PROTEIN QUALITY AND MOLECULAR EVENTS DURING ENERGY RESTRICTION

MOLECULAR EVENTS UNDERPINNING CHANGES IN TISSUE METABOLISM WITH WHEY AND SOY PROTEIN INGESTION IN ENERGY RESTRICTION IN OVERWEIGHT/OBESE ADULTS

By

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ABSTRACT

Energy restriction-induced weight loss results in the loss of both fat mass (FM) and fat free mass (FFM). Declines in FFM, comprised mostly of skeletal muscle, have the potential to impair metabolic health. A recent meta-analysis demonstrated higher protein hypo-energetic diets (≥ 1.25 g/kg/d) can help retain FFM (Weinheimer, Sands, & Campbell, 2010). This effect is attributed to protein ingestion induced stimulation of muscle protein synthesis (MPS), which is dependent on protein source (Tang, Moore, Kujbida, Tarnopolsky, & Phillips, 2009). However, protein source on modulating FFM preservation during ER remains understudied. In a double blind investigation, 40 adults (BMI 28-40) undertook a moderate 14 day hypo-energetic diet. Subjects were randomized to be supplemented twice daily with whey (WHEY) or soy (SOY) protein, or a carbohydrate (CHO) powder as part of their ER diet. Preliminary and post diet intervention testing included skeletal muscle biopsies, blood samples and DXA measures. Absolute changes in body mass (-2.3 ± 0.18 kg), FM (-1.2 ± 0.16 kg) and FFM (-0.8 ± 0.24 kg) did not significantly differ between groups. Basal serum cortisol was unchanged in WHEY but increased significantly in SOY and CHO ($p<0.05$). No changes were observed in the expression of genes associated with skeletal muscle protein turnover or mitochondrial metabolism. The lack of a treatment effect on body composition or changes in gene expression may be due to the limited time in which our subjects were hypo-caloric; however, the cortisol effect may provide a mechanism by which protein can spare FFM during ER.

PREFACE

Skeletal muscle is a highly plastic tissue critical for both force production and metabolism, as it represents one of the largest and most dynamic storage sites of energy in times of excess and dearth. Consequently, the maintenance of skeletal muscle mass is incredibly important for metabolic health measures. The importance of skeletal muscle's role in metabolism is reflected in resistance training studies within type 2 diabetics, who upon partaking in resistance training programs exhibit marked improvements in HbA1c levels, plasma glucose and insulin levels, as well multiple risk factors for CVD (Cauza, et al., 2005). Furthermore, an indirect comparison of metformin and resistance training in type 2 diabetics show similar improvements in HbA1c levels, further supporting the importance of skeletal muscle in metabolism (Bweir, et al., 2009; Schernthaner, Matthews, Charbonnel, Hanefeld, & Brunetti, 2004). In accordance with these findings it is not surprising that markers for skeletal muscle mass, i.e strength, have been shown to be strong predictors of all-cause and cancer related moralities (Ruiz, et al., 2008).

The plasticity of skeletal muscle, being sensitive to both loading and nutrition (Tang, Moore, Kujbida, Tarnopolsky, & Phillips, 2009), allows us to modulate this critical component of our metabolic health (LeBrasseur, Walsh, & Arany, 2011). As such, the primary focus of our lab has been to understand the mechanisms that control skeletal muscle plasticity. We specifically strive to understand how hypertrophic and atrophic factors regulate skeletal muscle protein turnover in the guidelines of improving an individual's metabolic health and strength. Ultimately, we hope our research will be used to guide recommendations for maximizing skeletal muscle mass (and strength) in athletes, the elderly, individuals recovering from disease or injury states and to help prevent muscle wasting during catabolic conditions such as caloric restriction, inactivity or disease.

"Sometimes, as I have stood contemplating the majesty of a locomotive by the platform of a railway station, I have thought of how meaningless would be the machine unless considered in respect of its activity, unless we think of it in terms of horse-power, or of the rate at which it will pull a certain load, or in some such terms apart from the fulfillment of its function, the engine is an agglomeration of curiously shaped pieces of metal. The condition of exercise is not a mere variant of the condition of rest, it is the essence of the machine."

Joseph Barcroft, -1935

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LIST OF ABBREVIATONS

ATP	Adenosine triphosphate
ANOVA	Analysis of variance
BCP	Bromochloropropane
CR	Caloric restriction
CRP	C-reactive protein
CVD	Cardiovascular disease
DEPTOR	DEP domain-containing mTOR interacting protein
DXA	Dual energy X-ray absorptiometry
eIF3f	Eukaryotic translation initiation factor 3 subunit F
ER	Energy restriction
FBX040	F-box protein 40
FFM	Fat free mass
FM	Fat mass
FOXO	Forkhead transcription factor
FOXO1	Forkhead transcription factor-1
FOXO3a	Forkhead transcription factor-3a
IGF-1	Insulin like growth factor 1
IL-6	Interleukin-6
LST8/GβL	Mammalian lethal with SEC13 protein
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
mSIN1	MAP kinase interacting protein 1
mTOR	Mammalian target of rapamycin
mTORC1	Mammalian target of rapamycin complex one
PGC-1α	Peroxisome proliferator-activated receptor-γ coactivator 1-α
PKB	Protein kinase B also called Akt
RICTOR	Rapamycin insensitive companion of mTOR
PRAS40	Proline rich Akt substrate
SEM	Standard error of the mean
SIRT1	Sirtuin 1
TG	Triacylglycerol
Trim32	Tripartite motif–containing protein 32
RAPTOR	Regulator associated protein of mTOR
TNF-α	Tumor necrosis factor alpha
TRAF6	Tumor necrosis factor (α) receptor adaptor protein 6
Ub	Ubiquitin
UCP3	Uncoupling protein 3
UPP	Ubiquitin proteasome pathway
YY1	Ying-yang 1

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CHAPTER 1: Literature Review

1.1 INTRODUCTION

Over the Last 20 years the number of obese individuals in the U.S. and Canada has risen by roughly 10 % in each country (Carrol, Ogden, & Margot, 2011). Current estimates place 34 % and 24 % of the respective U.S and Canadian populations as obese (Carrol, Ogden, & Margot, 2011). Consequently, obesity can justifiably be called an epidemic in North America. The health consequences of obesity include an increased risk for hypertension, hypercholesterolemia, some forms of cancer, cardiovascular disease (CVD) and type II diabetes (Solomon & Manson, 1997). According to the World Health Organization the leading cause of death in the U.S. as of 2004 was due to CVD complications accounting for 36 % of all U.S. deaths. When combining deaths attributed to CVD and diabetes mellitus this estimates reaches 40 % of all U.S. deaths in 2004. This suggests that a near majority of all deaths in the U.S. can be largely attributed to relatively preventable obesity related complications. Furthermore, with obesity resulting in an increased risk for morbidity and mortality, greater health care costs have consequently been reported for obese individuals as compared to their lean counterparts. A recent report from the Public Health Economics Program at RTI International, stated that medical spending for obese individuals is on average $ 1,429 greater, per year (42 % higher) than for non-obese Americans (Finkelstein, Trogdon, Cohen, & Dietz, 2009). Similar findings were observed for the Canadian population with obesity attributed health care costs accounting for an estimated $6 billion yearly or 4.1 % of total health care expenditures in 2006 (Anis, et al., 2010). If current trends in obesity are left unchecked, both the prevalence of the disease and the costs of treatment will pose a growing economic and health challenge for North Americans.

Current recommendations for the treatment of obesity are effective; however, they are not without repercussions. Weight loss requires being in a hypo-energetic state accomplished via a decrease in energy intake, an increase in energy expenditure or both. The American Heart Association endorses a caloric deficit between 500-1000 kcal/day, with macronutrient ratios of ≤ 30 % fat, ≥ 55 % carbohydrate (CHO) and ≤ 15 % protein (PRO) to achieve healthy weight loss (Krauss, et al., 2000). While effective, this method often results in the unwelcome loss of fat free mass (FFM; Note: fat-free mass or FFM as it referred to in

this thesis is usually derived from DXA and is obtained by subtracting bone and fat mass from total mass). Indeed individuals placed on a hypo-energetic diet (without exercise) typically observe between 20-30 % of their total body weight loss as the loss of FFM (Weinheimer, Sands, & Campbell, 2010). The loss of FFM results in a diminished resting metabolic rate (RMR), and therefore a decreased daily caloric requirement. This not only lowers the effectiveness of a hypo-energetic diet, but may increase the propensity for weight regain following the cessation of the diet (Amigo & Fernández, 2007). With an already low success rate for weight maintenance following weight loss (~20 %) (Wing & Hill, 2001), interventions to preserve FFM during weight loss are critical. Additionally, FFM plays an important role in mobility, metabolic health (including CVD markers and insulin and glucose regulation) and increases one's chances of survival during disease and injury states (Srikanthan & Karlamangla, 2011; Wolfe, 2006). Therefore, limiting the loss of FFM during a hypo-energetic diet may limit weight regain, result in improved metabolic health and preserve mobility. The development of dietary interventions which limit the loss of FFM are critical to achieve healthy weight loss.

One dietary intervention that has been shown to aid in the preservation of FFM during diet induced weight loss is an increased consumption of dietary protein at the expense of carbohydrate. A recent meta-analysis found higher protein diets (averaging 1.25 ± 0.17 g/kg/d), were able to better preserve FFM over standard protein (averaging 0.72 ± 0.09 g/kg/d) diets during diet induced weight loss (Wycherley, Moran, Clifton, Noakes, & Brinkworth, 2012).

The purpose of my review will be to focus on clinical trials examining the effects of *ad libitum* and hypo-energetic higher protein diets on changes in body composition and to discuss the potential mechanisms behind higher protein diets. Of the potential mechanism this review will cover the effects of protein in; the regulation of anabolic and catabolic pathways within skeletal muscle as well as the role of protein in regulating satiety, thermogenesis, and mitochondrial biogenesis.

1.2 HIGH PROTEIN DIETS: EFFECTS ON BODY COMPOSITION

Over the past century there has been much debate as to whether diet-induced weight loss can be optimized by altering the macronutrient composition of the diet. A

variety of fad diets recommending extreme, whether minimal or excessive, intakes of protein, fat and carbohydrate have cycled through the popular media. All of these diets have claimed to provide a quick and healthy means to weight loss; however, limited or no scientific data is available to support these claims. One of the first controlled long duration studies to compare common fad diets includes work by Gardner *et al.,* (2007) in which four different diets ranging from high to low carbohydrate intakes on weight loss and metabolic risk factors in overweight and obese women were studies over a 12 month period. These authors' observed that women on the low carbohydrate, higher protein diet (Aktins) lost more total body weight at 2 and 6 months than women on a moderate protein, high carbohydrate diet; nonetheless, this effect was diminished at 12 months as all groups had reverted back to their pre-intervention dietary intake (Gardner, et al., 2007). This study demonstrated that long term self-efficacy to adhere to any diet will ultimately determine the success of the diet. However, over the short term (<6months), while compliance is high, higher protein diets (or by default lower carbohydrate diets) appear to result in greater losses in body weight suggesting a physiological effect of protein on energy expenditure, caloric intake or both.

1.2.1 Preservation of Fat Free Mass during Caloric Restriction

The following section will briefly review some selective representative studies in which higher protein (HP) verse adequate protein (AP) hypo-energetic diets have been compared particularly with respect to the preservation of FFM. A more in-depth analysis of the effects of higher protein hypo-energetic diets on body composition can be found in a recent meta-analysis (Wycherley, Moran, Clifton, Noakes, & Brinkworth, 2012) and meta-regression (Krieger, Sitren, Daniels, & Langkamp-Henken, 2006). Both of the aforementioned studies demonstrated an effect of higher protein diets on the preservation of FFM during diet induced weight loss in interventions lasting 12 weeks or longer.

Work by Bopp *et al.,* demonstrated a strong correlation between protein intake and the absolute change in FFM during a hypo-energetic diet (Bopp, et al., 2008). In this study, seventy women were divided into two groups, aerobic exercise and sedentary controls, and were provided with meals to elicit a 400 kcal/d energy deficit over a twenty week period. The aerobic exercise intervention had no effect on measures of FFM, consequently all

participants were analyzed together. A significant correlation between protein intake and the absolute change in FFM (r=0.30 P=0.01) and appendicular FFM (r=0.41, P<0.001) was observed with a greater relative intake of protein associate with smaller losses in FFM (Bopp, et al., 2008). It should be noted, however, that the relative protein intake in this study ranged from 0.45 g/kg/d to the RDA's recommended protein intake of 0.8 g/kg/d. Despite the lower than recommended protein intake, ~32 % of total body weight losses were attributed to FFM losses, a value typical of most weight loss studies (Weinheimer, Sands, & Campbell, 2010). These data (Bopp, et al., 2008) suggest the existence of a correlation between protein intake and the retention of FFM during a hypo-energetic diet, a relationship which has been observed in highly controlled weight loss studies.

In a randomized control trial Soenen *et al.,* used a three compartment model (FM as determined by BodPod, total body water by $^{2}H_2O$ dilution) of body composition to accurately measure changes in body composition following weight loss with a higher versus a moderate protein diet (Soenen, Martens, Hochstenbach-Waelen, Lemmens, & Westerterp-Plantenga, 2013). Over the course of six weeks participants consumed 1.2 g/kg/d or 0.8 g/kg/d with caloric intakes at ~33 % of their daily energy requirements. A significant group by time effect was observed for absolute changes in FFM, with the higher protein diet group retaining 0.7 kg more FFM than the control group. The strengths of this study rest on the use of the 3 compartment model used to determine changes in body composition following weight loss. While duel energy X-ray absorptiometry (DXA) is often claimed to be the gold standard for measuring changes in body composition; DXA measures have been shown to overestimate FFM as compared to 3 and 4 compartment models of body composition (Visser, Fuerst, Lang, Salamone, & Harris, 1999). However, the relative ease of obtaining DXA scans if a unit is present combined and fast collection times allows for a convenient measure of body composition.

Frestedt *et al.,* (2008) examined the impact of a whey protein based and carbohydrate supplement on changes in body composition following a 12 week moderate hypo-energetic diet (-500 kcal/d). The whey protein (Prolibra) and the iso-caloric maltodextrin control supplements were consumed twice daily prior to meals. Following 12 weeks, the Prolibra group showed greater losses in body weight and FM with a significant group by time effect for both measures ($p<0.05$). In a prospective analysis participants

were divided into completers (body weight loss <2.25 kg) and responders (body weight loss >2.25 kg). Within the responders there was a significant group by time effect for FFM, with the Prolibra group observing a 1.34 kg greater preservation of FFM. It should be noted that the protein intake for the control group, being 0.6 g/kg/d, was below the RDA of 0.8 g/kg/d. This being said the percentage of FFM lost relative to total body mass losses was similar between the Prolibra and control groups with both groups experiencing an ~50 % loss of mass as FFM. While the control group's protein levels were below the RDA, it appears that the relative loss of FFM was not accelerated due to a lower protein intake.

Leidy *et al.,* placed 46 overweight or obese women on a twelve week moderate hypo-energetic diet (-750 kcal/d) with either a higher protein (1.4 g/kg/d) or adequate protein (0.8 g/kg/d) intake (Leidy, Carnell, Mattes, & Campbell, 2007). While no group by time effects were observed for DXA measures of body mass (BM) or FM a group by time effect was observed for changes in lean body mass (LBM; $P<0.05$). Following the 12 week hypo-energetic diet the HP group observed a ~1.25 kg greater preservation of LBM over the AP group. In a post hoc analysis, in which dietary groups were sub divided into pre obese (POB, BMI 25.0 - 29.9 kg/m^2) and obese (OB, BMI: 30 to 37 kg/m^2) participants, a time by obesity effect was observed in which AP-OB exhibited the greatest loss of LBM (-3.6 ± 0.6 kg) followed by HP-OB (-2.1 ± 0.4 kg), AP-POB (-1.7 ± 0.5 kg), and HP-POB (-0.7± 0.3 kg). Despite the equivalent caloric deficits and relative protein intakes achieved, the obese individuals observed greater absolute losses in LBM relative to their overweight counterparts. These data suggest that either a greater relative protein intake is required for obese individuals or that obesity may alter muscle protein turnover in either the postabsorptive or postprandial conditions resulting in the accelerated loss of muscle mass during energy restriction. The latter hypothesis will be covered in more detail in later sections of this review.

The work by Liedy *et al.,* (2007) provides support for the use of higher protein diets for the preservation of FFM during diet induced weight loss; however, there is a caveat as the protein sources used between the HP and AP groups were not balanced. The HP and AP group's protein source primarily consisted of pork and dairy based protein respectively. While little work has been done to directly compare pork and milk based proteins on energy expenditure and FFM changes during weight loss, it is possible that different protein

sources may have confounded the study's findings. Pervious animal work has shown a difference in 24 hr energy expenditure while consuming pork verse soy protein based diets (Mikkelsen, Toubro, & Astrup, 2000). Additionally, work by our lab has shown difference in the protein sources effect on muscle growth following periods of resistance training (Hartman, et al., 2007) and acute measures of muscle protein turnover (Tang, Moore, Kujbida, Tarnopolsky, & Phillips, 2009). These data suggest that not all protein sources have the same anabolic effects on skeletal muscle or daily energy expenditure and therefore protein sources should be balance during diet induced weight loss.

While the general consensus in the literature supports the increased consumption of protein to more effectively preserve FFM during a hypo-energetic state, a number of studies have contested these findings. For example, Belobrajdic *et al.,* (2010) conducted a 12 week weight loss study examining the role of higher protein diets on circulating IGF-1 following weight loss. Protein intake averaged 1.27 and 0.81 g/kg/d for the HP and the NP groups respectively. There was no significant group by time effect for changes in FFM despite the HP group losing an average of 1 kg less FFM than the AP group (P=0.172) (Belobrajdic, et al., 2010).

Similarly, work by Kasim-Karakas *et al.,* in which 24 women with polycystic ovary syndrome were placed on a moderate hypo-energetic diet (550-800 kcal/day deficit) for 8 weeks showed no diet effect on FFM losses (Kasim-Karakas, Almario, & Cunningham, 2009). Protein intake was 1.07 and 0.66 g/kg/d for the HP and AP groups respectively. Following the 8 week intervention the HP lost a total -0.3 ± 0.4 kg and the AP group lost -0.6 ± 0.3 kg of FFM with no significance group by time effect (P=.535). A FFM group by time effect may have been observed if the study had been extended to at least 12 weeks as suggested by Wycherley *et al.,* (Wycherley, Moran, Clifton, Noakes, & Brinkworth, 2012) allowing for greater absolute losses in FFM. In addition, the protein intake in the HP group was just at the cutoff point established by Krieger *et al.,* (Krieger, Sitren, Daniels, & Langkamp-Henken, 2006) in a meta-regression and below the average protein intake used in a meta-analysis conducted by Wycherley *et al.,* (Wycherley, Moran, Clifton, Noakes, & Brinkworth, 2012). Beyond changes in FFM, higher protein hypo-energetic diets have also been observed to results in greater loss of FM.

1.2.2 Augmentation of Fat Mass Losses during Caloric Restriction

The effect of higher protein hypo-energetic diets producing favorable changes in body composition does not appear to only result in a preservation of FFM but also may augment FM losses. Several studies from Layman et al., have shown an effect of higher protein hypo-energetic diets in augmenting FM losses (Layman, et al., 2009; Layman, et al., 2005). In a 2005 study Layman *et al*., examined the effects of exercise and protein intake (30 % verse 17 % energy from protein) on changes in body composition during a hypo-energetic diet over a 4 month period. Following the intervention there was a main effect for both diet and exercise in respect to total and % FM losses, with the higher protein groups losing on average 2 kg more FM than the control groups (Layman, et al., 2005). Similar findings were observed by layman *et al.,* in 2009, when comparing higher verse moderate protein hypo-energetic diets over a four month period without exercise (Layman, et al., 2009). While no difference in the absolute loss of BM was observed in the 2009 study the HP group lost on average 1 kg of FM more than the AP group, -5.6 ± 0.4 kg verse -4.6 ± 0.3 kg respectively ($P<0.04$) (Layman, et al., 2009).

Work from a variety of laboratories has consistently shown an effect of higher protein diets on the augmentation of FM losses during a hypo-energetic state. For example, Frestedt *et al.,* 2008 reported a significant augmentation of FM losses following a 12 week moderate hypo-energetic diet with protein intakes of 0.81 verses 0.61 g/kg/d. The completers (all participants) and high responders (participants who lost >2.25 kg of body weight) in the HP groups showed a ~1.2 and 1.5 kg, respectively, greater loss of FM over the AP group ($P<0.05$) (Frestedt, Zenk, Kuskowski, Ward, & Bastian, 2008). Fletchner-Mors *et al.,* 2010 (Flechtner-Mors, Boehm, Wittmann, Thoma, & Ditschuneit, 2010) conducted a one year study in which individuals with metabolic syndrome were placed on a moderate hypo-caloric diet with target protein intakes of 1.34 and 0.8 g/kg/d. As early as three months into the intervention those consuming the higher protein diet showed a significantly ($P<0.01$) greater reduction in BM and FM over the control group. With 80.5 % and 60.2 % of weight loss as the loss of FM in the HP verse AP groups respectively (Flechtner-Mors, Boehm, Wittmann, Thoma, & Ditschuneit, 2010). Josse *et al.,* examined the effects of protein, calcium content from dairy intake and exercise on changes in body composition during a 16 week hypo-energetic diet. While no group by time effect for BM was observed, the HP group lost ~1 kg more FM than the AP groups ($P<0.05$) (Josse, Atkinson, Tarnopolsky, &

Phillips, 2011). Together, these studies demonstrate a consistent effect for higher protein diets for the augmentation of FM losses during energy restriction, yet there are cases were the effects are not as apparent.

Work by Noakes *et al*., (Noakes, Keogh, Foster, & Clifton, 2005) initially failed to demonstrate a higher protein diet effect for the augmentation of FM losses during a hypo-energetic diet; however, upon a post hoc analysis a FM effect was observed when obese women were classified into high (≥1.5mmol/L) and low (<1.5mmol/L) plasma triacylglycerol (TG) groups (Noakes, Keogh, Foster, & Clifton, 2005). Women with elevated TG levels in the HP group observed a ~3 kg greater loss in FM over the AP group with elevated TG levels (P=0.035). This data suggests that higher protein diets maybe more effective in individuals with elevated blood markers for CVD. Still not all studies have quantified basal TG levels (Layman D. K., et al., 2009; Layman D. K., et al., 2005), making it difficult to determine if this is a novel or a consistent observation. While it remains unclear how high or low plasma TG levels are related to the effects of higher protein hypo-energetic diets on FM losses this may provide a clue as to why some studies failed to show an effect.

While any number of factors could be responsible for a lack of a main effect of higher protein diets (ranging from the duration of the diet, the caloric deficit and to the absolute amount of protein ingested), blood TG levels <1.5 mmol/L have been a consistent observation in multiple studies which failed to show a diet effect for measures of FM. Over the course of a 6 week hypo-energetic diet Soenen *et al*., (2013) placed 72 individual on either a HP (~1.2 g/kg/d) or AP diet (~0.8 g/kg/d). While a FFM effect was observe there was no significant difference between losses in FM (Soenen, Martens, Hochstenbach-Waelen, Lemmens, & Westerterp-Plantenga, 2013). Interestingly, the average basal plasma TG levels for both groups was 1.3 mmol/L, this being just below the 1.5 mmol/L cut off used by Noakes *et al*., (Noakes, Keogh, Foster, & Clifton, 2005). Similar results were observed in a 2003 study by Layman *et al.* in which participants basal TG levels ranged from 1.05 to 1.21 mmol/L with no diet effect on FM losses (Layman, et al., 2003). Leidey *et al.,* 2007 placed 46 overweight/obese women on a moderate hypo-caloric diet for 12 weeks with 30 to 18 % of calories from protein. Following the 12 week dietary protocol no effect of protein intake on changes in FM were observed (Leidy, Carnell, Mattes, & Campbell, 2007); again TG levels were below the 1.5mmol/L (1.37 mmol/L) cut off used by Noakes *et al*. (Noakes, Keogh,

Foster, & Clifton, 2005). While promising these findings are no more than correlations; consequently, more work will be required to determine if elevated TG levels or a co-factor, are a perquisite to observe a treatment by time effect for higher protein hypo-energetic diets on changes in FM. Nevertheless, despite the collection of studies showing no effect of higher protein hypo-energetic diets on FM losses a recent meta-analysis has demonstrated a consistent effect.

Wycherley *et al*., recently published a meta-analysis comparing the effects of energy restricted higher protein diets verses standard protein diets on changes in body composition measures (Wycherley, Moran, Clifton, Noakes, & Brinkworth, 2012). A total of 24 studies were examined with relative protein intakes averaging (Mean ± SEM) 1.25 ± 0.17 g/kg/d or 30.5 ± 2.5 % of energy intake from protein, verses 0.72 ± 0.09 g/kg/d or 17.5 ± 1.5 % of energy intake from protein for the higher protein and standard protein groups respectively. Ultimately, during energy restriction higher protein diets were shown to results in a ~0.8 kg greater loss in FM over standard protein diets for studies lasting less or more than 12 weeks. The increased consumption of protein during a hypo-energetic diet appears to provide a true benefit in respect to producing favorable changes in body composition during weight loss; however, for higher protein diets to be truly effective we have to determine if these diets are successful at maintaining weight losses or altering body composition during periods of energy balance.

1.2.3 *Ab libitum* Diets

Currently the success rate for individuals maintaining a weight loss of at least 10 % of initial body weight at one year follow diet induce weight loss is estimated to be ~20 % (Wing & Hill, 2001). This either results in the individual's cessation of their weight loss attempts or multiple repeated attempts to lose weight, termed yo-yo dieting. Multiple attempts at weight loss with subsequent weight regain may result in a greater metabolic risk as losses in FFM observed during diet induced weight loss could take extended periods of time to return to pre-diet levels (Lejeune, Kovacs, & Westerterp-Plantenga, 2005; Claessens, Van Baak, Monsheimer, & Saris, 2009). As cited perilously FFM, a component of which includes skeletal muscle, is a critical contributor to metabolic health (Wolfe, 2006). Therefore the creation of successful dietary interventions which help maintain lost weight and improve body composition are a critical component for healthy weight loss.

The benefits of the increased consumption of protein during a hypo-energetic state on changes in body composition have also been observed during period of weight maintenance following weight loss. Work by Claessens *et al.,* 2009 placed three groups of overweight or obese adults on a very low calorie diet (VLCD) for 5 weeks. All groups observed similar changes in body composition with average losses of -9.4 kg in BM, -7.4 kg of FM and -1.8 kg of FFM. In a 12 week follow up participants returned to their pre weight loss *ad libitum* dietary habits, but were given a whey protein, casein protein or a carbohydrate control supplement consumed twice daily. After 12 weeks the carbohydrate group had gained back 1.19 ± 0.9 kg in BM, of which 0.96 ± 0.38 kg and 0.24 ± 0.7 kg were FFM and FM respectively. Interestingly, the whey and casein groups lost an additional 1.09 ± 0.59 kg in BM, of which an increase of 0.88 ± 0.37 kg of FFM and an additional loss 1.96 ± 0.52 kg of FM was observed. Additionally, the whey group tended to show a slightly greater gain in FFM of 1.43 ± 0.49 kg over the casein groups gain of 0.16 ± 0.53 ($P=0.09$) (Claessens, Van Baak, Monsheimer, & Saris, 2009). In a similar study Lejeune *et al.,* 2005 placed 113 individuals on a 4 week VLCD resulting in an average loss of ~6.3 kg of BM. The VLCD was followed by six month of supplementing the participant's diets with or without 30 g of additional protein daily. After 6 months both dietary groups gained back ~0.85 kg of the ~2.2 kg of FFM lost during the VLCD with no significant group by time effect. However, a group by time effect was observed for changes in FM which increased by 1.8 kg in the control group but decreased by ~1 kg in the higher protein group ($P<0.05$) (Lejeune, Kovacs, & Westerterp-Plantenga, 2005). Similar findings were observed by Westerterp-Plantenga *et al.,* when examining changes in body composition in participants consuming higher (18% energy from protein) and moderate protein (15% energy from protein) *ad libitum* diets for 3 months, following a 4 week VLCD. A significant ($P<0.05$) time by treatment effect was observed for measures of BM, FM and body fat percent in which participants in the higher protein group observed additional losses in FM and gains in FFM while the moderate protein group observed gains in both FM and FFM (Westerterp-Plantenga, Lejeune, Nijs, Van Ooijen, & Kovacs, 2004). These data suggest that increasing the consumption of protein during periods of weight maintenance following weight loss will help individuals to maintain and potentially further augment preferable changes in body composition.

Even without prior weight loss the increased consumption of protein may result in favorable changes in body composition or metabolic risk factors during ad libitum diets. In a 23 week intervention Bear *et al*., 2011 took 90 overweight/obese free living adults and randomized them to have their diets supplemented with a whey protein, soy protein or carbohydrate powder. Participants were asked to consume the supplements twice daily, before meals with the whey and soy protein being isonitrogenous (56 g protein/day) and all supplements being isocaloric. Following the ~6 month intervention the soy group observed no changes in body composition while the whey group observed ~1 kg loss in FM and the control group gained ~1 kg in FM, with a significant ($P<0.005$) difference between the carbohydrate and whey groups only (Baer, et al., 2011). Similar results have been observed in other studies (Skov, Toubro, Rønn, Holm, & Astrup, 1999) with greater absolute losses in BM and FM in higher protein groups. Interestingly, a study similar to Bear *et al.,* 2011 failed to show a group by time effect for a whey protein, casein protein or a carbohydrate supplement during an *ad libitum* diet on measures of body composition over a 12 week period (Pal, Ellis, & Dhaliwal, 2010). Despite the lack of an effect on measures of body composition there was a significant effect of whey protein for decreases in total TG, fasting plasma insulin levels, total cholesterol, and LDL cholesterol over the control group (Pal, Ellis, & Dhaliwal, 2010). While a metabolic benefit was observed with the whey protein supplement the lack of a body composition effect may be attributed the shorter duration of the intervention in combination with the use of only DXA measures for body composition. The use of more precise measures of body composition, including a 4 compartment model, has produced a diet by time effects of higher protein *ad libitum* diets over the same duration (Soenen & Westerterp-Plantenga, 2010). Work by Soenen *et al.,* 2010 demonstrated a group by time effect for decreases in FM ($P<0.05$), and a trend towards increases in FFM ($P=0.05$) when comparing higher verses moderate protein *ad libitum* diets over a 12 week period (Soenen & Westerterp-Plantenga, 2010). While a meta-analysis examining the effects of higher protein diets on measures of body composition during periods of energy balance has yet to be completed, it is likely that a beneficial effect would be observed.

1.2.4 Protein Satiety and Thermogenesis

One mechanism by which higher protein diets may induce greater losses of FM includes both the greater satiating effect and thermic effect of protein over other

macronutrients (Halton & Hu, 2004). The greater thermic effect of protein over carbohydrates and fats is generally attributed to increases in energy costs associated with protein ingestions stimulation of protein synthesis, ureogenesis and gluconeogenesis (Volpi, et al., 1996). The role of protein in increasing the perception of satiety, while well characterized (Veldhorst, et al., 2008), the mechanisms of action are poorly understood in humans. This lack of an understanding is largely attributed to the interplay between a variety of satiety-related, adiposity-related, and psychological factors all regulating food intake (Woods, 2004). Both the increased measures of satiety and of thermogenesis represent two possible macroscopic mechanisms by which higher protein diets may help augment weight loss.

The satiating effect of protein to contribute to weight loss requires that protein ingestion results in a reduction in subsequent caloric intake in controlled and *ad libitum* conditions and that this effect is maintained during long duration higher protein diets. Multiple studies have demonstrated a satiating effect of protein by studying subsequent food intake preceded by a standardized higher protein meal balanced to control meals for caloric content and weight (Latner & Schwartz, 1999; Poppitt, McCormack, & Buffenstein, 1998; Dove, et al., 2009). However, when test meals are delivered *ad libitum* followed by measures food intake a satiating effect of protein is observed is some (Skov, Toubro, Rønn, Holm, & Astrup, 1999; Barkeling, Rössner, & Björvell, 1990) but not all (Blatt, Roe, & Rolls, 2011) studies. In addition, for a true satiating effect of protein to induce weight loss the satiating effect needs to be observed beyond acute measures. While limited data is available a select few studies have demonstrated a greater satiating effect of protein over an acute (<1 week) (Lejeune, Westerterp, Adam, Luscombe-Marsh, & Westerterp-Plantenga, 2006; Weigle, et al., 2005) and a 12 week dietary period (Weigle, et al., 2005). These data suggest that higher protein diets appear to provide a true satiating effect resulting in a reduced caloric intake following the acute or chronic consumption of higher protein meals and therefore may contribute to adherence to a diet or a reduce caloric intake during periods of energy balance or energy restriction.

Another reason for the success of higher protein diets in the augmentation of FM losses during periods of controlled energy restriction or ad libitum feeding maybe due to a greater thermic effect of protein. Energetically protein is the most 'expensive'

macronutrient as the energy costs to digest and store carbohydrates and lipids accounts roughly 5-10 % and 0-3 % of the caloric content of the food consumed respectively, whereas protein requires 20-30 % (Tappy, 1996). This suggests that of three isocaloric meals of solely fat, carbohydrate or protein the greatest net gain in caloric content would occur in the following hierarchical manor fat>carbohydrate>protein. In addition, the thermic effect of food is estimated to contribute to a considerable portion, 10-15 %, of total energy expenditure over a 24 hr period (Sims & Danforth Jr, 1987). Therefore it is possible that the greater thermic effect of protein could contribute to an elevated 24 hr energy expenditure when comparing higher protein to adequate protein diets. A recent review by Halton and Hu (2004) examined fifteen studies measuring the thermic effect of higher protein meals using a variety of methods. The authors concluded there was a significantly greater thermic effect of higher protein meals over standard protein meals (Halton & Hu, 2004). Comparisons between isocaloric diets containing 30 % or 15 % energy from protein, estimates that higher protein meals require an additional 23 kcal over standard protein meals due to the increased thermic effect of protein (Eisenstein, Roberts, Dallal, & Saltzman, 2002). While small, it is possible that over extended periods of time the greater thermic effect of protein could contribute to improved weight maintenance or potentially small amounts of weight loss. While the significance of the increased thermic effect of protein to augment body composition changes is debatable; it is possible that both the greater satiating and thermogenic costs of protein contribute to weight loss.

1.2.5 Summary

A majority of the literature, including a recent meta-analysis, support the theory that HP diets can result in favourable changes in body composition (including augmented FM losses and the preservation of FFM) over AP diets during periods of energy restriction and energy balance. The macroscopic mechanisms by which increased protein ingestion results in increases in FM losses may be attributed to a greater thermic effect and satiating effect of protein ingestion. At the microscopic levels increased protein ingestion induced preservation of FFM and augmentation of FM losses remains relatively unexplored and will be the focus of the next sections.

1.3 THE REGULATION OF SKELETAL MUSCLE MASS

1.3.1 Regulation of Muscle Protein Turnover: MPS and MPB

The maintenance of skeletal muscle mass is dependent on a balance between the rates of muscle protein synthesis (MPS) and muscle protein breakdown (MPB) (Rennie, Wackerhage, Spangenburg, & Booth, 2004). With MPS and MPB representing the rate at which amino acids are directly incorporated into or removed from skeletal muscle proteins respectively. In the postabsorptive state the rate of MPB exceeds the rate of MPS resulting in a net negative protein balance and the consequent loss of skeletal muscle mass (Tipton, Ferrando, Phillips, Doyle, & Wolfe, 1999). Conversely, in the postprandial state the rate of MPS increases 2-3 fold while the rate of MPB falls by 20-50 % resulting in a net positive protein balance and an accretion of skeletal muscle mass (Tang & Phillips, 2009) (see Figure I). With proper nutrient intake a net neutral balance in protein turnover is achieved, resulting in the maintenance of skeletal muscle mass. Fluctuations in the rates of MPS are 10-20 fold greater than fluctuations in MPB between the postabsorptive and postprandial states (Tang & Phillips, 2009); suggesting that MPS is the principle determine of skeletal muscle mass during anabolic and catabolic conditions (Rasmussen & Phillips, 2003; Phillips, Glover, & Rennie, 2009). Two of the most widely characterized stimuli known to increase MPS include both protein ingestion (Rennie, Wackerhage, Spangenburg, & Booth, 2004) and mechanical loading (Phillips, Tipton, Aarsland, Wolf, & Wolfe, 1997). Extensive work from our laboratory and others has focused on quantifying and optimizing the MPS and MPB responses to anabolic stimuli produced by protein ingestion and mechanical loading on skeletal muscle during both anabolic and catabolic conditions. While significantly headway has been made in this area the cellular mechanisms controlling MPS and MPB require much more attention.

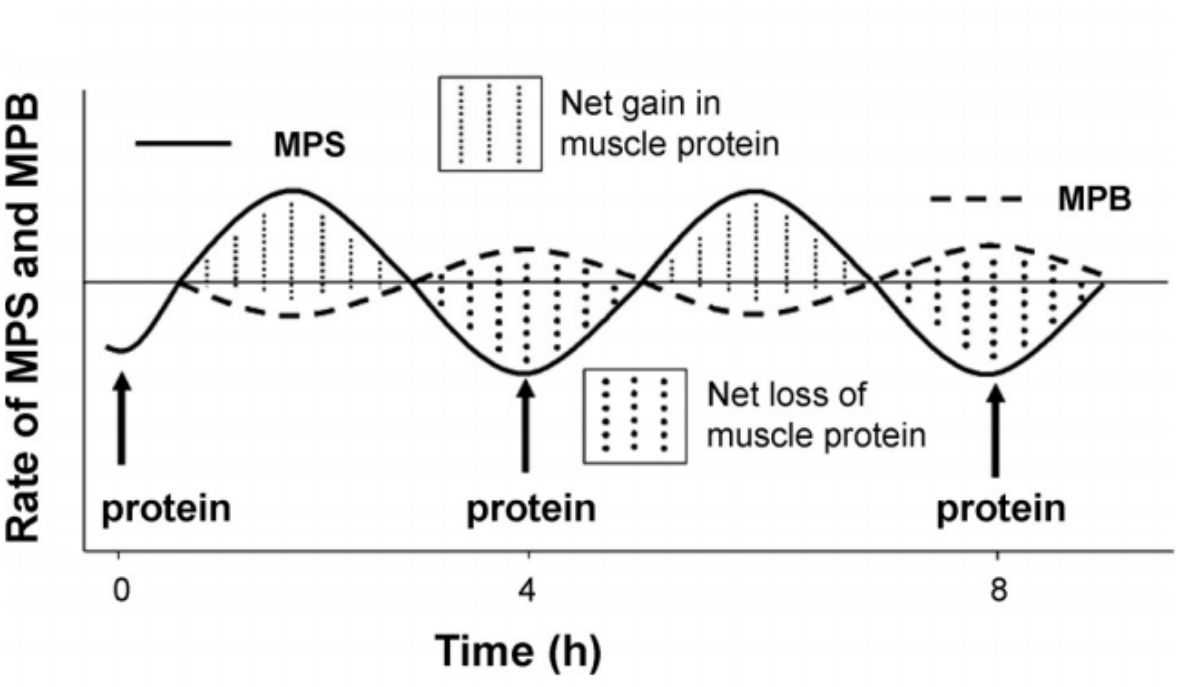

Figure I. Adapted from (Phillips, Glover, & Rennie, 2009). Schematic depiction of the diurnal fluctuations in muscle protein turnover attributed to MPS and MPB with regular protein ingestion approximating 3 equally spaced protein-containing meals.

1.4 MUSCLE PROTEIN SYNTHESIS AND THE mTOR PATHWAY

There are a large variety of anabolic stimuli for MPS, including amino acids, hormones, and mechanical loading but almost all provide a stimulus that, ultimately converges on the mammalian target of rapamycin (mTOR), which is node for the regulation of MPS and skeletal muscle mass (Adegoke, Abdullahi, & Tavajohi-Fini, 2012). The central protein, mTOR, is an evolutionarily well conserved serine threonine protein kinase regulating cell growth in organisms ranging from man, rodents and yeast. mTOR resides in two complexes (mTORC1 and mTORC2), with mTORC1 believed to be a key focal point in the regulation of MPS, cell growth and cell metabolism (Wullschleger, Loewith, & Hall, 2006). Complex one is rapamycin sensitive and is composed of multiple proteins including: regulator associated protein of mTOR (RAPTOR), mammalian lethal with SEC13 protein 8

(LST8/GβL), DEP domain-containing mTOR interacting protein (DEPTOR) and proline rich Akt substrate (PRAS40). In contrast mTORC2 is largely rapamycin insensitive and is composed of LST8, DEPTOR, PROTOR, mammalian stress activated MAP kinase interacting protein 1 (mSIN1) and rapamycin insensitive companion of mTOR (RICTOR). The activation of mTORC1 induces the phosphorylation of a number key proteins regulating translation including: ribosomal protein S6 kinase ($p70^{S6K1}$) and 4E-binding protein (4E-BP1) which ultimately results in an increase in translational efficiency, ribosomal biogenesis and global rates of protein synthesis (Wullschleger, Loewith, & Hall, 2006) (See Figure II, adopted from (Bodine S. C., 2006).

It has been proposed that mTORC1 is the primary regulator of MPS and muscle mass. Rates of MPS have been shown to correlate with gains in muscle mass following mechanical loading (Goldberg, 1968) and the phosphorylation of $p70^{S6K1}$ correlates with muscle growth in rodents (Baar & Esser, 1999), and in some (Terzis, et al., 2008) but not all (Mitchell, et al., 2012) human studies. The inhibition of mTOR with rapamycin results in impaired muscle growth in rodents during functional overload and following reloading after hind limb suspension (Bodine, et al., 2001). Human models have shown similar findings with rapamycin supplementation shown to inhibit acute increases in MPS follow a single bout of resistance exercise (Drummond, et al., 2009). Gain of function studies conducted by Bodine *et al.,* demonstrated that the injection of a genetic construct which expressed a constitutively active form of Akt, an upstream activator of mTOR, into the tibialis anterior of adult mice resulted in muscle hypertrophy in a rapamycin sensitive manor (Bodine, et al., 2001). Together these studies suggest an important role of mTOR in the regulation of MPS and muscle mass, the reader is direct to a number of reviews for more detail (Adegoke, Abdullahi, & Tavajohi-Fini, 2012; Sengupta, Peterson, & Sabatini, 2010)

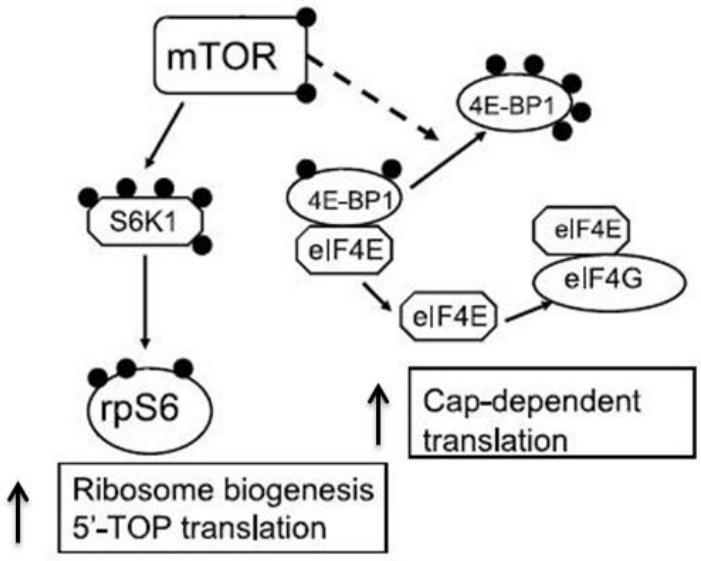

Figure II. Adapted from (Bodine S. C., 2006). Schematic depiction of a subset of the mTORC1 downstream signaling components regulating MPS.

1.4.1 Molecular Basis for MPS

Anabolic stimuli ranging from insulin and growth factors, amino acids and mechanical loading act through different but similar pathways to ultimately up regulate mTORC1 activity (Adegoke, Abdullahi, & Tavajohi-Fini, 2012). An extensive explanation of these pathways is beyond the scope of this review; however, the reader is directed to the following review articles detailing the pathways mediated insulin/growth factors (Proud, 2006), amino acids (Adegoke, Abdullahi, & Tavajohi-Fini, 2012) and mechanical loading (Hornberger, Sukhija, Wang, & Chien, 2007) induced activation of mTORC1. The anabolic effects of amino acid and insulin, primarily mediated through mTORC1, appear to be a primary mechanism mediating the effects of higher protein diets on the preservation of FFM and will be discussed in further detail.

1.4.2 Amino Acids and Insulin in the Regulation of MPS

The molecular mechanisms by which higher protein diets help to preserve FFM have been largely attributed to the role of amino acids and to a lesser extent insulin in the stimulation of MPS (Churchward-Venne, Murphy, Longland, & Phillips, 2013). A greater protein intake is clearly important to support FFM gains or retention during both anabolic and catabolic conditions. Extended periods of resistance training resulting in gains in

skeletal muscle mass are often augmented by the increased consumption of dietary protein (Hayes & Cribb, 2008; Josse, Atkinson, Tarnopolsky, & Phillips, 2011; Cermak, Res, de Groot, Saris, & van Loon, 2012). This effect is believed to be a direct result of increased protein ingestion and not due merely to the increase in caloric intake (Andersen, et al., 2005). In catabolic conditions, such as a hypo-energetic state, relative protein intake is negatively correlated with absolute losses in total and appendicular FFM (Krieger, Sitren, Daniels, & Langkamp-Henken, 2006). Conversely, while insulin's effect on skeletal muscle is anabolic it is shown to only be permissive for the anabolic effect of amino acids on MPS and that this effect is present at very low levels of insulin (Biolo, Fleming, & Wolfe, 1995; Fujita, Rasmussen, Cadenas, Grady, & Volpi, 2006). These data suggest that amino acids, and to lesser extent insulin, are important regulators of FFM changes during anabolic and catabolic conditions. Given this, the proper modulation of amino acids and insulin ingestion to produce a maximal anabolic effect should be considered in the context of preserving FFM during a hypo-energetic state.

Interestingly, different protein sources provide a varying degree of stimulation for protein anabolism in accordance with measures of their essential amino acid content and rates of digestion (Phillips, Tang, & Moore, 2009). The current standard rating of protein quality was established by a joint committee between the Food and Agriculture Organization and the World Health Organization and is termed PDCASS (Protein Digestibly Corrected Amino Acid Score). PDCASS is determined by measures of the percentage of the first limiting essential amino acid (EAA) in the target protein relative to a reference protein (egg) and the digestibility of the protein as determine by fecal extraction. However, recent work has contested the PDCASS system as a means to determine the anabolic effects of different protein sources (Phillips, Tang, & Moore, 2009; Schaafsma, 2005) since certain proteins contain EAA in an abundance greater than the reference protein. For example, we have shown that while whey, soy and casein proteins have an identical and maximal PDCASS value of 1.0 (reflecting the truncation of this score) their ingestion results in different rates of MPS. With a greater MPS response observed in the following hierarchical manner: whey>soy>casein (Tang, Moore, Kujbida, Tarnopolsky, & Phillips, 2009; Wilkinson, et al., 2007). Consequently, PDCASS scores maybe not be an ideal method for choosing a protein source to produce maximal changes in body composition during an anabolic or

catabolic state. Flaws in the PDCASS scoring system ultimately stem from a protein scores being truncated at 1.0 and thus fails to recognize that proteins with scores >1.0 (i.e., with EAA content greater than that of egg protein) may have beneficial effects on MPS. In the context of the postprandial MPS response to protein ingestion, the EAA content and digestibility of a protein source are 2 of 4 main factors regulating MPS. Our lab and others have demonstrated that the varying postprandial MPS response to different protein sources can be attributed to the absolute amount of protein ingested (Moore, et al., 2009), the leucine content of the protein (Smith, Barua, Watt, Scrimgeour, & Rennie, 1992), the rate of digestion/absorption of the protein (West, et al., 2011) and the timing of ingestion (Bohé, Low, Wolfe, & Rennie, 2001). Therefore, all four of these factors should be controlled for and optimized when examining changes in body composition during a higher protein hypo-energetic diet; and maybe responsible for a lack of an effect on FFM observed in some higher protein hypo-energetic studies.

Of the four components regulating the postprandial MPS response to protein ingestion the absolute amount of protein ingested and the EAA/leucine content of the protein are critical components. A clear dose response effect has been shown with protein and MPS following both intravenous infusion (Cuthbertson, et al., 2005; Bohé J., Low, Wolfe, & Rennie, 2003; Bohé J. J., Low, Wolfe, & Rennie, 2001) and ingestion post resistance exercise in young (Moore, et al., 2009) and older men (Yang, et al., 2012). These studies demonstrated that roughly 20-25 g of protein is enough to maximally stimulate MPS in healthy young adults whether at rest or following resistance exercise. The EAA have been shown to be the primary trigger of a net positive muscle protein balance as the ingestion of 40 g EAA was more effective than a 40 g supplement containing both non-essential amino acids (NEAA) and EAA (Tipton, Ferrando, Phillips, Doyle, & Wolfe, 1999). Of the EAA, leucine is a, if not the most, potent driver of MPS. In humans, an intravenous flooding dose of leucine results in an increase in MPS (Smith, Barua, Watt, Scrimgeour, & Rennie, 1992). Similarly, in rodent models the oral administration of leucine is able to increase MPS at levels independent of increases in plasma insulin (Crozier, Kimball, Emmert, Anthony, & Jefferson, 2005). Similar *in vitro* work demonstrated leucine was the most effective EAA in stimulating phosphorylation of known mTOR pathway components that regulate MPS (Atherton, Smith, Etheridge, Rankin, & Rennie, 2010). In a practical context the increased

consumption of leucine has often (Katsanos, Kobayashi, Sheffield-Moore, Aarsland, & Wolfe, 2006) but not always shown to increase rates of MPS (Churchward-Venne, et al., 2012). Together these data show that the absolute amount of protein, and its leucine content mediate the MPS response to protein ingestion.

The modulation of the proteins rate of absorption and timing of ingestion are two other prominent factors that regulate MPS. Our lab has previously shown that the rate of absorption of an ingested protein can affect the MPS response. The consumption of whey protein as a single large bolus (25 g protein) was more effective than when consumed in a pulse pattern (10 x 2.5 g protein) at stimulating MPS following resistance exercise in young men (West, et al., 2011). This finding was observed despite the same absolute amount of protein ingested and the same aminoacidemia and leucinemia between supplements over the 5.5 hr trial. The authors' concluded the greater stimulation of MPS observed in the whey bolus group was attributed to the larger initial spike in blood EAA and leucine content observed in the whey bolus group but not in the whey spread group (West, et al., 2011). In addition, the timing of ingestion of protein is another critical factor regulating MPS response to feeding. Upon the continuous intravenous infusion of AA, MPS is up regulated and sustained for 1-2 hrs. Thereafter, MPS returns to basal levels despite sustained hyperaminoacidemia (Bohé, Low, Wolfe, & Rennie, 2001). This same effect was also observed with protein ingestion which resulted in an elevated MPS rate for 46-90 min and then returned to baseline between 91-180 min, despite elevated blood EAA and leucine content up to and beyond 180 min respectively (Atherton, et al., 2010). While more work needs to be completed this data suggest postprandial rates of MPS rise for 1-2 hrs and then become refractory to further hyperaminoacidemia for an estimated 3-4 hrs. Together these studies demonstrate that MPS can be modulated by both the magnitude and duration of postprandial aminoacidemia.

While protein source and patterns of protein ingestion can regulate the postprandial MPS response, few studies have manipulated these components during a hypo-energetic or iso-energetic diet in the guides of maximizing changes in body composition. However, there is some evidence suggesting an efficacy of the use of whey protein over other protein sources in mediating optimal changes in body composition during weight loss. For example, Claessens *et al.,* demonstrated a trend (P=0.09) for whey protein over casein to produce a

greater regain in FFM after an initial losses during a hypo-energetic diet (Claessens, Van Baak, Monsheimer, & Saris, 2009). This result is in line with previous work demonstrating whey protein to be superior to casein in stimulating MPS at rest and following mechanical loading in young (Tang, Moore, Kujbida, Tarnopolsky, & Phillips, 2009) and older men (Pennings, et al., 2011). In contrast, Adechain *et al*., did not show a FFM sparing effect when examining four hypo-energetic diets varying protein source (whey or casein) and timing of ingestion (bolus or spread) (Adechain, et al., 2012). The lack of a FFM sparing effect may have been attributed the short duration of the study (6 wks) or that the protein intake at ~1 g/kg/d was insufficient to elicit a FFM sparing effect (Krieger, Sitren, Daniels, & Langkamp-Henken, 2006). Few other studies (Yamashita, Sasahara, Pomeroy, Collier, & Nestel, 1998; Abete, Parra, & Martinez, 2009) have directly compared protein sources on body composition changes during weight loss; however, because either body composition was not assessed (Yamashita, Sasahara, Pomeroy, Collier, & Nestel, 1998) or the relative protein intake differed significantly between groups (Abete, Parra, & Martinez, 2009) these studies cannot be used to determine the efficacy of varying protein sources during a hypo-energetic diet for the preservation of FFM. Due to the limited number of studies further work is required to determine if changes in body composition can be optimized during weight loss by varying the protein source, amount and timing of ingestion.

1.5. REGULATION OF SKELETAL MUSCLE MASS: MPB

The regulation of skeletal muscle mass is determined by a number of factors, but ultimately the algebraic difference between MPS and MPB to yield a net muscle protein balance will be a primary determinant of long-term changes. This section details the systems regulating MPB in skeletal muscle.

1.5.1 The Proteolytic Systems

The regulation of MPB is a complex and multifaceted system with multiple pathways mediating skeletal muscle atrophy. At least 4 major proteolytic pathways exists within skeletal muscle including the ubiquitin proteasome pathway (UPP) (Attaix, et al., 2005), the autophagy lysosome pathway (Sandri M. , 2010), Ca^{2+} dependent pathways i.e. calpains

(Bartoli & Richard, 2005), and the caspases (Ventadour & Attaix, 2006). The calpains are believed to primarily be activated by increases in cytosolic calcium levels resulting from cell injury (which can be induced by severe or unaccustomed exercise); thereafter the activation of calpains aids in the apoptotic response (Bartoli & Richard, 2005). Similarly, the caspases are a class of enzymes also believed to mediate the apoptotic process resulting in cleavage of selective proteins at aspartate residues (Boatright & Salvesen, 2003). The autophagy lysosome pathway removes dysfunctional proteins and organelles in the cytoplasm by engulfing them into double layered membrane vesicles, which are then delivered to the lysosome for degradation of their contents (Sandri M. , 2010). Much work remains to be done within the autophagy lysosome pathway but it may soon prove to be a critical component regulating skeletal muscle atrophy during catabolic conditions. The UPP is believed to be the primary regulator of skeletal muscle atrophy (Lecker, Goldberg, & Mitch, 2006); however, the UPP cannot degrade intake myofibrils (Solomon & Goldberg, 1996); thus it has been proposed that components of the calpain system (Fareed, et al., 2006) or caspases (Du, et al., 2004) are responsible for the initial cleavage of myofibrils allowing for their subsequent degradation by the UPP. This review will briefly cover the UPP and its role in skeletal muscle atrophy.

1.5.2 The Molecular Basis for MPB

The UPP mediates the destruction of many of skeletal muscle proteins through a multifaceted mechanism (Lecker, Goldberg, & Mitch, 2006). In brief, proteins are targeted for degradation by the 26S proteasome via the attachment of small chains of ubiquitin (Ub). The attachment of Ub to a target protein is a highly regulated process which occurs via three different types of enzymes (Lecker, Goldberg, & Mitch, 2006). These enzymes include an E1 (ubiquitin activating enzyme), E2 (ubiquitin conjugating enzymes), and E3 (ubiquitin ligating enzymes). Before Ub can be attached to a protein it must first be activated by the E1 Ub activating enzyme with the use of ATP to create a highly reactive Ub molecule. Once activated the Ub molecule is transferred from an E1 activating enzyme to an E2 conjugating enzyme. The E2 conjugating enzyme acts as a shuttle to deliver the Ub molecule to an E3 ligating enzyme. The E3 enzyme serves as a platform to hold and facilitate the transfer of the Ub molecule from the E2-Ub complex to the protein targeted for ubiquitination. Each E3

enzyme is highly specific, binding only a limited number of E2 and target proteins allowing for precise control of the system. This process is repeated until a polyubiquitin chain is created on the target protein. Once a protein is polyubiquitinated it is ultimately targeted for destruction within the 26S proteasome via multiple peptidases. The reader is directed to other review articles for a more detailed explanation of the UPP (Lecker, Goldberg, & Mitch, 2006; Attaix, et al., 2005). The UPP pathway is ultimately responsible for the degradation of a variety of contractile and signaling proteins within skeletal muscle. Proof of evidence in support of the UPP in muscle wasting can be observed with the use of correlative, loss and gain of function studies.

A variety of human and rodent models have demonstrate a significant role of the UPP in mediating skeletal muscle atrophy during catabolic conditions. In 2004 Lecker *et al.,* used microarrays to quantify common changes in skeletal muscle gene transcripts observed between multiple catabolic models (food deprivation, cancer cachexia, streptozotocin-induced diabetes mellitus, uremia and subtotal nephrectomy) in rodents (Lecker, et al., 2004). Between the 5 models studied there was shown to be consistent increases in the expression of genes associated with the UPP including many E3 ubiqutin ligases and multiple subunits of the 20s proteasome and its 19s regulator subunit. Furthermore, conditions of food deprivation in rodents have been shown to increase the number of Ub-conjugated proteins within skeletal muscle, an effect that was rescued upon refeeding (Wing, Haas, & Goldberg, 1995). The role of the UPP pathway in regulating muscle proteolysis is even more evident in knock out models of specific E3 ligases. Bodine *et al.,* demonstrated that two muscle specific E3 ligases, MuRF1 and MAFbx not only correlate with skeletal muscle atrophy but that their inhibition attenuates skeletal muscle loss following denervation (Bodine, et al., 2001). Similarly, during starvation skeletal muscles losses can be attenuated with the complete knockout of MAFbx but not MuRF1 (Cong, Sun, Liu, & Tien, 2010). In contrast, the overexpression of transcription factors controlling MuRF1 and MAFbx can induce muscle atrophy (Sandri, et al., 2004; Kamei, et al., 2004). Beyond MuRF1 and MAFbx other E3 ligases have been discovered including Trim32 (Cohen, Zhai, Gygi, & Goldberg, 2012), TRAF6 (Paul, et al., 2010) and Fbxo40 (Shi, Luo, Eash, Ibebunjo, & Glass, 2011). Of these additional E3 ligases knock out models of only TRAF6 and Fbxo40 have been shown to impact muscle protein turnover. Knocking out TRAF6

expression in mice results in a significant protection of muscle fiber CSA following 24 and 48 hrs of starvation. Similarly, Fbxo40 knockout models results in an augmentation of skeletal muscle mass (Shi, Luo, Eash, Ibebunjo, & Glass, 2011). These data suggest that the UPP plays a critical role in skeletal muscle atrophy within murine models and therefore may also be critical in human models. While this information provides a brief insight into the regulation of the underlying pathways controlling MPB, interventions to regulate the UPP and ultimately MPB need to be examined in greater detail. Specifically, we have to determine if findings from murine models of muscle wasting are appropriate for humans (Phillips, Glover, & Rennie, 2009) and if the inhibition or stimulation of MPB or MPS respectively, is the most appropriate means to preserve muscle mass during catabolic conditions.

1.5.3 Amino Acids and Insulin in the Regulation of MPB

The regulation of MPB is tightly controlled by nutrient availability, with postprandial increases in insulin believed to be the primary regulator of MPB. Diurnal rates of MPB fluctuate by as much as 50 % between the postabsorptive and postprandial states (Greenhaff, et al., 2008). The postprandial inhibition of MPB is primarily regulated by insulin, as increases in plasma insulin levels above those observed in the postabsorptive state (i.e., greater than 5 mU/L) up to ~15 mU/L result in a ~50 % decrease in measures of MPB. This effect is entirely insulin mediated as it could not be recapitulated when insulin levels were clamped at 5 mU/L and blood amino acid levels were tripled (Greenhaff, et al., 2008; Wilkes, et al., 2009). Correlation based studies have shown that the UPP is up regulated with food deprivation in rodents and normalized up re-feeding (Wing, Haas, & Goldberg, 1995). Furthermore, Larbaud *et al.,* compared the use of lysosomal and UPP inhibitors during a 3 hr hyperinsulinaemic clamp in rats. Insulin mediated inhibition of MPB was observed with the concurrent administration of lysosomal inhibitors but not with UPP inhibitors, suggesting that insulin inhibits MPB through the UPP (Larbaud, et al., 2001). Other work which failed to show an acute effect of insulin on the inhibition of the UPP induced proteolysis have observed effects after extended periods of re-feeding following food deprivation (Kee, et al., 2003). While insulin mediates the inhibition of MPB in the postprandial state, potential through the UPP, it does not tell us by what exact mechanisms this occurs. Correlative studies suggest that insulin mediated inhibition of MPB functions in

part through the inhibition of MAFbx gene expression in human skeletal muscle (Greenhaff, et al., 2008). While it is apparent insulin inhibits MPB the mechanism of action remains to be fully elucidated.

1.6 REGULATION OF MUSCLE PROTEIN TURNOVER DURING ATROPHY

1.6.1 MPS and MPB during Skeletal Muscle Atrophy

Skeletal muscle mass is regulated by the rates of MPS and MPB in the postabsorptive and postprandial state; therefore, the loss of muscle mass occurs as a result of a diminished rate of MPS, an exaggerated rate of MPB or a combination of the two in either or both states. To date only four studies have examined the rates of MPS in non-exercising adults during energy restriction in the postabsorptive or postprandial states; unfortunately, all four studies have shown conflicting findings. A 2009 study examined changes in MPS in the postabsorptive and postprandial state in women place on a hypo-energetic diet for 16 weeks with or without exercise (Campbell, et al., 2009). The non-exercising control group exhibited no change in the postprandial MPS response but observed an increase in postabsorptive rates of MPS during the hypo-energetic diet, relative to pre interventions levels. The increase in postabsorptive rates of MPS in the control group are difficult to reconcile without a concurrent increase in MPB, as these individual observed losses in FFM. Pasiakos *et al.,* 2010 examined postabsorptive rates of MPS in young adults following a 10 day weight maintenance and a 10 day hypo-energetic diet. Over the course the 10 day weight maintenance diet no change in body weight occurred whereas a ~1 kg loss in body weight was observed during the hypo-energetic diet. MPS rates in the postabsorptive state were observed to slightly, but significantly decrease by ~19 % (Pasiakos, et al., 2010). These data suggest that muscle loss during food deprivation can be attributed to decrease in postabsorptive rates of MPS. In contrast Villareal *et al.,* examined MPS rates in obese adults in both the postabsorptive and postprandial conditions before and during weight loss (Villareal, Smith, Shah, & Mittendorfer, 2011). Following weight loss no significant change in the postabsorptive rates of MPS were observed; however, the participants observed a greater MPS response in the postprandial period. This is difficult to reconcile with most known data as a greater MPS response during the postprandial state is suggestive of gains in FFM but not the losses observed in this study. This result has to be noted with caution as

these participants initially failed to illicit a MPS response to a test meal prior to weight loss and that their average BMI (38.3 ± 1.6 kg/m^2) was significantly higher than participants in previous MPS based studies. A further study was conducted by Pasiakos *et al.,* in 2013 in which participants' MPS rates were characterized during the postabsorptive and postprandial states following a 10 day weight maintenance period and a 21 day hypo-energetic diet. Participants were split into three groups consuming a relative protein intake of 0.8, 1.6 or 2.4 g/kg/d at a 40% energy deficit. This study did not confirm Pasiakos *et al*., 2010 work in that no change in postabsorptive MPS rates were observed following the hypo-energetic diet. However, it is interesting to note that during the energy deficit phase the participants consuming a relative protein intake of 0.8 and 1.6 g/kg/d failed to show a stimulation of MPS in the postprandial state as they had done pervious during the weight maintenance period. Only those with a protein intake of 2.4 g/kg/d were able to mount a significant MPS in response to feeding during the energy deficit; however, these same individuals were not able to mount an MPS response to feeding during the weight maintenance period (Pasiakos, et al., 2013). These data suggest that the MPS response to feeding is impaired during a hypo-energetic diet in healthy individuals consuming protein between 0.8-1.6 g/kg/d. While a protein intake of 2.4 g/kg/d appears to be beneficial in a hypo-energetic state the authors did not see a stimulation of MPS in the weight maintenance phase, this calls into question some of the authors' conclusions in this study. Interestingly, the diminished MPS response observed in 2 of the 3 groups during the postprandial state by Pasiakos *et al.,* 2013 is not dissimilar to other conditions of skeletal muscle wasting including: advanced age (Cuthbertson, et al., 2005) and immobilization (Glover, et al., 2008). However, not all models of muscle loss function through the same mechanisms making comparisons between models difficult to justify (Phillips, Glover, & Rennie, 2009). While much attention has been paid to examining changes in MPS, the rates of MPB also have to be considered.

If the rates of MPS are not influence by a hypo-energetic diet in either the postabsorptive or postprandial states, the loss of muscle must be attributed to an extended or accelerated rate of MPB in either state. While limited data is available for food deprivation induced atrophy, changes in MPB during disuse have been well quantified. In short, disuse atrophy in humans has been consistently attributed to a diminished MPS

response upon feeding and not to accelerated rates of MPB (Symons, Sheffield-Moore, Chinkes, Ferrando, & Paddon-Jones, 2009; Ferrando, Lane, Stuart, Davis-Street, & Wolfe, 1996). However, in extreme conditions of muscle wasting accelerated rates MPB drive muscle loss as observed in burn patients (Biolo G. , et al., 2002). To my knowledge no study has quantified MPB in humans during food deprivation using a 3 pool model of muscle protein turnover (Biolo G. , Fleming, Maggi, & Wolfe, 1995). While limited conclusions can be made from the aforementioned data, it is probable that changes in MPS in the postabsorptive or postprandial states may be the major contributors to muscle wasting during energy restriction. This does not, however, preclude the investigation and manipulation of the skeletal muscle proteolytic systems during weight loss. As either up regulating the MPS response or the inhibition of MPB may be able to improve skeletal muscle retention during a hypo-energetic state. More work will need to be done to determine the efficacy behind these two options to reduce skeletal muscle losses during catabolic conditions.

1.7. HIGHER PROTEIN DIETS AND MITOCHONDRIAL FUNCTION

1.7.1 Amino Acids and Mitochondrial Function

Skeletal muscle growth is tightly regulated by nutrient availability (Sengupta, Peterson, & Sabatini, 2010). The cross talk between cellular pathways that control cell growth and metabolism have been shown to inter-regulate one another thereby allowing for a balance between growth and metabolism. A number of energy sensitive signaling pathways converge on mTOR to down-regulate cell growth when limited nutrients are available (Sengupta, Peterson, & Sabatini, 2010). In contrast in the postprandial state mTOR appears to up regulate cell metabolism through what some have called the master regulator of mitochondrial biogenesis (i.e., the peroxisome proliferator-activated receptor-γ coactivator 1-α; PGC-1α) (Hock & Kralli, 2009). The transcriptional co activator PGC-1α, which upon activation, targets multiple transcription factors and nuclear receptors, resulting in an increased expression of nuclear and mitochondrial encoded genes (Dominy, Lee, Gerhart-Hines, & Puigserver, 2010). Activation of mTOR has been shown to directly up-regulate PGC-1α activity through the transcription factor yin-yang 1 (YY1) (Cunningham, et al., 2007). The inhibition of mTOR with rapamycin treatment results in a decrease in

muscle tissue expression of PGC-1α, ultimately resulting in a decrease in oxygen consumption (Cunningham, et al., 2007). Given the anabolic effect of EAA on mTOR activity, it is possible that a persistent EAA-mediated stimulation of mTOR would result in an increase in mitochondrial function. In theory, higher protein diets may up regulate FM losses not only by measures of satiety and thermogenesis but by an increase in mitochondrial biogenesis; however, limited work in humans has been done in this context and thus this remains highly speculative.

In support of a mTOR mediated regulation of mitochondrial function a number of rodent and cell culture studies have shown increases in mitochondrial content or function with the increased consumption of protein. For example, supplementation of rodents' diets with branch chain amino acids (BCAA) results in an increase in PGC-1α gene expression in both cardiac and skeletal muscle tissues within sedentary and exercise-trained rodents (D'Antona, et al., 2010). Supplementation with BCAA also results in increases in cardiac and skeletal muscles mitochondrial volume, an effect that was ultimately reflected with greater times to exhaustion during a treadmill exercise test (D'Antona, et al., 2010). While this finding has significant implications for promoting long term health, the researchers did not report a significant effect of BCAA supplementation on adiposity or body weight over the course of the rodents' life span (D'Antona, et al., 2010). However, other studies have shown an effect of leucine supplementation on FM. In rodent models leucine supplementation has been shown to limit FM gains and increase FM losses during high fat diets (Zhang, et al., 2007) and hypo-energetic diets (Donato Jr, Pedrosa, Cruzat, Pires, & Tirapegui, 2006) respectively. These data suggest that leucine is directly able to up-regulate energy expenditure regardless of the caloric balance. Work by Sun *et al.,* supports D'Antona findings in that leucine treatment of C2C12's and murine adipocytes results in an up regulation of mitochondrial mass, oxygen consumption, and multiple mitochondrial associated regulatory genes (Sun & Zemel, 2009; Sun & Zemel, 2007). Together these data suggest a genuine effect of protein ingestion on the up regulation of mitochondrial function in murine and cell culture models.

At this time there is limited data from human models demonstrating a strong connection between protein intake and mitochondrial function. Bohé *et al.,* demonstrated that the intravenous infusion of amino acids had a stimulatory effect on mitochondrial

protein synthesis (Bohé J. , Low, Wolfe, & Rennie, 2001); however, due to complications with analysis only two participants were used for this finding, suggesting much more work should be done. A recent review examined the health benefits of increased protein intake in humans (Valerio, D'Antona, & Nisoli, 2011). While the authors concluded that the increase consumption of protein improves the health of individuals during disease or advanced age, no studies demonstrated a clear protein effect on mitochondrial biogenesis in healthy young individuals (Valerio, D'Antona, & Nisoli, 2011). Clearly more work needs to done in this area to determine the impact of protein ingestion on mitochondrial function in humans.

1.8 CONCLUSION

In conclusion, an increased consumption of protein above recommended levels has the potential to augment FM losses while preserving FFM during a hypo-energetic state. The mechanisms of action for this effect are still largely unknown but are slowly being elucidated. At the dietary level, protein ingestion has been shown to provide an increased thermic and satiating effect over other macronutrients, resulting in an increased potential to reduce caloric intake and increase caloric requirements and thus augment FM losses. Losses in FFM are ultimately dependent on the rates of MPS and MPB in both the postabsorptive and postprandial conditions. While more work will need to be done to determine if and how changes in MPS and MPB occur in the postabsorptive or postprandial state during food deprivation, it is apparent that the anabolic effect of amino acids can preserve FFM during a hypo-energetic diet. Lastly, the cellular machinery regulating cell growth and metabolism appears to be connected suggesting that the anabolic effect of amino acids may result increases in mitochondrial function and energy expenditure and therefore maybe an additional mechanism by which higher protein diets augment FM losses. Future work should focus on understand amino acids effects on MPS, MPB as well as mitochondrial function in conditions of energy balance and energy deficit.

1.9 RATIONALE FOR RESEARCH

A recent meta-analysis has shown that the consumption of higher protein diets (1.25 ± 0.17 g/kg/d versus 0.72 ± 0.09 g/kg/d) results in a greater preservation of FFM and

augmentation of FM losses during periods of energy restriction lasting 12 weeks or longer. This effect is believed to be attributed to the known anabolic stimulation of MPS by protein ingestion. The ability of protein to stimulate MPS is not only dependent on the absolute amount of protein ingested but also the quality of the protein, primarily dictated by its rate of absorption and leucine content. Furthermore, leucine has been shown to have an inhibitory effect on genes associated with skeletal muscle protein breakdown and a stimulatory effect on genes associated with mitochondrial biogenesis and metabolism. Despite this knowledge, few studies have examined the role of protein quality on the changes in body composition or the aforementioned gene targets during energy restriction. Therefore, research to discover the mechanisms mediating the favorable effect of a higher protein diet on body composition will not only allow us to better understand muscle wasting but will allow us to optimize hypo-energetic dietary methods to maximize health benefits during weight loss.

1.10 STATEMENT OF RESEARCH QUESTION AND HYPOTHESIS

The primary aim of this study was to determine if measures of body composition could be more favorably changed (as determined by the preservation of LBM and augmentation of FM losses) during an acute period of energy restriction through an increase in protein intake via protein supplementation and if this effect could be augmented by protein quality (whey versus soy protein supplementation). To provide a mechanistic insight we also assessed the role of protein quality on the expression of gene transcripts associated with skeletal muscle atrophy and mitochondrial metabolism. We placed 40 overweight or obese individuals on an acute 14 day, moderate hypo-energetic diet supplemented with whey protein (WHEY), soy protein (SOY) or a maltodextrin, carbohydrate control (CHO) powder twice daily. Absolute changes in body composition, the expression of select skeletal muscle gene transcripts and serum hormone levels were assessed before and after weight loss. We hypothesized that a greater preservation of LBM and augmentation of FM losses would be observed in accordance with protein intake and quality in the following hierarchical manner: WHEY>SOY>CHO. Additionally, increases in the expression of genes associated with skeletal muscle atrophy would be limited, and those

associated with mitochondrial function would be augmented in the same hierarchical manner, i.e WHEY>SOY>CHO. Contrary to our hypothesis we did not observe any significantly differences in diet-induced changes in body composition between groups. Additionally, there was no time or group by time effect for skeletal muscle gene transcription associate with catabolic processes or mitochondrial function. However, protein quality did have a protective effect against increases in basal serum levels of the catabolic hormone cortisol during energy restriction. The lack of a main effect for protein quality on measures of body composition and select gene transcripts maybe due to the acute nature of the study. While limited conclusions can be drawn from these finding, these data suggest that higher protein hypo-energetic diets of sufficient duration may limit the loss of FFM, in part, through an inhibition of increases in serum cortisol levels.

CHAPTER II:

MOLECULAR EVENTS UNDERPINNING CHANGES IN TISSUE METABOLISM WITH WHEY AND SOY PROTEIN INGESTION IN ENERGY RESTRICTION IN OVERWEIGHT/OBESE ADULTS

2.1 INTRODUCTION

Globally, rates of obesity have increased over the past several decades (Consultation, 2000). Common morbidities associated with obesity include, but are not limited to, cardiovascular disease (CVD), type 2 diabetes, and an increased risk for certain types of cancers; all of which consequently result in an increased risk for mortality (Do Lee, Blair, & Jackson, 1999; Finer, 2006; Hubert, Feinleib, Patricia, & William, 1983). Approximately 40 % of annual deaths in the U.S are attributed to type 2 diabetes and CVD related complications, this being largely a result of the high prevalence of obesity, being 33% of the population, within the U.S (Jemal, Ward, Hao, & Thun, 2005). Given the increase rate of morbidity and mortality among obese individuals, health care costs are estimated to be 42 % higher for obese Americans over their lean counterparts (Finkelstein, Trogdon, Cohen, & Dietz, 2009). Similar findings are observed in Canada, where obesity is estimated at 24 % of population as of 2007-2009 (Shields, et al., 2010). Additionally health care cost for overweight/obesity related complications were estimated to contribute to $6 billion annually or 4.1 % of Canadian health care expenditures in 2006 (Anis, et al., 2010). Therefore, the development of effective nutritional strategies to combat obesity is critical to reduce both the physiological and economic impact of obesity across the globe.

The treatment of obesity is usually multifaceted with the general recognition that weight loss in the range of 5-10 % of initial body weight results in numerous benefits. The achievement of weight loss requires a net negative energy balance (i.e., energy expenditure > energy intake) as a result of a decrease in caloric intake and/or an increase in caloric expenditure. Although solely decreasing caloric intake induces a reduction in stored energy as fat mass (FM) there is often a simultaneous loss of fat free mass (FFM) comprised mostly of skeletal muscle (Weinheimer, Sands, & Campbell, 2010). The loss of FFM would, it is proposed, be a negative consequence of weight loss as metabolically, skeletal muscle serves as an important regulator of basal energy requirements and is the principle site of

postprandial glucose disposal (DeFronzo, Gunnarsson, Bjorkman, Olsson, & Wahren, 1985). Skeletal muscle is also an important regulatory organ in post-prandial lipemia, as ~80 % of its resting energy requirements are generated via fat oxidation (Dagenais, Tancredi, & Zierler, 1976). Therefore, the loss of skeletal muscle mass during diet-induced weight loss can have a detrimental impact on human metabolism and pose a potential health risk (Wolfe, 2006). As such, attenuating losses of FFM should be a primary goal of any weight loss intervention in an attempt to minimize any associated metabolic maladaptations.

A recent meta-analysis demonstrated that the consumption of higher protein diets (protein intakes ~1.25 ± 0.17 g/kg/d), during diet induced weight loss results in a significant preservation of FFM over standard protein diets (protein intakes of ~0.72 ± 0.09 g/kg/d) (Wycherley, Moran, Clifton, Noakes, & Brinkworth, 2012). This effect may be attributed to 'extra' protein ingestion, and the subsequent hyperaminoacidemia, having a direct anabolic effect on muscle protein synthesis (MPS) (Rennie, Wackerhage, Spangenburg, & Booth, 2004). Skeletal muscle mass is dependent upon the coordinated interaction and balanced rates of MPS and muscle protein breakdown (MPB), with muscle protein accretion occurring when MPS exceeds rates of MPB. The increased consumption of protein would therefore result in a greater stimulation of MPS and help to preserve skeletal muscle mass during diet induced weight loss.

The stimulation of MPS by protein ingestion varies between different protein sources (Tang, Moore, Kujbida, Tarnopolsky, & Phillips, 2009) an effect ultimately attributed to the quality of the protein as measured by its EAA content and digestibility. Our lab and others have shown the MPS response to protein ingestion is regulated by at least four factors including: the absolute amount of protein ingested (Moore, et al., 2009), the leucine content of the protein (Smith, Barua, Watt, Scrimgeour, & Rennie, 1992), the rate of digestion/absorption of the protein (West, et al., 2011) and the timing of ingestion (Bohé J. , Low, Wolfe, & Rennie, 2001). Of these factors protein quality is ultimately determined by the leucine/EAA content and the rate of absorption of the protein (Tang & Phillips, 2009). We have previously shown that whey protein is superior to soy protein in stimulating MPS following resistance exercise; an effect attributed to the higher quality of whey protein with respect to its greater leucine (and other EAA) content and greater rate of absorption (Bilsborough & Mann, 2006). Despite the known variances in the anabolic nature between

protein sources only a handful of studies, each showing conflicting findings, have directly compared the effects of protein quality or timing of ingestion in an attempt to augment changes in body composition during diet induced weight loss (Baer, et al., 2011; Adechain, et al., 2012; Claessens, Van Baak, Monsheimer, & Saris, 2009). Therefore, research comparing the effects of supplemental whey and soy protein on the evolution of body composition during a controlled hypo-energetic diet would provide valuable information on how protein type might aid in the promotion of FM losses and FFM retention during energy restriction.

Although significant developments in the understanding of the molecular pathways regulating the effects of inactivity and nutrition on skeletal muscle mass have been made, more work remains to be done. Recently, the expression of two muscle-specific ubiquitin ligases, muscle RING-finger 1 (MuRF1) and muscle atrophy F-Box (MAFbx also called Atrogin-1), have been shown to correlate with skeletal muscle atrophy in rodents (Bodine, et al., 2001). Furthermore, murine knock out models of MuRF1 and MAFbx show a 36 % and 56 %, respectively, prevention of skeletal muscle atrophy following muscle denervation (Bodine, et al., 2001). An effect also observed during starvation induced atrophy in mice with a siRNA mediated knock down of MAFbx (Cong, Sun, Liu, & Tien, 2010). The proteins that MuRF1 and MAFbx target for destruction make them prime regulators of muscle protein balance. MuRF1 expression is associated with MPB as targets include structural and contractile components of the myofibril such as: titin (McElhinny, Kakinuma, Sorimachi, Labeit, & Gregorio, 2002), troponin I (Kedar, et al., 2004) myosin binding protein, myosin light chain (Cohen, et al., 2009) and the myosin heavy chain slow and fast isoforms (Clarke, et al., 2007). Known MAFbx targets are centered around the regulation of MPS and the myogenic program with targets including: MyoD (Tintignac, et al., 2005), calcineurin (Li, et al., 2004) and the eukaryotic translation initiation factor 3 subunit F (eIF3f) (Lagirand-Cantaloube, et al., 2008). The most studied upstream activators of MuRF1 and MAFbx include the forkhead family of transcription factors (FOXO) 1 and 3a. Increased expression of FOXO1 has been observed during fasting in rodents (Lecker, et al., 2004) and has been shown to directly regulate the expression of both MuRF1 and MAFbx (Stitt, et al., 2004). The overexpression of FOXO1 results in severe skeletal muscle atrophy (Kamei, et al., 2004). Similarly, FOXO3a directly binds to the MAFbx promoter and up regulates its expression,

resulting in muscle atrophy *in vitro* (Sandri, et al., 2004). These data suggest that both MuRF1 and MAFbx could be key components regulating skeletal muscle atrophy in humans; however, this requires a thorough investigation given murine models of skeletal muscle atrophy often differ significantly from human models (with rodents exhibiting accelerated rates of muscle atrophy over humans) (Phillips, Glover, & Rennie, 2009). Beyond differences between species, muscle atrophy is regulated through a variety of different mechanism depending on the catabolic stimulus (Phillips, Glover, & Rennie, 2009). The ultimate goal of limiting skeletal muscle atrophy may be through the inhibition of muscle proteolysis or increasing anabolic processes, future work will have to determine which path will produce the safest measure.

Increased consumption of dietary protein and in particular proteins rich in leucine, have been shown to limit muscle wasting during atrophic conditions, potentially through the inhibition of MuRF1 and MAFbx. Leucine treatment of nutrient deprived C2C12's directly inhibits the expression of both MuRF1 and MAFbx (Herningtyas, et al., 2008). Rodents placed under hind limb suspension supplemented with leucine or branch chain amino acids, show a greater retention of lean mass and an inhibition of MuRF1 and MAFbx protein expression over non supplemented controls (Baptista, et al., 2010; Maki, et al., 2012). Similar work done by Paddon-Jones *et al*., demonstrated that the consumption a carbohydrates and essential amino acids supplemented during 28 days of bed rest was able to preserve human leg lean mass (+0.2 ± 0.3 kg) over a control group (−0.4 ± 0.1 kg) receiving standard meals (Paddon-Jones D. , et al., 2004); however, MuRF1 and MAFbx expression were not quantified. These findings (Paddon-Jones D. , et al., 2004) show that planned nutritional interventions can preserve FFM during atrophic conditions in humans. Together these correlative findings suggest that higher quality protein (i.e., rich in leucine) can be utilized to limit skeletal muscle loss, in part, through the inhibition of MuRF1 and MAFbx; however, it is important to recognize that these data are often obtained from cell culture and animal models that, although informative, provide little insight into the molecular control of human skeletal muscle atrophy. Therefore, future work in humans is now required to scrutinize and extend existing, animal-based hypotheses.

In the context of weight loss, protein quality may not only regulate skeletal muscle atrophy but may also influence FM losses by increasing energy expenditure. In murine

models leucine supplementation has been shown to limit FM gains and increase FM losses during high fat diets (Zhang, et al., 2007) and hypo-energetic diets (Donato Jr, Pedrosa, Cruzat, Pires, & Tirapegui, 2006) respectively. Cell culture models have demonstrated leucine treatment results in an increase in mitochondrial mass and oxygen consumption in C2C12 cells and cultured adipocytes (Sun & Zemel, 2009). This effect is believed to be attributed to leucine mediated increases in the expression of the peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α), a transcriptional co activator often termed the master regulator of mitochondrial biogenesis (Hock & Kralli, 2009) and Sirtuin 1 (SIRT1), a NAD+ dependent protein deacetylase which functions as an important regulator of PGC-1α (Gerhart-Hines, Rodgers, Bare, Lerin, & Kim, 2007). Rodent-based studies have demonstrated that the supplementation of dietary branch chain amino acids (BCAA) can stimulate an up-regulation of skeletal muscle PGC-1α gene expression (D'Antona, et al., 2010). Interestingly, leucine-mediated modulation of mitochondrial function is often associated with an increase in uncoupling protein (UCP) 3 expression within both skeletal muscle and adipose tissue (Zhang, et al., 2007; Sun & Zemel, 2009). These studies suggest that leucine may act to directly upregulate energy expenditure through an increase in mitochondiral flux. Despite these findings in murine models, data collected in humans has been inconsistent. Two human trials have examined mitochondrial biogenesis following weight loss, with both studies showing contradicting results (Civitarese, et al., 2007; Toledo, et al., 2008). While the mechanisms mediating the effect of leucine and weight loss on mitochondrial biogenesis and metabolism still remain to be fully elucidated; a meta-analysis has demonstrated FM losses can be augmented with the consumption of higher protein diets over standard protein diets during a hypo-energetic state (Wycherley, Moran, Clifton, Noakes, & Brinkworth, 2012).

Taken together, existing studies in cell culture and murine models have demonstrated potential mechanistic pathways by which higher quality proteins could preserve LBM, and augment FM losses during diet induced weight loss. While these studies provide novel data mechanistic work in human models should be undertaken. Therefore the purpose of my study was to determine the role of protein quality on: 1) changes in body composition during an acute hypo-energetic diet and 2) skeletal muscle gene transcripts associated with skeletal muscle atrophy and mitochondrial biogenesis. We hypothesized

that the supplementation of a hypo-energetic diet with whey protein (WHEY), soy protein (SOY) or a carbohydrate control (CHO) would produce a greater preservation in LBM and augmentation of FM losses in the following hierarchical manner: WHEY>SOY>CHO. Additionally, increases in the expression of genes associated with skeletal muscle atrophy would be limited and those associated with mitochondrial biogenesis would be augmented in the same hierarchical manner: WHEY>SOY>CHO.

2.2 METHODS

2.2.1 Participants

A total of 40 overweight/obese (BMI 28-40) participants, (19 men and 21 women), completed the study. Participants were recruited through posters and advertisements placed in the local newspaper. Questionnaires were used to assess daily activity levels, general health, current medications as well as food preferences and allergies. Inclusion criterion required participants to be between the ages of 35-65 (yr), sedentary, non-diabetic, nonsmokers, weight stable for at least 3 months prior to participation and to have a BMI between 28-40 kg/m^2. Prior to participation, all participants were asked to complete a 3 day *ad libitum* food intake record and were screened both verbally and through questionnaires to ensure inclusion requirements were met. Participants were randomly stratified into three dietary groups (WHEY, SOY and CHO) based on their age and BMI. All participants were informed of the potential risks and hazards before volunteering and gave their written, informed consent prior to participation. This study protocol was approved by the research ethics board of Hamilton Health Sciences and conformed to the standards for the use of human subjects in research according to the most recent update of the *Declaration of Helsinki.*

2.2.2 Study Design

In a double-blind, randomized parallel group trial, participants were required to attend the laboratory for testing a total of 2 times. The first visit was preceded by a 3 day weight maintenance diet and included a 6 hr infusion of stable isotopic tracers described below. Three hours prior to the start of the infusion participants consumed a standardized

meal relative to their body weight. At 3 hours into the infusion, a whey or soy protein or a carbohydrate control supplement corresponding to the participant's assigned dietary group was consumed. Within 1 week after the first laboratory visit participants began a 14 day hypo-energetic diet supplemented with whey or soy protein or a carbohydrate control. On day 15 participants returned to the laboratory for the same testing procedure. Muscle biopsies, venous bloods samples and a post infusion whole body dual energy X-ray absorptiometry (DXA) scan were obtained at both testing dates. Primary items assessed included relative changes in skeletal muscle gene transcripts, serum inflammatory and growth markers and body composition measures. A schematic illustration of the study design can be seen in Figure 1.

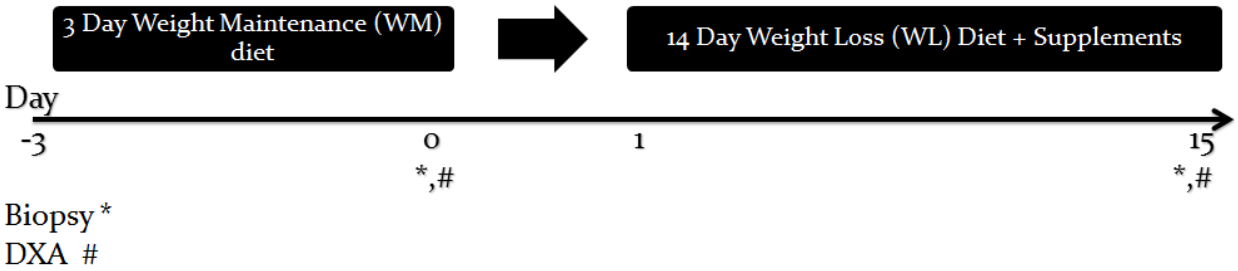

Figure 1. A schematic illustrating the experimental design.

2.2.3 Diet Interventions and Supplements

Participants' energy requirements were assessed using the Mifflin St. Jeor equation (GFiOUP, 2005) with a sedentary activity factor. Our in-house registered dietitian designed the 3 day weight maintenance (WM) and 14 day weight loss (WL) menus in 200 kcal increments. Each participant was assigned to a WM and WL menu which most closely reflected an energy balance and a caloric deficit of -750 kcal/d (versus calculated requirements) respectively. All food, consisting of pre-packaged frozen meals and snacks were provided to participants during the WM and WL dietary periods. Dairy intake was held constant for all participants at 2.5 servings/d. Experimental supplements consisted of a whey protein (Iso chill 800, Agropur, Montreal, QC), soy protein (soypro950M, International Trade Company, Roseville, CA) and a carbohydrate (maltodextrin) control. WHEY, SOY and CHO supplements were consumed twice daily, in-between meals in equal

portions providing a total of 54, 52 and 1.8 g protein/d respectively (Table 2). Participants' compliance to the dietary intervention was assessed with a daily food check-off list. Participants were also asked to record any extra foods consumed during the WM and WL dietary periods to ensure a proper estimate of macronutrient and caloric intake. All subjects were asked to refrain from taking vitamin or mineral supplements during the dietary protocol.

2.2.4 Infusion Protocol

Infusion trials were completed prior to (day 0) and immediately following (day 15) the 14 day hypo-energetic diet. On the morning of each infusion participants consumed a liquid meal providing 5.8 kcal/kg and 0.21 g protein/kg at ~5:00 hours. Participants reported to the laboratory at ~7:30 hours upon which a catheter was inserted into an antecubital vein for repeated blood sampling. Basal blood plasma and blood serum samples were collected in 4 ml heparinized and non-heparinized evacuated tubes respectively. Heparinized tubes were kept on ice until processing. All blood samples were stored at -20□C before later analysis at the Core Laboratory at the McMaster University Medical Centre.

After baseline blood samples were collected a 0.9% saline drip line was started to prevent obstruction of the catheter and to allow for repeated arterialized blood sampling over the course of the experimental trial. A second catheter was placed in the contralateral arm to allow for a 6 hour primed constant infusion of [ring-$^{13}C_6$] phenylalanine and glycerol (1,1,2,3,3-D_5, 99%) (Cambridge Isotope Laboratories, Woburn, MA, USA). Both infusates were passed through a 0.2 μm filter before entering the participant's bloodstream. Three hours after the start of the infusion (~11:00hr) participants consumed one serving of their assigned supplement (27, 26 and 0.9 g of protein for WHEY, SOY and CHO respectively). Data obtained from the stable isotopic tracers were used in a subsequent experiment and will not be discussed in this study.

Muscle biopsies were obtained from the *vastus lateralis* muscle using a 5 mm Bergström needle modified for manual suction under 2 % xylocaine local anesthesia. Muscle samples were freed from visible blood, fat and connective tissue, and immediately frozen in liquid nitrogen for later analysis. A total of 5 muscle biopsies were collected, 2

prior to (11:00 and 14:00 hours) and 3 (8:00, 11:00 and 14:00 hours) following the WL dietary period. Each muscle biopsy sample was obtained from a separate incision, 4–5 cm apart. Muscle biopsies 1 and 3, being the first samples taken pre and post dietary intervention respectively, were used for transcriptomic analysis.

2.2.5 Body Composition

Whole body DXA (QDR-4500A; Hologic, software version 12.31) scans were completed at day 0 and 15 to assess changes in total body mass (BM), FM, bone mass and lean body mass (LBM). Both pre and post DXA scans were conducted by the same trained technician following the 6 hour infusion protocol. All participants were asked to consume similar amounts of fluids during the infusion and to refrain from exercise for 48 hours prior to the infusion protocol. Following completion of the study, pre and post intervention DXA scans were analyzed to assess changes in abdominal fat mass. The abdominal region was isolated between lumbar spinal columns L1-L4 by the same trained individual as described previously (Josse, Atkinson, Tarnopolsky, & Phillips, 2011).

2.2.6 Analysis

2.2.6.1 Blood Analysis

Blood samples were spun at 1,500 *g* for 15 min at 4°C. Thereafter, aliquots of plasma and serum were frozen at −20°C for subsequent analysis. Blood Cortisol, tumor necrosis factor alpha (TNF-α), interleukin-6 (IL-6), C-reactive protein (CRP) and insulin like growth factor 1 (IGF-1) were analyzed at the Core Laboratory at the McMaster University Medical Centre using solid-phase, two site chemiluminescence immunometric assays (Immulite; Intermedico, Holliston, MA).

2.2.6.2 RNA Extraction and cDNA Synthesis

Skeletal muscle samples, 15-25 mg, were individually homogenized using a Tissue lyser II (Qiagen) with 500 µl of TRIzol® Reagent (Invitrogen, Canada) and one 5 mm retch ball. Muscle samples were homogenized at 28.5 Hz for 1 minute at -20 °C. Following homogenization 100 ul of bromochloropropane (BCP) was added; thereafter, the sample

was centrifuged at 15,000 G for 20 minutes. The supernatant was removed and repeated phenol/chloroform extract (1:1 supernatant: phenol/chloroform) were performed under the same centrifugation conditions. The Phenol/cholorform extraction was repeated until a clear and debris free interphase was observed, upon which the supernatant was removed and added to 100 ul of BCP, vortexed and centrifuged again. RNA was then precipitated with the addition of 1:1 isopropyl alcohol, inverted multiple times to mix and centrifuged. Upon removal of isopropyl alcohol, the RNA samples were washed with cold 80 % ethanol and centrifuged for 5 minutes at 15,000 G. Following the removal of the ethanol phase the samples were allowed to air dry for 5 minutes before reconstitution within 21.5 µl of RNAse free water. RNA concentrations were determined spectrophotometrically at 260-280 nm with a NanoDrop 2000 (Thermo Fisher Scientific). RNA levels were standardized to 50 ng/µl, thereafter cDNA was synthesized using a high capacity reverse transcription kit (Applied Biosystems), without the RNase inhibitor according to the manufacturer's instructions.

Quantitative real-time polymerase chain reaction (RT- PCR) was performed using an Eppendorf Mastercycler Realplex 2, SYBR Green (Sigma Aldrich), and custom-designed primers. PCR's were performed in 25 µl volume reactions in duplicate within a 96 well manually sealed PCR plate (Eppendorf). Each reaction contained 12.5 µl of SYBR green (Qiagen), 4 µl of forward and reverse primers and 1 µl of cDNA. Primer sequences are listed in Table 1.

Fold change in target gene expression were quantified using the Pfaffl method (Pfaffl, 2001). All target genes were normalized to the reference gene β_2-microglobulin, the use of which as a reference gene was confirmed as its expression did not differ from pre- to post-intervention trials or between dietary groups (data not shown). Primer amplification efficiencies were determined over a ~50 fold cDNA dilution and were matched to the reference gene amplification efficiency within ± 3%. All gene targets were expressed as fold changes from pre intervention mRNA expression values (mean ± SEM)

Table 1. The qRT-PCR primer sequences and corresponding NCBI reference sequences.

	Primer Sequences and NCBI Reference Numbers for Primers		
Gene Name	**Forward Primer**	**Reverse Primer**	**NCBI Reference Sequence**
SIRT1	5'-TAG CCT TGT CAG ATA AGG AAG GA-3'	5'-ACA GCT TCA CAG TCA ACT TTG T-3'	NM_012238.4
PGC-1α	5'-CAC TTA CAA GCC AAA CCA ACA ACT-3'	5'-CAA TAG TCT TGT TCT CAA ATG GGG A-3'	NM_013261.3
MuRF-1	5'-CTT CCA GGC TGC AAA TCC CTA-3'	5'-ATC CAT GAT CAC CTC GTG GC-3'	NM_032588.3
MAFbx	5'-GTG CCA TGC CAT TCC TCG G-3'	5'-TTG GTG GAA ATA CTG AGT TTT GGT-3'	NM_058229.3
FOXO1	5'-TCG TCA TAA TCT GTC CCT ACA CA-3'	5'-CGG CTT CGG CTC TTA GCA AA-3'	NM_002015.3
FOXO3a	5'-CGG ACA AAC GGC TCA CTC T-3'	5'-GGA CCC GCA TGA ATC GAC TAT-3'	NM_001455.3

Sirtuin 1(SIRT1), Peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α), Muscle specific RING finger 1 (MuRF1), Muscle atrophy F-box (MAFbx), Forkhead transcription factor 1, 3a (FOXO1, FOXO3a).

2.2.7 Statistics

Anthropometric measures and blood measures including cortisol, TNF-a, IL-6, CRP, IGF-1, and changes in gene expression using the ΔCt scores were asses by a two-factor (treatment × time) repeated measures ANOVA. Absolute changes in anthropometric measures and the aforementioned blood measures were compared using a one-factor (treatment) ANOVA. Values are displayed as mean ± S.E.M, with statistical significance set at ($P<0.05$). Significant group by time measures were followed by Tukey's HSD post hoc test with statistical significance set at ($P<0.05$). Correlations between the absolute change in basal serum cortisol and the participant's relative protein intake were calculated with using a one-tailed Pearson's correlation coefficient. All analysis was performed using SPSS 20 for windows (Chicago, IL, US).

2.3 RESULTS

2.3.1 Participant Characteristics

There were no significant differences amongst subjects' age (51 ± 1 yr), height (169 ± 2 cm), weight (102 ± 3 kg), BMI (35.5 ± 0.8 kg/m^2), LBM (62.2 ± 2.1 kg) or FM (37.5 ± 1.7 kg) between groups at baseline (Table 2). *Ad libitum* energy intake, percent fat and percent carbohydrate intake did not differ between dietary groups. *Ad libitum* protein intake as a percent of total calories was significantly lower in SOY over WHEY ($P<0.05$) (Table 2).

Table 2. Baseline characteristics and self-reported dietary intake of participants.

	Subject Baseline Characteristics and Dietary Intake		
	CHO	**SOY**	**WHEY**
Age (yr)	47 ± 2	52 ± 2	52 ± 2
Height (cm)	170 ± 2	171 ± 3	168 ± 2
Body Mass (kg)	104.9 ± 4.9	103.9 ± 6.6	98.3 ± 4.4
Lean Body Mass (kg)	62.6 ± 3.6	63.5 ± 3.9	60.6 ± 3.5
Fat Mass (kg)	39.6 ± 2.7	37.6 ± 3.3	35.5 ± 2.5
% Body Fat	37.9 ± 2.4	35.9 ± 1.7	36.0 ± 1.8
BMI	36.9 ± 1.2	34.8 ± 1.5	34.7 ± 1.1
Caloric intake (kcal)	2094 ± 368	2265 ± 185	2731 ± 531
Protein (% of energy)	18 ± 1.6 [a]	15 ± 0.5 [b]	20 ± 1.6 [a]
Carbohydrate (% of energy)	43 ± 1.3	48 ± 2.2	42 ± 3.4
Total Fat (% of energy)	41 ± 2.5	35 ± 1.5	33 ± 2.4
N	12	14	13

Values are depicted as Mean ± SEM.
Different letters [a/b] indicate a significant difference ($P<0.05$).

2.3.2 Dietary Analysis

Over the course of the WM period subjects consumed on average -146 ± 26 kcal/day below their estimated caloric requirements with no significant difference between groups. Total calorie (2321 ± 73 kcal), protein (97 ± 3 g), carbohydrate (312 ± 11 g) and fat (79 ± 3 g) intake did not differ between dietary groups. Protein intake averaged 1.01 ± 0.03, 0.96 ± 0.03 and 0.89 ± 0.03 (g/kg/d) for WHEY, SOY and CHO respectively.

Table 3 depicts the macronutrient composition of the supplements provided during the weight loss phase. Table 4 depicts the total caloric and macronutrient intake during the 14 day, WL protocol. The self-reported energy deficit for WHEY, SOY and CHO averaged -680 ± 37, -750 ± 38 and -832 ± 39 (kcal/d) respectively, with a significant ($P<0.05$) difference between WHEY and CHO only. As prescribed, protein intake was significantly ($P<0.05$) greater in WHEY [(29.4 ± 0.8 %; 1.28 ± 0.04 g/kg/d)] and SOY [(29.6 ± 1.4 %; 1.23 ± 0.05 g/kg/d)] over CHO [(19.5 ± 0.6 %; 0.74 ± 0.02 g/kg/d)] with no significant difference between WHEY and SOY. Carbohydrate intake as a percent of total energy was significantly ($P<0.05$) greater in CHO (56 ± 0.3 %) than WHEY (47 ± 0.8 %) and SOY (48 ± 0.8 %).

Similarly, total fat intake as a percent of energy was significantly (P<0.05) greater in CHO (26.8 ± 0.5 %) than WHEY (24.5 ± 0.4 %) and SOY (23.7 ± 0.7 %).

Table 3. Nutrient composition of supplements consumed during the 14 day weight loss intervention.

	Supplement Composition (1 serving)			
	Calories (Kcal)	**Protein (g)**	**Carbohydrate (g)**	**Fat (g)**
CHO	**117**	**0.9**	**24.8**	**2.4**
SOY	**170**	**26.1**	**15.4**	**0.9**
WHEY	**190**	**27.2**	**13.7**	**3.4**

Table 4. Macronutrient and caloric intake during the 14 d Weight Loss (WL) diet.

	WL Diet Characteristics		
	CHO	**SOY**	**WHEY**
Caloric Deficit (kcal)	-861 ± 39 [a]	-750 ± 38 [a,b]	-681 ± 37 [b]
Protein (g/kg/d)	0.74 ± 0.02 [a]	1.23 ± 0.05 [b]	1.28 ± 0.04 [b]
Protein (% of energy)	19 ± 0.6 [a]	30 ± 1.4 [b]	29 ± 0.8 [b]
Carbohydrate (% of energy)	56 ± 0.3 [a]	48 ± 0.8 [b]	47 ± 0.8 [b]
Total Fat (% of energy)	27 ± 0.5 [a]	24 ± 0.7 [b]	25 ± 0.4 [b]

Values are depicted as Mean ± SEM.
Different letters indicate a significant difference (P<0.05).

2.3.3 Body Composition

There was no significant group by time effect for any measures of body composition (Table 5). Bone mineral density remained unchanged due to the intervention (-0.03 ± 0.04 g/cm^2) (P=0.36). A significant (P<0.05) main effect for time was observed for the following measures of body composition, with participant's observing losses in BM (-2.3 ± 0.2 kg), FM (-1.5 ± 0.2 kg), LBM (-0.8 ± 0.2 kg) abdominal FM (-0.5 ± 0.1 kg) and percent body fat (-0.7 ± 0.2 %).

Table 5. Changes in body composition following the 14 day weight loss protocol.

	Changes in Body Composition		
	Pre	**Post**	**Total Loss**
Body Mass (kg)			
CHO	104.9 ± 4.4	102.8 ± 4.4 [a]	-2.1 ± 0.3
SOY	103.8 ± 6.6	101.1 ± 6.7 [a]	-2.7 ± 0.3
WHEY	98.8 ± 4.6	96.7 ± 4.7 [a]	-2.0 ± 0.3
Fat Mass (kg)			
CHO	39.6 ± 2.7	38.0 ± 2.8 [a]	-1.6 ± 0.2
SOY	37.6 ± 3.3	36.1 ± 2.2 [a]	-1.5 ± 0.2
WHEY	35.5 ± 2.5	34.1 ± 2.4 [a]	-1.4 ± 0.4
Lean Body Mass (kg)			
CHO	62.6 ± 3.6	62.2 ± 3.5 [b]	-0.5 ± 0.3
SOY	63.5 ± 3.9	62.2 ± 4.1 [b]	-1.3 ± 0.4
WHEY	60.6 ± 3.5	59.9 ± 3.7 [b]	-0.6 ± 0.5
% Body Fat			
CHO	37.9 ± 2.2	37.0 ± 2.3 [a]	-0.9 ± 0.2
SOY	35.9 ± 1.6	35.4 ± 1.6 [a]	-0.5 ± 0.2
WHEY	36.0 ± 1.9	35.4 ± 2.0 [a]	-0.6 ± 0.4
Abdominal Fat (kg)			
CHO	9.8 ± 1.0	9.3 ± 1.0 [a]	-0.5 ± 0.1
SOY	9.6 ± 1.3	9.2 ± 1.2 [a]	-0.5 ± 0.1
WHEY	9.1 ± 0.8	8.6 ± 0.8 [a]	-0.5 ± 0.1

Values are depicted as Mean ± SEM.

[a] indicates a significant ($P<0.001$) effect for time.

[b] indicates a significant ($P<0.05$) effect for time.

2.3.4 Blood Metabolites and Hormones

There was no significant time or group by time effect for serum TNF-α, IL-6, CRP, or IGF-1. However, there were trends for main effects of time for decreases in serum CRP (P=0.079) and IGF-1 (P=0.099). Serum cortisol remained unchanged in WHEY (-7 ± 20 nM) but increase significantly and in a hierarchical manor in SOY (98 ± 14 nM) and CHO (180 ± 32 nM), $F(2,36) = 17.04$, $P < 0.001$ (Table 6 and Figure 2). A negative correlation was observed between relative protein intake and the absolute change in basal serum cortisol levels (nM) ($r=-0.532$, $p<0.01$). A significant correlation remained between the relative protein intake (g/kg/d) and the change in basal serum cortisol levels (nM) when controlling for participants self-reported caloric deficit ($r=-0.483$, $p=0.001$) (Figure 3).

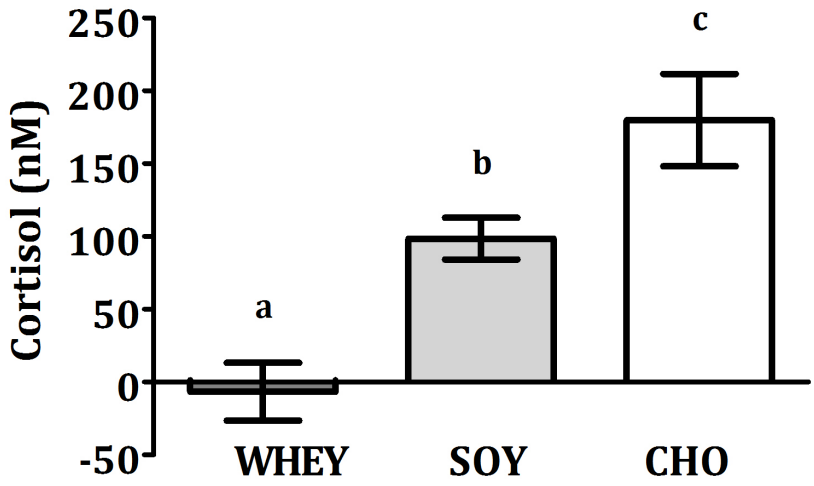

Figure 2. Absolute change in basal serum cortisol levels following the 14 d weight loss intervention. Letter difference ^{a,b,c} signifies a significant difference determined by tukeys HSD at ($P<0.05$)

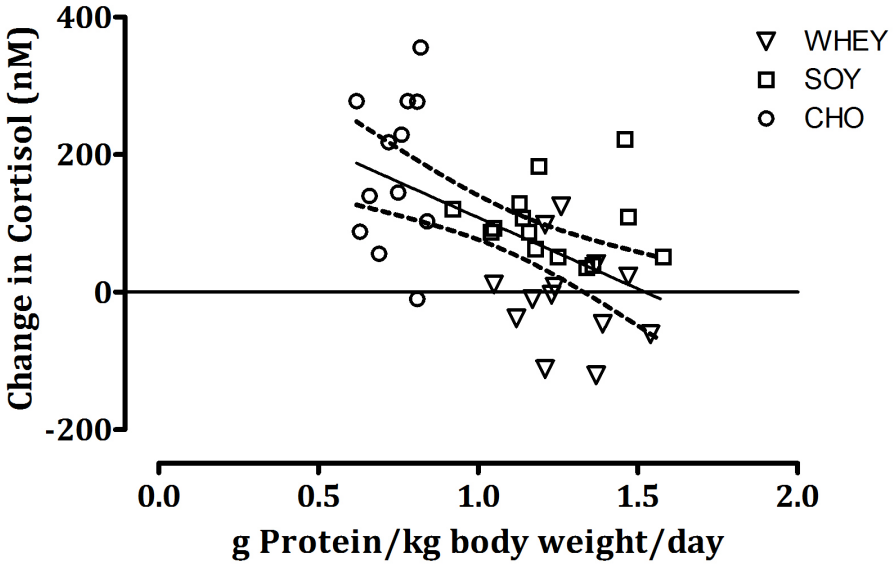

Figure 3. Correlation between the change in basal serum blood cortisol levels (nM) and participants relative protein intake after controlling for caloric deficit (r= -0.483, p=0.001).

Table 6. Depicts the preliminary, post and absolute change in select basal blood serum markers following the 14 day hypo-energetic diet.

	Changes in Basal Serum Markers Following Weight Loss								
	CHO			**SOY**			**WHEY**		
	PRE	**POST**	**CHANGE**	**PRE**	**POST**	**CHANGE**	**PRE**	**POST**	**CHANGE**
Cortisol (nM)	557 ± 9	736 ± 30	180 ± 32 [c]	550 ± 8	648 ± 15	98 ± 14 [b]	585 ± 12	579 ± 16	-7 ± 20 [a]
CRP (mg/L)	2.3 ± 0.2	2.4 ± 0.1	0.1 ± 0.2	2.6 ± 0.1	2.5 ± 0.1	-0.1 ± 0.1	2.4 ± 0.1	2.1 ± 0.1	-0.3 ± 0.1
IL-6 (pg/ml)	87 ± 5	92 ± 5	6 ± 7	98 ± 5	92 ± 5	-6 ± 7	90 ± 5	79 ± 3	-10 ± 5
IGF-1 (nM)	41 ± 1	38 ± 2	-3 ± 3	39 ± 2	40 ± 2	2 ± 2	43 ± 2	38 ± 2	-5 ± 2
TNF-a (pg/ml)	7.1 ± 0.3	6.9 ± 0.4	-0.2 ± 0.5	6.7 ± 0.3	6.9 ± 0.3	0.2 ± 0.5	7.0 ± 0.3	6.7 ± 0.3	-0.4 ± 0.6

All values are presented as mean ± SEM.

Letter difference indicates a significant difference at ($P<0.05$)

2.3.5 Gene Expression, RT-PCR

No time or group by time interaction was observed for the relative changes in the expression of SIRT1, PGC-1a, MAFbx, MuRF1, FOXO1 or FOXO3a (Table 7).

Table 7. Relative fold change in the expression of target genes following the weight loss protocol.

	Relative Fold Change in Gene Expression		
	CHO	**SOY**	**WHEY**
SIRT1	1.03 ± 0.13	1.25 ± 0.24	1.67 ± 0.48
PGC-1α	0.99 ± 0.15	0.69 ± 0.21	1.71 ± 0.36
MuRF1	1.03 ± 0.06	0.97 ± 0.17	1.36 ± 0.24
MAFbx	1.34 ± 0.14	1.29 ± 0.17	1.13 ± 0.16
FOXO1	0.71 ± 0.06	0.73 ± 0.11	1.43 ± 0.37
FOXO3a	1.28 ± 0.30	1.07 ± 0.13	2.22 ± 0.78

Values are depicted as Mean ± SEM. Sirtuin 1 (SIRT1), Peroxisome proliferator-activated receptor-γ coactivator 1-α (PGC-1α), Muscle specific RING finger 1 (MuRF1), Muscle atrophy F-box (MAFbx), Forkhead transcription factor 1, 3a (FOXO1, FOXO3a).

A 3 by 2 repeated measures ANOVA was completed on the ΔCT scores using the Pfaffl method.

No significant time or group by time interactions was observed.

2.4 DISCUSSION

The purpose of this study was to determine if protein per se and, more specifically, protein quality could augment favorable changes in body composition and the expression of gene transcripts associated with skeletal muscle atrophy and mitochondrial biogenesis/metabolism following an acute hypo-energetic diet. No group by time interaction was observed for the absolute amount or source of protein on changes in body composition during this study. Furthermore, no time or group by time interactions were observed for changes in the expression of SIRT1, PGC-1α, MuRF1, MAFbx, FOXO1 or FOXO3a. These novel data did show, however, that whey protein supplementation during a 14 d hypo-energetic diet can prevent increases in basal serum cortisol levels, over a soy or carbohydrate control supplement.

The lack of an acute difference between groups in body composition measures was contrary to our hypothesis that lean tissue and fat tissue declines would be the least and greatest, respectively, in the whey supplemented group. It is difficult to ascertain whether our hypothesis was not supported due to a *bona fide* mechanism or was due to the short-term nature of the study. A recent meta-analysis demonstrated higher protein hypo-energetic diets preserve FFM consistently in studies lasting 12 weeks or longer (Wycherley, Moran, Clifton, Noakes, & Brinkworth, 2012). The extension of our study to 12 weeks or more may have allowed us to observe a group by time effect; however, the primary measures for this study beyond body composition included changes in the expression of gene transcripts and measures of postabsorptive and postprandial rates of MPS (unpublished data). Both of the aforementioned measures are highly sensitive to nutritional and exercise stimuli. Therefore, extending the duration of the study may have resulted in compromised controls, mostly through a lack of participant adherence, and so obscuring any true experimental effects.

Despite the short duration of the study there was an imbalance within the self-reported caloric intake between the WHEY and CHO groups resulting in a significant difference in the estimated energy deficit achieved during the weight loss intervention (WHEY: -680 ± 37 kcal/d and CHO: -832 ± 39 kcal/d). Over the course of 14 days this caloric difference would be expected (assuming 1 kg of fat is 7350 kcal) to contribute to a 0.2 kg greater loss of FM in the CHO group. However, there were no significant differences in the DXA-measured losses of BW, FM, or LBM between the WHEY and CHO groups, irrespective of the greater caloric deficit achieved by CHO. Consequently, WHEY may have observed greater losses in body weight per unit caloric deficit accounting for the estimated 0.2 kg difference. This observation remains highly speculative and cannot be proven in this context given the inherent variability within self-reported food intake records, rates of weight loss between individuals and within DXA measures. An alternative thesis could be that the carbohydrate supplement, resulted in a significant retention of DXA measured LBM through an attenuation of muscle glycogen losses or an insulin mediated inhibition of MPB that would have spared lean tissue.

The caloric intake and macronutrient composition of a diet can significantly alter muscle glycogen stores and its associated water content resulting in the potential to impact

DXA measures of LBM. A period of five days of fasting can deplete muscle glycogen by as much as 40% (Hultman & Bergström, 1967). Similarly, the consumption of diets low in carbohydrates but high in fats and proteins resulted in a 30 % reduction in muscle glycogen after 7 days (Hultman & Bergström, 1967). On the contrary, an excessive intake of carbohydrate (≥10 g carbohydrate/kg body weight/d) can increase muscle glycogen content, even without prior glycogen depletion (Bussau , Fairchild, Rao, Steele, & Fournier, 2002). With muscle glycogen stores ranging from 1-1.4 g/100 g wet muscle mass in the deltoid and quadriceps muscles respectively (Hultman E. , 1967) total muscle glycogen for our participants is estimated to range from 400-560 g (assuming 40% body mass is skeletal muscle). Given 3-4 g of water are associated with every gram of glycogen (Olsson & Saltin, 1970) this suggests that 1-2 kg of muscle mass is attributed to glycogen and its associated water content in our participants. While our participants were neither completely food deprived or placed on excessively high protein or carbohydrate diets, it is possible losses in muscle glycogen content were regulated differently between groups. With the repeated ingestion of maltodextrin, the CHO group may have observed an attenuation of muscle glycogen losses and its corresponding water content. While DXA remains a robust method for the quantification of body composition, fluctuations in total body water have the potential to alter DXA measures of LBM or FM (Pietrobelli, Wang, Formica, & Heymsfield, 1998). Therefore any changes in muscle glycogen content and its associated water have the potential to increase the variability in DXA measures of LBM and may have limited our findings. Future studies solely using DXA measures for LBM should consider using a multiple compartment model of body composition to improve upon body compartment-specific changes (including total body water) (Heymsfield, et al., 1990) to help control for changes in glycogen content and its associated water. While the context of muscle glycogen stores remains to be explored, the role of insulin in the inhibition of MPB maybe an alternative hypothesis to explain the lack of a group effect for LBM retention.

While insulin is believed to have a permissive role in regulating MPS, it is a known potent inhibitor of MPB (Rasmussen & Phillips, 2003) an effect that may be altered during energy restriction induced skeletal muscle atrophy. To this author's knowledge, little work has been done to examine insulin mediated inhibition of MPB during conditions associated with muscle loss. Wilkes *et al.,* demonstrated that the insulin mediated inhibition of MPB is

diminished with advanced age (Wilkes, et al., 2009). While aging and hypo-energetic diet induced muscle atrophy are two very different models of muscle wasting, it is possible the inhibitory effect of insulin on MPB is diminished during energy restriction. If true, a decrease in MPB sensitivity to insulin could be compensated for by a greater hyperinsulinemia response resulting from the repeated ingestion of a carbohydrate supplement. As little work has been done in this area this assumes that the repeated ingestion of a carbohydrate supplement would result in a greater daily hyperinsulinemia. Yet, to this authors knowledge no study has been conducted that directly quantifies changes in MPB during a hypo-energetic state in humans. Nor has the effect of insulin on the rate of MPB during a hypo-energetic state been examined in either acute or chronic conditions. In contrast to this hypothesis, the general consensus in the literature states that skeletal muscle atrophy is primarily attributed to diminished rates of MPS in the postprandial period rather than changes in MPB (Phillips, Glover, & Rennie, 2009). Similarly, elevated serum cortisol levels, as observed in the CHO group versus the WHEY supplemented group, have previously been shown to inhibit insulin sensitivity. A study by Rizza *et al.,* demonstrated the infusion of cortisol over a 24 hr period, resulting in serum cortisol levels just slightly greater than those observed in the CHO group, post intervention, 37±3 μg/dl versus 27±1 μg/dl respectively, resulted in a significant decrease in insulin sensitivity (Rizza, Mandarino, & Gerich, 1982). Therefore, it is possible increases in serum cortisol could counter any advantage attributed to hyperinsulinemia mediated inhibition of MPB. Taken together these data suggest that the repeated ingestion of large qualities of carbohydrate may not ameliorate LBM losses as a result of the insulin mediated inhibition of MPB. This conclusion is further supported here as we observed the greatest increases in basal serum cortisol levels following weight loss in the CHO supplemented group.

We demonstrated that increases in basal serum cortisol levels had an inverse relationship with relative protein intake and that this increase following weight loss was attenuated significantly in accordance with protein quality in the following hierarchical manner: WHEY>SOY>CHO. These effects remained for both the relative protein intake and in accordance with protein quality after adjusting for each participant's estimated caloric deficit (data not shown). Being able to modulate serum cortisol levels remains a highly important finding in the context of preserving LBM. As stated previously muscle mass is

regulated by a balance between MPS and MPB in the postabsorptive and postprandial states. Skeletal muscle loss therefore results from a negative balance between MPS and MPB in one or both of these conditions. The infusion of cortisol has been shown to result in a greater net negative skeletal muscle protein balance in the postabsorptive state (Gore, Jahoor, Wolfe, & Herndon, 1993) but not in the postprandial state (Paddon-Jones D. , et al., 2003). While it remains unclear how protein quality affects the increase in serum cortisol during a hypo-energetic state, our finding is not novel as a single bolus of whey protein can lower blood cortisol levels for up to five hours in individuals with polycystic ovary syndrome (Kasim-Karakas, Cunningham, & Tsodikov, 2007). Therefore, the repeated consumption of whey protein may help preserve LBM, in part, via the inhibition of circulating cortisol levels and thus result in a less negative muscle protein balance in the postabsorptive state. The effect of protein quality on the inhibition of increases in basal serum cortisol levels during energy restriction is, at least, suggestive of a partial mechanism by which protein can help preserve LBM during a hypo-energetic state. While an effect of protein source was observed for serum cortisol other serum inflammatory and growth markers showed no significant changes resulting from the intervention.

No significant time or group by time effect was observed for CRP, IGF-1, TNFα or IL-6; however, both CRP and IGF-1 observed trends for decreases in basal blood serum levels following weight loss ($P<0.10$). CRP is used as a marker for systemic inflammation, as it has been shown to correlate with BMI (Visser, Bouter, McQuillan, Wener, & Harris, 1999) and is a risk factor for coronary heart disease (Danesh, Collins, Appleby, & Peto, 1998). A three year longitudinal study showed women with CRP levels above 1.5 mg/L had a 2-4.8 fold greater risk of myocardial infraction or stroke events (Ridker, Julie , Shih, Matias, & Hennekens, 1998:). While CRP has been shown to correlate with BMI the lack of a decrease in CRP following weight loss has been observed previously (Jean-Philippe, et al., 2000). Select dietary interventions over absolute FM losses maybe better suited to lower blood CRP levels; as suggested by work demonstrating that diets high in plant sterols and soy protein have been shown to decrease serum CRP levels more than diets high in animal proteins despite similar decreases in FM (Jenkins, et al., 2003). Clearly longer duration weight loss interventions should be conducted to determine if there is a protein source

effect on lowering CRP levels. Similar to CRP, serum IGF-1 levels observed a trend for a decrease with time.

IGF-1 is a well-established growth factor associated with obesity and may play a role in cancer prognosis. Serum levels of IGF-1 have been positively correlated with an increased risk of breast (Kaaks, et al., 2002) prostate (Stattin, et al., 2000) and colorectal (Kaaks, et al., 2000) cancers. The role of obesity as a well-established risk factor for cancer may be partially attributed to obesity induced increases in plasma glucose, insulin and potentially IGF-1, resulting in accelerated cell growth and an inhibition of apoptosis (Renehan, Frystyk, & Flyvbje, 2008). However, the regulation of free and total IGF-1 with obesity has been heavily debated. As some studies have shown elevated levels of free or total IGF-1 within obese individuals (Frystyk, Vestbo, Skjaerbaek, Mogensen, & Ørskov, 1995; Nam, et al., 1997) while others have shown no difference (Frystyk , Brick, Gerweck, Utz, & Miller, 2009; Nyomba, Michelle, Lori, & Murphy, 1999; Ricart & Fernández-Real, 2001) or a decrease (Gómez, et al., 2004). Changes in IGF-1 with weight loss are equally varied with some studies showing a decrease (Rasmussen, Juul, Lund, & Hilsted, 2006) and others showing an increase (Belobrajdic, et al., 2010). Regardless, insulin resistance appears to result in elevated levels of free IGF-1 (Nyomba, Michelle , Lori, & Murphy, 1999). Despite the inconsistencies between FM and circulating IGF-1 levels, IGF-1 may play a role in cancer prognosis (Peyrat, et al., 1993) and therefore interventions to regulate IGF-1 should be explored. While this study observed a trend for decreases in serum IGF-1 with weight loss, there appears to be no protein dependent effect on serum IGF-1 levels as observed here and previously (Belobrajdic, et al., 2010). Clearly further work needs to be done to better understand the regulation of IGF-1 with obesity and to develop interventions to lower IGF-1 levels in conditions of excess. Unlike CRP and IGF-1, serum IL-6 and TNFα appeared to remain unchanged following the intervention.

No significant time or group by time effect for changes in serum IL-6 or TNFα were observed over the course of the 14 d hypo-energetic diet. IL-6 and TNFα are inflammatory cytokines used a markers for systemic inflammation and overall health. Elevated blood levels of IL-6 and TNFα have been suggested to be risk factors for all-cause mortality, with epidemiological studies demonstrating IL-6 being a better marker (Harris, et al., 1999) than TNFα (Bruunsgaard, et al., 2003) for mortality in elderly individuals. Systemic levels of IL-6

and TNFα are in part regulated through their production within adipose tissue (Kershaw & Flier, 2004). IL-6 not only directly correlates with FM in humans but the production of IL-6 by adipose tissue is believe to account for 15-35% of systemic IL-6 blood serum levels (Mohamed-Ali, et al., 1997). Beyond IL-6's role in inflammation, IL-6 contributes to insulin resistance through an inhibition of IRS-1 and GLUT4 expression (Rotter, Nagaev, & Smith, 2003). Similarly, TNFα also produced by adipose tissue, has been shown to be elevated in the circulation of obese individuals (Bastard, et al., 2000). Like IL-6, TNFα contributes to insulin resistance (Uysal, Wiesbrock, Marino, & Hotamisligil, 1997) but through the inhibition of the insulin receptors phosphorylation (Feinstein, Kanety, Papa, & Lunen, 1993). Therefore, creating dietary interventions to achieve optimal improvements in metabolic health should consider both changes in body composition as well as markers of systemic inflammation. Pervious work has shown weight loss to reduce IL-6 (Belobrajdic, et al., 2010) and TNFα (Kern, et al., 1995) blood serum levels. However, little work has been done to examine the effect of a diets macronutrient composition on these markers. Here, we failed to show an effect of protein quality or weight loss on IL-6 and TNFα basal serum levels. This effect may have been attributed to the relatively small decrease in FM observed here (~-1.5 kg) as compared to previous studies (-6.5 kg) (Belobrajdic, et al., 2010). Therefore, observable changes in serum IL-6 and TNFα may require a longer duration intervention with greater absolute changes in body composition, a requirement which may also hold true for the gene targets quantified within this study.

There were no significant changes in the expression of genes associated with the ubiquitin proteasome pathway (UPP) including MuRF1, MAFbx, FOXO1 or FOXO3a. Correlative and loss of function studies demonstrate a significant role of MuRF1 and MAFbx in regulating skeletal muscle atrophy in rodents (Bodine, et al., 2001). Similarly, *in vitro* models have shown an equal importance of FOXO1 and FOXO3a as upstream transcription factors regulating MuRF1 and MAFbx expression and skeletal muscle atrophy (Sandri, et al., 2004). The lack of an effect here is in direct contrast to many murine models showing increases in these same targets during caloric restriction (Lecker S. H., et al., 2004; Furuyama, et al., 2002). This may be attributed to the time dependent nature of the expression of the aforementioned targets upon the onset of skeletal muscle atrophy and/or general disparities between human and murine models (Murton, Constantin, & Greenhaff,

2008). Generally, human (De Boer, et al., 2007; Abadi, et al., 2009) and rodent models (Krawiec, Frost, Vary, Jefferson, & Lang, 2005) of disuse atrophy show only an early acute increase MuRF1 and MAFbx transcription (<10 d) following the onset of atrophy. To this author's knowledge only one study has examined changes in MuRF1 and MAFbx expression during a hypo-energetic state in humans. Larsen *et al.,* observed no changes in the expression of either MuRF-1 or MAFbx within six healthy adults at 3 or 40 hrs following the onset of food deprivation (Larsen, et al., 2006). These finding suggest that the transcription of MuRF1 and MAFbx are time dependent or are regulated differently between murine and human models during catabolic conditions. Indeed, the mechanisms controlling skeletal muscle atrophy and the rate of muscle mass loss have been observed to vary between different atrophic models, species and even the muscle's anatomical location (Phillips, Glover, & Rennie, 2009). When comparing different models of skeletal muscle atrophy it is likely that models of disuse, ageing, disease or caloric restriction may function through different pathways to ultimately regulate muscle protein balance; this is especially true when comparing models of muscle atrophy in which a marked inflammatory response is observed (including: sepsis, cancer, HIV AIDS, renal failure and to a far lesser extent aging) and those in which an inflammatory response is not observed (simple disuse and mild energy restriction). Even among disuse- and energy restriction-mediated atrophy the mechanisms controlling muscle wasting may differ slightly (Jackman & Kandarian, 2004). All these factors make comparisons between different atrophic models and species difficult to justify. Thus, while MuRF1 and MAFbx expression appear to be important in multiple murine models of skeletal muscle atrophy, it is not clear if they are important in human models during energy restriction. Consequently, more work will need to be done to determine the importance of MuRF1 and MAFbx as well FOXO1 and FOXO3a in the regulation of skeletal muscle atrophy during a hypo-energetic state in humans. In accordance with a lack of change in the expression of MuRF1 and MAFbx as well FOXO1, FOXO3a we observed no change in the expression of genes associated with mitochondrial metabolism, an effect that may also be attributed to the inherent differences between human, murine and cell culture models.

Contrary to our hypothesis we observed no time or group by time effect for changes in SIRT1 or PGC-1α gene expression following weight loss. PGC-1α is a transcriptional co

activator, which upon activation targets multiple transcription factors and nuclear receptors resulting in an increased expression of nuclear and mitochondrial encoded genes (Dominy, Lee, Gerhart-Hines, & Puigserver, 2010). Moreover, conditions of fasting in rodents appear to up-regulate PGC-1α expression (Enzo, et al., 2005) and activity resulting in an increase in mitochondrial fatty acid oxidation (Gerhart-Hines, Rodgers, Bare, Lerin, & Kim, 2007). SIRT1, a class III NAD^+ dependent deacetylase, is not only energy sensitive but is also a direct regulator of PGC-1α activity (Gerhart-Hines, Rodgers, Bare, Lerin, & Kim, 2007). Similarly, SIRT1 gene expression and protein content appear to be up regulated with food deprivation (Nemoto, Fergusson, & Finkel, 2004; Kanfi, et al., 2008). Nutritional interventions are also proposed to regulate mitochondrial biogenesis. For example, work by Sun *et al.,* showed that leucine treatment of C2C12s not only up regulated SIRT1 and PGC-1α expression but also resulted in increases in mitochondrial biogenesis (Sun & Zemel, 2009). In theory, the combination of both energy restriction and the concurrent ingestion of a high quality protein (high in leucine) should result in an increase in SIRT1 and PGC-1α expression or activity. However, we did not observe increases in SIRT1 or PGC-1α expression within skeletal muscle following the 14 d hypo-energetic diet. This being said changes in SIRT1 and PGC-1α gene expression do not provide a complete picture of mitochondrial function. Future studies should measure the activity and concentration of mitochondrial proteins to determine the role of protein quality and food deprivation on mitochondrial biogenesis and metabolism.

In conclusion, we showed that the absolute amount and the source of protein had no effect on changes in DXA-measured body composition over a 14 d hypo-energetic diet. Similarly, no effect of weight loss or protein source was observed on the transcription of SIRT1, PGC-1α, MuRF1, MAFbx, FOXO1 or FOXO3a. However, during energy restriction protein quality did have a protective effect against increases in basal serum levels of the catabolic hormone cortisol. While limited conclusions can be drawn from this finding, these data suggest that higher protein diets of sufficient duration may limit the loss of LBM, in part, through an inhibition of a hypo-energetic diet induced increase in serum cortisol levels.

2.4.1 Future Directions

Future directions of this research include extending the duration of the study and to conduct a more in-depth mechanistic analysis focused on skeletal muscle protein balance as well as changes in gene and protein expression within both skeletal muscle and adipose tissue. A longer duration intervention and or the inclusion higher resolution systems to quantify changes in LBM may reveal if protein quality can augment changes in body composition during diet induced weight loss. Experimental controls for a longer intervention would require participants to regularly meet with a registered dietitian, quantitative measures of daily activity, continued provision of all food (to enhance compliance), and modifications of ongoing diets to ensure maximal compliance. Mechanistically two approaches can be taken to expand upon this research. The first includes examining the effects of a hypo-energetic diet on the rates of MPS in the postabsorptive and postprandial states. These outcomes were examined in conjunction within this study; however, the results are of currently still being analyzed. We hypothesized that hypo-energetic diet-induced skeletal muscle atrophy results from an impaired MPS response to protein ingestion and that this effect would be diminished with higher quality proteins in the following hierarchical manner: WHEY>SOY>CHO. If true, this would suggest: 1) that the consumption of the highest quality protein (as measured by leucine/EAA content and rate of absorption) would result in the greatest retention of LBM during diet induced weight loss, and 2) hypo-energetic diet induced muscle loss occurs through the inhibition of postprandial rates of MPS, as observed in other models of skeletal muscle atrophy (Phillips, Glover, & Rennie, 2009). This work can be further expanded upon with additional measures including the quantification of genes and proteins (including expression and activity) involved in the pathways regulating MPS, MPB and mitochondrial function. The quantification of the activity of proteins within the Akt-mTOR pathway should be completed as an indicator for MPS. In addition, pathways regulating MPB including the UPP and the autophagy-lysosomal pathway should be examined. Lastly, markers of mitochondrial biogenesis and metabolism within both adipose and skeletal muscle tissue can be quantified to assess the effect of weight loss and protein quality on mitochondrial function. This data will not only help to clarify disparities between murine and human models but will help define the mechanisms of action by which higher protein hypo-energetic diets can augment FM losses while preserving LBM. The main goal of this

work is to optimize and understand the mechanisms controlling higher protein hypo-energetic diets effects on body composition in hopes of improving the metabolic health of millions of overweight or obese individuals across the globe.

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LIST OF APPENDICES

Appendix A: Consent Form and Subject Questionnaires

EXERCISE METABOLISM RESEARCH GROUP
DEPARTMENT OF KINESIOLOGY, MCMASTER UNIVERSITY

INFORMATION AND CONSENT TO PARTICIPATE IN RESEARCH

Molecular Events Underpinning Changes in Tissue Metabolism with Whey and Soy Ingestion in Energy Restriction in Overweight/Obese Adults

Sponsored by The US Dairy Research Institute

You are being invited to participate in a research study conducted by the investigators listed below. Participation in this study is completely voluntary. In order to decide whether or not you want to participate in this research study, you should understand what is involved and the potential risks and benefits. Please read the **information below and** the attached "**Description of Medical Procedures**". In addition, you must **complete** the attached "**Subject Screening Questionnaire**", which asks some questions regarding your health.

Please take your time to make your decision and ask questions about anything that you do not understand before deciding if you want to participate. A researcher listed above will be available to answer your questions. Feel free to discuss it with your friends and family, and/or your family physician. Once you understand the study, you will be asked to sign this form if you wish to participate.

Unless otherwise stated, all testing procedures will be conducted in the laboratory of Dr. Stuart Phillips at the Ivor Wynne Centre (IWC) at McMaster University.

INVESTIGATORS	DEPARTMENT CONTACT
Stuart M. Phillips, Ph.D. x24465 or x27037	Kinesiology, IWC AB116
Cameron Mitchell, M.Sc. x27037	Kinesiology, IWC AB132

For Emergency Contact:
Dr. Stuart Phillips at (905) 525-9140 x 24465 (Day) or (905) 524-1262 (Night)

WHY IS THIS RESEARCH BEING DONE?

There are many lifestyle-oriented strategies for the treatment of obesity including dietary and physical activity-based approaches. Recent literature suggests the consumption of dairy products, specifically whey protein, is one such dietary approach that has been correlated with lower body weight. Whey protein produces a satiating effect, and may also retain muscle mass while stimulating fat loss in humans. Increasing or maintaining lean muscle mass, especially during periods of caloric restriction, would help to offset declines in metabolic rate often associated with dieting. The purpose of this study is to investigate changes in fat and lean mass in response to the ingestion of a fixed dose of whey or soy protein during a hypocaloric period of 14 days.

Participants initials: April 2012-Version 2

If you consent to participate in this research you will be one of 42 participants.

INCLUSION AND EXCLUSION REQUIREMENTS

Inclusion Requirements

In order to participate in this study, you must be a male or female between the ages of 35 to 55 years inclusive and you cannot be a smoker or user of tobacco products. In addition, you must have a body mass index (BMI) between 30 and 40 kg/m^2 inclusive.

Exclusion Requirements

You cannot participate in or will be released from this study if you meet any one of the following criteria:

- Have a body mass index less than 30 kg/m^2 or greater than 40 kg/m^2.
- Have health problems such as: heart disease, vascular disease, rheumatoid arthritis, diabetes, poor lung function, uncontrolled blood pressure, dizziness, thyroid problems, or any other health conditions for which you are being treated that might put you at risk for this study.

WHAT WILL MY RESPONSIBILITIES BE IF I TAKE PART IN THE STUDY?

If you choose to participate in this study, you will be required to commit a total of 2 (6 hour) days at in addition to consuming a provided diet for 14 days. The study will begin with a biopsy taken from the vastus lateralis muscle, and an adipose biopsy from the same incision, which will act as the baseline for gene microarray studies. This biopsy will take place at the Exercise Metabolism Research Laboratory (EMRL) in IWC A103. Subjects will be provided with a controlled diet for 14 days. In addition to this diet, subjects will be asked to ingest a 25-gram supplement twice daily (at ~0830h and ~1430h). This supplement is comprised of whey or soy protein, or a control (maltodextrin), and you will not know which you are ingesting. Additionally, each subject will be required to collect all urine produced over a 24hr period at the beginning and end of the diet protocol.

At the end of the 14-day dietary intervention, we will ask you to consume a standardized breakfast (the same as the breakfast consumed for the standardized diet) at ~0500h. Then, you will be asked to report to the testing center at 0800h and receive infusions of stable isotope labeled phenylalanine (*ring*-[$^{13}C_6$]phenylalanine) to measure muscle protein

synthesis. You will also receive an infusion of glycerol, which helps to determine the breakdown of fat. The infusion will last for 3 hours, and then both muscle and adipose tissue biopsies will be taken. This infusion in conjunction with muscle biopsies will allow for the measurement of muscle protein synthesis. We will also be taking periodic blood samples over the 3 hours to measure the rate of whole-body fat breakdown. You will then be asked to ingest your supplement, which contains stable isotopes to prevent declines in isotope enrichment. You will rest for another 3 hours before we take a second muscle biopsy. After this biopsy, you are free to go.

ATTIRE

It is best if you are dressed in comfortable exercise attire, i.e., loose shorts, t-shirts, and athletic socks and running / training shoes, for all sessions during this study.

WHAT ARE THE POSSIBLE RISKS AND DISCOMFORTS?

Muscle biopsies involve a small incision (~4-5 mm) in your skin in order to create an opening through which to put the biopsy needle into your thigh muscle. This technique may be associated with a transient dull muscle ache for 24-48 hours post biopsy. Further complications are typically rare provided that proper precautions are taken. Based on extensive experience performing biopsies by Dr. Tarnopolsky and Dr. Phillips, the following risks have been identified: 4/10,000 with a local skin infection, 1/200 with a fibrous lump at the site of biopsy (connective tissue), 1/1,000 with a small patch of numbness just past the biopsy incision due to cutting a small sensory nerve branch. In all cases, recovery was complete in 3 months or less.

BENEFITS AND REMUNERATION

In participating in this study you realize that there are no direct benefits to you. You will receive an honorarium of $300 upon the completion of the study to compensate you for your time commitment. In the case a subject is unable to fully complete the study, partial remuneration will be provided.

PLEASE NOTE: Remuneration will be in the form a cheque issued by McMaster University. The cheque will be mailed to you at the completion of the study for the full amount. While we will attempt to have the remuneration forwarded to you as soon as possible, it may take **up to three weeks** following the completion of the study for the cheque to be processed and the remuneration received.

WHAT AND HOW WILL INFORMATION BE KEPT PRIVATE?

Muscle, adipose, and blood samples will be used for this research project only. All data collected during this study will remain confidential and stored in offices and on computers to which only the investigators haves access. You should be aware that the results of this study will be made available to the scientific community through publication in a scientific journal, although neither your name nor any reference to you will be used in compiling or

publishing these results. Additionally, if you are interested you will have access to your own data, as well as the group data, when it becomes available.

CAN PARTICIPATION IN THIS STUDY END EARLY?

You may exercise the option of removing yourself or your data from the study at any time if you wish. You may also refuse to answer any questions posed to you during the study and still remain a subject in the study.

If you experience any of the side effects listed in the Potential Risks and Discomforts section or if you become ill during the research, you may need to be withdrawn from the study, even if you would like to continue. The investigators will make the decision and let you know if it is not possible for you to continue. The decision may be made to protect your safety and welfare, or because it is part of the research plan that people who develop certain conditions may not continue to participate.

RIGHTS OF RESEARCH PARTICIPANTS

Funding for this study is provided by the US Whey Protein Research Consortium. This agency is government funded and all research carried out under the auspices of this agency is covered by the Tri-Council Policy Statement, which is the over-riding national policy relating to research involving human participants. A copy of this policy statement can be viewed at http://www.pre.ethics.gc.ca/english/policystatement/policystatement.cfm. As a condition of the investigators
Participants initials:

April 2012-Version 2

listed on this application receiving funding from this agency, this research proposal has been reviewed by the Hamilton Health Sciences/Faculty of Health Sciences Research Ethics Board using the strict application of the ethical principles and the articles of this policy.

You will receive a completed (i.e. signed) copy of this consent form. You may withdraw your consent to participate in this study at any time, and you may also discontinue participation at any time without penalty. In signing this consent form or in participating in this study you are not waiving any legal claims or remedies. Finally, please realize that this study has been reviewed by the Hamilton Health Sciences/Faculty of Health Sciences Research Ethics Board. If you have any further questions regarding your rights as a research participant please feel free to contact:

Office of the Chair of the Hamilton Health Sciences/Faculty of Health Sciences Research Ethics Board at 905 521-2100, Ext. 42013

IF YOU HAVE ANY QUESTIONS

You will be able to contact Dr. Stuart Phillips at 905-525-9140 (x24465 or x27037) regarding any questions you may have about the study.

In the event of an emergency, please contact Dr. Stuart Phillips at (905) 525-9140, ext. 24465 during office hours or (905) 524-1262 after office hours.

If you have any questions regarding your rights as a research participant, you may contact the Office of the Chair of the Hamilton Health Sciences/Faculty of Health Sciences Research Ethics Board at (905) 521-2100, ext. 42013.

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CONSENT STATEMENT

I have read and understood the preceding information thoroughly.
I have read and understood the attached "Description of Medical Procedures" and completed the attached "Subject Screening Questionnaire".
I have had the opportunity to ask questions, and all of my questions have been answered to my satisfaction. I agree to participate in this study. I understand that participation in this study is voluntary and that I may withdraw from the study at any time without penalty. I understand that I will receive a signed copy of this form.

Signature of Participant
Date (dd/mm/yy)

Printed Name of Participant

WITNESS
My signature as witness, certifies that I witnessed the participant voluntarily sign this consent form in my presence.

Signature of Person Obtaining Consent
Date (dd/mm/yy)

Printed Name of Person Obtaining Consent

INVESTIGATOR
In my judgment, the participant is voluntarily and knowingly giving informed consent and possesses the legal capacity to give informed consent and participate in this research study.

---------- ----------
Signature of Investigator Date
(dd/mm/yy)

Printed Name of Investigator

Participants initials:

April 2012-Version 2

EXERCISE METABOLISM RESEARCH GROUP
DEPARTMENT OF KINESIOLOGY, MCMASTER UNIVERSITY

PARTICIPANT INFORMATION

Molecular Events Underpinning Changes in Tissue Metabolism with Whey and Soy Ingestion in Energy Restriction in Overweight/Obese Adults

Sponsored by The US Dairy Research Institute

Name _____

Email _____ **Phone number** _____

Address (to which all meals will be delivered)
_____
_____
_____

Date of Birth _____ **Sex M / F**

Height (cm) _____ **Body Weight (kg)** _____

If female, have you had one or more regular menstrual cycles in the past year?_____

How long (months or years) have you been at your current body weight?
_____

When was the last time you were on a diet? _____

Are you currently taking any medication for hyperlipidemia (high blood cholesterol)?
_____

Are you Diabetic? _____

Do you have any known thyroid problems? _____

What types of physical activity do you participate in?
_____
_____

On Average how many hours a week do you regularly complete the above physical activities?

0-1 1-2 2-3 3-4 4-5 5-6 6-7 7-8 8+

How many days a week do you exercise at low intensities (walking, gardening, house hold chores)?

0 1-2 2-3 3-4 5-6

How many days per week do you regular participants in high intensity activities (jogging, racket sports, swimming, weight lifting, dancing)?

0 1-2 2-3 3-4 5-6

Please list any medication your are currently taking.

_____ _____
_____ _____
_____ _____

Please list any allergies to medications you currently have.

_____ _____
_____ _____
_____ _____

Are you currently taking any Vitamin or mineral supplements? If so which ones?

_____ _____
_____ _____

EXERCISE METABOLISM RESEARCH GROUP
DEPARTMENT OF KINESIOLOGY, MCMASTER UNIVERSITY

Food Preference Checklist

Molecular Events Underpinning Changes in Tissue Metabolism with Whey and Soy Ingestion in Energy Restriction in Overweight/Obese Adults

Participant Name:

1) Do you have any food allergies?

   Yes No

   If yes please specify:

2) Do you have any special dietary requirements i.e. celiac disease, religious dietary requirements etc.?

   Yes No

   If yes please specify:

3) Are you a vegetarian?

   Yes No

4) Do you have any food dislikes?
   Yes No

   If yes please specify:

The study diet will be made up of the following foods.

- Red meat
- Chicken
- Turkey
- Fish
- Milk
- Cheese
- Soup (i.e. chicken noodle, carrot soup etc)
- Vegetables
- Apples
- Bananas
- Cereal bars

- Butter
- Oats

Would you be unable to eat any of these foods?

Yes No

If yes please circle which foods you would be unable to eat and specify why (i.e. strongly dislike, food allergy, religious reasons etc.) beside it.

Appendix B: Data Table – Participant Baseline Characteristics
Subject Baseline Characteristics are presented as Mean ± Standard Error

	Summary of Subject Baseline Characteristics		
	CHO	**SOY**	**WHY**
Age (y)	47 ± 2	52 ± 2	52 ± 2
Height (cm)	170 ± 2	171 ± 3	168 ± 2
Body Mass (kg)	104.9 ± 4.9	103.9 ± 6.6	98.3 ± 4.4
BMI	36.9 ± 1.2	34.8 ± 1.5	34.7 ± 1.1
N	12	14	13

Values are depicted as Mean ± SEM.

	Subject Baseline Characteristics: CHO			
Subject	**Age (y)**	**Body Mass (kg)**	**Height (cm)**	**BMI (kg/m^2)**
4	49	88.7	158	34.6
12	50	119.1	178.5	37.22
13	46	112.5	172	38.37
17	53	105.2	160	41.3
18	51	83.0	167	29.83
21	51	77.7	154.25	33.41
22	41	115.6	172	38.66
28	48	103.4	167	36.97
38	35	101.9	178	32.8
46	59	114.9	169	40.9
48	54	130.1	173	43
49	35	106.4	172	36
Mean	48	104.9	168.4	36.9
SEM	2	4.4	2.2	1.1

	Subject Baseline Characteristics: SOY			
Subject	**Age (y)**	**Body Mass (kg)**	**Height (cm)**	**BMI (kg/m^2)**
1	44	80.3	157	32.1
5	53	69.8	158	27.6
6	53	85.1	167.5	29.9
10	38	111.8	171	38.0
14	47	88.4	173	29.3
16	40	92.9	169	32.2
20	54	107.9	178	33.6
29	58	74.2	158	29.1
30	56	92.9	152	40.3
34	64	146.2	190	40.8
35	53	143.5	173.5	47.4
39	54	130.4	182.5	38.9
41	47	122.4	189	34.2
50	60	108.5	179	34.5
Mean	52	103.9	171.3	34.8
SEM	2	6.6	3.2	1.5

	Subject Baseline Characteristics: WHEY			
Subject	**Age (y)**	**Body Mass (kg)**	**Height (cm)**	**BMI (kg/m^2)**
2	48	87.8	159	34.9
7	53	105.4	172	35.2
8	59	82.1	159	32.4
19	47	89.9	168.25	29.7
23	60	98.9	155	41.2
24	45	110.4	169	38.3
31	35	123.7	185.5	36.1
36	59	135.1	176.25	44.8
40	51	72.0	152.5	30.7
42	54	92.9	170	32.4
44	62	91.3	172.5	30.7
45	63	99.8	169.5	34.9
47	52	96.7	172	33.1
Mean	53	98.9	167.7	34.9
SEM	2	4.7	2.5	1.2

One way ANOVA for baseline characteristics
Significant differences at P<.05

		Sum of Squares	df	Mean Square	F	Sig.
Age (y)	Between Groups	183.3	2	91.63	1.591	.218
	Within Groups	2073.1	36	57.59		
	Total	2256.4	38			
Body Mass (kg)	Between Groups	260.578	2	130.289	.336	.717
	Within Groups	13952.909	36	387.581		
	Total	14213.487	38			
Height (cm)	Between Groups	94.7	2	47.33	.488	.618
	Within Groups	3492.5	36	97.01		
	Total	3587.1	38			
BMI (kg/m^2)	Between Groups	34.3	2	17.15	.772	.470
	Within Groups	800.2	36	22.23		
	Total	834.5	38			

Appendix C: Data Table – Caloric Intake 3 Day Weight Maintenance (WM) Diet
Below is the dietary caloric and macronutrient breakdown during the 3 day weight maintenance (WM) diet for each group. Caloric balance (kcal) was determined by subtracting estimated caloric needs, using the Mifflin-St Jeor equation, from reported caloric intake.

	3 Day WM Diet: CHO					
Subject	**Caloric Intake (kcal)**	**Caloric Balance (kcal)**	**Protein (g)**	**Protein (g/kg/d)**	**CHO (g)**	**Fat (g)**
4	1904	-117	84.1	0.97	253.3	63.2
12	2404	-93	110.1	0.93	321.3	80.6
13	2502	-55	101.6	0.89	340.6	85.3
17	2032	-264	90.9	0.86	264.1	69.8
18	1536	-498	69.3	0.83	207.7	49.3
21	1850	-170	79.0	0.99	247.6	62.6
22	2674	-109	94.3	0.82	370.8	92.6
28	2337	-10	103.7	1.01	320.8	74.3
38	2788	8	103.1	0.99	386.0	96.6
46	2280	-435	82.1	0.70	322.4	77.2
48	2647	-303	106.5	0.83	366.3	88.3
49	2343	-419	92.8	0.87	314.3	82.2
Mean	2275	-205	93.1	0.89	309.6	76.8
SEM	27	12	0.9	0.01	4.0	1.0

	3 Day WM Diet: SOY					
Subject	**Caloric Intake (kcal)**	**Caloric Balance (kcal)**	**Protein (g)**	**Protein (g/kg/d)**	**CHO (g)**	**Fat (g)**
1	1897	-53	83.4	1.06	250.7	64.0
5	1780	28	77.3	1.13	240.0	59.0
6	1839	-210	86.7	1.03	231.8	64.6
10	2318	-251	99.4	0.89	304.3	80.9
14	2788	18	103.1	1.18	386.0	96.6
16	2245	-20	93.8	1.03	300.5	73.3
20	2459	-220	104.8	0.99	324.6	85.1
29	1609	-172	67.6	0.93	213.7	55.8
30	1822	-201	77.3	0.83	247.2	60.3
34	3089	-201	123.5	0.84	418.6	105.4
35	2724	-433	108.8	0.76	363.6	95.1
39	2886	-157	114.9	0.89	402.6	94.4
41	3008	-36	119.6	0.98	416.2	100.6
50	2805	97	106.5	0.96	390.3	95.4
Mean	2376	-129	97.6	0.96	320.7	80.8
SEM	37	10	1.2	0.01	5.4	1.3

	3 Day WM Diet: WHEY					
Subject	**Caloric Intake (kcal)**	**Caloric Balance (kcal)**	**Protein (g)**	**Protein (g/kg)**	**CHO (g)**	**Fat (g)**
2	1887	-175	80.1	0.91	241.2	69.0
7	2826	-149	114.4	1.10	385.6	94.9
8	1945	34	86.8	1.06	257.7	64.7
19	1573	-525	71.3	0.85	212.3	50.4
23	2058	-44	88.0	0.89	270.2	71.2
24	2352	-122	106.3	0.97	304.0	81.9
31	3224	97	126.8	1.02	443.6	106.8
36	2940	-135	114.4	0.82	407.0	100.3
40	1705	-50	75.8	1.06	222.1	58.4
42	2396	-37	106.8	1.14	313.0	82.8
44	2466	99	105.7	1.16	325.2	86.7
45	2366	-270	111.6	1.11	319.4	72.9
47	2218	-305	101.5	1.04	279.3	80.7
Mean	2304	-122	99.2	1.01	306.2	78.5
SEM	37	13	1.3	0.01	5.5	1.3

Summary table for 3 day WM diet data

	3 Day WM Diet: Summary					
Group	**Caloric Intake (kcal)**	**Caloric Balance (kcal)**	**Protein (g)**	**Protein (g/kg)**	**CHO (g)**	**Fat (g)**
WHEY	2304 ± 37	-122 ± 13	99 ± 1	1.01 ± 0.01 [a]	306 ± 5	79 ± 1
SOY	2376 ± 37	-129 ± 10	98 ± 1	.96 ± 0.01 [a]	321 ± 5	81 ± 1
CHO	2275 ± 27	-206 ± 13	93 ± 1	0.89 ± 0.01 [b]	310 ± 4	77 ± 1

Different letter difference indicates a significant difference at (P <.05)

ANOVA - Summary of effects
WM Diet One way ANOVA Summary Table

		Sum of Squares	df	Mean Square	F	Sig.
Caloric intake (kcal)	Between Groups	72396.168	2	36198.084	.166	.847
	Within Groups	7827408.643	36	217428.018		
	Total	7899804.811	38			
Caloric Balance (kcal)	Between Groups	53214.295	2	26607.148	1.004	.377
	Within Groups	954211.620	36	26505.878		
	Total	1007425.915	38			
Protein (g)	Between Groups	245.235	2	122.617	.489	.617
	Within Groups	9030.849	36	250.857		
	Total	9276.084	38			
Protein (g/kg/d)	Between Groups	.090	2	.045	3.819	.031
	Within Groups	.423	36	.012		
	Total	.513	38			
CHO (g)	Between Groups	1564.020	2	782.010	.168	.846
	Within Groups	167321.574	36	4647.821		
	Total	168885.594	38			
Fat (g)	Between Groups	101.177	2	50.589	.196	.823
	Within Groups	9274.513	36	257.625		
	Total	9375.691	38			

Post Hoc Tukey HSD for WM Diet, one way ANOVA

Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Protein(g/kg/d)	WHEY	SOY	.046	.042	.524	-.056	.148
		CHO	.11917*	.043	**.025**	.013	.225
	SOY	WHEY	-.046	.042	.524	-.148	.056
		CHO	.073	.043	.211	-.031	.178
	CHO	WHEY	-.11917*	.043	**.025**	-.225	-.013
		SOY	-.073	.043	.211	-.178	.031

Tukeys HSD shows a significant difference between the relative amount to of protein intake (g/kg/d) between WHEY and CHO

Appendix D: Data Table – Caloric Intake 14d Weight Loss (WL) Diet
Caloric balance determine by subtracting estimated caloric needs (st joer eq) from reported caloric intake.

	14d WL Diet: CHO						
Subject	**Estimated Caloric Requirement**	**Caloric intake (kcal)**	**Caloric deficit (kcal)**	**Protein (g)**	**Protein (g/kg)**	**CHO (g)**	**Fat(g)**
4	2021	1248	-773	67.7	0.78	174.6	34.7
12	2497	1641	-856	85.5	0.72	227.9	47.0
13	2557	1388	-1169	70.2	0.62	183.8	43.9
17	2296	1468	-829	80.8	0.76	200.8	39.8
18	2034	1262	-772	67.5	0.81	177.8	35.1
21	2020	1239	-781	67.2	0.84	174.6	33.9
22	2783	2065	-718	85.3	0.75	293.8	64.8
28	2347	1389	-958	71.3	0.69	190.9	41.1
38	2779	2044	-736	85.4	0.82	294.3	61.7
46	2714	1739	-975	73.9	0.63	240.1	57.1
48	2950	1955	-995	85.1	0.66	273.5	61.4
49	2762	1993	-769	86.9	0.81	278.0	62.9
Mean	2480	1619	-861	77.2	0.74	225.8	48.6
SEM	89	88	36	2.2	0.02	12.9	3.2

	14d WL Diet: SOY						
Subject	**Estimated Caloric Requirement**	**Caloric intake (kcal)**	**Caloric deficit (kcal)**	**Protein (g)**	**Protein (g/kg)**	**CHO (g)**	**Fat(g)**
1	1950	1029	-921	82.0	1.04	123.1	25.6
5	1753	1206	-547	108.4	1.58	133.9	27.7
6	2049	1312	-736	114.5	1.36	151.5	30.5
10	2570	1910	-660	130.9	1.18	224.3	53.5
14	2770	2088	-682	128.3	1.46	267.2	58.7
16	2266	1448	-817	114.2	1.25	166.1	37.2
20	2679	1943	-736	142.1	1.34	232.4	50.7
29	1781	1119	-662	106.7	1.47	121.3	25.6
30	2022	1189	-833	108.0	1.16	132.4	27.9
34	3290	2725	-566	167.9	1.14	350.3	73.4
35	3157	2453	-704	149.6	1.05	314.7	69.9
39	3043	1981	-1063	119.5	0.92	257.6	64.8
41	3043	2136	-907	138.2	1.13	263.8	60.7
50	2708	2039	-669	131.9	1.19	263.1	53.5
Mean	2506	1756	-750	124.4	1.23	214.4	47.1
SEM	142	143	38	5.7	0.05	20.3	4.7

	14d WL Diet: WHEY						
Subject	**Estimated Caloric Requirement**	**Caloric intake (kcal)**	**Caloric deficit (kcal)**	**Protein (g)**	**Protein (g/kg)**	**CHO (g)**	**Fat(g)**
2	2062	1222	-840	93.0	1.05	145.5	33.7
7	2975	2200	-775	142.3	1.37	276.1	61.6
8	1910	1438	-473	120.4	1.47	157.6	39.4
19	2098	1372	-726	115.2	1.37	158.0	32.8
23	2102	1462	-641	120.1	1.21	165.3	37.4
24	2473	1822	-652	132.4	1.21	210.5	51.3
31	3128	2559	-568	153.5	1.24	322.0	76.5
36	3076	2476	-600	156.3	1.12	319.8	66.9
40	1755	1215	-540	90.1	1.26	123.4	31.7
42	2433	1543	-890	109.8	1.17	179.9	42.6
44	2368	1807	-561	140.9	1.54	202.7	49.3
45	2636	1923	-713	140.0	1.39	223.0	53.5
47	2523	1652	-871	120.7	1.23	193.8	45.3
Mean	2426	1745	-681	125.8	1.28	206.0	47.8
SEM	122	123	37	5.8	0.04	17.8	3.9

Summary table for WL diet

Group	Estimated Caloric Requirement	Caloric intake (kcal)	Caloric deficit (kcal)	Protein (g)	Protein (g/kg/d)	CHO(g)	Fat(g)
CHO	2480 ± 89	1619 ± 88	-861 ± 36 [a]	77.2 ± 2.2 [a]	0.74 ± 0.02 [a]	225.8 ± 12.9	48.6 ± 3.2
SOY	2506 ± 142	1756 ± 143	-750 ± 38 [b]	124.4 ± 5.7 [b]	1.23 ± 0.05 [b]	214.4 ± 20.3	47.1 ± 4.7
WHEY	2480 ± 122	1745 ± 123	-681 ± 37 [b]	125.8 ± 5.8 [b]	1.28 ± 0.04 [b]	206.0 ± 17.8	47.8 ± 3.9

All values are depicted as Mean ± SEM, significant differences at P(.05) are depicted by letter differences

ANOVA – Summary of Effects for WL Diet

ANOVA summary table of WL diet

		Sum of Squares	df	Mean Square	F	Sig.
Caloric Deficit (kcal)	Between Groups	205069.4	2	102534.7	5.374	**.009**
	Within Groups	686883.9	36	19080.1		
	Total	891953.3	38			
Protein (g/kg/d)	Between Groups	2.2	2	1.1	52.817	**.000**
	Within Groups	0.8	36	0.0		
	Total	3.0	38			
Protein (g)	Between Groups	19021.4	2	9510.7	28.520	**.000**
	Within Groups	12005.2	36	333.5		
	Total	31026.6	38			
Fat (g)	Between Groups	14.5	2	7.3	.033	.968
	Within Groups	7923.8	36	220.1		
	Total	7938.3	38			
CHO (g)	Between Groups	2477.4	2	1238.7	.298	.744
	Within Groups	149837.7	36	4162.2		
	Total	152315.1	38			

Post hoc test for WL diet characteristics

Tukeys HSD Post Hoc Test for WL Diet Characteristics

Dependent Variable			Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
						Lower Bound	Upper Bound
Caloric Deficit (kcal)	**WHEY**	SOY	69.7	53.2	.399	-60.4	199.7
		CHO	180.16103*	55.3	**.007**	45.0	315.3
	SOY	WHY	-69.7	53.2	.399	-199.7	60.4
		CHO	110.5	54.3	.119	-22.3	243.3
	CHO	WHY	-180.16103*	55.3	**.007**	-315.3	-45.0
		SOY	-110.5	54.3	.119	-243.3	22.3
Protein (g/kg/d)	**WHEY**	SOY	0.0	0.1	.694	-0.1	0.2
		CHO	.53840*	0.1	**.000**	0.4	0.7
	SOY	WHY	0.0	0.1	.694	-0.2	0.1
		CHO	.49274*	0.1	**.000**	0.4	0.6
	CHO	WHY	-.53840*	0.1	**.000**	-0.7	-0.4
		SOY	-.49274*	0.1	**.000**	-0.6	-0.4
Protein (g)	**WHEY**	SOY	1.3	7.0	.981	-15.9	18.5
		CHO	48.51821*	7.3	**.000**	30.6	66.4
	SOY	WHY	-1.3	7.0	.981	-18.5	15.9
		CHO	47.20095*	7.2	**.000**	29.6	64.8
	CHO	WHY	-48.51821*	7.3	**.000**	-66.4	-30.6
		SOY	-47.20095*	7.2	**.000**	-64.8	-29.6

Appendix E: Data Table - Changes in Basal Serum Cortisol

	Serum Cortisol levels: CHO		
Subject	**Pre Cortisol (nM)**	**Post Cortisol (nM)**	**Change (nM)**
4	569	847	278
12	606	824	218
13	578	856	278
17	514	743	229
18	505	782	277
21	576	679	103
22	539	684	145
28	545	601	56
38	523	879	356
46	573	661	88
48	590	730	140
49	564	554	-10
Mean	556.8	736.7	179.8
SEM	9.1	30.2	31.6

	Serum Cortisol levels: SOY		
Subject	**Pre Cortisol (nM)**	**Post Cortisol (nM)**	**Change (nM)**
1	554	641	87
5	527	578	51
6	529	568	39
10	605	668	63
14	553	775	222
16	525	576	51
20	552	587	35
29	555	664	109
30	592	679	87
34	537	645	108
35	582	675	93
39	558	679	121
41	520	649	129
50	504	687	183
Mean	549.5	647.9	98.4
SEM	7.7	15.0	14.4

	Serum Cortisol levels: WHEY		
Subject	**Pre Cortisol (nM)**	**Post Cortisol (nM)**	**Change (nM)**
2	575	586	11
7	627	506	-121
8	604	627	23
19	590	631	41
23	647	535	-112
24	575	673	98
31	530	538	8
36	631	593	-38
40	519	644	125
42	522	512	-10
44	567	506	-61
45	576	530	-46
47	645	641	-4
Mean	585.2	578.6	-6.6
SEM	12.3	16.7	19.9

Appendix F: Data Table - Change in Basal Serum CRP.

	Serum CRP levels: CHO		
Subject	**Pre CRP (mg/L)**	**Post CRP (mg/L)**	**Change (mg/L)**
4	2	2.3	0.3
12	2.9	2.5	-0.4
13	2.1	2.4	0.3
17	1.8	2.6	0.7
18	2.9	2.4	-0.5
21	2.8	2.5	-0.3
22	3	2.3	-0.7
28	2.8	2.5	-0.3
38	1.9	2.1	0.2
46	1.7	2	0.3
48	1.9	2.3	0.4
49	1.8	2.2	0.4
Mean	2.3	2.3	0
SEM	0.1	0	0.1

	Serum CRP levels: SOY		
Subject	**Pre CRP (mg/L)**	**Post CRP (mg/L)**	**Change (mg/L)**
1	2.3	2.7	0.4
5	2.4	2.1	-0.3
6	2.9	2.1	-0.9
10	2.9	2.2	-0.7
14	2.7	2.9	0.3
16	2.5	2.2	-0.3
20	2.8	2.1	-0.6
29	2.5	3	0.5
30	2.7	2.9	0.2
34	2	2.5	0.4
35	2.9	2.4	-0.5
39	2.7	2.5	-0.2
41	2.1	2.2	0.1
50	2.5	2.8	0.3
Mean	2.6	2.5	-0.1
SEM	0.1	0.1	0.1

	Serum CRP levels: WHEY		
Subject	**Pre CRP (mg/L)**	**Post CRP (mg/L)**	**Change (mg/L)**
2	1.9	2.1	0.2
7	2.1	2.2	0.1
8	3.0	2.2	-0.8
19	2.6	2.1	-0.4
23	2.7	2.0	-0.7
24	2.1	2.1	0.0
31	2.8	2.1	-0.6
36	2.6	2.1	-0.5
40	2.1	2.2	0.1
42	2.3	2.2	-0.1
44	2.7	2.1	-0.7
45	2.6	2.0	-0.6
47	2.3	2.1	-0.2
Mean	2.4	2.1	-0.3
SEM	0.1	0.0	0.1

Appendix G: Data Table - Change in Basal Serum IGF-1

	Serum IGF-1 levels: CHO		
Subject	**Pre IGF-1 (nM)**	**Post IGF-1 (nM)**	**Change (nM)**
4	49	32	-17
12	41	33	-8
13	40	32	-8
17	39	43	4
18	42	41	-1
21	45	43	-2
22	37	43	6
28	44	36	-7
38	34	38	5
46	46	34	-12
48	36	49	13
49	39	32	-7
Mean	41	38	-3
SEM	1	2	2

	Serum IGF-1 levels: SOY		
Subject	**Pre IGF-1 (nM)**	**Post IGF-1 (nM)**	**Change (nM)**
1	30	41	11
5	35	45	10
6	45	38	-7
10	37	32	-5
14	44	36	-8
16	36	37	2
20	37	48	11
29	32	46	14
30	35	31	-4
34	34	31	-4
35	44	42	-2
39	47	47	0
41	40	42	2
50	47	50	3
Mean	39	40	2
SEM	1	2	2

	Serum IGF-1 levels: WHEY		
Subject	**Pre IGF-1 (nM)**	**Post IGF-1 (nM)**	**Change (nM)**
2	40	46	6
7	43	43	0
8	43	31	-12
19	49	38	-10
23	34	33	-1
24	49	43	-5
31	48	31	-17
36	37	40	3
40	38	42	4
42	48	36	-12
44	50	31	-19
45	38	38	0
47	50	45	-5
Mean	43	38	-5
SEM	2	2	2

Appendix H: Data Table - Change in Basal Serum TNF-a

	Serum TNF-α levels: CHO		
Subject	**Pre TNF-α (pg/ml)**	**Post TNF-α (pg/ml)**	**Change (pg/ml)**
4	7.2	8.7	1.5
12	7.0	5.4	-1.6
13	7.0	8.6	1.6
17	6.4	8.5	2.1
18	5.8	7.4	1.6
21	6.6	5.9	-0.7
22	6.9	8.1	1.2
28	8.5	6.0	-2.5
38	7.1	5.2	-1.9
46	5.4	6.6	1.2
48	8.7	6.6	-2.1
49	8.3	5.7	-2.6
Mean	7.1	6.9	-0.2
SEM	0.3	0.4	0.5

	Serum TNF-α levels: SOY		
Subject	**Pre TNF-α (pg/ml)**	**Post TNF-α (pg/ml)**	**Change (pg/ml)**
1	5.1	5.7	0.6
5	8.4	7.9	-0.5
6	6.6	5.3	-1.3
10	6.6	7.9	1.3
14	5.6	8.6	3.0
16	7.3	6.7	-0.6
20	5.5	6.4	0.9
29	8.5	6.3	-2.2
30	7.0	5.6	-1.4
34	5.8	7.0	1.2
35	5.2	7.3	2.1
39	6.2	9.0	2.8
41	8.5	7.1	-1.4
50	7.2	5.9	-1.3
Mean	6.7	6.9	0.2
SEM	0.3	0.3	0.5

	Serum TNF-α levels: WHEY		
Subject	**Pre TNF-α (pg/ml)**	**Post TNF-α (pg/ml)**	**Change (pg/ml)**
2	7.4	6.5	-0.9
7	8.4	5.9	-2.5
8	6.9	8.1	1.2
19	5.1	8.5	3.4
23	6.8	7.6	0.8
24	8.0	6.5	-1.5
31	5.0	6.3	1.3
36	8.6	5.5	-3.1
40	8.8	5.8	-3.0
42	6.3	5.7	-0.6
44	6.9	7.3	0.4
45	5.7	7.4	1.7
47	7.6	5.5	-2.1
Mean	7.0	6.7	-0.4
SEM	0.4	0.3	0.6

Appendix I: Data Table - Change in Basal Serum IL-6

	Serum IL-6 levels: CHO		
Subject	**Pre IL-6 (pg/ml)**	**Post IL-6 (pg/ml)**	**Change (pg/ml)**
4	98	109	11
12	85	111	26
13	95	69	-26
17	105	85	-21
18	64	82	19
21	107	75	-32
22	61	92	30
28	77	107	31
38	82	65	-17
46	78	98	20
48	70	110	39
49	118	106	-12
Mean	87	92	6
SEM	5	5	7

	Serum IL-6 levels: SOY		
Subject	**Pre IL-6 (pg/ml)**	**Post IL-6 (pg/ml)**	**Change (pg/ml)**
1	120	115	-5
5	102	103	0
6	108	105	-3
10	79	95	17
14	117	81	-36
16	68	110	41
20	113	64	-50
29	88	61	-27
30	109	104	-5
34	115	104	-12
35	97	93	-4
39	73	110	37
41	93	77	-17
50	92	72	-20
Mean	98	92	-6
SEM	4	5	7

	Serum IL-6 levels: WHEY		
Subject	**Pre IL-6 (pg/ml)**	**Post IL-6 (pg/ml)**	**Change (pg/ml)**
2	100	85	-16
7	117	71	-46
8	87	92	5
19	117	83	-35
23	95	87	-9
24	83	73	-10
31	66	81	15
36	94	72	-22
40	67	63	-5
42	111	85	-26
44	63	83	20
45	95	64	-31
47	85	95	10
Mean	91	79	-11
SEM	5	3	6

Appendix J: ANOVA Table – Changes in Blood Serum Markers

One way ANOVA Summary Table on the Changes in Serum Markers

		Sum of Squares	df	Mean Square	F	Sig.
Cortisol	Between Groups	219091.52	2	109545.76	17.040	**.000**
	Within Groups	231438.17	36	6428.84		
	Total	450529.69	38			
CRP	Between Groups	0.82	2	0.41	2.295	.115
	Within Groups	6.40	36	0.18		
	Total	7.21	38			
TNF-α	Between Groups	2.59	2	1.30	.373	.691
	Within Groups	125.13	36	3.48		
	Total	127.72	38			
IGF-1	Between Groups	343.61	2	171.81	2.735	.078
	Within Groups	2261.43	36	62.82		
	Total	2605.05	38			
IL-6	Between Groups	1907.78	2	953.89	1.699	.197
	Within Groups	20215.99	36	561.56		
	Total	22123.77	38			

Tukeys HSD for Change in Basal Serum Cortisol levels.

		Mean Difference (I-J)	Std. Error	Sig.	95% Confidence Interval	
					Lower Bound	Upper Bound
WHEY	SOY	-105.04396*	30.9	.005	-180.5	-29.6
	CHO	-186.44872*	32.1	.000	-264.9	-108.0
SOY	WHEY	105.04396*	30.9	.005	29.6	180.5
	CHO	-81.40476*	31.5	.037	-158.5	-4.3
CHO	WHEY	186.44872*	32.1	.000	108.0	264.9
	SOY	81.40476*	31.5	.037	4.3	158.5

*. The mean difference is significant (P<0.05)

Appendix K: RNA Isolation

The following RNA extraction protocol has been taken form Thomas Jensen (tj@medical-prognosis.com)

1. Clean working area, pipettes etc with RNAseZap. Use gloves. Spray work area and gloves with RNAseZap. Note: Be careful with gloves when changing rooms.
2. Prepare 500 µl Trizol in 2ml eppendorf tubes.
3. Add tissue to the 500 µl TRIzol (tissue may not exceed 10% of the volume of TRIzol - if so add extra TRIzol). Skeletal muscle samples used should range from 20-30mg.
4. Add 1, 5 mm retch ball to sample and homogenize in TissueLyser II at 28.5 Hz for a sufficient duration to completely dissolve the tissue. Often is 1 to 3 minutes enough, however roughly 1 minute is required for a 10-50mg skeletal muscle sample. (Ensure that the TissueLyser II block is equilibrated at -20C before use).
5. Add 100 µl bromochloropropane "BCP" (20% or 1:1.5 ratio of BCP to trizol) BCP and vortex 15 sec.
6. Incubate 2-3 minutes at RT.
7. Centrifuge 15,000 G for 20 min (at 4˚C).
   Note: can spin for up to 60 min if desired but 20min will suffice.
8. Transfer the supernatant, containing RNA, to a new RNA free 1.5ml centrifuge tube. Be careful not to disturb the interface. Typically supernatant volumes range from 250-400µl.
9. Perform repeated phenol/chloroform extractions (by mixing 1:1 the supernatant with phenol/chloroform – (remember to go below the buffer line when collecting the phenol/chloroform solution. In short, the phenol/cholorform solution will tend to develop and upper *aq* buffer phase. To avoid this phase aim to pipet up from the lower middle portion of the container). Vortex thoroughly (10-15 sec). Spin 15,000 G, 20 minutes, 4 degrees. Take the supernatant, without disturbing the interface, and transfer to a new RNA free tube. Repeat until a very distinct and debris-free interface appears (typically three times).
10. Remove the supernatant, without disturbing the interface, and transfer to a new RNA free 1.5ml tube.
11. Add 1:1 BCP and vortex thoroughly (10-15 sec).
12. Spin for 20 minutes at 15,000 G, at 4˚C (NOTE: be sure to line up the hinges of the tubes to face the outside of the centrifuge. This way the pellet will be directly below the hinge of the tube) remove the supernatant.
    a. NOTE: The next step needs to be done as soon as possible!
13. Precipitate by adding 1:1 cold isopropyl alcohol (from freezer -20˚C). Invert tube 3-4 times (DO NOT VORTEX). Spin 20 minutes at 15,000 G. Remove supernatant. Do so by first using a 500 ul tip, followed by a short spin then a 200 ul tip.
14. Wash with cold 500 µl 80% EtOH. Spin for 5 minutes @ 15,000 G.
15. Remove as much EtOH as possible and then allow to air-dry the pellet. It is important to get rid of all the EtOH.
16. Dissolve in 21.5 µl of RNA free water. Pipet up and down multiple times to re-suspend the RNA.
17. Standardize all RNA samples to 50 ng/µl

Appendix L: cDNA Generation

Kit used: High capacity cDNA reverse transcription kit, applied Biosystems

1. Starting RNA concentrations at 50 ng/µl with target cDNA concentration at 25 ng/µl.
2. Allow Kit components to thaw on ice.
3. Prepare Reverse transcription master mix without the RNase inhibitor as shown in the below table.

RT Master Mix Components

Component	Volume (µL)
10 ✕ RT Buffer	2
25✕ dNTP Mix (100 mM)	0.8
10 ✕ RT Random Primers	2
MultiScribe™ Reverse Transcriptase	1
RNase Inhibitor	-
Nuclease-free H_2O	4.2
Total per Reaction	10

4. Pipette 20 µl of RNA sample and 20 µl of the RT master mix into a .5 µl RNA free tube. Mix by pipetting for RNA samples starting at 50 ng/µl.
5. Briefly centrifuge samples and keep on ice.
6. Use below time and temperature conditions for Reverse transcription within eppendorf master cycler.
7. Briefly spin and store in freezer.

Reverse Transcription Conditions

	Step 1	Step 2	Step 3	Step 4
Temperature (°C)	25	37	85	4
Time (min)	10	120	5	∞

Appendix M: Communication of Research – Poster, European College of Sport Sciences (ECSS). June, 2013
Presented: Saturday, June 29th, 2013

Molecular Events Underpinning Changes in Tissue Metabolism with Whey and Soy Ingestion in Energy Restriction in Overweight/Obese Adults

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*Both authors contributed equally to the project

Introduction

❑Diet-induced weight loss results in the loss of both fat mass (FM) and fat free mass (FFM).

❑Skeletal muscle, being the principle component of FFM, is the primary regulator of lipidemia and glycemia. Therefore, decreases in skeletal muscle mass can negatively impact metabolic health.

❑A meta analysis by Wycherley *et al.*, 2012 demonstrated that higher protein diets can limit FFM losses during diet induced weight loss. An effect thought to be attributed to the ability of protein to stimulate muscle protein synthesis (MPS).

❑ As our lab and others have shown previously, the stimulation of MPS by protein is dependent on protein quality, leucine content, the rate of absorption as well as the absolute amount of and timing of protein ingestion.

❑To date, little work has been done to understand the effects of protein quality/source on the preservation of FFM during diet induced weight loss. Nor have the mechanisms behind this effect been fully explored.

Purpose and Hypothesis

❑ The purpose of this study was to **investigate the effects of whey (WHEY) protein, soy (SOY) protein and a carbohydrate (CHO) control supplement on body composition and skeletal muscle gene expression following a 14-day hypo-energetic diet.**

❑ It was hypothesized that a greater preservation of FFM and augmentation of FM losses would be observed in the following hierarchical manner WHEY>SOY>CHO.

❑ The expression of skeletal muscle genes associated with mitochondrial metabolism would increase while increases in atrophy associated genes would be impaired in the same hierarchical manor WHEY>SOY>CHO.

Methods

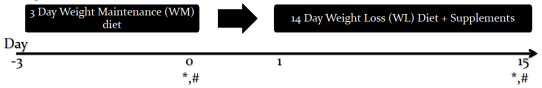
Participants
- 40 overweight/obese (BMI 28-40) subjects, (19 men and 21 women) participated in the study.

Dietary intervention and supplements
- Subjects energy requirements were assessed using the Mifflin St. Jeor equation with a sedentary activity factor. A registered dietitian designed the 3-day weight maintenance (WM) and 14d weight loss (WL) diets in 200kcal increments. Each participant was assigned to a WM and WL diet which most closely reflected an energy balance and a caloric deficit of -750kcal/d respectively. All food stubs were provided for the duration of the study.
- Supplements were consumed twice daily, between meals and consisted of whey protein (Iso chill 800, Agropur), soy protein (soypro950M, International Trade Company) or carbohydrate (maltodextrin). WHEY, SOY and CHO supplements provided a total of 54, 52 and 1.8 g protein/d respectively.

Body Composition
- Body composition was assessed using Whole body dual-energy X-ray absorptiometry (DXA) (QDR-4500A; Hologic, software version 12.31). Scans were completed on days 0 and 15 immediately prior to and following the hypo-energetic diet.

Methods

Study Time Line



Gene Expression
- RNA was isolated from skeletal muscle biopsies taken on day 0 and day 15 using TRIzol and a modified phenol/chloroform method.
- RNA concentration was determined spectrophotometrically at 260-280nm and then standardized to 50 ng/ul. Thereafter cDNA was synthesized using the high capacity reverse transcription kit (Applied biosystems).
- Real time qPCR was performed using a Eppendorf Mastercycler Realplex 2 with Syber green and custom designed primers.
- The Pflaffl method was used to quantify changes in gene transcripts with all values expressed as a fold change relative to pre intervention expression levels.

Statistics
- Changes in anthropometric and blood measures were compared using a one-factor (treatment) ANOVA.
- Changes in gene expression were quantified using the ΔCt scores with a 3 by 2 factor (group by time) repeated measures ANOVA.
- Values are displayed as mean ±S.E., with statistical significance set at $p < 0.05$.

Results

Participants
❑ There were **no significant** differences between subject's age (50.8 ± 1.2 yr), height (169.2 ± 1.6 cm), body weight (102.4 ± 3.1 kg), BMI (35.5 ± 0.8), FFM (62.2 ± 2.1 kg) or FM (37.5 ± 1.7 kg) between groups at baseline.

Dietary intervention
❑During the WM period total calorie (2,321 ± 73 kcal/d), protein (96.8 ± 2.5 g/d), carbohydrate (312.5 ± 10.7 g/d) and fat (78.8 ± 2.5 g/d) intake did not differ between dietary groups.

❑Self-reported energy deficit during the 14d weight loss period for WHEY, SOY and CHO averaged -680 ± 37, -750 ± 38 and -832 ± 39 (kcal/d) respectively, with a significant ($P < 0.05$) difference between WHEY and CHO.

❑**Protein intake during the WL period was significantly (P<0.05) greater** in WHEY (1.28 ± 0.04 g/kg*d)] and SOY (1.23 ± 0.05 g/kg*d)] over CHO (0.74 ± 0.02)] with no significant difference between WHEY and SOY.

Changes in Body Composition
❑ There were **no significant group versus time effects** for any body composition measure ($P > 0.10$).

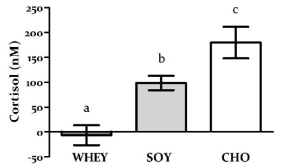
❑ A **significant (P<0.05) main effect for time was observed for all measures of body composition** with all subjects exhibiting an average change in body mass (-2.29 ± 0.18 kg), fat mass (-1.49 ± 0.16 kg), fat free mass (-.81 ± 0.24 kg), abdominal fat mass (-0.53 ± 0.06 kg) and body fat percent (-0.66 ± 0.16 %).

Results

Gene expression
❑ **No significant effects** for changes in gene expression of SIRT-1, PGC-1$^{\alpha}$, MuRF-1, MAFbx, Foxo1, Foxo3a.

Blood Markers
❑ There was **no significant time or group effect for serum TNF-a, IL-6, CRP, or IGF-1.**
❑ Serum **cortisol** remained **unchanged in WHEY** (-6.6 ± 19.8 nM) but **increased significantly** and in a hierarchical manner **in SOY** (98.4 ± 14.4 nM) **and CHO** (179.8 ± 31.6) with a significant group effect ($P<0.05$) (figure 1, different letters $^{a/b/c}$ represents a significant effect at $P<0.05$).

Change in Serum Cortisol Levels Following Weight Loss



Discussion

❑ There was no effect of WHEY or SOY supplementation on body composition measures. The acute nature of this experiment maybe responsible for a lack of treatment effect as Wycherley *et al.*, 2012 only demonstrated a main effect for higher protein diets on the preservation of FFM over prolonged periods (≥12wk).

❑ The stepwise increase in basal cortisol levels within the WHEY, SOY and CHO conditions may reflect a mechanism by which whey protein can help preserve FFM during caloric restriction (CR). As cortisol is a known catabolic agent, the infusion of which has been shown to increase MPB (Gore *et al.*, 1993).

❑ There was no time or treatment effect for changes in genes associated with mitochondrial metabolism. This finding may again be due to the acute nature of the study as contrasting findings have been observed with longer durations (Civitares *et al.*, 2007).

❑ Similarly the lack of a time or treatment effect in the expression of MuRF1, MAFbx, FOXO1 and FOXO3a is in contrast to many murine models of energy restriction. Further investigation into the regulation of these genes and the regulation of MPB in human models of energy restriction is required.

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