 48 hours in healthy older men consuming a habitual diet (59). Similarly, Murphy et al., showed that one bout of RE augmented MPS after 72 hours in healthy older men consuming both the RDA for protein (0.8 g/kg/day) or a high protein, leucine supplemented diet (1.2 g/kg/day) (60). Given that the RE protocols in Study 3 were similar in nature to the studies by Bell et al., and Murphy et al., (60-65% of 1RM, 3 sets until volitional fatigue), it is surprising that we were not able to capture a representative RE response with CP over 6 days (last exercise bout was 72 hours prior to the last biopsy). However, both populations in the aforementioned studies were healthy older men whereas the population in Study 3 was healthy older women. Indeed, Devries et al., (6) showed no elevation in rates of MPS 72 hours after RE in healthy older women consuming a control beverage mimicking the blend of proteins typically found in a nutritional support provision (6). Thus it could be speculated that protein quality is of greater importance for the stimulation of integrated MPS with RE in older women in comparison to older men.

Not surprisingly, both acute and integrated MPS were augmented in response to WP supplementation alone, and with RE. Further, rates of MPS were higher with feeding and exercise in the WP group compared to the CP group both acutely, and when integrated over 6 days. These results are in line with numerous studies examining the effects of WP to stimulate MPS in older adults (7, 48, 49, 61). Recently, Devries et al., showed a significant increase in acute (4 hours) and integrated (6 days) rates of MPS with the consumption of 25g of whey in healthy older women (6). Further, when WP was ingested following a bout of RE, MPS was greater than feeding alone both acutely (4 hours) and 72 hours following RE (6). Whey protein is acid soluble meaning that is rapidly digested resulting in pronounced hyperaminoacidemia, a necessary component for the stimulation of MPS (62). Whey is scored at 1 using the PDCAAS (the highest achievable value on this scale) and at 1.09, below milk protein concentrate (1.18) using the DIAAS method (63). Whey is also a rich source of leucine which has been shown to independently stimulate the mechanistic target of rapamycin complex-1 (mTORC-1) pathway that is involved in the up regulation of MPS (44). Indeed, protein sources with a higher leucine content stimulate rates of MPS to a greater extent compared to supplements with a lower leucine content in healthy older adult populations (45, 47, 61). Supplementation with leucine alone (with the ingestion of a mixed meal) has also been shown stimulate MPS in healthy older adults (60, 64). Interestingly, Devries et al., compared 25 g of WP to 10 g of enriched milk protein that was matched for total leucine content (3 g) (50) and showed similar acute and integrated MPS responses indicating the importance of leucine content as opposed to over the all protein content of a supplement.

A major limitation of Study 3 was that we did not include a control group consuming an isocaloric non-nitrogenous supplement for comparison of exercise alone in rates of acute and integrated MPS. However, given that exercise combined with CP supplementation did not augment integrated rates of MPS in older women consuming a high protein diet (1.6 g/kg/day) over 6 days, it can be hypothesized that exercise alone would have resulted in a similar effect. A major contribution to the literature of Study 3 is its demonstration that augmenting total dietary nitrogen content via a low quality protein is not sufficient to increase rates of MPS in healthy older women. Augmenting total dietary nitrogen via the addition of high quality protein that is rich in leucine, should be considered in an effort to improve muscle anabolism.

5.5 Conclusions and future directions

Nutrition and physical activity are potent modulators of skeletal muscle health. Optimizing nutrition, levels of physical activity/exercise, and improving recovery from bouts of physical inactivity are crucial elements to enhance skeletal muscle functionality and prolong independence into older age reducing the burden of aging on the healthcare system. Study 1 and 2 examined for the first time, the effects of an energy restricted, high protein diet during step reduction as well as the effects of protein supplementation alone on recovery in healthy older adults. Episodic, brief bouts of muscle disuse are a pivotal but underappreciated issue in the aging population and our findings contribute substantially to the literature in demonstrating the negative whole body, systemic effects of inactivity in otherwise healthy individuals. The existing literature is unclear as to the

benefits of low quality but highly marketed, collagen peptides to improve muscle health in older persons. Study 3 provides meaningful, albeit acute data, to the null impact of hydrolyzed collagen on skeletal muscle protein synthesis. Therefore, at least in healthy older adults, increasing total dietary nitrogen by the ingestion of low quality protein is not sufficient to improve indices of muscle protein synthesis however future work should evaluate its efficacy in a frailer or more physically compromised population. Given that the life expectancy of older adults will increase by 2 years in men and 2.9 years in women by 2036, strategies to enhance or maintain skeletal muscle health with age are of significant importance to both health care expenditures as well as to extending years of independence and wellbeing in aging Canadians.

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(Chapter 2)

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(Chapter 3)

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