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The Role of Dairy Protein and Dietary Calcium in Weight Regulation and Glucose Homeostasis

by

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Abstract

Objective: To determine the role of complete dairy foods, individual dairy protein fractions (casein, whey, and leucine), and calcium (Ca) on weight management and glucose homeostasis in Sprague-Dawley diet-induced obese (DIO) rats.

Methods: Three main studies were performed. Dietary interventions that varied in protein source (complete dairy, casein, whey, or casein+leucine), Ca level [0.67% (LC) or 2.4% (HC)], and energy density [normal energy (NE) or high fat/high sucrose (HFHS)] were tested. Rats consumed the experimental diets either *ad libitum* or energy restricted to 70% of control *ad libitum* (ER) intake. Body composition was measured via dual-energy x-ray absorptiometry (DEXA). A meal tolerance test (MTT) or oral glucose tolerance test (OGTT) and/or an insulin tolerance test (ITT) were performed. A euglycemic-hyperinsulinemic clamp was also utilized in the final study to measure whole body glucose uptake. Tissues were collected and gene expression was assessed using real-time PCR and microarray.

Results: Consistently within and between all studies, complete dairy treated rats had improvements with body weight and composition. During *ad libitum* feeding, the complete dairy treatment promoted greater fat loss and during ER complete dairy accelerated weight loss with the maintenance of lean body mass (LBM). Additionally, supplementation with HC resulted in increased benefits to body composition. Measurements of glucose homeostasis and insulin sensitivity varied between protein source and Ca, with leucine improving whole body glucose clearance, complete dairy improving baseline measurements of insulin and glucose, and HC resulting in lower

glucagon secretion. Hepatic expression of genes related to metabolism (PPARγ, PGC1α, PEPCK, FAS, ACC, and SREBP1c) were altered by protein source and Ca level.

Conclusions: In Sprague-Dawley DIO rats, complete dairy and Ca attenuated weight gain during *ad libitum* feeding and accelerated weight loss during ER. The precise bioactive component in complete dairy foods responsible for the metabolic changes observed is unknown, as the individual protein fractions of dairy (casein and whey) did not appear to have analogous effects on metabolic parameters. A diet including complete dairy foods may provide some protection against susceptibility to obesity and improve weight loss during restriction.

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Dedication

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Thank you for your support.

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List of Symbols, Abbreviations and Nomenclature

Symbol	Definition
$[Ca]_i$	Intracellular calcium concentration
1, 25-$(OH)_2$ - D	1,25-dihydroxy vitamin D
AA	Amino acid
ACC	Acetyl-coenzyme A carboxylase alpha
ACEi	Angiotensin converting enzyme inhibitor
AIN	American Institute of Nutrition
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
AUC	Total area under the curve
BCAA	Branched chain amino acid
BMD	Bone mineral density
BMI	Body mass index
BP	Blood pressure
BSS	Behavioural sequence of satiety
Ca	Calcium
CCK	Cholecystokinin
DEXA	Dual energy x-ray absorptiometry
DIO	Diet-induced obese
EE	Energy expenditure
ER	Energy restriction
ERK1	Mitogen activated protein kinase 1
FAS	Fatty acid synthase
Fat OX	Fat oxidation
FF	Fecal fat excretion
FM	Fat mass
G6Pase	Glucose - 6- phosphatase
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase

GK	Glucokinase
GLP-1	Glucagon-like peptide 1
GSK3β	Glycogen synthase kinase 3 beta
HC	High calcium diet (2.4%)
HFHS	High fat high sucrose diet (4.6 kcal/gram)
HOMA-IR	Homeostatic model of assessment (insulin resistance)
ITT	Insulin tolerance test
KEGG	Kyoto Encyclopaedia of Genes and Genomes
LBM	Lean body mass
LC	Low calcium diet (0.67%)
MetS	Metabolic syndrome
mTOR	Mammalian target of rapamycin
MTT	Meal tolerance test
NE	Normal energy density diet (3.6 kcal/gram)
NHANES	National Health and Nutrition Examination Survey
OGTT	Oral glucose tolerance test
PEPCK	Phosphoenolpyruvate carboxykinase
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator-1 alpha
PPAR	Peroxisome proliferator-activated receptor
PTH	Parathyroid hormone
PYY	Peptide tyrosine tyrosine
RCT	Randomized control trial
RT	Reverse transcription
rt-PCR	Real time polymerase chain reaction
SEM	Standard error of the mean
SREBP1c	Sterol regulatory element binding protein 1c
TA	Taste aversion
TG	Triglyceride
UCP	Uncoupling protein

Chapter One: Introduction

1.1 Background

Obesity affects people of all age, race and socioeconomic status with the prevalence increasing at an alarming rate [1, 2]. It is the most common metabolic condition in developed countries and has become a problem of worldwide significance. The World Health Organization (WHO) has consequently labelled obesity a global epidemic. In Canada, almost 2/3 of adults are classified as overweight or obese with 36.1% of adults being overweight and 23.1% being obese [3]. This is comparable to rates found in The United States, The Middle East, Central Europe, and Eastern Europe [2]. In addition to obesity, the metabolic syndrome (MetS), a conglomeration of medical disorders that increase the risk of developing cardiovascular disease and type 2 diabetes, has also become increasingly prevalent; with an estimated 14.4% of the Canadian population being affected [4]. The increased prevalence of metabolic disease has been shown to have serious economic implications. In Canada, it was estimated that health care costs associated with obesity were $4.3 billion in 2005 [5]. The majority of this spending is related to the treatment of co-morbidities such as type 2 diabetes, hypertension, and coronary artery disease.

Obesity is defined as an excess of body fat [2], simply quantified by the body mass index (BMI), which is a ratio of body mass (kg) to height squared (m^2) [6]. A BMI between 20 and 25 is considered healthy, a BMI greater than 25 is considered overweight, and a BMI of 30 or greater is considered obese [6]. A BMI greater than 25 is associated with increased health risks, decreased quality of life, increased morbidity, and

premature death [1, 2, 7]. Medical complications associated with obesity include, but are not limited to: hypertension, type 2 diabetes, obstructive sleep apnea, cancer, arthritis, respiratory dysfunction, and dyslipidemia [7, 8]. The etiology of obesity is multi-factorial and occurs as a result of environmental, genetic, social, cultural, physiological, and psychological causes [8-11]. Despite this complexity, increases in body weight manifest only when energy intake exceeds energy expenditure [12]. The contribution of lifestyle towards the development and maintenance of obesity is well documented. A lack of physical activity and an increased consumption of foods that are calorically dense, high in fat, and high in simple sugar, are major contributing factors [1, 9, 13-15]. Although balancing energy consumption with energy expenditure is, in theory, a simple process, it has been shown to be surprisingly difficult – the failure rate of weight loss diets is extremely high with over 90% recidivism and failure to maintain the weight loss [12]. There is an urgent need for nutritional strategies that effectively support body weight management but do not require or produce a drastic negative energy balance. Furthermore, it is important that loss of lean body mass (LBM) during weight loss is reduced in order to minimize decreases in basal metabolic rate.

Metabolic syndrome (MetS) is a cluster of symptoms that predispose an individual to cardiovascular disease and type 2 diabetes. Clinical diagnosis of MetS generally includes presentation with three of the following: abdominal obesity, hypertension, fasting hyperglycaemia, or dyslipidemia (elevated triglycerides and LDL cholesterol and/or decreased HDL cholesterol) [16]. The onset of MetS is intimately linked to diet and lifestyle. A recent study found that a Western style diet (high red meat, fried food, processed meats, and refined grain intake) was associated with an increased

risk of MetS. Interestingly, a diet rich in whole grains, nuts, and fruits and vegetables showed no relationship to the onset of MetS, but those who consumed more dairy foods were less likely to develop MetS [17].

Milk and other dairy foods comprise an important component of Canadian diets. Dairy foods not only provide a good source of high quality protein, but also a rich assortment of micronutrients. Dairy products are the major source of calcium (Ca) in the North American food supply, providing greater than 70% of dietary Ca [18]. Dairy foods also contribute substantial amounts (>10% of the daily value) of phosphorus, riboflavin, potassium, zinc, magnesium, and vitamins A, B_{12}, and D [18]. Results from the Canadian Community Health Survey [19] found that the majority of Canadian adults do not meet their adequate intake (AI) for Ca (1000mg/day), suggesting that there is low consumption of dairy foods. There has been considerable epidemiological data published that suggests low dairy food intake and/or low Ca intake is associated with greater fat mass (FM) and predicts risk of metabolic disturbances [17]. The concomitant increase in consumption of soda confounds the relationship of declining dairy food intake to deteriorating metabolic health [18].

The concept that dietary intake of dairy foods and calcium (Ca) modulate energy metabolism and may attenuate symptoms of MetS has grown in the last six years with supporting evidence ranging from observational studies to randomized control trials (RCT). Dietary Ca is now a well recognized factor in modulating various physiological processes associated with metabolic health [20-24]. The relationship between Ca and weight control was first observed by Zemel *et al*, in a clinical trial that examined the effect of eating dairy products (yogurt) in hypertensive African-American men [21]. This

study reported that men with the greatest Ca intake - 1000mg as compared to 400mg - also had the least FM. Subsequent to this study, epidemiological and observational studies, but no RCT, have shown an inverse relationship between the amount of Ca consumed and adiposity, type 2 diabetes risk, and blood pressure (BP) [24-27]. Of interest, when studies examined dairy sourced Ca, the effects on body weight regulation were even more pronounced than when solely supplementing with elemental Ca [22, 23, 28], suggesting a synergistic effect between dairy foods and Ca. Currently, many questions remain regarding the possible mechanisms through which dairy and Ca intake affect obesity and why Ca consumed specifically as a component of dairy food might be more efficacious than Ca from other sources.

In summary, there is convincing evidence that dairy foods and Ca play a role in health and disease status. The particular components of dairy associated with these effects are unknown and require identification. This knowledge would allow for the development of updated dietary recommendations designed for the improvement of metabolic health.

1.2 Significance of Study

This body of work was designed to provide increased insight into the bioactive compounds found in dairy foods and the potential mechanism(s) behind their effectiveness in improving metabolic health. A deeper understanding of this relationship has the potential to aid in the development of new dietary and pharmacological agents for health concerns associated with MetS and obesity. Furthermore, once the mechanism of action is determined, an updated recommended amount of dairy food and Ca can be

prescribed to promote healthy body weights. With obesity and its associated co-morbidities increasing across the Canadian population, it is of vital importance to establish simple, non-restrictive dietary interventions that will help promote healthier body weights. It is well established that large changes to diet are difficult to maintain in the long term; however, small changes in food intake including foods containing bioactive molecules that assist with the prevention of weight gain could be one widespread approach.

1.3 Objectives

The objectives of this work were:

(1) to examine the role of various dairy proteins (complete dairy, casein, or whey) and Ca supplementation in preventing weight gain during high fat, high sucrose *ad libitum* feeding in a diet-induced obese rat (DIO) model;

(2) to examine the role of various proteins (complete dairy, casein, whey, or soy) and Ca supplementation in promoting weight loss in an energy restricted DIO model; and

(3) to determine the role of dairy proteins and/or amino acids (complete dairy protein, casein, and casein supplemented with leucine) combined with Ca supplementation on insulin sensitivity in a DIO model.

1.4 Hypotheses

Recent evidence suggests that dairy foods have bioactive components that improve body weight and insulin sensitivity. It is hypothesized that a component of the milk protein is responsible for these beneficial effects by means of partitioning nutrients towards lean mass and away from fat mass. Specifically, whey protein will improve insulin sensitivity and assist with the maintenance of a healthy body weight via alterations in metabolic pathways associated with lipid and protein metabolism. Additionally it is hypothesized that leucine, a branched chain amino acid, found in high concentrations in whey protein is responsible for the beneficial metabolic effects. Moreover, it is thought that the diets supplemented with high calcium will further enhance weight management.

Together, the three experiments described within will provide insight into how individual protein fractions of dairy (casein, whey, and leucine) promote metabolic health compared to complete dairy foods. Furthermore, the potential synergistic effect of Ca on these protein sources will be examined.

1.5 Presentation

This dissertation contains several chapters arranged in the following manner: Chapter 1 is a brief introduction to the thesis and presents the significance, objectives and hypotheses; Chapter 2 is a review of the literature; Chapter 3 is a published manuscript summarizing the results of pilot work regarding taste aversion to Ca and satiety responses to various proteins in Sprague-Dawley rats. Chapter 4 represents a published manuscript summarizing the role of dairy food and Ca consumption in the prevention of weight gain

during *ad libitum* high fat feeding; Chapter 5 is a manuscript currently awaiting a decision on a revision, summarizing the effect of dairy food and Ca consumption on the promotion of weight loss during energy restriction; Chapter 6 is a manuscript outlining the role of leucine, dairy foods, and Ca supplementation in insulin sensitivity. Chapter 6 also summarizes gene data from a microarray study examining the role of leucine and dairy foods in hepatic gene expression. Chapter 7 provides an overall discussion and conclusions to the thesis and presents suggestions for future research. While Chapters 3 and 4 have been published, Chapter 5 is currently submitted and awaiting a final decision on acceptance. Each chapter provides an introduction, details of the methods, results, and a discussion. To supplement the material provided in Chapters 1 – 7, there are five appendices. Appendix A provides a concise summary of all published human RCT (between 1994-2009) that had an *a priori* hypothesis of how dairy foods and Ca affect metabolic parameters. Appendix B provides detailed dietary composition for all experimental diets used during studies presented in this thesis. Appendix C is a list of primers used for real-time polymerase chain reaction. Appendix D is a summary of microarray data for all metabolic genes altered by treatment with complete dairy protein or leucine. Appendix E is a schematic of hepatic genes that were altered by dietary treatments. Appendix F outlines the diet-induced obese rat selection.

Chapter Two: Literature Review

2.1 Introduction

This literature review examines the potential link between dairy foods and dietary calcium (Ca) and the treatment and prevention of obesity. It will begin with a summary of human studies, followed by animal studies and then proposed mechanisms of action. Throughout this dissertation, various terms are used to describe Ca and dairy foods. To differentiate between sources of Ca, the following terminology has been used: Ca or elemental Ca refers to Ca consumed without any dairy foods (found in supplements and non-dairy foods such as fortified orange juice); dairy-sourced Ca, dairy foods, or dairy Ca refers to any Ca that is consumed as part of dairy foods (milk, yogurt, or elemental Ca enrichment of dairy foods). Complete dairy refers to treatments made with skim milk powder as the protein source.

2.2 Current Knowledge

2.2.1 Evidence from Human Studies

In the early 1980's McCarron *et al* [29] first reported a relationship between dietary Ca intake and metabolic health in humans. This group analysed the National Health and Nutrition Examination Survey-I (NHANES-1) data and showed that there was an inverse relationship between Ca intake and body weight. Subsequently, a randomized control trial (RCT) by Zemel *et al* examining the role of yogurt in BP regulation serendipitously found that an increase in dietary dairy-sourced Ca was associated with

lower adiposity [30]. Since the publication of this study, there has been a rapid increase in the number of human studies examining the role of dairy foods and/or Ca in weight regulation and in components of the MetS.

The evidence from human studies comes from a variety of sources including longitudinal and prospective examinations of cohort data to RCT and meta-analyses. The following review will discuss only human studies originally designed with weight, body composition, energy metabolism, MetS, or weight regulatory mechanisms as end points. Many studies designed with other outcome variables (i.e. bone mineral density) have analyzed the role of dairy foods and/or Ca in metabolic parameters *post-hoc*. These studies will not be included in this review as studies performed with other *a priori* outcome variables are often not designed to adequately test metabolic variables and are inappropriate for comparison. For example, a weight change of 1 kg/year may be important for weight management, but large groups and lengthy trials are required for sufficient power. Many of the trials performed with bone health do not meet these criteria.

There is extensive epidemiological data supporting the role of dairy foods and Ca in the treatment and prevention of the MetS. Following the initial findings of the NHANES-I data, Zemel *et al* [21] found the same inverse relationship when examining the NHANES-III data. Moreover, a dose response to Ca was found with those individuals with the greatest Ca intake being the leanest, and those who consumed the least Ca showing the highest risk of being overweight or obese [21]. More recently, Pereira *et al* [26] examined the associations between dairy intake and general health as part of the Coronary Artery Risk Development in Young Adults (CARDIA) Study. This

is an American, multi center, longitudinal study that began in 1985 and includes 5115 subjects. Using four week diet history questionnaires to assess dietary intake it was shown that individuals who were overweight consumed significantly less Ca than their lean counterparts [26]. It was also demonstrated that each daily serving of low fat dairy products resulted in a ~21% reduction in risk for developing insulin resistance. This effect was seen in overweight individuals but not in normal weight individuals, and was similar for men and women. Even more impressive are two large prospective studies linking dairy consumption to a reduction in insulin resistance and incidence of type 2 diabetes. The Health Professionals Follow-up Study that prospectively examined 41,254 men found that for each additional serving of low-fat dairy product per day, risk of developing type 2 diabetes decreased by 9% [31]. Similarly, in 37,183 women, prospective risk of type 2 diabetes was decreased by 4% per daily serving of low fat dairy food [32]. Additionally, Lin *et al* [25] found that following a two year exercise intervention designed to look at the effects of exercise and diet on body composition, women aged 18-31 who had the highest Ca intake gained less weight; however, this only applied if they fell below the caloric median (<1876 kcal/day) for energy intake, suggesting that the effect of Ca is dependent on caloric intake.

Some epidemiological studies have examined dairy food and/or elemental Ca consumption and found no relationship between Ca intake and obesity or symptoms of MetS. Therefore, it is possible that consumers of dairy food are simply making better food choices and have an increased awareness of what constitutes a healthy diet. Additionally, those who drink more milk may also drink less soda and other calorie laden beverages [33]. However, based on data from the majority of cross-sectional,

prospective, and observational studies, it is reasonable to propose that low Ca intake is associated with an increased risk of being overweight or obese.

This hypothesis has spurred a recent surge in RCT. Since 2000, there have been 30 human RCT – 10 with crossover design and 20 with parallel design – which have examined the role of dairy foods and/or Ca in regards to metabolic parameters (Appendix A). Of the 10 randomized crossover studies [34-44] (Table A.1), seven found consumption of Ca or dairy foods to be beneficial. Of all the outcome variables measured in these studies, increased fecal fat excretion (FF) (3 out of 3 studies) [34, 35, 37] and improved blood lipids were most consistently demonstrated (2 out of 2 studies) [41, 44], and were found for both dairy foods and elemental Ca. Less convincing is the data on 24-hour energy expenditure (EE) and fat oxidation (fat OX), as they do not appear to be regulated by either dairy foods or Ca. The longest study in duration (five weeks; n=10), found no difference in fat OX or EE with consumption of an 800mg Ca supplement once per day versus a placebo [43]. The crossover design of these studies provides compelling evidence because individual subjects act as their own controls. Due to the short-term nature of most of these studies, however, none of them used body weight or body composition as an endpoint. To adequately determine the role of dairy foods and Ca in long-term weight management, RCT with parallel design have also been used. These studies are usually much longer in duration and provide a better representation of long-term outcome.

From 1994-2009, there have been 20 human RCT – with parallel design – that have focused on metabolic regulation by Ca and/or dairy foods (Appendix A, Table A.2). Of these studies, 10 had treatments with only elemental Ca supplementation [45-54], nine

had treatments with only dairy products [55-63], and only one had multiple treatment groups comparing dairy Ca and elemental Ca. In the 10 studies that examined elemental Ca with weight as an endpoint, all found a positive or neutral role for Ca. Six of these studies found no difference [45, 47, 50, 52, 54, 64]; in two studies, subjects gained less than expected based on energy intake [48, 51], and in the final two studies, if the subjects were initially low Ca consumers, they lost weight [46, 49]. This is similar to the nine studies using dairy foods: one study showed no difference [61]; three studies had subjects who gained less weight than expected [50, 59, 62]; and five studies demonstrated weight loss or improvement in body composition [55-58, 60]. In the study examining dairy and Ca, there was a synergistic effect with greater weight loss occurring when Ca was consumed with dairy foods [65]. In all of the parallel studies, there was a dose response with higher Ca consumption resulting in greater weight loss, regardless of source (elemental or dairy).

Two unique RCT using milk as the sole food supports the role of dairy foods in nutrient partitioning. Summerbell *et al* [55] found that in obese outpatients, a 16-week diet consisting solely of milk resulted in significantly greater weight and fat loss than two other eucaloric diets. This unique design allowed for examination of data with limited confounders. A similar study in 1989 found that obese women (n=22) who consumed a diet solely of milk compared to an isocaloric liquid control diet for 24 weeks maintained more LBM during weight loss than the control group. The study required subjects to have their jaws wired shut to ensure strict compliance [66]. At the end of this study, no nutritional deficiencies were identified in subjects despite having consumed strictly milk for six months.

More relevant to free-living individuals, Barr *et al* [59] recently examined the health effects of dairy foods for elderly adults by adding three 8-ounce servings of skim or 1% milk to their usual diet [65]. The control group maintained their normal diet. After 12 weeks, the dairy intervention group had improved nutritional status and an increase in weight of 0.6 kg compared to the control group. The 0.6 kg weight gain was less than expected for the extra calories consumed from the 24 ounces of milk per day [59]. This study suggests that Ca intake – or more specifically dairy consumption – may assist in weight management and the partitioning of nutrients in favour of lean versus fat mass.

Similarly, a RCT by Zemel *et al* [60] demonstrated that obese subjects, when provided with an energy reduced diet (-500 kcal per day) and 1100 mg of Ca per day, experienced 22% greater weight loss compared to a low Ca (<500 mg Ca/day) control group. The higher Ca treatment also resulted in a 61% increase in fat loss. However, the benefits were only seen in subjects who had low habitual Ca consumption prior to the treatment. A second study by this group used a similar cohort (obese, low dietary Ca consumers) and randomized them to either a control group that consumed less than one serving of dairy food per day, or to a treatment group that consumed three servings of dairy per day. The high-dairy group experienced a twofold greater weight and fat loss compared to the low-dairy treatment. Although most trials have found a positive role of dairy and Ca in weight management, some have not. A recent study with overweight and obese adults found that 1500 mg of elemental Ca supplementation for two years did not result in any change to the metabolic parameters examined, including prevention of weight gain [45].

In conclusion there is good, although not unanimous evidence for the role of dairy and elemental Ca in regulation of metabolic parameters. Future studies need to concentrate on addressing the limitations of current RCT. First, many studies have used a single daily Ca supplement (i.e. 800-1000 mg/dose), but because of incomplete absorption which occurs with once a day high Ca supplementation, this amount of Ca may not contribute towards the desired effect [67]. Smaller, more frequent doses result in better Ca bioavailability. Second, measurements of serum Ca concentrations would be beneficial for determining the degree of Ca absorption given that a portion of Ca intake is lost through FF; not a single study has examined serum Ca. As well, many studies use *either* elemental Ca or dairy foods. It is rare that a study has used both treatments and compared the response of dairy foods to elemental Ca. This is a major weakness, as there is evidence of a synergistic effect of dairy foods and Ca. Studies also need to address the relationship between low Ca consumers and dose response, as there is significant evidence from RCT showing that only individuals who were habitually low Ca consumers (<500mg Ca/day) prior to the study will see an improvement in metabolic parameters with increased dairy foods and Ca consumption. Finally, there are very few studies examining the potential mechanisms of action. More detailed studies focusing on mechanistic roles are needed to design future RCT with appropriate outcome measures.

2.2.2 Evidence from Animal Studies

Experimental support for an association between Ca intake and obesity has also been obtained from various rat and mouse models [20] [22]. The following is a brief summary of studies that have examined the role of elemental Ca or dairy sourced Ca in weight regulation.

Shi *et al* [20] examined the role of both elemental and dairy Ca in weight regulation of the aP2-*agouti* mouse model. This animal model over-expresses the *agouti* gene that is normally involved in coat pigmentation in mice; the wild type *agouti* gene produces black fur with subapical yellow coloring. When the *agouti* gene is mutated at the locus, it is expressed in an ectopic manner, which causes the mutated mice to develop pure yellow coats. More importantly, these mutants also become obese, insulin resistant, and hyperinsulinemic [68]. The *agouti* gene is also expressed in human adipocytes which allows *agouti* mouse research to be theoretically applied to humans [28]. Six-week old aP2-*agouti* mice were provided *ad libitum* a low Ca (0.4%), high fat and high sucrose (HFHS) diet for six weeks [20]. Over this period, body weight increased 29% and total fat pad mass increased twofold. The mice also had a 100% increase in adipocyte intracellular calcium $[Ca]_i$ demonstrating that diet could alter this intracellular marker of homeostasis. $[Ca]_i$ is directly related to excess fat gain in this mouse model and it has been suggested that elevated adipocyte $[Ca]_i$ may promote lipogenesis and inhibit lipolysis [28]. Following the six-weeks of *ad libitum* feeding, mice were energy restricted provided with one of the following diets: 0.4% elemental Ca, 1.2% elemental Ca, 1.2% dairy Ca, or 2.4% dairy Ca. The 1.2% Ca group experienced greater reductions in body weight when the Ca was consumed with dairy. With the 1.2% elemental Ca diet, the mice had a 19% reduction in body weight and in the 1.2% dairy Ca diet, the mice lost 25% [20]. The 2.4% dairy group lost 29% of their body weight. A decrease in adipocyte $[Ca]_i$ which was correlated to Ca intake and weight loss was also observed. These data allude to the presence of synergistic compounds in dairy foods that work together with Ca to promote weight loss. As a measurement of adipocyte lipid turnover,

this study also measured fatty acid synthase (FAS), an enzyme that inhibits lipolysis and promotes lipogenesis. In the 1.2% elemental Ca group, FAS activity decreased 35% and in the 1.2% and 2.4% dairy Ca groups, FAS was further reduced by 63% and 62% respectively. Furthermore, it was noted that as dietary Ca increased, lipolysis also increased. Although the largest increase in lipolysis was seen in the 2.4% dairy Ca group [20], there was still an increase in the 1.2% elemental Ca group, with the 1.2% dairy Ca being intermediate. This would suggest that although elemental Ca plays a role in regulation of lipogenesis, dairy Ca is of greater importance. In conclusion, this study found that the greatest weight loss was seen in the mice that consumed a diet high in dairy Ca, implicating a bioactive component(s) in dairy foods in weight regulation.

This study by Shi *et al*, however, did not provide data on body composition or body size. This is an important consideration given that the mice were young and undergoing rapid growth. Consequently, it is unknown whether the changes in body mass are due to a restriction of normal growth or loss of lean body mass (LBM) during the high Ca protocol or whether the observations were strictly due to changes in fat mass (FM)

More recently, Sun and Zemel [22] examined the effects of elemental Ca and dairy Ca on preventing weight regain during *ad libitum* HFHS re-feeding in the aP2-*agouti* mouse. Six-week old mice were provided *ad libitum* HFHS food for a six-week obesity induction period. This was followed by six weeks of 70% energy restriction (ER) with a diet consisting of normal calorie density (NE) and 1.3% elemental Ca. The final six-weeks involved *ad libitum* consumption of one of five diets: i) HFHS 0.4% Ca, ii) NE 1.3% Ca, iii) NE 1.3% dairy Ca (milk), iv) NE 1.3% dairy Ca (yogurt), or v) NE

0.4% Ca. Although there was no statistical difference in food consumption during the weight regain phase there was significant variability between the 0.4% Ca and 1.3% Ca groups in terms of weight gain and fat pad mass. Mice consuming the 0.4% Ca diets experienced a rapid regain of weight (27%) and fat pad mass (487%) [22]. Conversely, mice in the 1.3% Ca group showed only ~50% of this weight regain. Furthermore, the diets containing 1.3% dairy Ca (milk or yogurt) were associated with less weight gain than any other group and had a significantly lower accumulation of fat pad mass. It was also demonstrated that dietary Ca intake was inversely proportional to adipocyte $[Ca]_i$ [22]. Increasing dietary Ca stimulated muscle UCP3 and adipose UCP2 expression resulting in an increase of core body temperature and thermogenesis. In addition, adipocyte FAS mRNA decreased with increased Ca consumption.

Similar to the study by Shi *et al* [20], this investigation did not provide measures of body size or mass following the ER phase (phase ii). Resultantly, with no baseline measurements, it is not possible to determine whether there was a difference in weight regain between the groups, only whether the absolute body weights were different. Additionally, body composition was not measured, therefore the relative contribution of lean and fat mass to the weight gain is not known. Furthermore, body composition was not reported following each phase in the study which would have confirmed the intended outcome in each of the phases of fat gain and fat loss. Finally, the study did not provide post weight loss (phase ii) fat pad mass, therefore their measurement of fat pad regain does not accurately provide detail on regain, only the absolute amount after all three phases. Based on the results of this study, it can be concluded that diets high in Ca may assist in the prevention of weight regain, with dairy sourced Ca being more effective in

inhibiting weight regain than elemental Ca; however, these data must be interpreted with care as per the limitations discussed above.

To build upon the this work, Zhang *et al* [69] proposed a study that was designed to investigate the role of Ca on weight regulation and was intended to be more rigorous than the studies from Sun and Zemel [22] and Shi *et al* [20]. There were two principle components to this study: Part 1 involved 90 female, C57BL/6J mice divided into six groups - a normal (NE) or high energy (HFHS) diet supplemented with 0.2%, 0.6%, or 1.8% elemental Ca. The mice were 36 weeks old and had consumed a NE or HFHS diet since weaning. Mice were divided into groups according to their previous diets (NE or HFHS). Prior to the Ca supplementation, mice fed the HFHS diet were significantly heavier than the NE diet mice. After six-weeks of Ca supplementation, there was no significant difference found for body weight or body fat (%) between Ca groups within each of the NE or HFHS diets.

Part 2 used 12-week-old female Sprague-Dawley rats that were fed a NE or HFHS diet (0.6% Ca) for 10 weeks. Thirty-six rats from the NE group were then subdivided into three groups of 12 that continued to eat a NE diet with 0.2%, 0.6%, or 1.8% elemental Ca. The 36 rats that had been fed the HFHS diet were then also subdivided into three similar groups of 12 and fed for 52 days.

Rats in the HFHS group with 1.8% Ca gained significantly less weight than the rats in the HFHS group with 0.2% Ca and 0.6% Ca. Caloric intake was not significantly different between the HFHS groups. Interestingly, the group fed the NE diet with 1.8% Ca not only failed to exhibit lower weight gain, but also had significantly less carcass protein than the groups fed NE diets supplemented with 0.2% Ca and 0.6% Ca. Fat mass

(FM) was not significantly different within the NE groups or within the HFHS groups. Additionally, the rats fed the 1.8% Ca had significantly higher concentrations of plasma 1,25-dihydroxy vitamin D (1, 25-$(OH)_2$ – D) and significantly lower concentrations of parathyroid hormone (PTH) than rats fed the 0.2% Ca and 0.6% Ca diet. Furthermore, rats fed the low Ca, HFHS diet had lower PTH and 1, 25-$(OH)_2$ - D than the equivalent Ca group in the NE groups. This would suggest that total calorie intake may influence calcitrophic hormone levels.

To attempt to control for growth confounders, Zhang *et al* [69] used adult rodents. Resultantly, this study looked at whether the diets reduced body weight gain by acting on body composition as opposed to retarding natural growth and development. Unfortunately, Zhang *et al* [69] did not provide a measurement of body size that is independent of fat gain. This additional data would be of interest given that absolute body weight did not change significantly but protein content did. The results suggest that prevention of weight gain was greater in lean rats than obese rats, which is similar to the studies by Shi *et al* [20] that show that Ca supplementation with ER causes greater weight loss. Zhang *et al* [69] did not test a 2.4% Ca diet; therefore the dose response of Ca that was seen in Shi *et al* [20] and Sun and Zemel [22] was not examined.

Other groups have published studies focusing on the specific component of dairy that may be affecting weight regulation. Pilvi *et al* [70] examined the role of casein and whey on body weight in C57BI/6J mice. Nine-week old mice were divided into three groups and provided *ad libitum* one of the following diets for 21 weeks: (i) 1.8% Ca/whey protein, (ii) 1.8% Ca/casein protein, and (iii) 0.4% Ca/casein protein. At the end of the 21-weeks, the 1.8% Ca/whey group had lower total weight gain and body fat

than both other groups, even with greater food intake. The total amount of fecal fat excretion (FF) was greater for the whey treatment; and serum concentrations of $1,25(OH)_2-D_3$ and PTH were lower in the two 1.8% Ca treatments (casein and whey) compared to the 0.4% Ca treatment. This well-designed study provides important insight into understanding the role that various components of dairy foods and Ca play in weight regulation. A study with complete factorial design (1.8% Ca for both whey and casein and 0.4% Ca for both whey and casein) would have been a positive addition.

Pichon *et al* [71] further demonstrated the role of whey or whey sourced bioactive components in weight regulation. Twelve-week old Wistar rats were provided with one of 10 diets designed to examine the relationship between various milk fractions (complete dairy, whey, or β-lactoglobulin-enriched fraction) on weight regulation. After 25 days of *ad libitum* feeding, the data, taken together, suggest that the whey fraction and the β-lactoglobulin performed best at maintaining LBM while still promoting fat loss. The weight loss profile of these two diets was also associated with improved plasma insulin and leptin levels.

In conclusion, it is apparent that in animal models, there is evidence for a role of dairy foods and elemental Ca in energy homeostasis and maintenance of a healthy metabolic state.

2.3 Potential Mechanisms of Action

The evidence to support the beneficial role of dairy foods in metabolic parameters is strong, however, the mechanism of action responsible for these benefits is less clear. There are various processes that have been hypothesized to improve metabolic health,

and include: fecal fat excretion (FF); bioactivity of specific protein fractions of milk; regulation of adipocyte $[Ca]_i$; and alteration of satiety signals and hunger regulation.

2.3.1 Fecal Fat Excretion

Increased fecal fat excretion (FF) is beginning to gain popularity as the primary reason for enhanced weight loss with Ca supplementation. Dietary Ca alters fat absorption in the small intestine by binding with fatty acids or bile acids to form insoluble and indigestible soaps. The bound fats are then excreted with no known contribution towards energy intake [72]. Of the RCT that have examined this variable, all have found an increase in FF with Ca intake (Appendix A). A recent meta-analysis examining FF following dairy food and Ca supplementation found that FF is likely contributing to weight change. The authors found that consumption of 1241mg of Ca per day resulted in an additional 5.2±1.6 g of FF per day. Although no clear dose effect of Ca was found, the authors feel that there was sufficient FF to reduce weight re-gain and to attenuate weight loss [72]. This is an attractive and simple theory, however, it does not explain the body weight effects or various metabolic effects in full.

2.3.2 Bioactivity

A bioactive substance is defined as any food component that has a physiological effect beyond the pure nutritional value of the food [73]. The fat and the protein fractions of dairy foods are rich sources of bioactive components. Dairy fat contains conjugated linoleic acid (CLA) which is produced during the natural biohydrogenation of linoleic acid (18:2) by bacteria in the gut of ruminates [74]. Dairy foods are our richest source of CLA, providing about 70% of total dietary CLA. The potential bioactive effects of CLA include positive regulation of body composition via promotion of protein

synthesis, decreased lipogenesis, and anti-diabetogenic effects. The evidence in both human and animal models supporting these claims is equivocal and further research is needed to clarify the role of CLA in regulation of metabolic parameters. Milk protein also provides a wide array of bioactive peptides that are inactive within the protein sequence until enzymatically processed within the gastrointestinal tract [75-77]. The bioactive proteins of dairy foods are intriguing and quite vast, with roles that include immune system modulation, antimicrobial effects, anti-hypertensive effects, and mineral binding. There are three specific bioactive peptides of dairy foods that will be examined as part of this review since their role in metabolic health is well established: angiotensin converting enzyme inhibitors (ACEi), the branched chain amino acids (BCAA), and dairy derived opioid agonists. The bioactivity of milk protein might explain why many studies have found dairy Ca to have a greater role in weight management than elemental Ca alone [22-24].

2.3.2.1 Angiotensin Converting Enzyme Inhibitors

An inverse relationship between dairy foods and blood pressure (BP) was suggested in the early 1980's following many epidemiological studies that found individuals who consumed more dairy foods generally had lower BP. There is much evidence that Ca and potassium, two nutrients found in physiologically relevant amounts in dairy food, have a positive role in regulating BP. Examination of the NHANES-I data found that in over 10,000 adults, those who consumed >1000mg of dairy sourced Ca per day had a 40-50% reduction in the prevalence of hypertension. Of the 17 nutrients examined in this analyses, only Ca and potassium were significantly related to BP regulation (reviewed by Huth *et al* [18]). Many RCT have since assessed the role of Ca

and dairy foods in BP regulation and have found that dairy food, but not elemental Ca supplementation, is beneficial for hypertension. The seminal Dietary Approaches to Stop Hypertension (DASH) diet found that when mildly hypertensive Americans consumed one of three diets for eight-weeks, the diet that was rich in fruits, vegetables, and low fat dairy foods resulted in improved BP compared to diets rich only in fruits and vegetables or a typical "North American" diet. Subsequent work has further shown that dairy foods have an anti-hypertensive effect in some human populations [18, 78-81].

Angiotensin converting enzyme (ACE) is a regulatory peptidyl-dipeptidase of the rennin-angiotensin system that is a key player in BP and fluid and electrolyte balance [75]. ACE acts on angiotensin I to form angiotensin II. Angiotensin II is a potent smooth muscle vasoconstrictor and activation results in elevations in BP. By reducing ACE, the net effect is improved BP. Of relevance, many ACE-inhibitory proteins (ACEi) are formed during the digestion of dairy foods, both from casein (casokinins) and whey protein (lactokinins). Therefore BP is most likely modulated by Ca, potassium, and ACEi that are found in dairy foods.

Although the effect of ACEi may appear to be much more relevant to hypertension and not weight management, it has been shown that adipocytes have an autocrine/paracrine rennin-angiotensin system and that adipocyte lipogenesis is partially regulated by angiotensin II [82]. Moreover, angiotensin receptor blockers are capable of modulating adipose tissue in a rat model of type 2 diabetes (Otsuka Long-Evans Tokushima Fatty rats). When these rats were treated with an angiotensin receptor blocker (ARB L158809) for six months, various metabolic parameters improved. Fasting glucose, cholesterol, triglycerides (TG), and adiponectin levels increased and insulin

resistance decreased. It was found that adipocytes had a phenotypic change with an increase in small, newly differentiated fat cells. These small cells have been shown to be more insulin sensitive than older larger adipocytes. Since adipocytes are regulated via the angiotensin system, the ACEi bioactive peptides found in dairy foods may influence fat metabolism as well as blood pressure regulation [83].

2.3.2.2 Branched Chain Amino Acids

In the typical North American diet, branched chain amino acids (BCAA) make up about 20% of the amino acid content [84]. Dairy foods are a particularly rich source of the branched chain amino acids (BCAA) – leucine, isoleucine, and valine – making up 26% of total AA content. BCAA are essential for protein synthesis and serve as an energy source. They are also responsible for various other non-essential bioactive functions which include, but are not limited to glucose homeostasis [84] and respiratory system regulation. The actions of BCAA are limited by their availability and increasing the plasma concentration of BCAA is a useful method for increasing bioactivity. For example, when there is an excess of dietary BCAA, they may be used for bioactive functions, but when there are insufficient levels, only the essential physiological processes are completed [84].

The BCAA make up about 20% of an individuals diet but represent about 70% of the AA leaving the liver after a meal [84]. This is of significance because the three BCAA are used preferentially as fuel by muscle tissue. BCAA are unique in that skeletal muscle possesses a dehydrogenase that is specific for BCAA allowing the transamination and oxidation of the BCAA for muscle fuel. All other AA are metabolized by the liver prior to entering systemic circulation. BCAA are essential AA’s and resultantly are not

endogenously produced. Thus, consuming an adequate amount of these AA is important for protein balance.

It is not known exactly how BCAA uptake is regulated in the muscle, but it is hypothesized that an increase in circulating insulin may play a role [84]. Interestingly, the quantity of BCAA in skeletal muscle is representative of many physiological processes affecting protein metabolism such as: amino acid (AA) uptake from circulating blood to tissue, AA release from the tissue to the circulation, utilization for protein synthesis, and AA production from protein degradation. Whey is an interesting protein in that it has a high concentration of BCAA and is insulinotropic. Resultantly, following whey consumption, post-prandial insulin release may increase uptake of BCAA into skeletal muscle. Moreover, whey is a rich source of leucine (14% of total AA). Leucine may be the most relevant BCAA for protein synthesis [84] and some studies have suggested that leucine may play a role in energy metabolism [84]. Since dairy and whey are rich sources of leucine, an alteration in nutrient partitioning may occur that favours maintenance of LBM during ER. More over, recent evidence suggests that leucine directly activates the mammalian target of rapamycin (mTOR) pathway. The mTOR pathway is responsible for cell growth and proliferation, protein synthesis, glucose homeostasis, and many other basic cell survival roles.

2.3.2.3 Opioid Agonist Activity

Bovine milk is one of the richest food sources of exogenous opioid agonists or antagonists [85]. There are six specific proteins found in milk that after enzymatic breakdown produce a peptide sequence that demonstrates an opioid effect. The best studied is β-casein and its breakdown product the opioid agonist, β-casomorphin. β-

casomorphin appears to regulate gastrointestinal function by increasing transit time and enhancing water and electrolyte absorption; in turn creating the potential for greater vitamin, mineral, and bioactive peptide absorption. The role of β-casomorphins outside of the intestinal lumen is not known; after ingestion of β-casein, β-casomorphins are released into the lumen, but are not found in the plasma of human adults [85].

2.3.3 Role of Adipocyte Intracellular Calcium

Serum Ca concentrations are modulated by two calcitrophic hormones, 1, 25-$(OH)_2$ - D and parathyroid hormone (PTH). 1, 25-$(OH)_2$ - D is the active form of vitamin D and acts to regulate Ca absorption and bone mineral mobilization, and is vital to maintaining serum Ca concentrations [86]. In the event of a decrease in blood Ca ion concentration, signalling in the parathyroid glands increase the production and secretion of PTH. PTH then causes osteoclasts to release bone minerals through the process of bone resorption, which results in a higher serum Ca level. Additionally, when blood Ca levels decrease, endogenous production of 1, 25-$(OH)_2$ – D is increased in the kidneys. Although a main function of 1, 25-$(OH)_2$ - D is Ca homeostasis, 1, 25-$(OH)_2$ - D has been shown to have several other target tissues such as the central nervous system, the immune system, and reproductive organs [86]. More recently, the cell membranes of human adipocytes have also been shown to have 1, 25-$(OH)_2$ - D receptors [21]. Consequently, serum levels of 1,25-(OH_2)-D may modulate adipocyte homeostasis. Zemel *et al* [28] have shown in rodent models that in conditions of low dietary Ca there are elevated levels of PTH and 1, 25-$(OH)_2$ - D that result in an increase of adipocyte $[Ca]_i$. Interestingly, *agouti*-expressing, transgenic mice have metabolic and hormonal responses that mimic this response to low dietary Ca [21]. In the presence of elevated levels of

agouti protein, there is an increase in adipocyte $[Ca]_i$. Although the pathways leading to elevated $[Ca]_i$ are different, the result is the same, as overexpression of the *agouti* protein and high serum 1, 25-$(OH)_2$ - D both cause an influx of Ca into rodent adipocytes [28]. This influx of Ca is dose dependent and the response is based on the circulating 1, 25-$(OH)_2$ - D or amount of *agouti* protein expressed [21, 68]. Studies from Zemel's research group have additionally shown in rodents that the level of adipocyte $[Ca]_i$ is directly related to adiposity, as an increase in $[Ca]_i$ is related to an increase in fat mass [28]. They have demonstrated that increasing adipocyte $[Ca]_i$ levels results in a simultaneous decrease in lipogenesis and increase in lipolysis in obese *agouti* mice. The mice exhibit increases in both steady state $[Ca]_i$ and Ca influx in several tissues and this closely correlates with both the degree of ectopic *agouti* expression and body weight [68]. The conclusion from these studies is that the *agouti* protein stimulates Ca influx into various cells which is similar to the effect of elevated 1, 25-$(OH)_2$ – D [68]. This results in increased lipogenesis, increased FAS transcription, and a decrease in lipolysis. In support of these animal models, Draznin *et al* [87] found that obese human patients have elevated adipocyte $[Ca]_i$ and the increased $[Ca]_i$ results in activation of Ca channels that stimulate expression and activity of fatty acid synthase (FAS). This sequence of events can be reversed by Ca channel antagonism.

2.3.3.1 Adipocyte $[Ca]_i$ and Fatty Acid Synthase

Fatty acid synthase (FAS) is a ubiquitous enzyme that is responsible for *de novo* synthesis of fatty acids. FAS catalyzes the condensation of acetyl-CoA and malonyl-CoA to form long-chain fatty acids in the cytoplasm. Studies have shown that inhibition of FAS reduces food intake and resultantly causes extreme weight loss [88].

It is suggested that *agouti* protein stimulates Ca influx and promotes energy storage (via lipogenesis) by stimulating the expression and activity of FAS [68]. This is similar to the response that occurs in the presence of elevated 1, 25-$(OH)_2$ – D. High concentrations of 1, 25-$(OH)_2$ – D are in turn associated with increased FAS expression in adipocytes [28]. A decrease in FAS activity with high Ca diets has been demonstrated and this response was even more pronounced when the Ca was dairy sourced [28]. FAS appears to be modulated by $[Ca]_i$ as it can be inhibited when the cell is treated with a Ca antagonist [89]. When *agouti* mice were treated with nifedipine, a Ca channel antagonist, there was a reduction in FAS and a decrease of fat pad mass of 18% over four weeks compared to control [90]. Conversely, FAS expression can be stimulated by receptor or voltage mediated Ca channel activation [89].

The control of the expression of FAS is vital to fat deposition, as decreasing levels of FAS reduces lipogenesis and resultantly lowers fat mass. Therefore any substance that potentially alters the levels of FAS would play an important role in regulating body composition.

2.3.3.2 Adipocyte $[Ca]_i$ and Uncoupling Protein 2

It has also been suggested that an increase in $[Ca]_i$ could upregulate uncoupling protein 2 (UCP2) which would result in changes to metabolic efficiency. Mitochondrial uncoupling proteins (UCP) are transporters in the mitochondrial inner membrane responsible for regulating the discharge of the proton gradient that is created in the electron transport chain. The energy-dissipating effects of UCP serve many functions such as thermogenesis, maintenance of redox balance, or reduction in reactive oxygen

species. A decrease in the expression of UCP2 has been linked with the development of obesity and hyperinsulinemia [91].

When adipocyte $[Ca]_i$ was raised in mice, there was a concurrent increase of UCP2 in the adipocytes. This resulted in an increased core temperature of ~0.5°C compared to the basal group [20]. UCP2 has been shown to stimulate mitochondrial proton leak and therefore may exhibit a potential role in thermogenesis, energy metabolism and obesity prevention [20]. 1, 25-$(OH)_2$-D is thought to inhibit UCP2 mRNA expression [20]. Suppression of 1,25$(OH)_2$-D via increased dietary Ca could potentially upregulate UCP2 and switch metabolism from energy storage to energy use. While it has been shown that Ca can upregulate UCP2 in white adipose tissue, the precise mechanism for the regulation of UCP2 expression remains known. Furthermore, given that only one group has consistently found an alteration in adipose expression of UCP2 mRNA, further research is required on the topic.

2.3.4 Satiety Hormones and Subjective Hunger

Following protein consumption, there is a postprandial release of cholecystokinin (CCK) and glucagon-like peptide (GLP-1), both of which act in regulation of satiety and appetite and are stimulated by the entry of nutrients into the small intestine [92]. GLP-1 is a peptide hormone secreted in the gastrointestinal tract that assists in blood glucose regulation by increasing insulin secretion and suppressing food intake through regulation of appetite [92]. Similarly, CCK is a peptide hormone, which among its various physiological roles, regulates satiety and appetite [93]. Different dietary proteins such as fish, red meat, and poultry each have a unique effect on GLP-1 and CCK release. This is hypothesized to be related to the different amino acid (AA) profiles of the proteins [94].

Bovine milk is composed of two major protein groups: casein (80%) and whey (20%). These two proteins are distinguishable by their respective solubility – whey is soluble and casein is insoluble at a pH of 4.6 and temperature of 20°C [76]. Consequently, when casein is consumed, it coagulates in the stomach while whey remains in solution. This characteristic alters digestion and absorption rates resulting in a slower passage and longer digestion time for casein compared to whey and a resultant slower release of postprandial plasma AA [95]. Casein also slows transit time due to release of β-casomorphins [96]. Because of this response, Boirie coined whey a "fast" protein and casein a "slow" protein [97]. Hall *et al* [98] found that whey was more satiating than casein, an observation most likely attributed to digestive properties as GLP-1 was increased by 60% in the high whey meal. Furthermore, plasma AA profiles differed following a high whey or a high casein meal [98]. These results suggest that the acute differential hormonal response caused by whey and casein may determine the satiating effect of these proteins.

2.4 Summary of Current Knowledge

In recent years, there has been mounting evidence that dairy foods and elemental Ca have a role in regulation of metabolic parameters [20, 21, 25, 28, 99]. Increasing dietary Ca appears to augment weight loss during ER [20, 22, 23] and to attenuate weight gain during *ad libitum* conditions [22, 59]. This occurs both in animal models [20, 22] and in human studies [22, 59]. When dairy foods are the source of the Ca, the effects are more pronounced suggesting that specific bioactive components found in dairy foods work together with Ca to affect weight and fat loss [20, 22-24]. The discovery and

development of these dairy-sourced, putative bioactive compounds provides a potential anti-obesity food for the general public. Additional rigorous studies are needed to determine the extent of the effect of elemental Ca and dairy foods on metabolic regulation. The potential mechanisms by which dairy products may reduce the risk for developing obesity-linked symptoms of MetS remain unclear and require elucidation.

Chapter Three: Pilot Study – The role of taste aversion and satiety [1]

3.1 Introduction

Dietary intake of calcium (Ca) is vital for many mammalian physiological processes including DNA synthesis, membrane permeability, cell signalling, and in the development and maintenance of bone health. More recently, adequate Ca intake has also been implicated in many diseases including hypertension, type 2 diabetes, heart disease, obesity, among other metabolic related conditions [100].

Currently, there are still many questions regarding the mechanisms through which Ca intake affects obesity and why Ca consumed, as a component of dairy foods may be more efficacious. The concept that dietary intake of dairy foods and Ca modulate energy metabolism and may lower obesity risk has grown in the last six years with supporting evidence ranging from observational studies to RCT. Dietary Ca is now a well-recognized factor in various physiological processes that assist with weight regulation [23, 99]. Zemel *et al* first observed the relationship between Ca and weight control in a clinical trial examining the effect of eating dairy products (yogurt) in hypertensive African-American men [21]. This study reported that men who had the greatest Ca intake - 1000mg as compared to 400mg - also had the least FM. Subsequent to this study, many other epidemiological and observational studies have shown an inverse relationship between the amount of Ca consumed and adiposity although not unanimously [23, 25-

[1] A version of this chapter has been published. Lindsay K. Eller and Raylene A. Reimer. *Attenuation in weight gain with high calcium and dairy enriched diets is not associated with taste aversion in rats: a comparison with casein, whey, and soy.* J. Med. Foods. *In Press.*

27]. Interestingly, when studies used dairy sourced Ca, the weight modulating effects were even greater [22, 23, 99], suggesting a synergistic effect between dairy protein and Ca. Many questions still remain regarding the possible mechanisms by which dairy and Ca intake affects obesity and how Ca consumed, as a component of dairy food may be more effective. To date, studies designed to determine the molecular mechanism of action have been completed in animal models such as the *agouti* mouse or other rodent models [22, 69]. Diets are commonly supplemented with a varying concentration of Ca and type of protein; however, the role of texture, flavour, and palatability in food consumption of these diets has not been determined. The role of dairy and Ca on weight management is generally examined as a physiological issue. Consequently, the role of behaviour in this area is often overlooked. It is well documented that supraphysiological levels of Ca are not palatable to humans and are avoided in some animals [101, 102]; according to Zhang and Tordoff [69], rats potentially exhibited an aversion to elevated Ca (1.8%) in the diet. Moreover, it has been shown that rats do have protein preferences, which depend on age and sex [103].

As a systematic evaluation of the effects of Ca and protein source on food intake and taste aversion (TA) in rats is lacking, the purpose of this research was two fold: (i) to determine if Sprague Dawley rats display a TA to standard rat chow supplemented to 2.4% Ca; and (ii) to determine if short (24-hour) and long-term (weekly) food intake and weight gain is altered when provided with diets containing various proteins (casein, whey, complete dairy, or soy).

3.2 Materials and Methods

3.2.1 Animals and Housing

These studies were approved by The University of Calgary Animal Care Committee and conformed to the Guide for the Care and Use of Laboratory Animals. Eight-week old male Sprague-Dawley rats were obtained from Charles River (St. Constant, Quebec, Canada) and individually housed on a 12-hour light-dark cycle (18:00-06:00), in a temperature and humidity controlled room in the University of Calgary Animal Resource Center.

3.2.2 Diets

Diet ingredients were obtained from Dyets, Inc (Bethlehem, PA) and were mixed in-house. All diets were designed to meet the nutritional requirements of adult rats with 12.3% of total calories derived from protein [casein, whey, soy or complete dairy (non-fortified skim milk powder)]; 10.0% of calories derived from fat [soybean oil]; and 77.7% of calories derived from carbohydrate [cornstarch, dextrinized cornstarch, and sucrose] (Appendix B, Table A.4). Calcium carbonate ($CaCO_3$) supplementation of the low Ca (LC) diets was: 4.3 g/kg in 0.67% casein, soy, and whey diets; skim milk powder has naturally occurring Ca at 0.67% by weight so no $CaCO_3$ was supplemented to the adequate Ca dairy diet. $CaCO_3$ supplementation of the high Ca (HC) diets was: 47.5 g/kg in 2.4% casein, 2.4% soy and 2.4% whey diets. The HC dairy diet was supplemented with 43.0 g/kg adjusting for the naturally occurring Ca content. All diets provided 3.6 kcal/g of diet. Free access to water was available throughout the experimental period. In the pre-experimental period, food was provided from 18:00 – 16:00 (22 hours) in a

stainless steel bowl fitted with a donut-shaped lid. The powder-like consistency of the diet, combined with the lid helped to minimize spillage, and prevented the rats from collecting the food and hoarding it elsewhere [104]. Paper bedding was used to facilitate collection of any spilled food.

3.2.3 Experiment 1. Ca Taste Aversion

Rats (n=6) were randomly assigned to one of two *ad libitum* diet groups. A crossover design was used to ensure each rat consumed each test diet in random order (Figure 3.1). Each group was assigned to a LC or HC diet for one week and then switched to the other diet for one week (Figure 3.1). This was repeated twice for a total of four test weeks and each rat was used in both conditions and was therefore its own control. Both diets were prepared as described above, with the AIN-93M specification of 12.3% of calories derived from protein (casein) with substitution of $CaCO_3$ for cornstarch to increase Ca content [69] (Appendix B, Table A.4). The concentrations of Ca were selected based on previous work as 0.67% Ca is naturally occurring in experimental skim milk diets and 2.4% Ca has been shown to be efficacious in weight management studies [20, 21]. A control group (n=8) consumed standard AIN-93M diet [105] over the four-week period to provide data on normal food consumption. Standard AIN-93M diet consists of 0.4% (wt/wt) Ca and 12.3% of total calories from protein in the form of casein.

3.2.4 Experiment 2. Protein Consumption

Animals (n=8) were randomly assigned to one of four *ad libitum* diet groups. A crossover design was used and ensured each rat consumed each test diet for one week, in random order (Figure 3.1). Each group was rotated through diets that had 12.3% (% kcal)

protein from casein, whey, complete dairy (unfortified skim milk powder), or soy for four one-week periods (Appendix B, Table A.4); each rat was used in all conditions and was therefore its own control. Diets were controlled for vitamin D content as the casein, whey, soy, and skim milk powder were not fortified and did not contain significantly different amounts. Free access to water was available throughout the experiment. Diets were flavoured with 50g/kg of banana flavouring (Dyets, Bethlehem, PA, USA) to mask the individual flavours of the protein to ensure taste preference was not observed. A control group consumed AIN-93M diet over the four-week period to provide data on normal food consumption.

3.2.5 Taste Aversion (TA)

On the first and last day of each diet period, when rats were given their fresh diet at 18:00, a trained professional observed them for one hour. Subsequent behaviours were categorized into grooming, eating, activity, resting, and other [106]. Rats have a normal behavioural progression from feeding to non-feeding activities followed by rest. This sequence of behaviours is referred to as the behavioural sequence of satiety (BSS). Any variation to the normal BSS is indicative of orexigenic or anorexigenic stimuli and/or TA [104].

3.2.6 Food Intake

Food intake was measured on a daily basis by weighing each food cup to the nearest 0.1g and subtracting this weight from the previously measured weight. All spillage was collected and accounted for.

Figure 3.1 Schematic of Study Design for Calcium and Protein

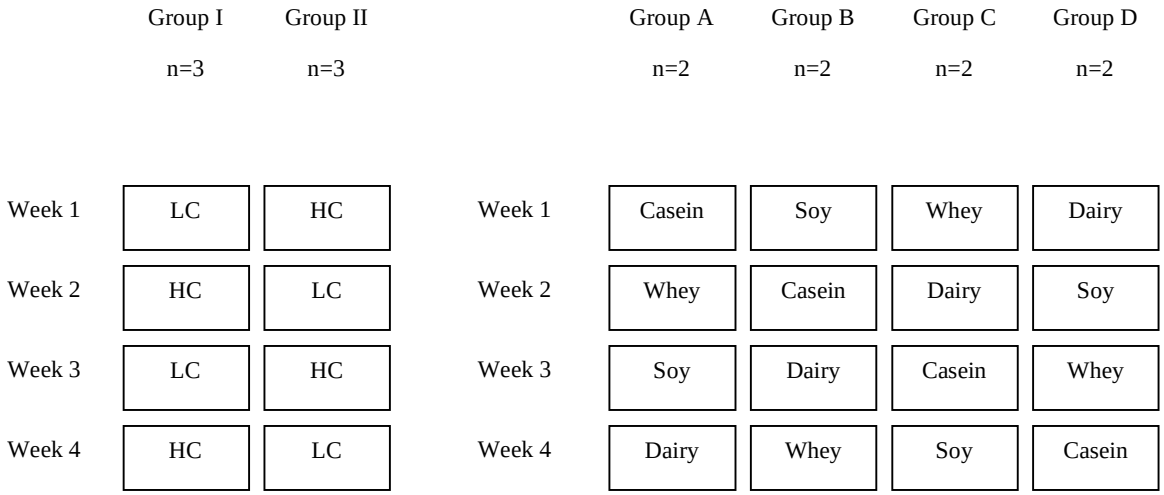


Figure 3.1: Schematic of study design for calcium (Groups I and II) and protein (Groups A, B, C, and D). The protein source for Groups I and II was casein. Calcium level was 0.67% for groups A-D.

3.2.7 Body Weight

Body weight of all animals was measured using an electronic scale prior to feeding experiments and subsequently every two days.

3.2.8 Data Analysis

All data is presented as mean ± SEM. Differences between the treatments were established using a one-way ANOVA with Tukey's multiple comparison *post-hoc* test. Repeated measures were used where appropriate. Statistical analyses were performed using SPSS v 15.0 software (SPSS Inc., Chicago, IL).

3.3 Results

3.3.1 Calcium Aversion

There was no significant difference in weekly food consumption (g or kcal) between the control, LC, or HC diets (Table 3.1). When food intake was analyzed on a daily basis, there was no difference in food intake, including the day when LC rats were switched to the potentially aversive HC diet. Although there was no significant difference in weekly weight gain during HC or LC diets, there was a tendency ($p=0.2$) for reduced weight gain during consumption of the HC diet (Table 3.1). Rats showed no behavioural activities that would suggest taste aversion (TA) as there was no significant difference in the percentage of time spent feeding, resting, doing activity, or grooming following introduction of either of the Ca diets (Figure 3.2 B and C).

3.3.2 Protein Choice

There was no significant difference in weekly food consumption (g or kcal) between the diets varying in protein source (Table 3.2). Daily food intake was the same, even on the first day of the new diet – there was no immediate decrease or increase in feeding between any of the groups upon introduction of a new diet. There was a significance difference in weight gain between the control, casein, and soy diets when compared to the dairy diet, with dairy protein attenuating weekly weight gain (Table 3.2). There was no significant difference in the percentage of time spent feeding, resting, doing activity, or grooming following introduction of any diet (Figure 3.2 D – G).

Table 3.1 Food Consumption and Weight Gain (Calcium)

Table 3.1: Weekly food consumption and weight gain for low calcium (LC) and high calcium (HC) treatments and the control group.

	Weekly food consumption (grams)	Weekly Weight Gain (grams)
Control	156.4 ± 6.8	31.5 ± 2.2
LC	166.8 ± 6.2	32.0 ± 4.9
HC	163.8 ± 6.8	25.4 ± 4.3

All values represent mean ± SEM, n=6/treatment. There was no significant difference in food consumption or weekly weight gain ($p>0.05$). Control diet was AIN-93M diet (0.4% Ca); LC is 0.67% Ca; and HC is 2.4% Ca. The protein source in all diets was casein.

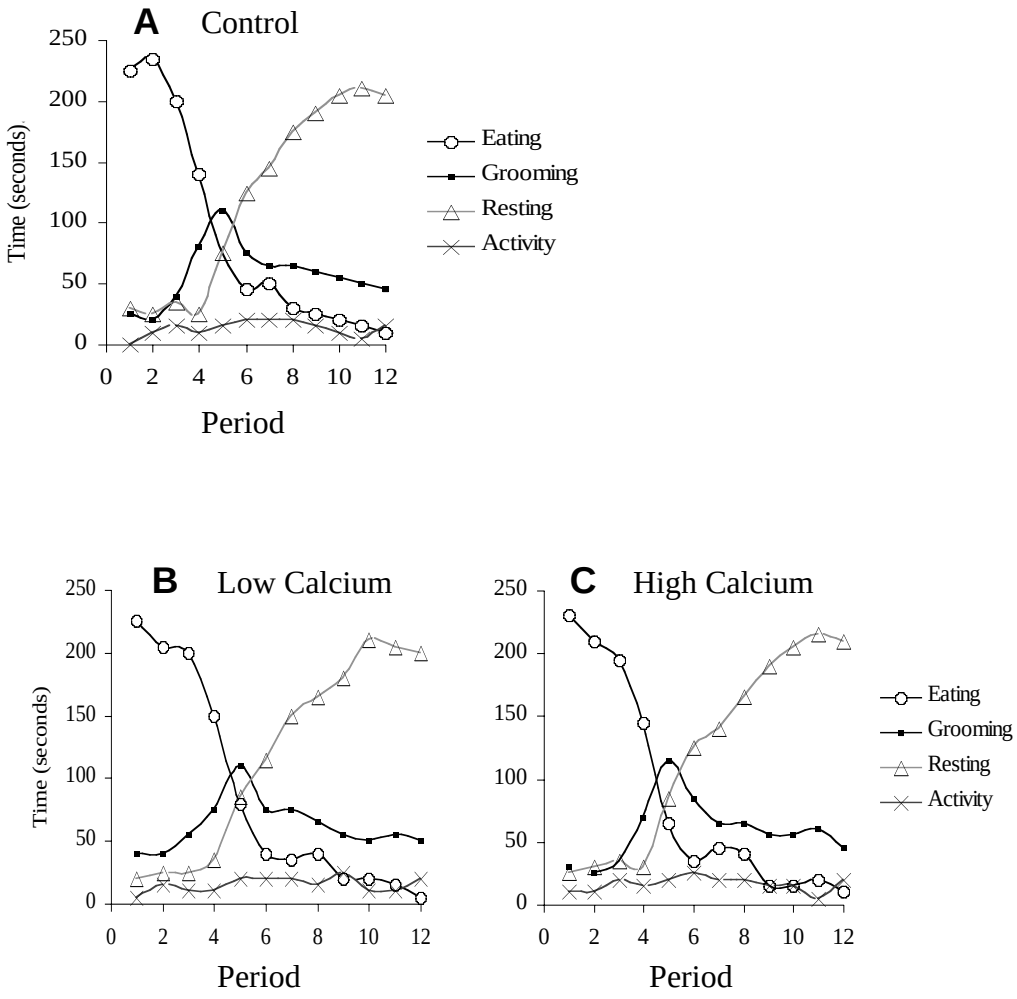
Table 3.2 Food Consumption and Weight Gain (Protein)

Table 3.2: Weekly food consumption and weight gain for the various protein (casein, dairy, soy, and whey) treatments and the control group.

	Weekly food consumption (grams)	Weekly Weight Gain (grams)
Control	156.4 ± 6.8	31.5 ± 2.2
Casein	155.8 ± 7.2	30.8 ± 5.5*†
Dairy	156.8 ± 6.1	14.4 ± 5.0
Soy	149.8 ± 6.4	26.9 ± 6.6*†
Whey	137.5 ± 5.4	21.2 ± 3.7*

All values represent mean ± SEM, n=8/treatment. * $p<0.05$ versus dairy; † $p<0.05$ versus whey. Control diet was AIN-93M (casein as a protein source and 0.4% Ca content). The Ca content in the experimental diets (complete dairy, casein, soy and whey) was 0.67% (LC).

Figure 3.2 Behavioural Satiety Sequence



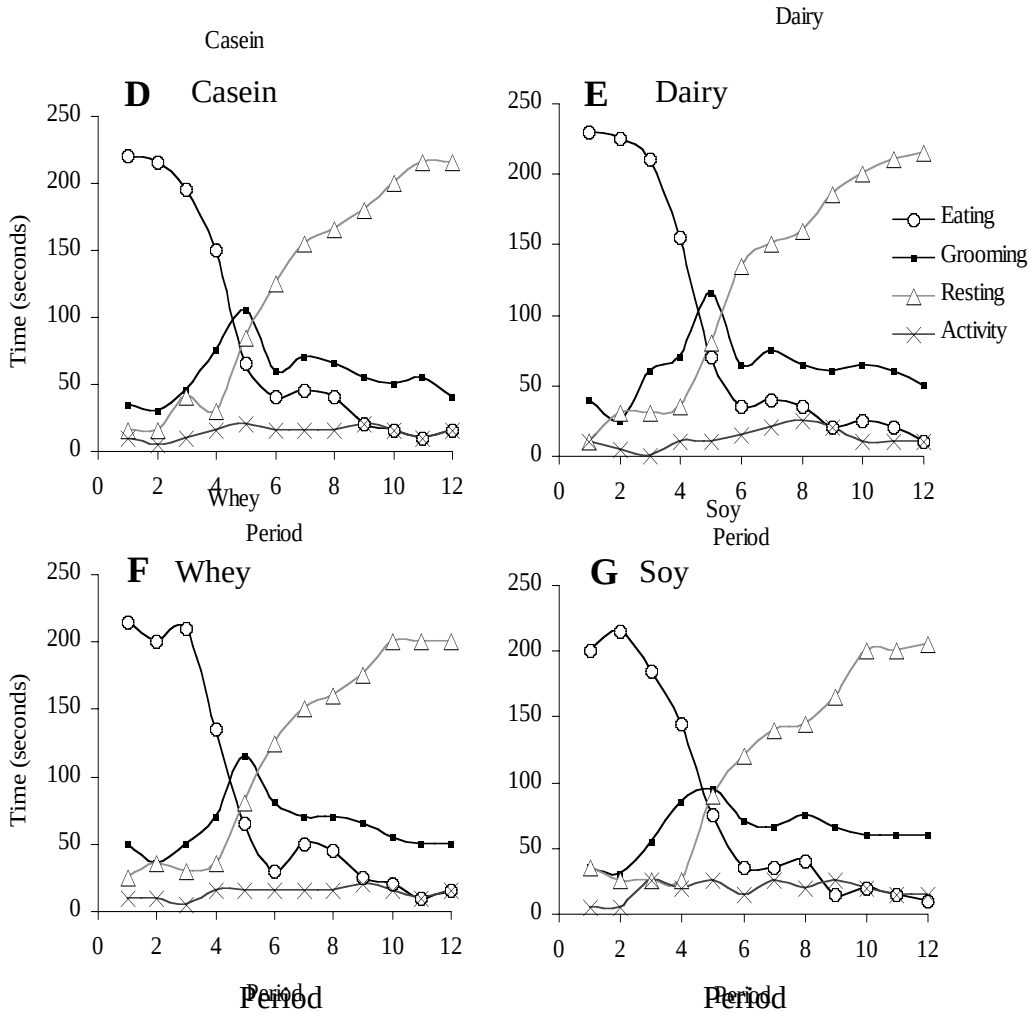


Figure 3.2: The behavioural satiety sequence for the control group (panel A) and the effect of low calcium (LC) and high calcium (HC) (panels B and C) and casein, complete dairy, whey, and soy (panels D – G). There were no significant differences between treatment groups for time spent during each five-minute period on eating, grooming, resting, or activity.

3.4 Discussion

The purpose of this study was to determine if adult, male Sprague Dawley rats have an aversion to, or altered satiety response to dietary Ca (LC or HC) or various proteins (casein, whey, soy, or complete dairy). There are three primary findings of this study: (i) 2.4% Ca (HC) does not cause a TA when it is integrated into standard AIN-93M diet; (ii) protein source does not affect overall food intake when protein flavour is masked; and (iii) protein source but not Ca level affects weight gain when caloric intakes are matched.

In male Sprague-Dawley rats, HC does not cause a TA or alter short-term (24 hour) or long-term (weekly) food intake (Table 3.1). Foremost, total food intake was not decreased, whereas this would have been the case after the habituation period in the event of a typical TA. This characteristic was confirmed by analysis of the behavioural sequence of satiety (BSS) [107] (Figure 3.2 B and C). Indeed, when eating the control and the LC diet, the four main behaviours appeared in the well-defined sequence of eating and grooming and/or activity and resting. The HC did not modify this sequence or period of time spent on each activity. Moreover, we did not see an acute decrease in food consumption when diets were switched from a LC to a potentially aversive HC diet. The crossover design (Figure 3.1) allowed for examination of feeding behaviour when rats were switched from a HC to a LC and a LC to a HC diet, on two occasions. Typically when animals are provided with an unfavourable food, there is an initial decrease in food intake with a future rebound; our behavioural data suggests that rats did not detect a change in the diets. This observation is important and provides support for the argument

that rats are not losing weight in studies utilizing up to 2.4% Ca due to reduction in food intake or stress related to consumption of the HC diet.

The results of this study are consistent with Zhang *et al* [69] who determined that when rodents are given chow supplemented with 1.8% Ca, they will consume it in equal amounts to a low Ca (0.2% or 0.6%) chow over a 52-day period. Moreover, this data is in agreement with other long-term feeding studies that examined chow supplemented with various amounts of Ca (≤ 2.4%) [20, 21, 108, 109]. This is contrary, however, to previous work which suggests that rodents will preferentially choose a lower Ca diet over a higher Ca diet when provided with two diets; or that rats dislike drinking water supplemented with Ca [69].

The second finding of this study was that rats consumed equal amounts of diets varying in protein source when protein flavour was masked (Table 3.2). In addition, rats that consumed various proteins did not show variations in BSS [107] (Figure 3.2 D – G). These findings suggest that there was no influence of organoleptic properties of the diet on food intake and changes in satiety or appetite were likely not playing a role in food intake. Although we did not measure meal duration, frequency, or size, the overall per day food intake did not differ whereas this would have been the case in the event of a physiologically relevant, long-term change in appetite. This is in contrast to other groups who have found a difference in satiety and/or subsequent food intake when various proteins are consumed during one meal. For example, previous work has shown that casein and whey may have varying effects on acute satiety due to digestive properties [97, 98] and satiety hormone release [98]; however, these studies have not examined the longer-term effects of these proteins and whether daily, weekly or longer feeding

behaviour is altered. The data from this study suggest that protein source does not alter 24-hour or one-week food consumption in rats (Table 3.2). Consistent with food intake seen in this study, further work in our laboratory suggests that when obese rats are fed long-term (>8 weeks) diets with protein sourced from skim milk powder, whey, or casein, there is no difference in GLP-1, amylin, ghrelin, or leptin response to a meal tolerance test [110]. The difference in food intake found in other studies might be explained by the use of higher amounts of protein in these acute meal studies [97, 98, 111]. Protein levels in our diets were 12.3% of calories and this may not be sufficient to elicit the same effects. Our observed lack of change in BSS is in contrast to when rats are provided with a high-protein diet. Herein there is a sharp decrease in food intake, which after the first day begins a return to previous levels and in the presence of other food choices shows that rats will select the lower-protein diet.

The final serendipitous finding of this study was that dairy and whey protein attenuated weight gain more so than casein or soy (Table 3.2). This is in agreement with previous studies where protein source appeared to affect weight gain [99]. This adds to the growing body of evidence that a factor – bioactive substance, protein composition, or functional component – found in dairy food plays an active role in modifying body weight. Several factors found in dairy have been suggested to regulate body composition. These include the AA composition, Ca levels, vitamin D status, lactose and various other immunoproteins [77, 112]. Our diets were matched for both vitamin D levels and Ca, suggesting that protein alone or in combination with another component of dairy may be a contributing factor. In contrast to previous work, Ca did not play a significant role in weight gain ($p>0.05$); however, if the study had been of longer duration, the trend ($p=0.2$)

towards reduced weight gain may have become significant. This weight difference is possibly attributable to FF [72] or to an alteration of UCP in the adipose tissue [22].

We chose to do these studies together due to the current overwhelming interest in dairy foods and Ca in weight management. There appears to be a link between Ca intake and dairy food consumption in relation to weight control; however how these products alter feeding behaviour in rats in unknown. The authors are aware of certain limitations of this study. Meal frequency, duration, or sizes were not measured. This therefore did not provide details of acute food intake; we were primarily concerned with 24-hour and weekly food consumption as long-term feeding is more physiological relevant than single meal bouts. Moreover, we only examined diets that were provided sequentially and not presented at the same time. In support of this decision, Sunday [113] has shown that when given meals with differing palatability sequentially as opposed to simultaneously, the meal frequency, size, and duration are not significantly different. Additionally, we did not conduct a study examining the flavours (i.e. no banana flavouring) of various proteins because it has been shown that even with diets up to 70% protein, there is no TA [106]. In our diets that consist of 12.3% protein, the flavour differences are minimal between diets, and certainly do not reflect the same flavour, texture or palatability issues that a 70% protein diet would.

In conclusion, our data suggests that rats do not have an aversion to 2.4% Ca (HC) and that diets consisting of 12.3% protein – either with casein, soy, whey, and complete dairy – do not alter 24-hour or one-week food intake. Finally, weight gain is altered significantly by protein source. Taken together this work establishes important foundational data which suggests that the loss of weight documented in rats placed on

diets high in Ca or dairy proteins is not due to reduction in food intake or stress related to consumption of the HC diet.

Chapter Four: The Role of Dairy Foods and Calcium in the Prevention of Weight Gain During High Energy Feeding [2] [3]

4.1 Introduction

The concept that dairy products and calcium (Ca) modulate energy metabolism and improve weight control has emerged in the last decade with evidence ranging from observational studies to RCT. Dairy foods have been proposed to play a role in various physiological processes associated with weight management and MetS [99]. Increased dietary Ca appears to augment weight loss during ER [20, 22, 23] and to attenuate weight gain during *ad libitum* feeding, both in animals [20, 22] and humans [23]. Ca alone does not appear to elicit the same magnitude of weight regulatory effects as dairy foods and likely acts in concert with other bioactive components found in milk-derived products.

Dairy consumption is also linked to a reduction in insulin resistance and incidence of type 2 diabetes. The prospective Coronary Artery Risk Development in Young Adults (CARDIA) study identified an ~21% decreased risk of insulin resistance in 3157 young adults with each daily serving of low fat dairy food [26]. This effect was seen in overweight individuals but not in normal weight individuals, and was similar for men and women. The Health Professionals Follow-up Study that prospectively examined 41,254 men, found that for each additional serving of low-fat dairy product per day, risk of

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[2] A version of this chapter has been published. Eller LK and Reimer RA. *Dairy Protein Attenuates Weight Gain in Obese Rats Better Than Whey or Casein Alone.* Obesity, 2009. September 24 Epub ahead of print.

[3] A portion of this work was presented in part at Experimental Biology, April 2006. *The effect of dairy protein and calcium on the prevention of weight gain in Sprague-Dawley Diet-Induced Obese (DIO) rats*. FASEB J. 20: A992-b.

developing type 2 diabetes decreased by 9% [31]. Similarly, in 37,183 women, prospective risk of type 2 diabetes was decreased by 4% per daily serving of low fat dairy food [32].

Many questions remain unanswered regarding the mechanisms by which Ca intake affect MetS, and why Ca consumed with dairy products tends to be more effective in its management. Dairy products may contain specific bioactive compounds that work together with Ca to promote these effects [114]. The protein fraction of dairy is composed of ~80% casein and ~20% whey. Both of these proteins have relatively high concentrations of BCAA, however whey is particularly rich in leucine [115]. Furthermore, specific bioactive fractions of milk proteins have been shown to produce unique physiological effects. For example, β-casomorphins in casein increase intestinal transit times; whey protein has been shown to increase satiety signals; and leucine may affect insulin regulation [77, 96, 116, 117].

In addition to particular fractions of the protein that may be biologically active, Zemel *et al* [21] have proposed that increased dietary intake of Ca results in decreased 1, 25-$(OH)_2$ - D levels, in turn decreasing adipocyte $[Ca]_i$. This decrease is hypothesized to stimulate adipocyte lipolysis and inhibit lipogenesis via decreased fatty acid synthase (FAS) and increased uncoupling protein 2 (UCP2). A further suggestion for the decreased weight associated with Ca intake is increased FF. While these may be contributing factors, the above theories do not completely explain the weight management or anti-diabetic effects of dairy. Specifically, no research to date has examined the individual protein fractions of dairy to determine if distinct effects on weight control exist. Our primary objective, therefore, was to determine the difference between various dairy

proteins (complete dairy, casein, or whey) and Ca supplementation in preventing weight gain in a diet-induced obese (DIO) rat model. Secondarily, we sought to evaluate satiety hormone secretion and the expression of genes related to satiety hormones and metabolism in liver, stomach, duodenum, jejunum, ileum, colon, and white adipose tissue (WAT)

4.2 Materials and Methods

4.2.1 Animals and Housing

Ethical approval was obtained from The University of Calgary Animal Care Committee. Six-week old male Sprague-Dawley rats were obtained from Charles River (Charles River, St. Constant, QC, Canada) and maintained on a 12-hour light-dark cycle in a temperature and humidity controlled room (n=128). Rats were provided *ad libitum,* a high fat/high sucrose (HFHS) diet for six weeks to induce obesity. From previous work, it has been demonstrated that in outbred Sprague-Dawley rats, approximately 50% develop diet-induced obesity (DIO) when fed a high-energy diet; the remaining 50% resist weight gain and gain weight and fat mass at a rate similar to chow-fed (NE) controls. The divergence of the population is evident after a minimum of two weeks of feeding a high-energy diet [118]. At 12 weeks of age, after six weeks of HFHS feeding, the rats in the upper 50% of body weight were selected as the DIO rats and then individually housed for the remainder of the study (n=64).

4.2.2 Experimental Diets

Obese rats were randomly assigned to one of eight, *ad libitum,* diet groups for eight weeks (Figure 4.1). The diets had varying protein source [complete dairy (skim

milk powder), casein, or whey], Ca concentrations (0.67% or 2.4%), and energy density [high fat/high sucrose (HFHS) or normal (NE)]. All HFHS diets were prepared with 10% protein content (by kcal) and the NE diets had 12% protein content (by kcal). The complete dairy diets had a greater amount of skim milk powder added due to the lower protein content of skim milk compared to casein and whey (Appendix B) (by weight). By design the HFHS diets had higher fat content than the NE diets and the respective energy densities were 4.6 kcal/gram and 3.6 kcal/gram. The NE diets were selected to provide a benchmark for adulthood weight gain on the standardized, nutritionally complete diet recommended for the maintenance of rodents, the AIN-93M diet [105] which utilizes casein as the protein source. Conversely, the HFHS diets were selected to metabolically challenge the animals with an energy dense diet and promote weight gain. The concentration of Ca [0.67% Ca (LC) and 2.4% Ca (HC)] was selected based on previous work in rodents [21]. The LC dose is the amount naturally found in the complete dairy diet and 2.4% has previously been utilized in weight control studies in rats [20]; calcium carbonate ($CaCO_3$) was used to increase Ca content of the diets [69]. All diets were designed to meet nutritional requirements of rats with amounts of L-cystine, AIN-93M vitamin mix, AIN-93M mineral mix, α-cellulose, and choline bitartrate consistent with recommendations [105] (Appendix B, Tables A.3 and A.4). All diets were controlled for vitamin D content as the casein, whey, and skim milk powder were not fortified and did not contain significantly different amounts. Free access to water was available throughout the experiment.

Figure 4.1 Schematic of Experimental Diets

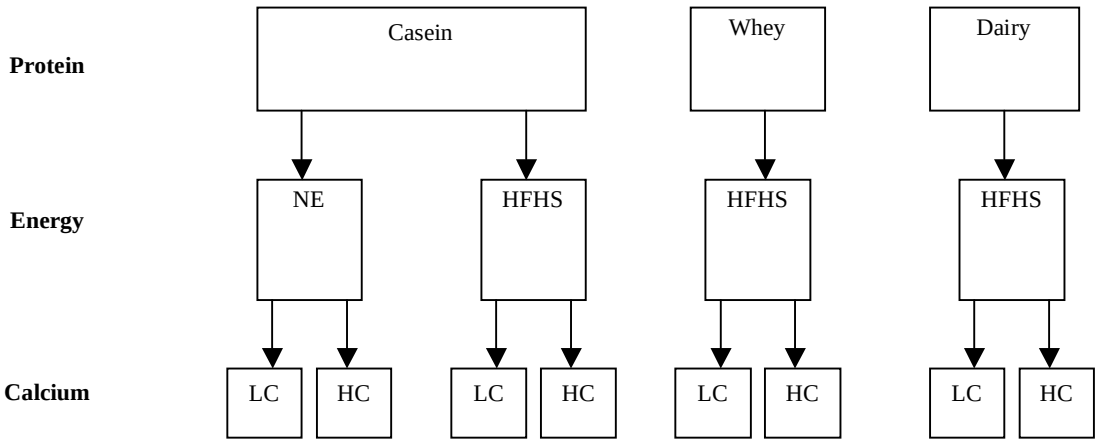

Figure 4.1: The eight experimental diets used to determine the role of various proteins and calcium on the prevention of weight gain in *ad libitum* fed, diet-induced obese rats. Diets differ in protein source (casein, whey, or complete dairy), energy density [normal energy (NE) or high fat, high sucrose (HFHS)], and calcium level [0.67% (LC) or 2.4% (HC)]

4.2.3 Measurements

4.2.3.1 Food Intake

Food intake was measured daily by weighing each food cup to the nearest 0.1 g. All spillage was collected from the bottom of the cages and accounted for. Daily, weekly, and total caloric intake of each experimental group was calculated from this data.

4.2.3.2 Body Weight and Composition

Body weight of all animals was measured prior to feeding experiments and subsequently once per week. Two days prior to testing, rats were lightly anaesthetized and body composition and bone mineral density (BMD) measured via dual energy x-ray absorptiometry (DEXA) with software for small animal analysis (Hologic QDR 4500, Hologic, Inc., Bedford, MA).

4.2.3.3 Meal Tolerance Test

A meal tolerance test (MTT) was performed according to previous work using cardiac blood sampling [119]. Briefly, following a 12-hour overnight fast, animals were given a MTT with their respective experimental diet. Animals were anaesthetized with isoflurane and a baseline blood sample collected via cardiac puncture. Rats were then allowed to waken and were given a 3g oral gavage of their respective diet emulsified in water to a total volume of 3mL. Following the gavage, rats were anaesthetized for the remainder of the sampling. Subsequent blood samples were collected at 15, 30, 60, and 90 minutes post-gavage. Blood for the analysis of insulin, leptin, active GLP-1, amylin, ghrelin and glucagon was collected into a vacutainer (BD Biosciences, Mississauga, ON, Canada) with the addition of EDTA (1mg/mL), aprotinin (5×10^5 KIU/L; Sigma,

Oakville, ON, Canada) and diprotin A (34µg/ml; MP Biomedicals, Irvine, CA). Plasma was collected and stored at -80˚C until analysis.

4.2.3.4 Tissue Collection

Immediately following the 90-minute blood draw, rats were over-anaesthetized with isoflurane and the aorta cut. 100 mg of liver, stomach, duodenum, jejunum, ileum, colon, and retroperitoneal adipose tissue was excised and immediately flash frozen in liquid nitrogen. Tissues were stored at -80˚C until analysis.

4.2.3.5 Blood Analysis

Blood glucose concentrations were measured via OneTouch Blood Glucose Meter (BD Biosciences, Mississauga, ON, Canada). Insulin, leptin, amylin, and glucagon were determined using a commercial rat endocrine Lincoplex Kit (Linco Research, Missouri). Ghrelin and active GLP-1 were measured via commercial ELISA (Millipore Corporation, Billerica, MA). Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated to provide an estimate of insulin resistance [HOMA-IR = (baseline insulin x baseline glucose)/22.5] [120].

4.2.3.6 Gene Expression

Total RNA was extracted from tissues using Trizol Reagent as per the manufacturer's directions (Invitrogen, Carlsbad, CA). RNA was quantified using Ribogreen and reverse transcription (RT) performed using the Omniscript RT Kit (Qiagen, Mississauga, ON, Canada). Gene expression analysis was performed in triplicate and quantified using a Bio-Rad Thermal iCycler (Bio-Rad, Mississauga, ON, Canada). The following genes were examined: proglucagon, PYY, and cholecystokinin (CCK) in the duodenum, jejunum, and ileum; peroxisome proliferator-activated receptor

γ (PPARγ), fatty acid synthase (FAS), sterol regulatory element binding protein (SREBP1c), and acetyl coenzyme carboxylase (ACC) in the liver; ghrelin in the stomach; and UCP2 in the white adipose tissue. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) or β-actin was used as the reference housekeeping gene and data was analyzed using the $2^{-\Delta CT}$ method [121]. Primers used for rt-PCR are provided in Appendix C (Table A.5).

4.2.4 *Data Analysis*

All data is presented as mean ± SEM. Differences between the treatments were established using a one-way ANOVA with Tukey's multiple comparison *post-hoc* test. A mixed model analysis was also performed with Ca level, protein source, and energy density as the fixed effects with the individual animal representing the random effect factor. Use of mixed model statistical analysis allows for the determination of the contribution of the fixed effects (Ca level, protein source, and energy density) to the model for each parameter examined. HOMA-IR and total area under the curve (AUC) were calculated according to previous work [120]. Pearson's correlation coefficients were determined for plasma leptin concentrations and body fat. Significance was set at $p<0.05$.

4.3 Results

4.3.1 Food Intake

Following eight weeks of *ad libitum* feeding, there was no significant difference in daily or total food intake (grams) between the two normal energy (NE) diet groups that differed only in Ca content (Table 4.1) ($p>0.05$). There was also no significant difference

between the six HFHS groups, which differed in protein source and Ca content ($p>0.05$). As expected, based on the energy density of the diets, the two NE groups consumed significantly fewer calories ($p<0.05$) than the HFHS groups with the exception of the two whey/HFHS groups which did not differ from the casein/NE diets ($p>0.05$) (Table 4.1). Mixed model analysis, which distinguishes the contribution of Ca level, protein source, and energy density to caloric intake, confirmed a significant contribution of energy density ($p=0.009$) to the differences observed in average calorie intake.

4.3.2 Anthropometrics

4.3.2.1 Body Weight

Initial body weights (all: 598.2±9.1) and both initial and final crown to rump lengths (all: 19.7±0.2 cm) were not significantly different between diet groups ($p>0.05$). Weight gain data is presented in Figure 4.2. At the end of the experimental feeding period, the dairy/HFHS/LC group had significantly lower weight gain compared to all other diets except the dairy/HFHS/HC group. Mixed model analysis revealed a significant contribution of protein source ($p<0.001$) and energy density ($p<0.001$) to the change in body weight.

Body weight data is presented in Table 4.1. The dairy/HFHS/LC group had a lower final body weight compared to all casein and whey HFHS groups ($p<0.05$) but was not significantly lower than the two NE diet groups (casein/LC and casein/HC). The group with the highest final body weight was the casein/HFHS/LC ($p<0.05$). Protein source and energy density were significant contributors to the model effects for final body weight (both: $p<0.001$).

4.3.2.2 Body Composition

Body fat (%) was significantly lower in both dairy groups compared to all other groups – including the NE diets ($p<0.01$) (Table 4.1). Protein source ($p<0.001$) and energy density ($p<0.05$) were significant contributors to the model effects for body fat (%). Absolute LBM (grams) was not significantly different between the groups, however, expressed as a percent of total body weight, LBM was significantly greater in both dairy diets compared to all other diets (Table 4.1). Mixed modeling revealed a role of energy density ($p=0.041$) and protein ($p<0.001$) in contributing towards LBM (%).

Figure 4.2 Total Weight Gain

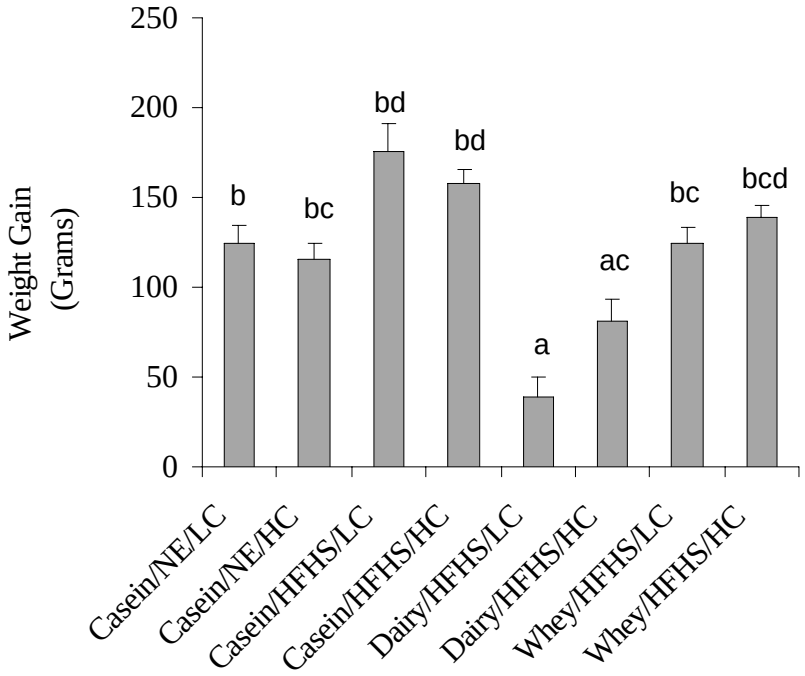

Figure 4.2: The total weight gain over the eight-week *ad libitum* period for the two normal energy (NE) diet groups [casein with low (LC) or high (HC) calcium] and the six HFHS diets (casein, complete dairy, or whey with LC or HC). All values represent mean ± SEM, n=6-10/treatment. Bars with different letters are significantly different from each other, $p<0.05$.

Table 4.1 Feeding and Anthropometric Data

Table 4.1: Energy intake and final body composition for the eight-week feeding period for the normal energy (NE) and the high energy (HFHS) diets made with casein, complete dairy, or whey and supplemented with low (LC) or high calcium (HC).

		Casein NE	Casein HFHS	Dairy HFHS	Whey HFHS
Energy Intake, kcal/d	LC	71.5±2.5[a]	85.3±1.8[b]	83.6±2.8[b]	80.1±1.7[ab]
	HC	74.3±1.7[a]	83.3±3.0[b]	86.0±2.2[b]	78.1±1.2[ab]
Final Weight, g	LC	716.0±8.2[ac]	773.4±23.5[d]	658.6±23.1[a]	723.9±13.8[bcd]
	HC	711.2±8.7[ac]	754.4±10.5[cd]	666.0±5.0[ab]	730.3±6.0[cd]
Body Fat, %	LC	29.7±1.0[a]	34.4±2.6[a]	19.9±1.7[b]	30.9±1.4[a]
	HC	28.4±1.1[a]	30.2±1.4[a]	20.3±1.4[b]	28.7±1.5[a]
LBM, g	LC	512.8±108	504.7±14.0	515.5±8.2	500.0±13.1
	HC	504.3±7.8	532.1±8.5	543.5±12.7	522.1±6.4
LBM, %	LC	70.3±1.0[a]	65.6±2.7[a]	80.1±1.7[b]	69.1±1.4[a]
	HC	71.6±1.1[a]	69.8±1.4[a]	79.7±1.4[b]	71.3±1.5[a]

BMD, g/cm^3	LC	0.167±0.003	0.186±0.002	0.188±0.002	0.187±0.002
	HC	0.180±0.003	0.185±0.003	0.190±0.003	0.179±0.003

All values represent mean ± SEM, n=6-10/treatment. Values with different superscript letters are significantly different ($p<0.05$) from each other (across all eight diets within a given outcome measure).

4.3.3 Plasma Analysis

4.3.3.1 Glucose

Baseline glucose and the postprandial MTT responses are presented in Table 4.2 and Figure 4.3, respectively. Baseline glucose was significantly lower ($p<0.05$) in dairy/HFHS/LC compared to whey/HFHS/HC (Table 4.2). Glucose levels at individual time points during the MTT did not differ between groups ($p>0.05$).

4.3.3.2 Plasma Hormones

Baseline insulin levels were not significantly different between any of the diet groups (Table 4.2) ($p>0.05$). At 30 minutes in the MTT, insulin was higher with LC versus HC in the NE casein group and the HFHS whey and HFHS casein groups (Figure 4.4; $p<0.05$). Mixed model analysis revealed a role for Ca in the insulin response ($p=0.008$). Baseline leptin levels were significantly lower in casein/NE/HC rats compared to casein/HFHS/HC rats (Table 4.2). Baseline leptin correlated with percent body fat ($R= 0.3$; $p<0.05$). Plasma GLP-1, amylin, glucagon, and ghrelin were not different between treatments at baseline or during the MTT ($p>0.05$).

Table 4.2 Baseline Glucose, Insulin, and Leptin Concentrations and HOMA-IR

Table 4.2: Baseline levels of glucose, insulin, and leptin and HOMA-IR at the end of the eight-week feeding period for rats consuming normal energy (NE) and the high energy (HFHS) treatments made with casein, complete dairy, or whey and supplemented with low (LC) or high calcium (HC).

		Casein NE	Casein HFHS	Dairy HFHS	Whey HFHS
Baseline Glucose, mmol/L	LC	7.3±0.4^{ab}	7.0±0.7^{ab}	5.1±0.3^{a}	6.5±0.7^{ab}
	HC	6.5±0.4^{ab}	7.1±0.5^{ab}	6.6±0.6^{ab}	8.1±0.3^{b}
Baseline Insulin, pM	LC	13.2±2.5	6.8±1.4	8.7±2.4	22.6±6.1
	HC	14.3±3.6	12.5±2.4	10.1±0.6	19.4±3.9
Baseline Leptin, pM	LC	114.1±18.2^{ab}	132.9±48.5^{ab}	82.0±18.6^{ab}	91.0±28.3^{ab}
	HC	64.7±9.6^{a}	174.4±34.0^{b}	80.0±14.8^{ab}	111.8±15.0^{ab}
HOMA-IR	LC	2.9±0.4^{c}	1.9±0.5^{c}	1.5±0.5^{c}	5.7±1.1^{ab}
	HC	3.5±1.0^{bc}	3.2±0.6^{ab}	2.7±0.4^{bc}	6.8±1.2^{a}

All values represent mean ± SEM, n=6-10/treatment. Values with different superscript letters are significantly different ($p<0.05$) from each other (across all eight diets within a given outcome measure).

Figure 4.3 Glucose Response During Meal Tolerance Test

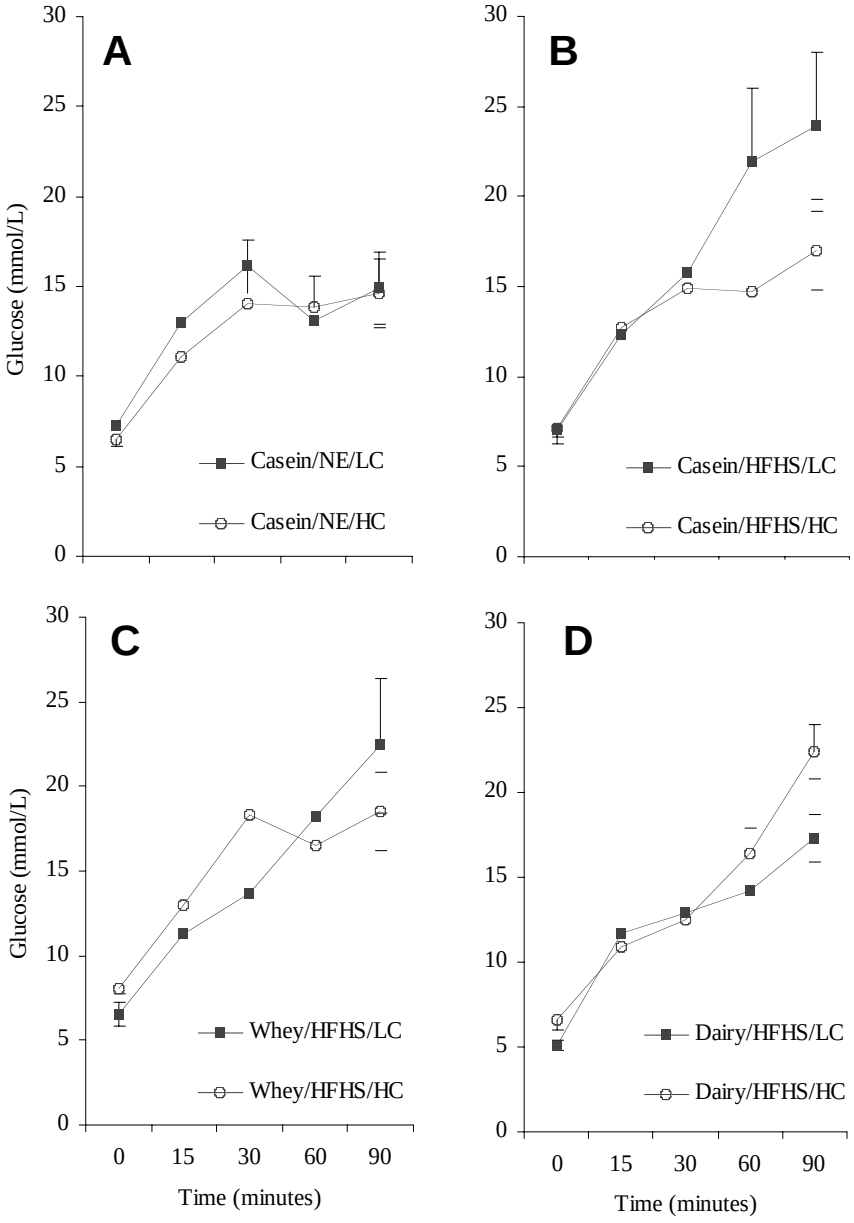

Figure 4.3: Postprandial glucose responses at the end of the eight-week feeding period for rats consuming (A) normal energy (NE)/casein, (B) high energy (HFHS)/casein, (C) HFHS/whey, and (D) HFHS/complete dairy and supplemented with low (LC) or high calcium (HC). All values represent mean ± SEM, n=6-10/treatment. There was no significant difference between the LC or HC diets or between proteins (casein, complete dairy, or whey) for individual time points at 0, 15, 30, 60, or 90 minutes postprandial.

Figure 4.4 Insulin Response During Meal Tolerance Test

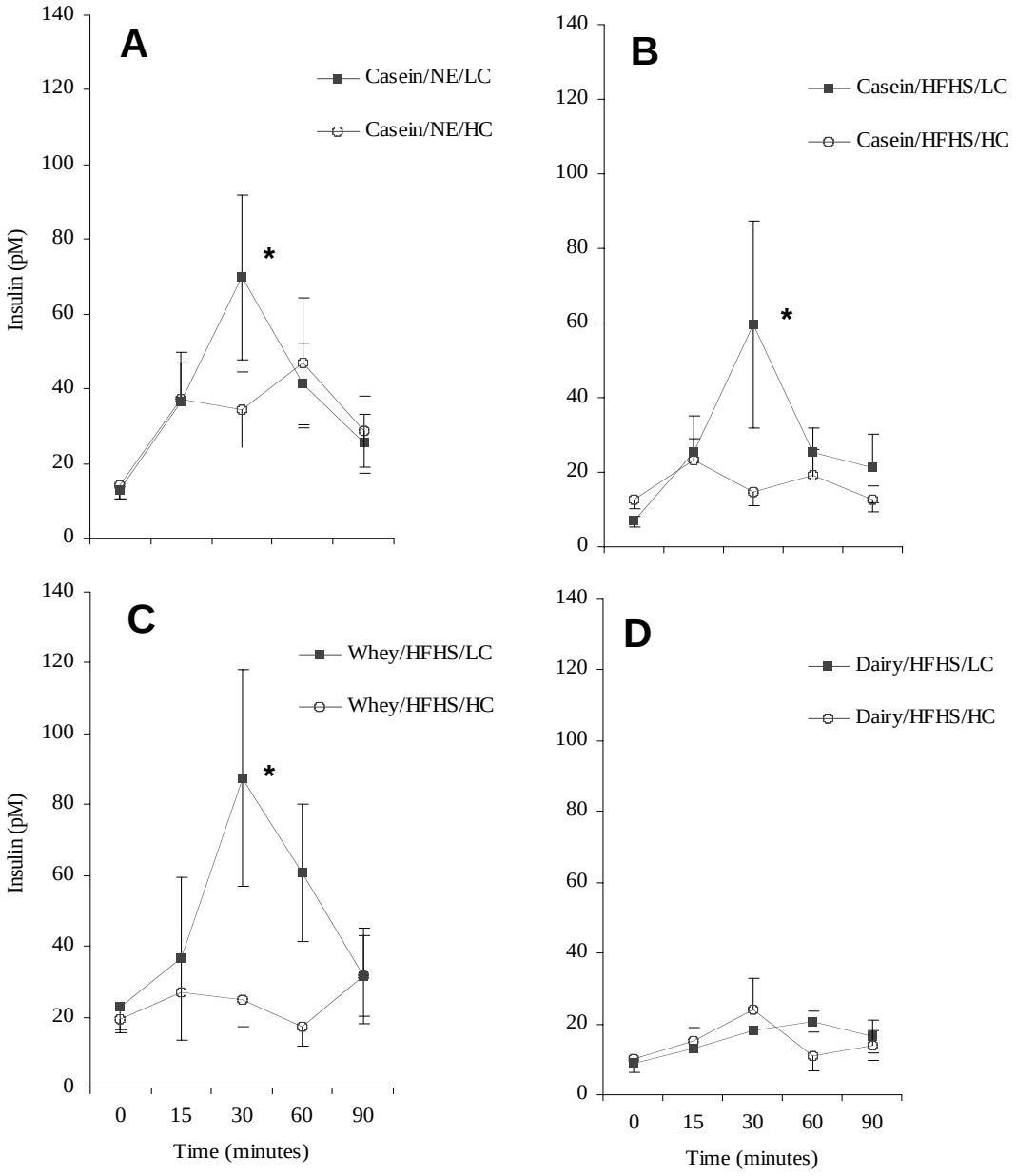

Figure 4.4: Postprandial insulin responses at the end of the eight-week feeding period for rats consuming (A) normal energy (NE)/casein, (B) high energy (HFHS)/casein, (C) HFHS/whey, and (D) HFHS/complete dairy and supplemented with low (LC) or high calcium (HC). Results are presented as mean ± SEM (n=6-10). Asterisks represent a significant difference ($p < 0.05$) between LC and HC diets at 30 minutes. Mixed model analysis indicates a role of calcium in the insulin response ($p=0.008$).

4.3.3.3 Homeostatic Model of Assessment - Insulin Resistance

HOMA-IR, an index of insulin resistance, was significantly higher for the whey/HFHS/HC diet when compared to all other diets except the casein/HFHS/HC diet and whey/HFHS/LC diet (Table 4.2; $p<0.05$). The dairy/HFHS/LC diet had the lowest (best) HOMA-IR and was significantly lower than the casein/HFHS/HC diet and both whey diets ($p<0.05$). Mixed model analysis revealed that protein source ($p=0.005$) contributed to the differences seen in HOMA-IR.

4.3.4 mRNA Expression

Hepatic SREBP1c and PPARγ mRNA were both significantly down regulated with the whey and dairy HFHS/LC diets when compared to casein HFHS/LC ($p<0.05$) (Figure 4.5). mRNA expression of white adipose FAS, UCP-2 and PPARγ; small intestine (duodenum, jejunum, and ileum) proglucagon, PYY, and CCK; and stomach ghrelin was not different between treatment groups ($p>0.05$, n=6-10/treatment).

Figure 4.5 Hepatic Gene Expression of SREBP1c and PPARγ mRNA

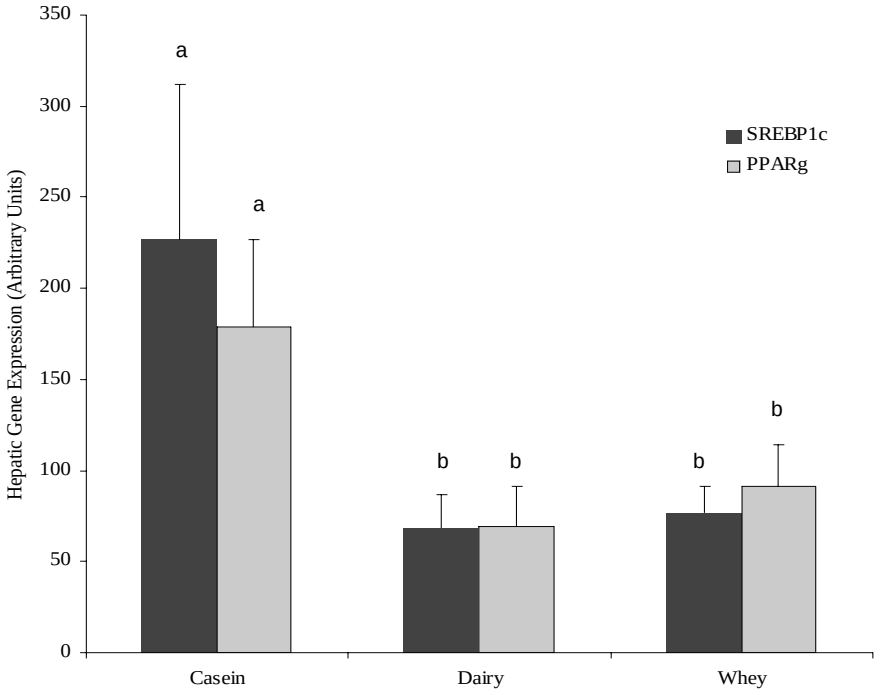

Figure 4.5: Hepatic gene expression of SREBP1c and PPARγ at the end of the eight-week feeding period for rats consuming high energy (HFHS) treatments made with casein, complete dairy, or whey and low calcium (LC). Results are presented as mean ± SEM, n=6-10/protein treatment. Treatments with different letters are significantly different ($p<0.05$) as determined with ANOVA and Tukey's *post hoc* analysis. PPARγ, peroxisome proliferator–activated receptor-gamma; SREBP1c, sterol regulatory element–binding protein.

4.4 Discussion

The results of this study provide further evidence for an important role of dairy foods and Ca in preventing excess weight gain during periods of high calorie food intake. In addition to changes in body composition and glycaemia, we examined mechanisms related to satiety hormone secretion and expression of genes related to thermogenesis and lipogenesis. The novel findings of this study are that: (1) consumption of complete dairy protein prevented fat mass (FM) accumulation more so than casein or whey during *ad libitum* HFHS feeding; (2) greater LBM and reduced HOMA-IR with dairy intake may predict enhanced insulin sensitivity; and (3) while satiety hormones do not seem to play a role in regulating weight gain in this study, hepatic expression of SREBP1c and PPARγ, two genes involved in hepatic lipid metabolism, were down-regulated by dairy.

The most striking finding of this study is the marked reduction in weight gain seen with the dairy/HFHS diets compared to all other diets examined. Weight gain with the two NE diets, of which rats consumed significantly fewer calories, was slightly higher and not significantly different from the complete dairy HFHS diets. The reduction in weight gain was accompanied by an improvement in body composition in the dairy groups, specifically lower FM (%) and greater LBM (%) compared to all other groups (Table 4.1 and Figure 4.2). Given that the LBM (g) was not different between the groups, the increased percentage of LBM in the dairy rats would predictably improve overall energy homeostasis [122]. The attenuation of weight gain occurred gradually over the period of eight weeks and as expected the lower body fat levels were related to lower circulating leptin concentrations. Using linear mixed model analysis, protein source and

energy density but not Ca was found to contribute significantly to the differences seen in FM (%) and LBM (%) between the groups, with complete dairy providing the overall greatest improvements.

Several mechanisms have been proposed to explain the greater weight loss seen with dairy intake, including regulation of $[Ca]_i$, increased thermogenesis via UCP2 upregulation, increased FF, and enhanced satiety hormone secretion [99]. We were unable to demonstrate any difference in the secretion of GLP-1 or ghrelin in this study. This lack of difference in these two well-known hormones that decrease and increase food intake respectively, is supported by our data showing no difference in food intake between the diet treatments. Due to the energy density of the diets, the NE rats did consume significantly fewer calories compared to the HFHS rats with the exception of the whey HFHS rats, which were not different from NE. This may reflect evidence in humans where the effect of whey protein on reducing short-term food intake is greater compared to casein or other proteins including soy protein and egg albumin [123].

While there were no marked changes in energy intake between our treatments, there appears to be an effect on nutrient partitioning with dairy rats exhibiting the greatest LBM (%) and lowest FM (%) (Table 4.1). Several components of dairy products have been proposed to play a role in body composition, including Ca, protein and AA composition, and vitamin D [124]. The branched chain amino acids (BCAA), particularly leucine, play an important role in maintenance of LBM [84] and although whey is high in BCAA, the rapid transit time of whey may reduce intestinal absorption of BCAA. When whey is ingested together with casein, as occurs naturally with dairy foods, it is plausible that transit time may be slowed and thus allow for greater BCAA absorption. In support

of the current study showing differences between casein versus complete dairy, is data showing that supplementing a casein diet with leucine in the drinking water of rats improves body composition [69]. Studies such as this indicate that a factor inherent in dairy protein, such as leucine, could play an important role in improvements to body composition. Future work addressing the specific role of leucine is warranted.

In addition to changes in total body weight, protein source appears to have an effect on insulin sensitivity, which may be mediated via increased LBM (Table 4.2). HOMA-IR calculations suggest that insulin sensitivity was significantly greater with the complete dairy treatment compared to whey and casein. This observation would support the two large epidemiological studies by Choi *et al* and Lui *et al* linking dairy consumption to a decrease in type 2 diabetes risk [31, 32]. In addition, while few studies have examined the association of Ca intake with insulin sensitivity, a recent clinical trial with 31 patients with type 2 diabetes did find higher insulin sensitivity in those patients randomized to 1500 mg of oral Ca supplementation daily for eight weeks compared to placebo [125]. In our study it would appear that protein source (namely complete dairy versus whey and casein) played a greater role in reducing insulin resistance compared to Ca given that minimal differences in HOMA-IR existed between LC and HC levels.

In our model, there does not appear to be a role for altered adipocyte UCP2 or FAS expression in reducing weight gain, which is similar to findings by Parra *et al* [126]. More recently, Zemel *et al* [22] demonstrated that high dairy/Ca re-feeding in obese mice was associated with an increase in skeletal muscle PPARα expression which could increase fat oxidation (fat OX) and improve insulin sensitivity. In our study, we observed a down-regulation of both hepatic SREBP1c and PPARγ in dairy and whey compared to

casein in a HFHS diet (Figure 4.5). In the liver, lipogenic genes (including FAS and ACC), are regulated primarily by SREBP1c [127] and secondarily by PPARγ [128]. One of the hallmarks of the development of insulin resistance is hepatic steatosis or fat accumulation in the liver [129]. Both SREBP1c and PPARγ are upregulated in rodent models of insulin resistant obesity and hepatic steatosis [130] which would be in keeping with our DIO rats who were consuming a HFHS diet. Further work is needed to establish whether the reduction in HOMA-IR observed in our dairy-fed rats is directly linked to reduced SREBP1c and PPARγ mRNA.

While numerous studies have examined the effects of complete dairy and whey protein on body weight regulation separately; to our knowledge this is the first study to provide a comprehensive direct comparison of complete dairy, whey, and casein with LC or HC. In addition to this strength, we are also aware of certain limitations of the study. This study was designed to examine the prevention of weight gain in animals consuming diets with various proteins and various levels of Ca. Therefore, the glucose and insulin data that was obtained only provides an indirect measurement of insulin sensitivity [120]. Future studies should employ a more robust protocol for determining insulin sensitivity (i.e. euglycemic-hyperinsulinemic clamp). Although in theory, 12 diet groups could have been examined to fully elucidate the role of protein source, and Ca in both NE and HFHS diets, our primary objective was to examine the potential for milk-related proteins to prevent further weight gain and therefore the high energy density diets were our chief focus. The NE diets were included as a benchmark for on going weight gain in adult rats. The Ca levels selected for this study reflects doses used in previous studies that have demonstrated efficacy in preventing weight gain in rodents. Zemel *et al* [20] have

previously used 0.4%, 1.2%, and 2.4% Ca and found a direct positive relationship between prevention of weight gain in aP2-*agouti* transgenic mice and Ca intake. Our HC dose (2.4% Ca), has been used repeatedly in the literature in rats and mice [69] to examine body weight regulation and used in similar high doses to examine blood pressure regulation [131].

In conclusion, we demonstrate that complete dairy exerts a significant protective effect against increased adiposity during high energy, *ad libitum* feeding compared to casein and whey independently. Partitioning of nutrients toward LBM instead of FM may partly explain the reduction in HOMA-IR, an indicator of insulin resistance, seen with the complete dairy diets. Reductions in hepatic SREBP1c and PPARγ mRNA may be one mechanism by which energy homeostasis is improved in dairy-fed animals. Future studies will be needed to elucidate the full extent to which individual bioactive ingredients in dairy contribute to body weight regulation as our data would suggest that it is not one of the major protein fractions, whey or casein, acting in isolation. The utility of this work lies in the tremendous increase in the prevalence of MetS and the need to identify dietary strategies for weight management that are affordable and acceptable across a wide population base.

Chapter Five: The Role of Dairy Foods and Calcium in the Promotion of Weight Loss during Energy Restriction [4] [5]

5.1 Introduction

Dairy foods are characterized by high quality protein, a rich micronutrient base, and a variety of bioactive compounds [18]. Recently, several studies have reported a positive role of dairy foods in the management of obesity and its related comorbidities. Analysis of the National Health and Nutrition Examination Survey (NHANES-III) found that those individuals with the highest Ca intake had an 85% lower chance of being in the highest quartile for BMI [21]. Moreover, many RCT, although not unanimously, have found that dietary Ca and dairy foods accelerate fat loss during energy restriction (ER). Zemel *et al* randomized obese individuals with habitual low Ca intake to either a control group consuming less than one serving of dairy food per day or to a treatment group consuming three servings of dairy products per day. Both groups followed prescribed diets with 500kcal/day ER. At the end of 24 weeks, the group with high-dairy product intake had twofold greater weight and fat loss compared to the low-dairy product treatment [58].

Although most observational and many experimental studies have shown a positive association between dairy foods and weight management, the topic remains controversial [99, 132]. It is not known what factors in dairy foods promote weight loss, although the

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[4] A portion of this work was presented and published in part at The Obesity Society Annual Meeting, 2007. Lindsay K. Eller and Raylene A. Reimer. 2007. *Differential effects of various proteins supplemented with calcium on the promotion of weight loss in calorie-restricted Sprague-Dawley Diet-Induced Obese rats.* Obesity 15 (Suppl):A155.

[5] A version of this chapter has been submitted to the Journal of Nutrition and is currently awaiting a decision on acceptance of the revised manuscript. Lindsay K. Eller and Raylene A. Reimer. *Dairy protein promotes weight loss with maintenance of lean body mass better than casein, whey, or soy in energy restricted diet induced obese rats.*

protein and AA composition and the level of Ca have been postulated to play a role. Furthermore, many questions remain unanswered regarding the mechanistic processes through which dairy food and Ca intake influence weight control. Although previously thought to play a major role in the regulation of body weight, Ca alone does not appear to cause the same degree of weight regulatory effects as dairy foods and likely acts together with other bioactive components found in milk-derived products [99].

The protein found in dairy foods consists of ~80% casein and ~20% whey protein. We have previously demonstrated that casein, whey, and complete dairy products (skim milk powder) have differential effects on weight gain during *ad libitum* HFHS feeding with complete dairy (skim milk powder) attenuating weight gain in obese rats better than whey or casein alone. Whether the individual protein fractions of dairy foods (casein and whey) also have distinct effects on weight loss remains to be determined. Our primary objectives, therefore were: (i) to determine the difference among diets where protein is derived from various sources (complete dairy, casein, whey, and soy) on the promotion of weight loss in energy restricted DIO rats; and (ii) to determine if a high Ca diet alters weight loss in the same rat model. Secondary objectives were to examine metabolic biomarkers and gene expression related to glucose homeostasis.

5.2 Materials and Methods

5.2.1 Animals and Housing

The experimental protocol was approved by the University of Calgary Animal Care Committee and conformed to the Guide for the Care and Use of Laboratory Animals. Six week old male Sprague-Dawley rats (n=144) were obtained from Charles

River (Charles River, St. Constant, QC, Canada) and maintained on a 12 hour light-dark cycle in a temperature and humidity controlled room. For six weeks, rats consumed a HFHS diet *ad libitum* to induce obesity. At 12 weeks of age, the rats (n=72) in the upper 50th percentile of body weight were selected as the DIO rats [118] and then individually housed for the remainder of the study.

5.2.2 Experimental Diets

Obese rats were divided into nine weight-matched groups, which included one *ad libitum* group as a reference group for normal food intake and weight gain, and eight energy restricted (ER) groups (Figure 5.1). The diets varied in protein source [complete dairy (skim milk powder), casein, whey, or soy]. Casein and whey were selected as protein sources because they are the major protein fractions of skim milk powder making up 80% and 20% of its protein content respectively. In addition to the milk proteins (complete dairy, casein, and whey), soy protein was also utilized. Soy protein is a plant protein of equal protein quality to milk and has previously been reported as being effective for reducing body weight [133]. No other study has directly examined the effectiveness of soy protein versus the various milk proteins during ER in obese rats.

All NE diets had 12.3% of total energy derived from protein and are outlined in Appendix B (Table A.4). Diets were designed and prepared as described in Chapter 3. All diets were controlled for vitamin D content as the casein, whey, soy, and skim milk powders were not fortified and did not contain significantly different amounts. Free access to water was available throughout the experiment. The *ad libitum* rats consumed AIN-93M to provide a reference value for normal, non-restricted food intake [20]. Food intake for this control group was measured daily by weighing each food cup to the

nearest 0.1 g. ER rats were given 70% of the daily *ad libitum* (control) food intake for four weeks.

Figure 5.1 Schematic of Experimental Diets

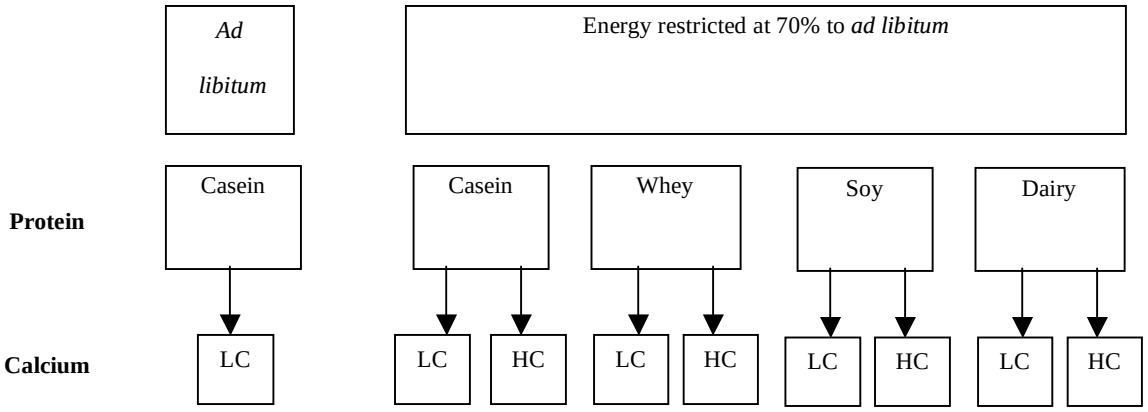

Figure 5.1: The nine experimental diets used to determine the role of various proteins and calcium on the promotion of weight loss during energy restriction (ER) in diet-induced obese rats. The control group consumed casein with low calcium (LC) *ad libitum* and the eight energy restricted diets differed in protein source (casein, whey, soy, or complete dairy) and calcium level [0.67% (LC) or 2.4% (HC)]. All diets were normal energy density (NE) and ER was at 70% to *ad libitum* intake for control rats.

5.2.3 Measurements

5.2.3.1 Body Weight and Composition.

Body weight of all rats was measured at baseline and subsequently once per week. Two days prior to the meal tolerance test (MTT), rats had body composition measured via DEXA as previously described (Chapter 4).

5.2.3.2 Meal Tolerance Test

Following 12 hours of food deprivation, rats were given an oral gavage of their respective diet (3 g of diet/kg body weight) emulsified in water to a total volume of 3 mL. Subsequent blood samples were collected at 15, 30, 60, and 90 minutes post-gavage via cardiac puncture. Blood for the analysis of insulin, leptin, active GLP-1, and glucagon was collected in an EDTA-coated vacutainer (BD Biosciences, Mississauga, ON, Canada) with the addition of aprotinin (5×10^5 KIU/L; Sigma, Oakville, ON, Canada) and diprotin A (0.1mmol/L; MP Biomedicals, Irvine, CA). Plasma was collected and stored at -80˚C until analysis.

5.2.3.3 Tissue Collection

Immediately following the final blood draw, rats were over-anaesthetized with isoflurane and the aorta cut. The liver was weighed and a 200 mg sample of liver was flash frozen in liquid nitrogen. 100mg tissue samples of duodenum, ileum, jejunum, colon, stomach, and white adipose were also collected and flash frozen in liquid nitrogen. Tissue was stored at -80˚C until analysis.

5.2.3.4 Blood Analysis

Blood glucose concentrations were measured with a OneTouch Blood Glucose Meter (BD Biosciences, Mississauga, ON, Canada). Plasma active GLP-1, leptin, insulin,

amylin, and glucagon were measured using a commercial rat endocrine Lincoplex Kit (Millipore Corporation, Billerica, MA). Ghrelin was measured using a commercial ELISA (Millipore Corporation, Billerica, MA). The degree of insulin resistance was determined using HOMA-IR [134].

5.2.3.5 Hepatic Triglyceride and Cholesterol Content

Triglyceride and cholesterol content in liver samples was measured as previously described [110]. Briefly, 25mg of wet liver tissue was weighed and triglycerides extracted with KOH-EtOH solution. Samples were heated at 70^{o}C for one hour and then allowed to rest overnight. Sample volume was brought up to 500µl with 2M Tris-HCl and diluted 1:5 with Tris-HCl. Triglyceride was quantified using a colourmetric assay with a triacylglycerol (GPO) liquid reagent set (Point Scientific, Inc. Lincoln Park, Michigan, USA). 1 mL of GPO was added to a tube for each standard or sample and heated to 37^{o}C for 5 minutes. Standard or sample was added to the GPO and heated for an additional 5 minutes. 200µl of each was added to a plate and read at 500nm. Triacylglycerol content in mmol/L was determined based on the linear curve (Protocol provided by DH Wasserman's lab, Vanderbilt University School of Medicine, Nashville, TN).

Approximately 10mg of wet liver tissue was used for the extraction of liver cholesterol. Following sonication in 200µl of chloroform-Triton X-100 solution, the extract was spun at top speed in a microcentrifuge for 10 minutes. The organic phase was collected and dried at 50^{o}C followed by a 30 minute vacuum dry. The dried lipids were dissolved in Triton X-100 and 200µl of cholesterol reaction buffer. For determination of cholesterol, a colorimetric assay (BioVision Research Products, Mountain View, CA, USA) was used. Cholesterol standard was diluted to create a standard curve. The reaction

mix was added to the standard and test samples followed by incubation for one hour at 37^{o}C. Optical densities of the samples were measured at 570nm. For calculations, the background was first subtracted and then cholesterol concentration was generated based on the standard curve.

5.2.3.6 Gene Expression

Total RNA was extracted from the liver using Trizol Reagent (Invitrogen, Carlsbad, CA). RNA was then quantified using Ribogreen and reverse transcribed as previously described (Chapter 4). Real-time PCR analysis was performed in triplicate using a Bio-Rad Thermal iCycler (Bio-Rad, Mississauga, ON, Canada). GAPDH or β-actin was used as the housekeeping gene and data analyzed using the $2^{-\Delta Ct}$ method [121]. Hepatic genes of interests included: fatty acid synthase (FAS), glucokinase (GK), peroxisome-proliferator-activated receptor gamma co-activator 1 alpha (PGC1α), peroxisome proliferator-activated receptor gamma (PPARγ), phosphoenolpyruvate carboxykinase (PEPCK), and glucose-6-phosphatase (G6Pase). Additionally, the following genes were examined: proglucagon, PYY, and cholecystokinin (CCK) in the duodenum, jejunum, and ileum; ghrelin in the stomach; and UCP2 in the white adipose tissue. Primers used for rt-PCR are provided in Appendix C (Table A.5).

5.2.4 Data Analysis

All data is presented as mean ± SEM. The *ad libitum* reference group was excluded from all statistical analysis. HOMA-IR and total area under the curve (AUC) were calculated according to previous work [120, 135]. Dependent variables were analyzed by two-way ANOVA to examine the effect of Ca (LC or HC), protein (casein, whey, soy, or complete dairy), and their interaction. If protein or calcium independently

affect the outcome variable, statistically, there are main effects of the treatment. Tukey's *post hoc* analysis was performed between protein treatments only when significant main effects were found. However, if the protein and calcium have an interaction, there is an interaction effect. To determine the specific interactions of the treatments, simple main effects testing allows for the determination of the effects between two variables (protein and calcium) that are not independent. Simple main effects' testing was performed when interaction effects were found.

5.3 Results

5.3.1 Food Intake

By design, ER groups were provided with and consumed 70% of the food eaten by the reference control rats. Over the four week period, control rats ate a mean of 29.8 ± 3.4 g/d (107.4±11.8 kcal/d) and ER rats consumed 20.9± 2.2 g/d (75.2±8.1 kcal/d). There was no significant difference in food intake between the eight ER treatment groups ($p>0.05$).

5.3.2 Body Composition

5.3.2.1 Body Weight

Initial body weight (all: 622.6 ± 7.6 g) and both initial (all: 20.6 ± 0.5 cm) and final (all: 20.9 ± 0.3) crown to rump length were not significantly different between groups ($p>0.05$). Analysis of final weight (g) revealed a significant interaction effect of Ca x protein ($p=0.027$) (Table 5.1). In the complete dairy and the soy protein groups, HC resulted in a final weight that was less than the LC treatment (Figure 5.2A). Ca level had

no effect with the casein treatment, whereas the whey treatment was associated with weight gain with HC (Figure 5.2A).

5.3.2.2 Weight Loss

Analysis of weight change (g) found an interaction effect of Ca x protein ($p=0.035$) (Table 5.1). When consumed with HC, soy protein and complete dairy resulted in greater weight loss whereas there was no effect of Ca level when consumed with casein; and less weight loss with HC when consumed with whey (Figure 5.2B).

5.3.2.3 Body Composition

There were significant main effects of both protein ($p=0.028$) and Ca ($p=0.006$) on FM (%). Treatment with complete dairy resulted in less FM (%) compared to all other treatments ($p=0.07$) (Table 5.2) and the HC treatment resulted in significantly less FM (%) than the LC treatment ($p<0.05$) (Figure 5.3A, Table 5.3). Absolute FM (g) had a significant Ca x protein interaction ($p=0.027$) (Table 5.1). The combination of complete dairy and HC resulted in the greatest decrease in FM (g) (Figure 5.2C). Casein and soy protein also had a similar trend of decreased FM with HC; however, whey treatment with HC resulted in minimal change in FM. LBM (g) showed main effects of protein source, with complete dairy being significantly greater than whey ($p=0.028$) (Figure 5.2B, Table 5.2).

Table 5.1 Body Composition and Parameters of Glucose Metabolism

Table 5.1: Body composition and parameters of glucose metabolism at the end of the four-week ER period in Sprague-Dawley DIO rats fed casein, soy, whey, or complete dairy diets containing either low (LC) or high calcium (HC).

			Protein Source				Significance, p		
	Ad Lib		Casein	Soy	Whey	Dairy	P	C	P x C
Final Weight, g	644.3± 9.6	LC	571.1±8.3^{ab}	592.0±6.4^{a}	542.6±15.0^{b}	575.6±11.0^{ab}	0.02	0.06	0.02
		HC	568.4±4.7^{ab}	559.4±4.7^{ab}	557.1±7.0^{ab}	553.8±6.7^{ab}			
Weight Change, g	23.4± 9.7	LC	-57.0±6.7^{ab}	-40.2±6.4^{a}	-89.7±9.3^{b}	-55.9±10.8^{ab}	0.01	0.04	0.03
		HC	-60.9±6.7^{ab}	-74.0±4.6^{ab}	-77.4±6.2^{b}	-78.0±10.6^{ab}			
Fat, %	26.2± 1.4	LC	21.0±1.4^{b}	21.9±2.7^{b}	17.7±0.9^{ab}	19.8±1.7^{ab}	0.03	0.01	0.18
		HC	19.5±1.3^{ab}	18.4±0.9^{ab}	17.5±0.4^{ab}	13.1±1.8^{a}			
FM, g	149.8± 5.3	LC	122.6±7.8^{b}	130.1±16.6^{b}	94.1±6.0^{ab}	115.0±11.4^{b}	0.01	0.01	0.02
		HC	107.3±5.3^{ab}	106.6±4.4^{ab}	106.2±4.3^{ab}	72.5±10.3^{a}			
LBM, g	490.5± 7.9	LC	448.5±8.0^{ab}	461.9±15.5^{ab}	446.6±8.8^{a}	460.6±3.3^{ab}	0.03	0.11	0.31
		HC	459.2±5.5^{ab}	452.8±4.4^{ab}	451.2±7.8^{ab}	481.2±9.1^{b}			

Baseline Glucose, mmol/L	6.3± 0.2	LC	5.8±0.5	4.4±0.1	4.4±0.2	4.4±0.3	0.01	0.08	0.70
		HC	5.9±0.4	4.9±0.3	4.7±0.5	5.4±0.2			
Baseline Insulin, pM	144.6± 5.4	LC	155.3±12.0^{b}	200.8±42.0^{ab}	114.6±25.9^{b}	97.1±14.8^{b}	0.001	0.07	0.72
		HC	176.3±21.4^{ab}	239.6±19.2^{a}	150.9±4.9^{b}	110.0±28.0^{b}			
Baseline Glucagon pM	58.4± 7.0	LC	46.2±9.2^{a}	27.1±2.6^{ab}	9.3±2.0^{b}	16.7±4.2^{b}	0.001	0.39	0.09
		HC	28.0±4.6^{ab}	27.5±4.6^{ab}	15.9±2.1^{b}	15.0±4.8^{b}			
Glucose AUC, mM/90 min	1.2± 0.1	LC	1.2±0.1^{ab}	1.6±0.2^{b}	1.3±0.1^{ab}	1.4±0.1^{ab}	0.01	0.01	0.17
		HC	0.8±0.04^{a}	1.1±0.05^{ab}	1.0±0.1^{ab}	1.6±0.2^{b}			
Insulin AUC, nM/90 min	14.3± 0.8	LC	14.7±0.6	11.2±1.3	10.2±1.2	12.0±1.5	0.01	0.18	0.09
		HC	14.5±0.6	12.8±1.0	14.2±0.4	10.7±1.7			
Glucagon AUC, nM/90 min	1.3± 0.08	LC	2.2±0.5	1.9±0.5	2.0±0.0.3	2.3±0.3	0.76	0.01	0.66
		HC	1.1±0.2	1.1±0.2	1.6±0.2	1.2±0.1			
HOMA-IR	4.1± 0.3	LC	4.0±0.4^{ab}	3.9±0.8^{ab}	2.2±0.5^{b}	1.8±0.3^{b}	0.01	0.04	0.86
		HC	4.5±0.6^{ab}	5.3±0.7^{a}	3.2±0.2^{ab}	2.6±0.6^{b}			

Results are presented as mean ± SEM, n=8/treatment. Means with different superscripts, $p<0.05$ as determined by one-way ANOVA. The *ad libitum* (control) group was excluded from analysis.

Table 5.2 Body Composition and Parameters of Glucose Metabolism by Protein

Table 5.2: Body composition and parameters of glucose metabolism at the end of the four-week ER period in Sprague-Dawley DIO rats fed casein, soy, whey, or complete dairy diets.

	Casein	Soy	Whey	Dairy
Fat, %	20.2±0.9	19.7±1.2	17.6±0.4	16.5±1.6
Lean Body Mass, g	453.6±5.0^{ab}	456.2±6.2^{ab}	446.0±6.0^{b}	471.0±5.6^{a}
Baseline Glucose, mmol/L	5.9±0.3^{a}	4.7±0.2^{b}	4.6±0.2^{b}	4.9±0.3^{ab}
Baseline Insulin, pM	166.4±12.5^{b}	226.7±18.7^{a}	140.5±8.7^{bc}	103.5±15.2^{c}
Baseline Glucagon, pM	36.1±5.2^{a}	27.4±3.0^{a}	13.5±1.7^{b}	15.9±3.0^{b}
Glucose AUC, M/90 min	1.0±0.08^{b}	1.2±0.1^{ab}	1.1±0.1^{ab}	1.5±0.1^{a}
Insulin AUC, nM/90 min	14.6±0.4^{a}	12.2±0.8^{ab}	12.3±0.8^{ab}	11.4±1.1^{b}
HOMA-IR	4.2±0.3^{a}	4.9±0.6^{a}	2.9±0.3^{b}	2.2±0.4^{b}

Results are presented as mean ± SEM, n=16/protein treatment. Means with different superscripts, $p<0.05$

Table 5.3 Body Composition and Parameters of Glucose Metabolism by Calcium

Table 5.3: Body composition and parameters of glucose metabolism at the end of the four-week ER period in Sprague-Dawley DIO rats fed low (LC) or high calcium (HC) diets.

	LC	HC
Fat, %	20.3±0.9	17.6±0.7*
Glucose AUC, M/90 min	1.3±0.08	1.1±0.08*
Glucagon AUC, nM/90 min	2.1±0.2	1.3±0.1*
HOMA-IR	3.2±0.3	4.0±0.3*

Results are presented as mean ± SEM, n=32/calcium treatment. * Different from LC, $p<0.05$.

Figure 5.2 Simple Effects of Protein and Calcium

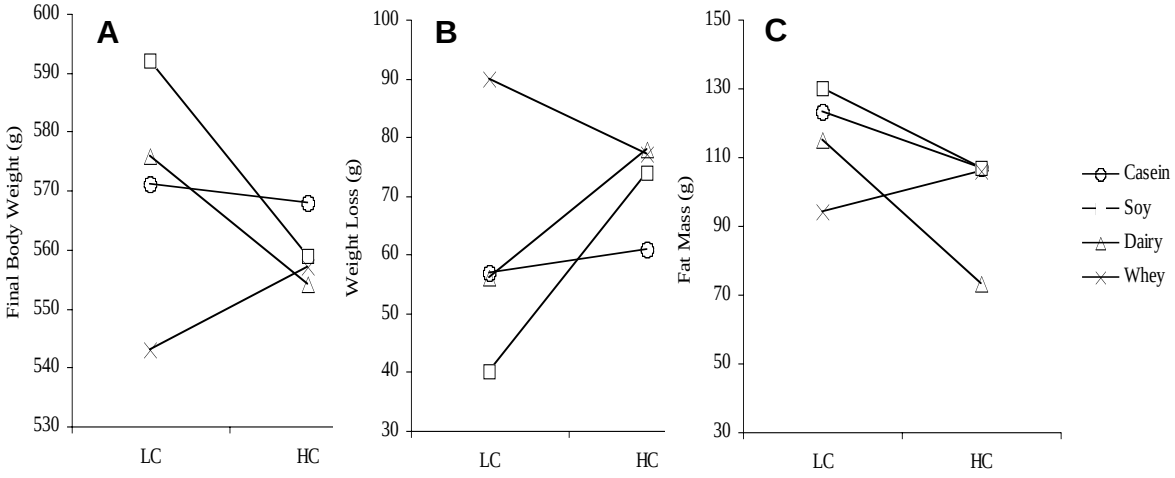

Figure 5.2: Representation of the simple main effects of significant protein x Ca interactions found with (A) final body weight in grams; (B) weight loss in grams; and (C) final fat mass in grams. Results are presented as mean, n=8/treatment

Figure 5.3 Anthropometrics

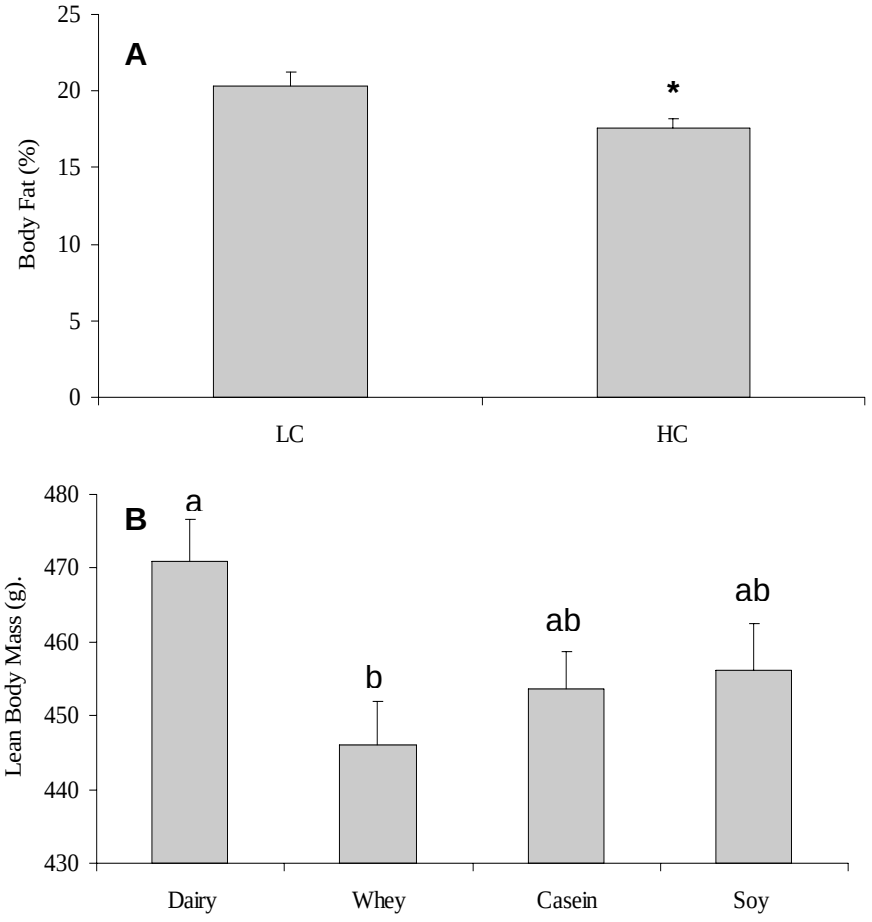

Figure 5.3: Anthropometric measurements at the end of the four-week ER period. (A) final body fat percentage for low (LC) and high calcium (HC). All values represent mean ± SEM, n=32/calcium treatment * Different from LC ($p<0.05$); (B) lean body mass in grams for protein treatments (casein, soy protein, whey, and complete dairy. All values represent mean ± SEM, n=16/protein treatment. Bars without a common letter differ, $p<0.05$.

5.3.3 Plasma Hormones and Glucose

5.3.3.1 Glucose

Analysis by two-way ANOVA indicated a main effect of protein on baseline (food deprived) blood glucose levels ($p=0.001$) with the casein treatment having significantly greater glucose than all other groups ($p<0.05$) (Table 5.2). Glucose responses at individual time points during the MTT were not significantly different among ER groups (Figure 5.4). However, total glucose AUC had main effects of both protein (Table 5.2) and Ca ($p<0.05$) (Table 5.3).

5.3.3.2 Insulin

Analysis by two-way ANOVA indicated a main effect of protein on baseline insulin ($p<0.001$) and total insulin AUC ($p=0.012$) (Table 5.2). In the complete dairy treatment, baseline insulin levels were significantly less than casein and soy protein ($p<0.05$) and total insulin AUC for complete dairy was significantly less than casein ($p<0.05$) (Figure 5.4).

5.3.3.3 Glucagon

There was a main effect of protein ($p<0.001$) on baseline glucagon (Table 5.2). Complete dairy and whey had significantly lower baseline glucagon than casein or soy (Table 5.2). Total glucagon AUC demonstrated a main effect of Ca with HC reducing total AUC ($p=0.001$) (Table 5.3) (Figure 5.4).

5.3.3.4 Ghrelin, Leptin, Amylin, and GLP-1

There were no significant differences in ghrelin, leptin, amylin and GLP-1 when measured at individual time points or when calculated as total AUC. Feed deprived

baseline values for GLP-1, ghrelin, amylin, and leptin for all rats combined were 18.8±0.9, 14.0±4.3, 5.7±0.8, and 581.7±239.1 pM, respectively.

5.3.4 Homeostatic Model of Assessment – Insulin Resistance (HOMA-IR)

Analysis of HOMA-IR showed main effects of both Ca ($p=0.04$) and protein ($p=0.001$) (Table 5.1). There was a significant reduction (improvement) in HOMA-IR with complete dairy and whey compared to casein and soy (Table 5.2) and with LC compared to HC ($p<0.05$) (Table 5.3).

5.3.5 Liver Triglycerides and Cholesterol

Analysis of hepatic triglyceride (TG) content demonstrated a main effect of protein ($p=0.011$) with the complete dairy group (33.0±6.9 mg/g wet liver weight) having significantly less TG than whey (77.3±9.8 mg/g wet liver weight) ($p=0.007$). Casein and soy were intermediate (53.5±12.0 and 45.1±9.8 mg/g wet liver weight, respectively). There was no significant effect of Ca on TG and there was no significant effect of protein or Ca on liver cholesterol content.

Figure 5.4 Plasma Glucose, Insulin, and Glucagon

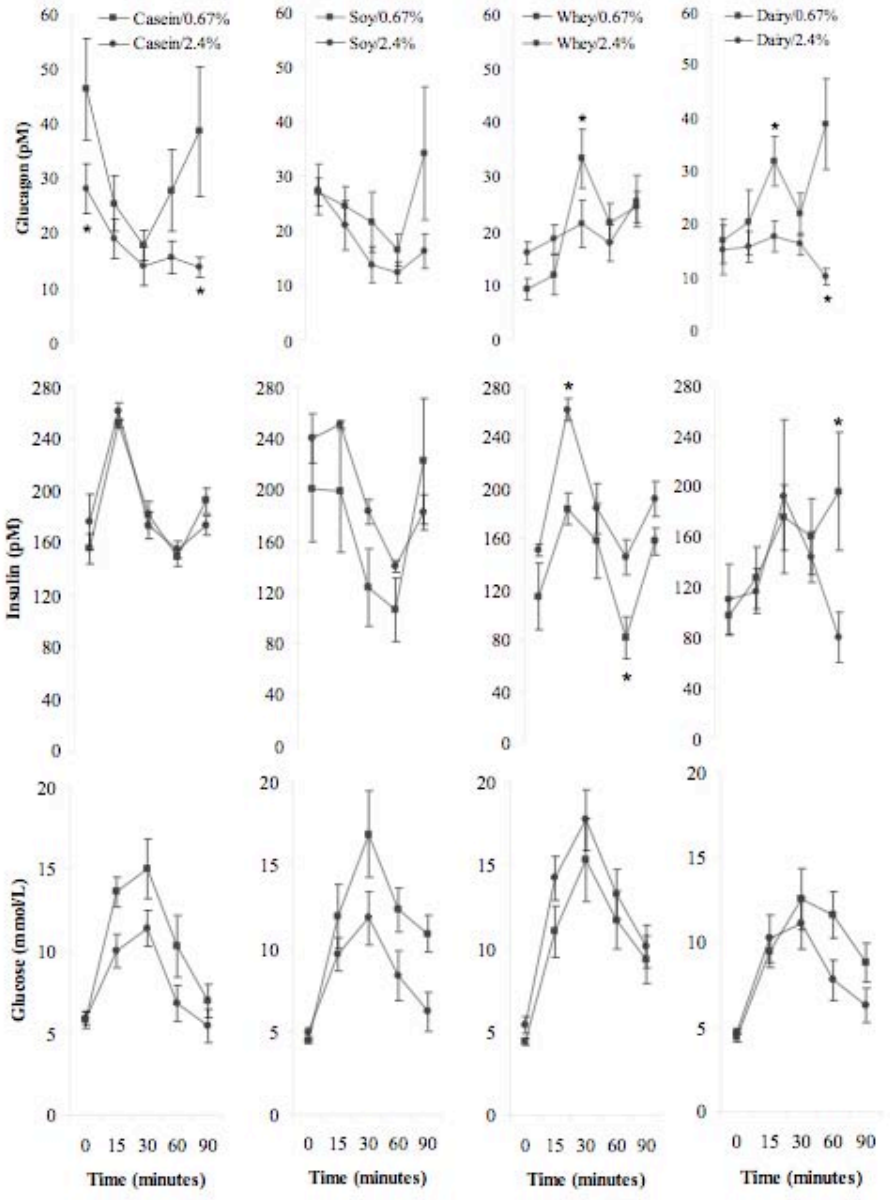

Figure 5.4: Postprandial glucose, insulin, and glucagon responses at the end of the four-week energy restriction period for rats consuming treatment diets. Results are presented as mean ± SEM, n=8/treatment.

5.3.6 Gene Expression

There was no effect of dietary treatments on gut, stomach, or white adipose gene expression. However, hepatic gene expression was altered by both protein source and Ca level.

5.3.6.1 Main Effects of Protein on Hepatic Gene Expression

Analysis of FAS and PGC1α gene expression revealed a main effect of protein (Table 5.4, Figure 5.5A). FAS and PGC1α mRNA expression was significantly greater with soy protein (LC and HC) compared to casein, complete dairy, and whey.

5.3.6.2 Main Effects of Calcium on Hepatic Gene Expression

There was a main effect of Ca level on G6Pase mRNA expression with reduction occurring in HC treatments (Table 5.4, Figure 5.5B).

5.3.6.3 Interaction Effects of Calcium and Protein on Hepatic Gene Expression

PPARγ, PEPCK, and GK all had significant protein x Ca interactions when analyzed by two-way ANOVA (Table 5.4, Figure 5.6). PPARγ was upregulated with the HC treatment only when consumed with the soy protein; expression of PPARγ following casein, whey, and complete dairy treatment was not altered by Ca level (Figure 5.6A). PEPCK was downregulated with the HC diet in the soy protein, complete dairy, and whey treatments; but upregulated with the casein treatment (Figure 5.6B). Protein also altered PEPCK expression with greater expression in the soy and dairy LC groups and lower expression in the casein LC group (Figure 5.6B). GK expression was down regulated in the casein and whey treatments when consumed with HC (Figure 5.6C).

Conversely, soy protein with HC upregulated GK compared to LC. There was no effect of Ca level on the complete dairy treated rats. Protein also altered GK gene expression with casein and whey having greater expression than complete dairy and soy (Figure 5.6C).

Table 5.4 Hepatic Gene Expression

Table 5.4: Gene expression (expressed as arbitrary units) at the end of the four-week ER period in Sprague-Dawley DIO rats fed casein, soy, whey, or complete dairy diets containing either low (LC) or high calcium (HC).

		Protein				Statistical Significance, p		
		Casein	Soy	Whey	Dairy	P	C	P x C
FAS	LC	17.2±4.1	126.0±20.2	35.2±10.8	16.7±4.8	<0.001	0.529	0.156
	HC	20.2±9.9	86.8±33.9	13.6±2.6	47.0±16.4			
PPARγ	LC	15.9±5.1	7.9±3.4	5.5±1.9	8.5±2.0	0.010	0.027	0.004
	HC	11.2±1.3	39.6±10.8	14.2±4.7	5.6±1.1			
G6Pase	LC	4.6±2.5	14.2±3.7	12.8±5.2	12.1±1.7	0.452	0.017	0.076
	HC	8.6±4.6	7.6±2.3	1.4±1.0	5.6±1.3			
PEPCK	LC	9.4±2.5	159.2±29.8	59.5±19.9	143.2±23.8	<0.001	0.199	0.012
	HC	75.7±11.5	101.1±25.1	41.6±14.5	79.0±19.5			
PGC1α	LC	21.9±3.9	24.0±5.0	7.9±4.3	16.0±2.5	<0.001	0.103	0.275
	HC	9.5±2.6	25.3±6.5	5.7±1.0	12.9±0.9			
GK	LC	91.6±32.5	16.8±3.8	53.2±10.2	9.6±0.4	0.001	0.050	0.010
	HC	28.4±4.3	34.6±9.3	30.2±13.6	10.2±1.8			

Results are presented as mean ± SEM, n=8/treatment. Statistical significance was set at $p<0.05$. FAS, fatty acid synthase; PPARγ, peroxisome-proliferator-activated receptor gamma; G6Pase, glucose-6-phosphatase; PEPCK, phosphoenolpyruvate carboxykinase; PGC1α, peroxisome-proliferator-activated receptor gamma co-activator 1 alpha; GK, glucokinase.

Figure 5.5 Hepatic Gene Expression

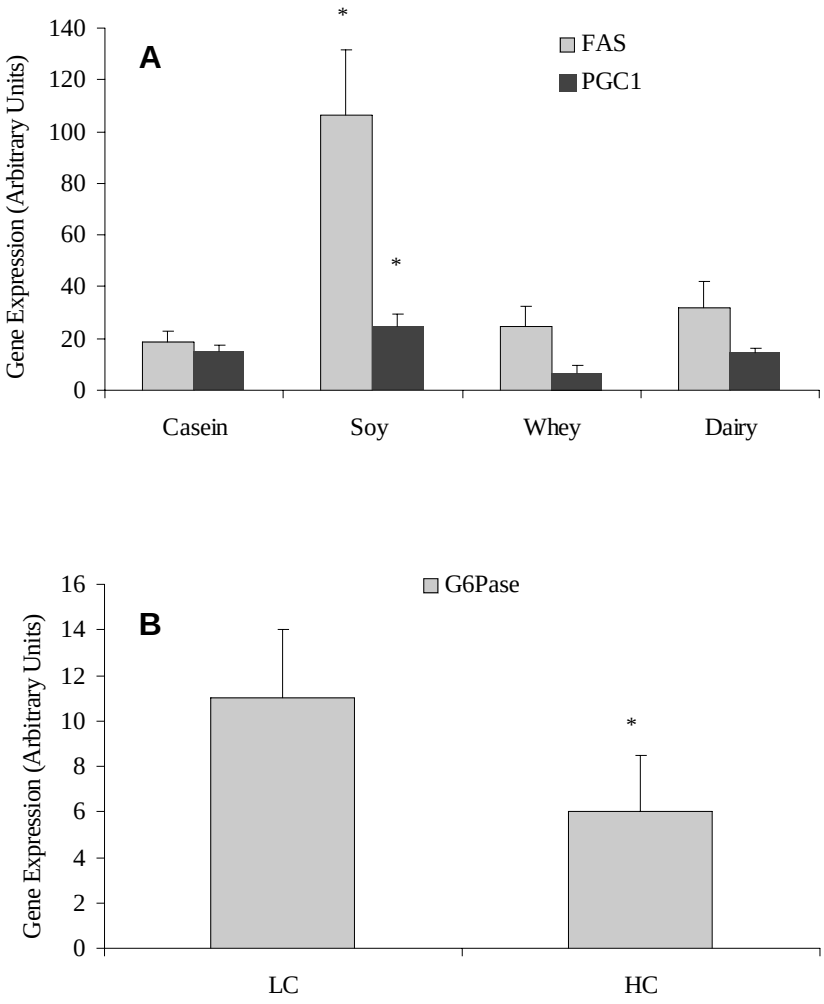

Figure 5.5: Hepatic expression of those genes that had significant ($p<0.05$) main effects of protein or main effects of Ca (A) FAS and PGC1α, n=16; (B) G6Pase, n=32. Results are presented mean ± SEM. * Different from other treatments, $p<0.05$. FAS, fatty acid synthase; PGC1, peroxisome-proliferator-activated receptor gamma co-activator 1 alpha; and G6Pase, glucose-6-phosphatase.

Figure 5.6 Hepatic Gene Expression (Simple Effects of Protein x Calcium Interaction)

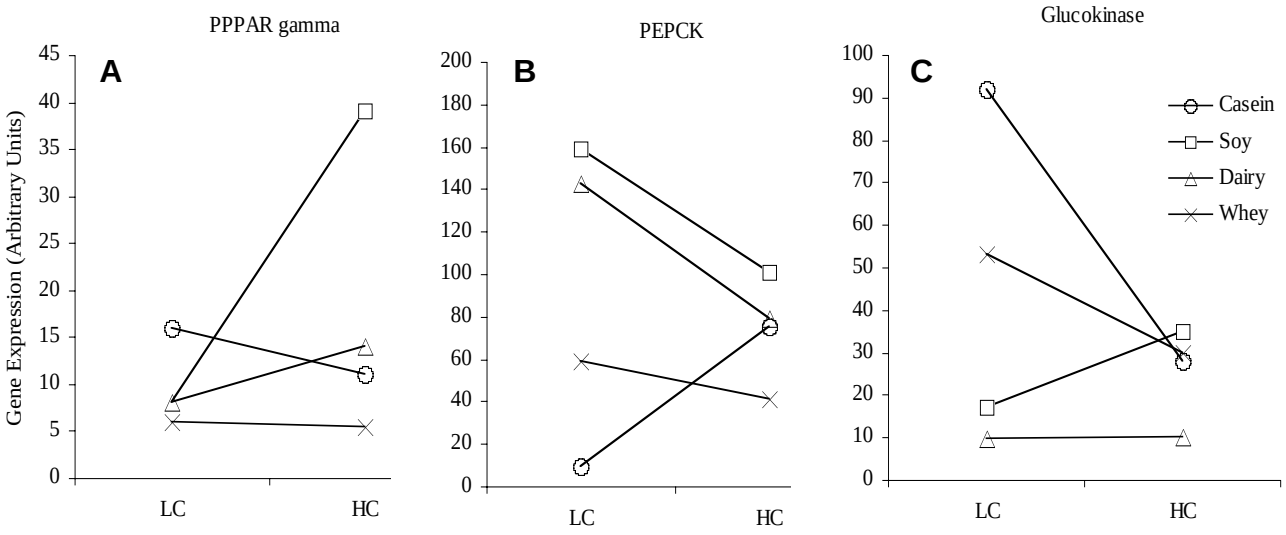

Figure 5.6: Representation of the simple main effects for significant protein x Ca interactions for gene expression of (A) peroxisome proliferator-activated receptor gamma (PPARγ); (B) phosphoenolpyruvate carboxykinase (PEPCK); and (C) glucokinase (GK). Results are presented as mean, n=8/treatment.

5.4 Discussion

In this study evaluating the effect of protein source (casein, whey, soy, and complete dairy) and Ca on weight loss during ER we demonstrate that: (i) there is a significant protein – Ca interaction that affects weight loss and body composition; (ii) consumption of complete dairy during ER preserves LBM when compared to whey protein; and (iii) dietary protein and Ca both have significant main effects on parameters of glucose homeostasis.

Our first major finding is that there is a significant interaction of protein source with Ca in regards to final body weight, weight loss, and final FM (Table 5.1, Figure 5.2). The soy protein and the complete dairy treatments were associated with lower final body weight (Figure 5.2A), greater weight loss (Figure 5.2B), and a decrease in FM (Figure 5.2C) when consumed with HC than when with LC. This is in contrast to casein, which was not affected by Ca level, and whey protein that showed the opposite effect (Figure 5.2). This is consistent with literature that suggests dairy foods assist with weight management and that Ca found in dairy foods enhances the weight regulatory effect [99]. Although both complete dairy and soy protein, when consumed with HC, promoted greater weight loss (Figures 5.2A, 5.2B), complete dairy with HC showed a greater interaction effect on FM (g) (Figure 5.2C). This would further the evidence that dairy products are beneficial in weight management, due to Ca and other unknown components found in complete dairy products.

Several factors found in dairy products have been suggested to regulate body composition. These include the AA composition, micronutrients such as vitamin D, Ca,

or phosphorus, various other immunoproteins, and lactose [18]. Our diets were matched for vitamin D levels and Ca; therefore suggesting a protein-linked factor or one of the other unique bioactive ingredients found in complete dairy is likely the contributing factor. Dairy protein is a high quality protein consisting of ~80% casein and ~20% whey. Resultantly, it is rich in the branched chain amino acids (BCAA): leucine, isoleucine, and valine. The BCAA are thought to play an important role in maintenance of LBM [115]. Dietary leucine has been shown to improve body composition in rats that consume leucine in their drinking water [69]. Similarly, our data showed significant maintenance of LBM with ER in the complete dairy treated rats (Table 5.2 and Figure 5.3B). Of relevance to these findings, are the digestive properties of casein (slow protein) and whey (fast protein). It is plausible that as dairy protein is digested, the casein coagulates in the stomach resulting in slower transit time and a resultant greater absorption of the whey fraction and respective whey-sourced bioactive components. This may cause the complete dairy treatment to have an increased intestinal absorption of one of the BCAA [97]. Future work on the role of leucine and the absorption of the BCAA from dairy products is warranted since it is known that consumption of various proteins in humans results in different postprandial plasma AA profiles; whey protein generating greater leucine and isoleucine levels in the blood than casein or soy [133, 136]

One of the major clinical concerns with weight loss via ER is the concurrent decrease of LBM with the desired loss of FM. While other isolated dairy ingredients, such as lactose, have been shown to reduce FM in rats fed a HFHS diet [137] the preservation of LBM seen with the complete dairy treatment in this study suggests that nutrient partitioning to LBM is enhanced. This beneficial distribution of tissues may

assist with future weight loss and/or weight maintenance by preservation of basal metabolic rate [122].

Several other mechanisms have been proposed to explain the enhanced weight loss seen with a complete dairy or HC diet. These include satiety hormone regulation; modulation of $[Ca]_i$ levels; increased thermogenesis via UCP2 upregulation; and increased FF via binding to Ca [72, 99]. We did not find a difference in amylin, ghrelin, or GLP-1 in this study. This is in contrast to many studies that have found a difference in satiety hormone expression among whey, soy, and/or casein [136]. A potential explanation for lack of change in our study could be the standard protein content (12.3% of energy) in our diets. Many studies noting a difference in satiety hormones have used high-protein diets or meals and a dose-dependent effect of protein on hormone secretion has been shown [136] [138]. The weight loss seen in our study is greater than can be attributed solely to FF, however it is plausible that it is a contributing factor since there was a significant main effect of Ca on FM (%) (Table 5.3). An increase in adipocyte UCP2 and a change in $[Ca]_i$ levels also cannot be ruled out as potential mechanisms of action as they were not measured in this study [99].

Our third major finding is that consumption of diets with protein derived from various sources alters parameters of glucose homeostasis, including glucose, insulin, glucagon, and HOMA-IR. Baseline levels of glucose, insulin, and glucagon all had significant main effects of protein (Table 5.2). Baseline glucose was significantly lower with all protein treatments compared to casein, yet total AUC for casein was less than the other protein sources. The largest glucose AUC occurred in the complete dairy treatment, even though baseline glucose, insulin, and glucagon and HOMA-IR for complete dairy

would suggest improved glucose homeostasis. Postprandial insulin response was also reduced in the complete dairy treatment. A more direct measurement of insulin sensitivity such as a euglycemic-hyperinsulinemic clamp would be warranted to further examine how protein and Ca affect parameters of glucose homeostasis.

HOMA-IR for the complete dairy and whey treatments was less than the casein and soy groups, suggesting improved insulin sensitivity. This observation supports two recent large epidemiological studies where dairy intake was positively linked to insulin sensitivity [31, 32]. Furthermore, data from our lab evaluating prevention of weight gain [110], showed similar findings of an improvement in insulin sensitivity in complete dairy fed rats compared to casein or whey. In addition to an improvement in body fat, hepatic TG content was also reduced in the complete dairy treatment, which may play a role in improving insulin sensitivity. A study by Wat *et al* [139], found that hepatic steatosis was reduced in mice fed a HFHS diet when it was enriched with dairy milk extract. Consistent with these findings is the greater expression of hepatic FAS and PPARγ in the soy protein treatment (Table 5.4, Figures 5.5 and 5.6). FAS and PPARγ are both upregulated in obesity and contribute to worsening of insulin sensitivity [140].

In addition to the protein effects, Ca significantly reduced both baseline and postprandial glucagon response. Secretion of glucagon by the ∝-cells of the pancreas is critical for glucose homeostasis. In diabetes, an excess of glucagon relative to insulin appears to be partially responsible for hyperglycaemia. Hepatic glucose output is elevated in part due to a lack of suppression of glucagon during hyperglycaemia [141]. The authors are not aware of any study to date that has found regulation of glucagon by dietary Ca, however other cations such as zinc are known to play a role in glucagon

secretion [142]. It is plausible that an increase in dietary Ca could regulate pancreatic ∝-cell function; this requires more thorough examination. Glucagon promotes glycogenolysis in the liver, which in turn increases blood glucose. G6Pase is an enzyme that is critical for regulation of this pathway and is positively regulated by glucagon levels. Hepatic gene expression of G6Pase was reduced with HC, which is consistent with our finding that plasma glucagon was less with HC. However, it is not known whether it was glucagon or the direct effect of Ca that altered this gene. Numerous pharmacological treatments for diabetes (imidazolines, GLP-1, GLP-1 mimetics, and DPP4 inhibitors) either indirectly or directly stimulate insulin and inhibit glucagon secretion [143-145].

The data regarding glucose homeostasis and insulin sensitivity is not consistent among groups in this study. There are factors that would suggest that complete dairy improves insulin sensitivity (HOMA-IR), yet other measures (total glucose AUC) contradict this. Additionally, the main effects of Ca also suggest this; the greater HOMA-IR is not consistent with the AUC and body composition data (Table 5.3). The disparity in this data may be due to the protein – Ca interaction effects shown with expression of PPARγ, PEPCK, and GK, the main effects of protein on FAS and PGC1α, and/or the main effects of Ca on G6Pase (Figures 5.5, 5.6). PGC1α, PEPCK, GK, and G6Pase are all key factors in the regulation of glucose homeostasis with PGC1α, PEPCK, and G6Pase down regulating glycolysis and glycogenesis and up regulating gluconeogenesis; GK does the opposite [146]. A schematic of these genes and associated pathways can be found in Appendix E. Our data demonstrates that Ca and protein alter expression of these genes differently and therefore may result in inconsistencies when examining glucose

homeostasis. Further examination of dietary factors that alter hepatic genes related to glucose metabolism is warranted to fully determine if complete dairy and Ca consumption are beneficial for glycemic control. Future work should also focus on the role of particular AA in regulating hepatic genes related to glucose and lipid metabolism and direct measurements of portal and systemic AA concentrations

In conclusion, many studies to date have examined the role of dairy foods in body weight regulation; this is the first study to our knowledge to provide a direct comparison of casein, whey, soy, and complete dairy supplemented with HC or LC on the promotion of weight loss during ER. We have shown that the complete dairy diet exerts a protective effect on LBM during ER. The partitioning of nutrients towards LBM instead of FM may explain the improvement of HOMA-IR found with complete dairy. The decrease in glucagon seen with HC and the significant protein – Ca interactions may help to explain the apparent synergistic effect of dairy sourced Ca found both in this work and previous work. Future studies are needed to examine the role of specific bioactive protein fractions found in dairy food as our data suggests that neither casein nor whey alone match the metabolic benefits seen with complete dairy products.

Chapter Six: The Role of Complete Dairy and Leucine on Insulin Sensitivity [6] [7]

6.1 Introduction

The important role of dairy foods and calcium (Ca) in bone health has long been known; however more recent studies have demonstrated that both dairy foods and elemental Ca may be significant regulators of body weight and insulin sensitivity [18]. Many epidemiological (cross-sectional, retrospective, and prospective) studies have found a positive role of dairy foods and Ca in weight regulation, body composition, and risk of developing insulin resistance. Recently, two large prospective studies have both found that the risk of insulin resistance decreases significantly with dairy food consumption in men (n=41,254) and women (n=37,183) [31, 32]. After adjusting for confounders including BMI, physical activity, and various other lifestyle confounders, it was found that with each additional serving of low fat dairy foods, the risk of developing type 2 diabetes decreased by 9% in men and by 4% in women.

There have also been many randomized experimental studies, both in animals [110, 147] and humans, which have found similar results. Barr *et al* advised free-living elderly individuals to consume three cups of milk per day or maintain their usual diet. While the subjects consuming the extra milk gained 0.6 kg more than the standard care

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[6] A portion of this work was presented and published at: The Obesity Society Annual Meeting, 2008 (Phoenix, AZ, USA). Lindsay Eller, Jane Shearer, and Raylene Reimer. *Insulin sensitivity is improved with leucine supplementation in diet induced obese rats.* Obesity 16 (Suppl 1):207-P.

[7] A portion of this work was presented as part of the Keystone Symposium: Type 2 Diabetes and Insulin Resistance (January, 2009, Banff, AB, CAN). Lindsay Eller, Jane Shearer, and Raylene Reimer. *Leucine, not calcium improves insulin sensitivity in obese rats.* Abstract #154.

group, this was less than would be predicted based on the increased energy intake of the milk group [59]. Similarly, in obese adults who restricted energy intake (500 kcal/day) for 12 weeks and consumed either three yogurts/day or one yogurt/day, the high yogurt consumers lost 22% more weight with a larger proportion derived from body fat [60]. In most intervention studies examining the impact of dairy foods and Ca on metabolic outcomes, Ca consumed in dairy foods seems to have a greater effect than equal amounts of Ca consumed as a supplement (elemental Ca). This would suggest that a component of dairy food is working synergistically with Ca to enhance these effects.

Although there is a significant body of literature pointing to a positive role of Ca and more specifically, dairy foods, in regulating body weight and metabolism, the mechanism by which this occurs is not known. Dairy foods are a rich source of essential AA and BCAA, particularly leucine. In addition to acting as a stimulus for protein synthesis, leucine activates the mammalian target of rapamycin (mTOR). mTOR is a serine/threonine kinase that alters metabolism, insulin sensitivity and other physiological processes related to growth [115]. Studies have shown that when obese energy-restricted rodents are supplemented with leucine; leucine promotes greater weight loss compared to a control diet [148]. Moreover, leucine supplementation has been shown to favourably regulate glucose metabolism and slow the progression of diet-induced obesity [69].

It is evident that dairy foods and Ca play a role in health and certain disease states. The particular components of dairy that contribute to these effects are unknown. Increasing our understanding of the specific bioactive components of dairy would strengthen existing evidence for the role of dairy foods in promoting health. The primary aims of this study were: (i) to compare the effects of diets with protein derived from

casein, casein supplemented with leucine, and complete dairy on body composition and insulin sensitivity; and (ii) to determine if there is a synergistic effect of dietary Ca and protein source on body composition and insulin sensitivity. Secondarily, we used microarray analysis to examine the effect of casein, leucine, or complete dairy containing diets on the expression of hepatic genes related to lipid and glucose metabolism.

6.2 Materials and Methods

6.2.1 Animals

Ethical approval was obtained from The University of Calgary Animal Care Committee. Rats had free access to water at all times and were kept on a 12-hour light-dark cycle. Six-week-old male Sprague-Dawley rats (n=132) were obtained from Charles River (Charles River, St. Constant, QC, Canada). For six weeks rats consumed *ad libitum* a HFHS diet to induce obesity. As previously described (Chapter 4), at 12 weeks of age, DIO rats were selected and individually housed for the remainder of the study (n=66). Rats were divided into six weight-matched groups and assigned to one of six *ad libitum* HFHS diets for eight weeks.

6.2.2 Experimental Diets

All diets were prepared in-house as previously described (Chapter 3). All diets had an energy density of 4.6 kcal/gram and provided 10% of total energy from protein [casein, complete dairy (skim milk powder), or leucine-supplemented casein (7.1% from casein plus 2.9% from leucine)]. Within each type of protein, calcium (Ca) levels of 0.67% (LC) or 2.4% (HC) were used based on previous work (Figure 6.1) [20, 21, 69]. The total leucine content of the respective diets was 0.81%, 1.13% and 3.71% of total

energy in casein, complete dairy and leucine-supplemented diets. Fat in the diet was supplied as 100g/kg of lard and 100g/kg of soybean oil and each diet contained 513 g/kg of sucrose (Appendix B, Table A.3). *Ad libitum* food intake was measured daily and all spillage was collected from the bottom of the cages and measured.

Figure 6.1 Schematic of Experimental Diets

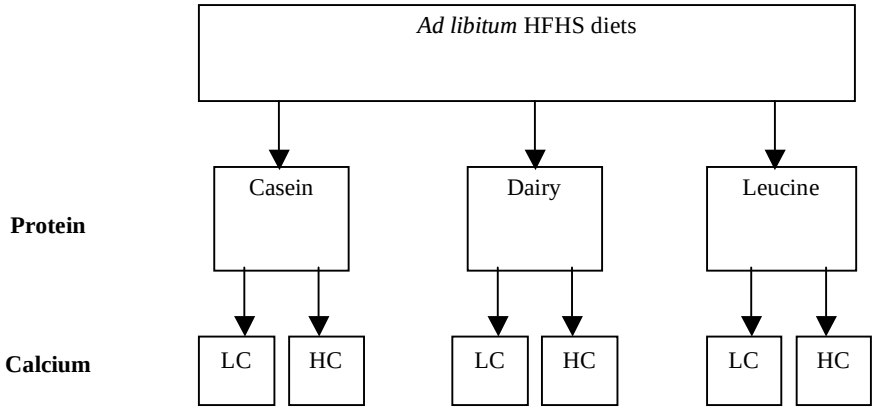

Figure 6.1: The six experimental diets used to determine the role of various proteins and calcium on the prevention of weight gain and insulin sensitivity in *ad libitum* fed, diet-induced obese rats. Diets differ in protein source (casein, complete dairy, casein+leucine) and calcium level [0.67% (LC) or 2.4% (HC)]. All diets are high energy density (HFHS).

6.2.3 Measurements

6.2.3.1 Body Weight and Composition

Body weight of all rats was measured prior to feeding experiments and subsequently once per week. Immediately prior to catheterization surgery, body composition was measured via DEXA as described in Chapter 4.

6.2.3.2 Indirect Measures of Insulin Sensitivity

Ten days prior to the euglycemic-hyperinsulinemic clamp, rats were randomized to receive an oral glucose tolerance test (OGTT) or an insulin tolerance test (ITT). For the OGTT, rats were food deprived for 12 hours and then baseline blood glucose was obtained via tail nick. Rats were then orally gavaged with 2g/kg glucose and subsequent blood samples collected via tail bleed at 10, 20, 30, 45 and 60 minutes post-gavage. For the ITT, rats were food deprived for two hours and then injected subcutaneously with 0.5U/kg of human insulin (Eli Lilly, Toronto, ON, Canada) and blood glucose measured at 30 minutes post-injection via tail bleed [149]. After a 48-hour washout period, rats were randomized to their second test (either OGTT or ITT) and the protocol repeated.

6.2.4 Euglycemic Hyperinsulinemic Clamp

6.2.4.1 Catheterization Surgery

Rats were catheterized five days prior to euglycemic-hyperinsulinemic clamp experiments. Rats were anaesthetized using isoflurane and the left common carotid artery and jugular vein were unilaterally catheterized using medical grade PE 50 tubing (0.023” ID x 0.038” OD; 22 gauge). The free catheter ends were tunnelled under the skin to the back of the neck and externalized with a stainless steel plug. Lines were flushed daily with ~200 μl of heparinized saline (100U/mL). Rats were individually housed

following surgery and body weight measured daily. Animals who lost >5% of pre-surgery weights were excluded from the study.

6.2.4.2 Euglycemic-Hyperinsulinemic Clamp

At 07:00, after 12 hours of food deprivation, rats were individually placed in a small personal-sized plastic container with bedding and a house. Catheters were connected to tubing that extended from the cage to calibrated pumps. Rats were allowed to acclimatize for two hours. At 09:00, a baseline blood sample was collected. The insulin clamp began at 09:15 with a primed continuous infusion of human insulin (Eli Lilly, Toronto, ON, Canada) at a rate of 40mU/kg/min. Glucose was infused to maintain blood glucose levels at 7.0 mmol/L. Euglycemia was maintained by measuring blood glucose (sample size: 10μl) every 5 minutes from 0-60 minutes and then every 10 minutes from 60 minutes to 120 minutes. 30 minutes of clamp data was collected for analysis of GIR.

6.2.4.3 Blood Analysis

Blood glucose concentrations were measured via OneTouch Blood Glucose Meter (BD Biosciences, Mississauga, ON, Canada). Baseline levels of glucagon and insulin were measured using a Lincoplex Kit (Linco Research, Missouri). Homeostatic model assessment of insulin resistance (HOMA-IR) was calculated to provide an estimate of insulin resistance [120].

6.2.5 Gene Expression

6.2.5.1 Tissue Collection and RNA Extraction

Immediately following the clamp, rats were over-anaesthetized with isoflurane and the aorta cut. The liver was excised and 100 mg immediately flash frozen in liquid nitrogen. Tissue was stored at -80˚C until analysis.

Total RNA was extracted from tissues using Trizol Reagent (Invitrogen, Carlsbad, CA) and purified using the Qiagen RNeasy Miniprep (Qiagen, Mississauga, ON, Canada). RNA was quantified using Ribogreen and RT performed using the Omniscript RT Kit as per manufacturers directions (Qiagen, Mississauga, ON, Canada).

6.2.5.2 Microarray

Four representative animals from each LC protein diet group (casein, complete dairy, or leucine) were selected for microarray analysis. The Affymetrix GeneChip Rat Gene 1.0 ST Array was used (Affymetrix, Santa Clara, CA) and provided expression levels of 27,342 genes. The Affymetrix protocol for one-cycle cDNA synthesis was followed. Briefly, cRNA was synthesized and first cycle cleanup performed. cDNA was synthesized and cleanup performed. Single stranded DNA was then fragmented and labelled and hybridization controls added. Gene chips were loaded with labelled DNA and allowed to hybridize for 17 hours in a 45°C oven. The chip was washed and stained as per Affymetrix protocol using an Affymetrix Fluidics 450 wash, stain, and scan station. The chips were processed at the University of Calgary Affymetrix Core Facility (Calgary, AB, Canada).

6.2.5.3 Microarray Analysis

Each individual chip was quality checked for background, quality control genes, outliers, and image abnormalities. The data was then further analyzed using Genesifter Analysis (Geospiza, Seattle, WA). Changes in relative gene expression were calculated using robust multi-array average (RMA) calculations [150] with analysis for the Rat Gene 1.0 ST Array being selected. A pair-wise analysis was performed using a Student's t-test. Casein was utilized as the control diet with complete dairy and leucine being the treatments. Only genes with significant differences in signal intensity were used for analysis ($p<0.05$). All microarray data were submitted in compliance with Minimal Information about Microarray Experiments (MIAME).

6.2.5.4 Real-Time Reverse Transcription Polymerase Chain Reaction (rt-PCR)

rt-PCR was performed for select genes to verify expression found in the microarray. Gene expression analysis was performed in triplicate and quantified using a Bio-Rad Thermal iCycler (Bio-Rad, Mississauga, ON, Canada). β-actin was used as the housekeeping gene and data analyzed using the $2^{-\Delta Ct}$ method [121]. Genes for verification of microarray data included: glucokinase (GK), peroxisome-proliferator-activated receptor gamma co-activator 1 alpha (PGC1α), and peroxisome proliferator-activated receptor gamma (PPAR γ). Primers used for rt-PCR are provided in Appendix C (Table A.5).

6.2.6 Data Analysis

All data is presented as mean ± SEM. Dependent variables were analyzed by two-way ANOVA to examine the effect of Ca (HC or LC), protein (casein, complete dairy, and leucine), and their interaction. Tukey's *post hoc* analysis was performed

between protein treatments when significant main effects were found. HOMA-IR and total area under the curve were calculated according to previous work [134, 135]. Significance was set at $p<0.05$.

6.3 Results

6.3.1 Food Intake

Daily food intake was not significantly different between groups (Table 6.1).

6.3.2 Body Composition

Initial body weight (all: 587.9±10.2 g) was not significantly different between groups ($p>0.05$). Analysis by two-way ANOVA demonstrated significant main effects of both protein and Ca on final body weight ($p<0.05$); however no protein x Ca interaction (Table 6.1). The rats fed the complete dairy and leucine diets had significantly lower final body weight than the casein treated rats (Table 6.2). The HC diet also significantly lowered final body weight compared to LC (Table 6.3). Weight gain over the eight-week period showed main effects of protein and Ca with no protein x Ca interaction (Table 6.1). Casein treated rats gained more weight than the complete dairy and the leucine rats (Table 6.2). The HC diet significantly reduced weight gain compared to LC (Table 6.3). Analysis by two-way ANOVA showed main effects of protein and Ca on total FM with no significant interaction between protein and Ca (Table 6.1). The casein treated rats had significantly greater FM than the complete dairy or the leucine treatments (Table 6.2) and the LC treatment was associated with greater FM compared to the HC treatment (Table 6.3). LBM showed no significant main effects of protein or Ca and no protein x Ca

interaction (Table 6.1). Bone mineral density (BMD) showed main effects of Ca, with HC promoting greater BMD than LC (Table 6.3).

6.3.3 Baseline Glucose, Glucagon, and Insulin

There were no main or interaction effects of protein and Ca on baseline levels of glucose, glucagon, or insulin (Table 6.1). Baseline insulin to glucagon ratio showed significant main effects of protein (Table 6.1), with leucine significantly lower than complete dairy (Table 6.2).

6.3.4 Oral Glucose Tolerance Test

There were no significant main effects of protein or Ca on individual time points of the OGTT or for calculated total AUC (Table 6.1).

6.3.5 Insulin Tolerance Test

There were significant main effects of protein for glucose response during the ITT (Table 6.1). The ITT_{30} glucose levels were significantly lower in complete dairy compared to casein, with leucine being intermediate (Table 6.2). The ITT_0 to ITT_{30} change in glucose level also showed a main effect of protein with complete dairy and leucine being significantly greater than casein (Table 6.2).

6.3.6 Euglycemic-Hyperinsulinemic Clamp

During the euglycemic-hyperinsulinemic clamp, euglycemia was maintained at 7.0±0.1 mmol/L. Glucose infusion rate (GIR) showed main effects of protein with leucine having significantly greater rates of infusion compared to dairy (Table 6.2). Casein was intermediate.

Table 6.1 Body Composition and Parameters of Glucose Metabolism

Table 6.1: Body composition and parameters of glucose metabolism at the end of the eight-week feeding period in rats fed casein, complete dairy, or leucine supplemented HFHS diets containing either low (LC) or high calcium (HC).

		Casein	Dairy	Leucine	Statistical Significance, p		
					P	C	P x C
Final Weight, g	LC	751.0±9.9	655.3±14.7	695.5±18.5	0.01*	<0.001*	0.884
	HC	707.3±18.6	627.4±20.3	653.2±21.0			
Weight Gain, g	LC	163.0±12.5	60.2±12.4	94.9±3.4	<0.001*	0.010*	0.867
	HC	133.7±11.9	38.2±16.2	58.2±16.3			
Fat Mass, g	LC	307.4±16.2	208.5±17.8	243.7±16.8	<0.001*	0.015*	0.849
	HC	259.9±17.1	175.2±16.3	215.8±21.8			
Lean Body Mass, g	LC	443.6±10.7	446.8±10.7	451.8±11.7	0.901	0.852	0.637
	HC	447.3±11.2	452.2±7.4	437.3±16.0			
BMD, g/cm^3	LC	0.1818± 0.002	0.1861± 0.007	0.1789± 0.006	0.181	<0.001*	0.219
	HC	0.1922± 0.007	0.1933± 0.007	0.1932± 0.007			

Daily Food Intake, g	LC	22.2±0.6	21.6±1.8	19.2±1.3	0.095	0.198	0.379
	HC	21.7±1.4	25.6±2.1	20.8±2.3			
Baseline Glucose, mmol/L	LC	7.3±0.1	7.1±0.3	6.5±0.3	0.112	0.600	0.810
	HC	7.0±0.4	7.4±0.4	7.1±0.2			
Baseline Glucagon, pM	LC	27.7±1.6	25.9±5.2	35.8±4.9	0.125	0.187	0.657
	HC	36.5±7.5	27.7±8.2	41.0±5.1			
Baseline Insulin, pM	LC	643.9±70.0	513.7±100.3	575.9±50.8	0.460	0.745	0.832
	HC	672.7±69.8	624.4±42.1	536.3±46.2			
ITT_{30} Glucose, mmol/L	LC	6.4±0.3	5.1±0.5	5.0±0.2	0.009*	0.119	0.120
	HC	5.3±0.4	4.5±0.3	5.4±0.3			
ITT change, mmol/L	LC	-0.9±0.4	-2.4±0.3	-1.5±0.4	0.003*	0.141	0.756
	HC	-1.7±0.5	-2.9±0.5	-1.6±0.3			
Insulin: Glucagon	LC	25.6±3.0	28.4±5.4	18.3±3.9	0.05*	0.578	0.927
	HC	22.9±5.2	28.3±5.6	14.5±1.9			
GIR, mg/kg/min	LC	22.5±1.2	21.8±1.3	26.9±2.5	0.100	0.189	0.972
	HC	24.7±1.7	25.1±1.3	29.2±2.9			
GIR, mg/min	LC	38.1±2.8	30.1±1.5	38.8±3.3	0.037*	0.632	0.674

	HC	37.0±2.1	33.9±3.1	39.4±3.2			
Glucose AUC, mM/90 min	LC	678.3±31.0	627.6±22.6	597.7±22.0	0.073	0.371	0.812
	HC	687.1±24.0	639.7±38.7	640.3±28.0			

Results are presented as mean ± SEM, n=9-11/treatment; analyzed by two-way ANOVA. (*) indicates $p<0.05$.

Table 6.2 Body Composition and Parameters of Glucose Metabolism by Protein Source

Table 6.2: Body composition and measures of glucose metabolism that showed main effects of protein source at the end of the eight-week *ad libitum* HFHS feeding period in rats fed casein, complete dairy, or leucine.

	Casein	Dairy	Leucine
Final Body Weight, g	729.1±11.3[a]	641.3±12.6[b]	674.3±14.4[b]
Weight Gain, g	148.4±9.1[a]	49.2±10.3[b]	76.5±10.3[b]
Fat Mass, g	283.7±12.6[a]	191.8±12.3[b]	229.8±13.8[b]
ITT_{30} Glucose, mmol/L	5.9±0.3[a]	4.8±0.3[b]	5.3±0.2[ab]
ITT Change, mmol/L	-1.3±0.3[a]	-2.7±0.3[b]	-1.6±0.2[b]
Insulin:Glucagon	24.1±3.1[ab]	28.3±3.9[b]	16.9±2.5[a]
GIR, mg/min	37.5±1.7[ab]	32.1±1.8[a]	39.1±2.2[b]

Results are presented as mean ± SEM, n=18-22/protein treatment; analyzed by two-way ANOVA. Means within the same row with different superscripts, $p<0.05$.

Table 6.3 Body Composition Data by Calcium Level

Table 6.3: Body composition data that showed main effects of calcium at the end of the eight-week HFHS feeding period in rats fed either low (LC) or high calcium (HC).

	LC	HC
Final Body Weight, g	700.6±10.8	662.6±12.6*
Weight Gain, g	107.1±10.4	76.7±11.1*
Fat Mass, g	253.2±11.9	217.0±12.0*
BMD, g/cm^3	0.1823±0.006	0.1929±0.007*

Results are presented as mean ± SEM, n=27-33/calcium treatment; analyzed by two-way ANOVA. (*) Different from LC, $p<0.05$.

6.3.7 Gene Expression

Changes in gene expression were identified via microarray analysis. mRNA expression of three genes was verified with rt-PCR (GK, PPARγ, and PGC1α). Directionality and relative change were consistent for PPARγ, GK, and PGC1α (Figure 6.2). Only treatment groups containing 0.67% Ca (LC) were used for microarray analysis given our main focus of comparing the effects of complete dairy and leucine on glucose homeostasis.

In response to the complete dairy diet, there were 2107 hepatic genes significantly changed (7.3% of probes): 1121 genes were up regulated and 986 genes were down regulated. In response to the leucine diet, there were 1217 (4.2% of probes) hepatic

genes altered with 578 significantly up regulated and 639 down regulated. The complete dairy diet significantly changed 108 (12%) genes related to Kyoto Encyclopaedia of Genes and Genomes (KEGG) metabolic pathways (Appendix D, Table A.6), whereas the leucine diet only altered 37 (4%) genes (Appendix D, Table A.7). 13 genes were altered by both complete dairy and leucine (Table 6.4). Additionally, in response to both complete dairy and leucine diets, genes associated with the insulin signalling, mTOR, and PPAR signalling pathways were also altered (Table 6.5). The complete dairy diet was associated with the down regulation of six genes related to fatty acid metabolism and alterations to five genes in the adipokine pathway; leucine did not alter any genes associated with fatty acid metabolism or adipokine signalling (Table 6.6).

Figure 6.2 rt-PCR Gene Expression Data for Verification of Microarray Data

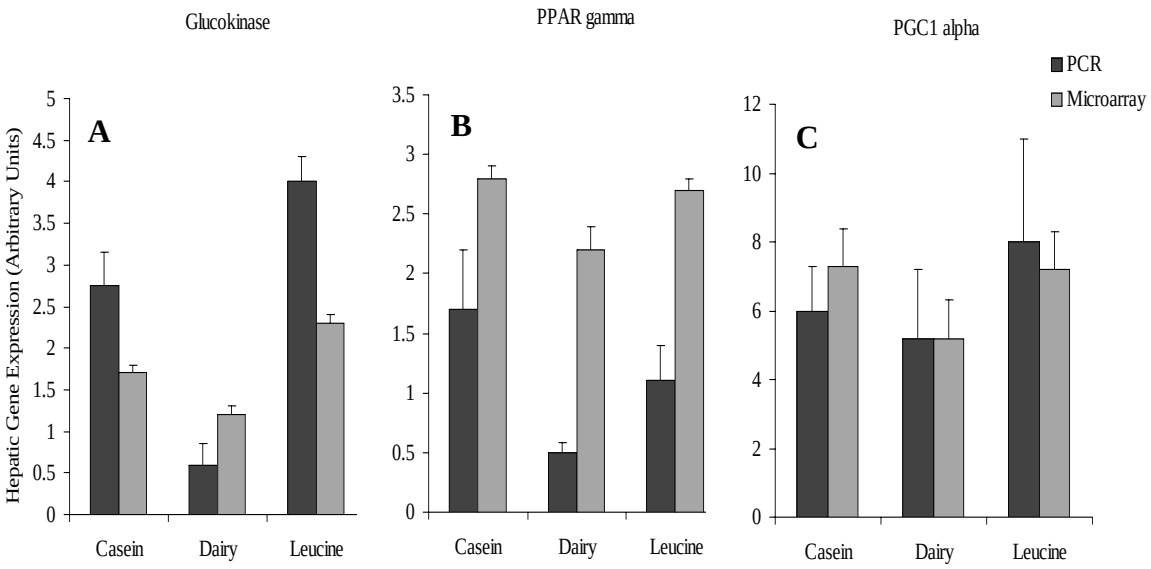

Figure 6.2: Verification of microarray data with gene expression obtained via rt-PCR for glucokinase, PPARγ, and PGC1α. Data is presented for directionality and not statistical analysis. Data is presented mean ± SEM, n=4/protein treatment.

Table 6.4 Expression of Genes Altered in Response to Leucine and Complete Dairy (KEGG Metabolic Pathway)

Table 6.4: Gene expression for those genes significantly altered by both complete dairy and leucine in the KEGG Metabolic Pathway at the end of the eight-week feeding period in rats fed casein, complete dairy, or leucine diets.

Casein	Dairy*	Leucine*	Gene ID	Gene Name
7.41±0.23	6.47±0.17	6.72±0.05	Alg5	Asparagine-linked glycosylation 5, dolichyl-phosphate beta-glucosyltransferase homolog (S. cerevisiae)
8.89±0.13	9.59±0.10	9.29±0.12	Atp5o	ATP synthase, H+ transporting, mitochondrial F1 complex, O subunit
9.71±0.04	9.44±0.07	9.32±0.11	Cox17	Cytochrome c oxidase, subunit XVII assembly protein homolog (S. cerevisiae)
5.00±0.55	7.99±0.31	7.69±0.26	Dut	Deoxyuridine triphosphatase
7.62±0.10	6.79±0.23	7.35±0.10	Galk2	Galactokinase 2
8.95±0.16	9.63±0.14	9.77±0.06	Prdx6	Peroxiredoxin 6
8.37±0.05	8.14±0.07	8.00±0.07	Pigt	Phosphatidylinositol glycan anchor biosynthesis, class T
9.81±0.05	9.60±0.04	9.51±0.07	Shmt2	Serine hydroxymethyltransferase

				2 (mitochondrial)
4.45±0.11	3.93±0.14	3.54±0.24	LOC497978	Similar to diacylglycerol kinase epsilon
3.67±0.02	4.27±0.13	3.92±0.07	-	Similar to Mdes protein
7.81±0.19	8.82±0.17	8.68±0.08	LOC685322	Similar to ubiquinol-cytochrome c reductase complex 7.2kDa protein isoform b
9.49±0.11	10.05±0.13	9.86±0.07	Smpd1	Sphingomyelin phosphodiesterase 1, acid lysosomal
5.04±0.09	4.62±0.10	4.43±0.12	Uprt	Uracil phosphoribosyltransferase (FUR1) homolog (S. cerevisiae)

Data is presented as mean ± SEM, n=4/protein treatment. * Denotes all listed genes, $p<0.05$ from the control (casein). KEGG, Kyoto Encyclopaedia of Genes and Genomes.

Table 6.5 Expression Data for Genes in the KEGG Insulin Signalling, mTOR, and PPAR pathways

Table 6.5: Gene expression in the KEGG Insulin Signalling, mTOR, and PPAR pathways at the end of the eight-week HFHS *ad libitum* feeding period in rats fed casein, complete dairy, or leucine diets.

Casein	Dairy	Leucine	Gene ID	Gene Name
Insulin Signalling				
7.83±0.07[a]	7.44±0.10[b]	7.92±0.12[ab]	Pdpk1	3-phosphoinositide dependent protein kinase-1
8.14±0.14[a]	7.35±0.20[b]	8.07±0.22[ab]	Gsk3b	Glycogen synthase kinase 3 beta
8.55±0.09[a]	8.16±0.13[b]	8.51±0.10[ab]	Mapk1	Mitogen activated protein kinase 1 (ERK1)
8.67±0.08[a]	9.01±0.07[b]	8.72±0.06[ab]	Map2k2	Mitogen activated protein kinase kinase 2 (MEK1/2)
5.24±0.34[a]	4.17±0.08[b]	4.48±0.28[ab]	Ppargc1a	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC1α)
5.75±0.13[a]	6.68±0.24[b]	4.89±0.10[ab]	Pck2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial) (PEPCK)
7.34±0.13[a]	6.58±0.18[b]	7.12±0.12[ab]	Phkb	Phosphorylase kinase, beta

9.35±0.14[a]	8.94±0.09[b]	9.30±0.14[ab]	Prkaa2	Protein kinase, AMP-activated, alpha 2 catalytic subunit (AMPK)
3.15±0.10[a]	3.16±0.05[ab]	2.81±0.06[b]	SHC	SHC (Src homology 2 domain containing) family, member 4
7.14±0.15[a]	6.67±0.16[ab]	6.67±0.11[b]	Socs4	Suppressor of cytokine signaling 4
mTOR Pathway				
7.83±0.07[a]	7.44±0.10[b]	7.92±0.12[ab]	Pdpk1	3-phosphoinositide dependent protein kinase-1
5.88±0.18[a]	7.06±0.23[b]	6.71±0.39[ab]	Ddit4	DNA-damage-inducible transcript 4
9.76±0.02[a]	9.44±0.09[b]	9.67±0.14[ab]	Eif4b	Eukaryotic translation initiation factor 4B
11.89±0.14[a]	11.41±0.12[b]	11.98±0.08[ab]	Igf1	Insulin-like growth factor 1
8.55±0.09[a]	8.16±0.13[b]	8.51±0.10[ab]	Mapk1	Mitogen activated protein kinase 1 (ERK 1)
9.35±0.14[a]	8.94±0.09[b]	9.30±0.14[ab]	Prkaa2	Protein kinase, AMP-activated, alpha 2 catalytic subunit (AMPK)
3.96±0.14[a]	4.14±0.06[ab]	4.03±0.03[b]	Rps6ka2	Ribosomal protein S6 kinase polypeptide 2

PPAR Pathway				
7.83±0.07[a]	7.44±0.10[b]	7.92±0.12[ab]	Pdpk1	3-phosphoinositide dependent protein kinase-1
9.52±0.11[a]	9.01±0.09[b]	9.57±0.08[ab]	Acsl4	Acyl-CoA synthetase long-chain family member 4
9.69±0.12[a]	9.56±0.20[ab]	10.41±0.09[b]	Acox2	Acyl-Coenzyme A oxidase 2, branched chain
10.32±0.08[a]	9.69±0.12[b]	10.24±0.23[ab]	Cyp27a1	Cytochrome P450, family 27, subfamily a, polypeptide 1
11.37±0.11[a]	10.34±0.027[b]	11.00±0.25[ab]	Ehhadh	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase
9.11±0.03[a]	8.85±0.22[ab]	8.70±0.03[b]	Ilk	Integrin linked kinase
5.75±0.13[a]	6.68±0.24[b]	4.89±0.10[ab]	Pck2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial) (PEPCK)
6.71±0.09[a]	6.56±0.17[ab]	6.17±0.07[b]	Slc27a1	Solute carrier family 27 (fatty acid transporter), member 1
7.53±0.21[a]	8.09±0.45[ab]	8.11±0.05[b]	Ubc	Ubiquitin C

Data is presented as mean ± SEM, n=4/protein treatment. Means within a row with different superscripts are significantly different from each other, $p<0.05$. KEGG, Kyoto Encyclopaedia of Genes and Genomes.

Table 6.6 Expression of Genes Altered only by Dairy in the Fatty Acid Metabolism and Adipokine Pathways

Table 6.6: Expression for genes altered only by complete dairy treatment in the KEGG Fatty Acid Metabolism Pathway and Adipokine Pathway at the end of the eight-week feeding period in rats fed casein or complete dairy.

Casein	Dairy*	Gene ID	Gene Name
Fatty Acid Metabolism Pathway			
9.52±0.11	9.01±0.09	Acsl4	Acyl-CoA synthetase long-chain family member 4
7.27±0.35	5.40±0.49	Adh6	Alcohol dehydrogenase 6 (class V)
6.93±0.13	6.10±0.23	Adh7	Alcohol dehydrogenase 7 (class IV), mu or sigma polypeptide
10.69±0.1	10.23±0.1	Aldh7a1	Aldehyde dehydrogenase 7 family, member A1
11.37±0.1	10.34±0.3	Ehhadh	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase
6.91±008	7.17±0.04	Mcat	Malonyl CoA:ACP acyltransferase (mitochondrial)
Adipokine Pathway			
9.52±0.11	9.01±0.09	Acsl4	Acyl-CoA synthetase long-chain family member 4
5.24±0.34	4.170.08	Ppargc1a	Peroxisome proliferator-activated receptor gamma, coactivator 1 alpha (PGC1α)
5.75±0.13	6.68±0.24	Pck2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial) (PEPCK)
4.11±0.12	4.49±0.08	Pomc	Proopiomelanocortin

9.35±0.14	8.94±0.09	Prkaa2	Protein kinase, AMP-activated, alpha 2 catalytic subunit (AMPK)

Data is presented as mean ± SEM, n=4/protein treatment. * Denotes all listed genes, $p<0.05$ between casein (control) and complete dairy treatments. KEGG, Kyoto Encyclopaedia of Genes and Genomes.

6.4 Discussion

We examined the effect of complete dairy foods, casein, and leucine supplemented with Ca on the prevention of weight gain in Sprague-Dawley DIO rats. Consistent with other studies we found that complete dairy enhanced weight regulation [99] and leucine influenced insulin sensitivity [84]. The main novel findings of this study are that (i) consumption of leucine improves whole body insulin sensitivity as measured by euglycemic-hyperinsulinemic clamp compared to casein; (ii) complete dairy prevents weight gain more so than leucine or casein with HC preventing weight gain more than LC, and (iii) alterations in hepatic gene expression in insulin signalling and related metabolic pathways supports the role of dairy foods in altering weight and glucose homeostasis.

The primary finding of this study is that leucine enhances whole body insulin-dependent glucose uptake as measured by euglycemic-hyperinsulinemic clamp (Tables 6.1 and 6.2). This is consistent with other studies which found leucine supplementation in the drinking water of mice improves insulin sensitivity [69]. However it is in contrast to another similar study by Nairizi *et al* [151] who found that leucine supplementation in drinking water does not cause a change in insulin sensitivity. The variation between the

current study and above mentioned studies could be due to methodological issues in measuring insulin sensitivity; both of the previous studies provided only indirect measures of insulin sensitivity and the methodology differed between studies. The data from the present study show that although glucose infusion rate (GIR) measured during the clamp procedure was greatest in the leucine-fed rats, insulin sensitivity measured indirectly by OGTT and ITT could lead to alternative interpretations. The OGTT data suggests that the complete dairy-fed rats had improved glucose clearance compared to casein and leucine, and the ITT data suggests that both leucine and complete dairy had greater insulin sensitivity than the casein diet (Table 6.1). Taken together, this data suggests that although complete dairy and leucine both appear to play a role in glucose homeostasis, they do so in different manners. The insulin sensitivity data are of interest because the leucine-fed rats had equal LBM to the complete dairy and casein rats but more fat than the complete dairy rats. It is plausible that the extra FM was used for glucose disposal, providing more glucose storage area and therefore greater insulin sensitivity. Further work examining the physiological characteristics of the adipose tissue in leucine-fed rats is warranted [152].

The data from the microarray analysis suggest that there are alterations in the KEGG insulin-signalling pathway that also might explain the observed variations in insulin sensitivity (Table 6.5). The complete dairy diet was associated with a decreased expression of suppressor of cytokine signalling 4 (SOCS4), 3-phosphoinositide dependent protein kinase-1 (PDPK1), glycogen synthase kinase 3β (GSK3β), and phosphorylase kinase, beta (PHKβ) (Table 6.6). This set of genes is responsible for regulation of starch and sucrose metabolism. Down regulation as seen with the complete

dairy diet, has the potential to result in lower hepatic accumulation of glycogen. Treatment with complete dairy also resulted in a down regulation of hepatic AMP-activated protein kinase (AMPK). AMPK is responsible for signalling of mitogen activated protein kinase 1 (ERK 1), PGC1α and PPARγ, three key factors in the regulation of glucose homeostasis and insulin sensitivity [153] (Appendix E).

The second main finding of this study is consistent with other studies that have shown complete dairy foods attenuate weight gain during *ad libitum* HFHS feeding more than the individual dairy proteins (casein and whey) [110]. In this study, the change in weight was reflected by a decrease in fat mass (FM) and not a change in LBM (Tables 6.1 and 6.2). The attenuation of weight gain in this study was not as a result of decreased energy consumption, as all groups ate similar amounts (Table 6.1). This is consistent with other work from our lab which showed no changes in food intake or alterations in satiety hormone levels [110].

Gene expression data from microarray analysis further supports a role of complete dairy in regulating factors associated with metabolic pathways (Appendix D, Table A.6) and specifically with fatty acid metabolism (Table 6.5). Genes responsible for regulation of β-oxidation were consistently down regulated in the complete dairy treatment compared to the control (casein), which may reflect a change in nutrient partitioning. Future work is necessary to determine if the metabolic rate and substrate utilization of the complete dairy rats differs from casein rats. Previous work from our lab has shown a down regulation of acetyl-coA carboxylase (ACC) in response to the complete dairy diet [110]. β-Oxidation is down regulated in response to insulin via ACC; although there was no significant difference in ACC expression as shown in microarray data, this might

contribute to the alteration in expression of genes associated with fat metabolism. Furthermore, our data showed down regulation of acyl-CoA synthetase long-chain family member 4 (ACSL4) with complete dairy. This enzyme is responsible for conversion of fatty acids to long chain acyl-CoA. In human fatty liver disease, there is generally an increase of this gene and is hypothesized to be related to increased fat storage [154].

Previous work has attributed the fat loss seen with dairy food consumption to FF [72]. Although FF was not measured, it is plausible that Ca did play a role in prevention of weight gain via an increase binding of dietary fat to Ca to form insoluble salts. Dietary Ca dose showed main effects on final body weight, weight gain, and fat mass, with HC improving body composition when compared to LC (Table 6.3). However, the greater BMD associated with the HC diet would suggest that these rats were eating and absorbing more Ca than the LC rats. It is plausible that the increase in dietary Ca not only contributes to FF but is also bioavailable to the rat for other roles such as those related to bone health.

In conclusion we have identified several roles for complete dairy and leucine in modulating energy metabolism and glucose homeostasis at the hepatic level. Hepatic KEGG pathways that were altered by both leucine and complete dairy were the insulin signalling, mTOR, PPAR, and metabolic pathways (Table 6.4). Pathways strictly altered by complete dairy were the fatty acid metabolic and adipokine pathway (Table 6.6). Analysis of microarray data suggests that complete dairy and leucine have very different effects on hepatic pathways related to metabolism. Evaluation of animal phenotype also supports significant differences at a genotype level as complete dairy and leucine rats had significantly different body weights and insulin responses. It is clear from this study that

leucine is not the only factor found in dairy foods that is linked to a change in metabolic parameters; however, it most likely plays a role in insulin signalling. The microarray analysis provides meaningful data that demonstrates the metabolic phenotypes seen with complete dairy and leucine are most likely from differential expression of hepatic genes related to weight control and insulin signalling.

Chapter Seven: Conclusions and General Discussion

7.1 Introduction

Nutritional interventions can assist with the maintenance of a healthy body weight; however, large changes to diet are difficult to maintain in the long term making these programs prone to failure. Foods that contain bioactive molecules have been shown to alter metabolic profiles – interventions that utilize these foods, and promote small changes in food intake or nutrient assimilation could be one solution for improving the success rate of nutritional interventions. With the increasing global incidence of obesity and related co-morbidities, it is important to establish simple dietary interventions that will help promote healthier body weights. The three main studies described herein (Chapters 4, 5, and 6) were designed to provide an increased understanding of bioactive compounds contained in dairy foods, and the potential mechanisms behind their apparent effectiveness in improving metabolic health.

The objectives of this work were to examine how various dietary proteins and Ca prevent weight gain during *ad libitum* feeding and promote weight loss during ER. Secondarily, we examined how these proteins alter insulin sensitivity. The principle findings were that (1) complete dairy food reduces weight gain during *ad libitum* feeding and accelerates weight loss during ER with the maintenance of LBM; (2) diets high in Ca increased any weight effects seen with complete dairy; and (3) protein source altered expression of hepatic genes related to glucose metabolism and insulin sensitivity.

This final chapter will begin with an overview of the strengths, limitations, and rationale for the methodology and then finish with an overall summary of findings and an interpretation of the results and their significance.

7.2 Strengths and Limitations of the Experimental Protocols

7.2.1 Animal Models

A polygenic model of obesity, specifically the Sprague-Dawley diet-induced obese (DIO) rat, was selected for studies presented in this thesis. This model of obesity has many similar features of common human obesity. These include the propensity to become obese in response to a high fat and palatable diet, insulin resistance, and resilience to weight loss. This model was selected over a monogenic model of obesity because it more closely mimics the progression of human obesity. Many studies examining the effect of dairy foods and Ca on metabolic parameters have used specific genetic models of obesity (ie. the *agouti* mouse). Although this provides interesting data, the applicability to common obesity has been questioned.

Outbred Sprague-Dawley rats have two distinct metabolic phenotypes: obesity resistant and obesity prone, the latter often termed diet-induced obese (DIO) [118]. When provided *ad libitum* with HFHS, DIO rats become obese whereas resistant rats maintain a lean phenotype. To select for DIO rats, twice the required number of rats were purchased from Charles River (Charles River, Quebec, Canada). Rats were obtained at six weeks of age and then provided *ad libitum* a HFHS diet for six weeks. At 12 weeks of age, the rats in the upper 50^{th} percentile of weight gain were selected for participation in the studies outlined in Chapters 4, 5, and 6 [118]. The rats were selected

based on their susceptibility to weight gain in an obesogenic environment and are thus representative of obesity prone animals. Although no body composition data is available to specifically quantify body fat of these rats, the selection process ensured that all rats were above the mean for susceptibility to weight gain (Figure A.2). The rats in the lower 50^{th} percentile were used for other studies. The DIO selection procedure ensured that only adult rats (>12 weeks of age) were used. Resultantly, the influence of dietary interventions on growth and development was eliminated as a confounder.

For this body of work, only male rats were used. Female rats were not also used to eliminate the need for more animals in case of a sex effect. Future work is warranted in female animals as it is well documented that male and female pattern fatness have different responses to dietary interventions [15] [60].

7.2.2 Dietary Interventions

Diets used for this study were formulated based on American Institute of Nutrition (AIN) guidelines [105] and were mixed in-house. In the four studies, there were a total of 16 diets that varied by energy density [normal – 3.6kcal/g (NE) or high – 4.6 kcal/g (HFHS)], protein source (casein, whey, complete dairy, soy, or casein+leucine), and level of Ca [0.67% (LC) or 2.4% (HC)] (Figures 3.1, 4.1, 5.1, and 6.1) (Appendix B).

7.2.2.1 Energy Density

In Chapters 4 and 6, we metabolically challenged rats with a HFHS diet (4.6 kcal/g) to encourage weight gain and associated metabolic dysfunction. Energy density for the HFHS was based upon previous work with Sprague-Dawley rats. Lard (100 g/kg) and soybean oil (100 g/kg) were used to increase the energy density of the HFHS diet.

As well, the majority of the cornstarch in the NE diets was replaced with sucrose for the HFHS diets (Appendix B, Table A.3). All diets within each energy density were matched for macronutrient content (percentage of carbohydrate, protein, and fat).

7.2.2.2 Protein Source

All diets provided between 10 – 12% of total energy from protein: the NE diets were 12% protein and the HFHS diets were 10% protein. This is consistent with human dietary requirements of 0.8 g/kg of body weight and falls within the acceptable macronutrient range for protein in humans, which is set at 10-35% of total energy [155]. Since complete dairy (skim milk powder) is ~38% protein, and whey, casein, and soy are 85% protein, we used a greater amount of skim milk powder than the casein, whey, or soy protein. This was to ensure all diets were isonitrogenous. This thesis examined how *complete dairy foods* affect metabolic parameters and whether the individual protein fractions (casein, whey, or leucine) act synergistically or if one specifically is responsible for the metabolic effects of complete dairy protein. Therefore we feel justified in the use of skim milk powder, despite the fact that the ratio of lactose to sucrose was different between diets. As well, complete dairy foods are a well-recognized rich source of bioactive components. The complexity of this food, while very intriguing from a nutritional perspective, also limits the number of factors that can strictly be controlled for in experimental diets. Future studies could use pure milk protein precipitated from skim milk, to control for the composition difference of simple sugars.

7.2.2.3 Calcium Level

When choosing Ca levels for our diets, we considered (1) naturally occurring levels of Ca in the complete dairy diet, and (2) concentrations used in previous studies. In

the diets that varied by Ca level, we substituted calcium carbonate ($CaCO_3$; 40% elemental Ca) for cornstarch. The complete dairy diet naturally contains 0.67% Ca by weight. Therefore, the casein, whey, soy, and leucine diets were all Ca-matched to 0.67% Ca and represented the low Ca (LC) diets. 2.4% Ca was considered our high Ca (HC) level and was based on previous work that found significant effects of Ca on metabolic parameters [20].

An obvious question regarding the experimental diets is how the levels of protein and Ca translate into meaningful amounts in humans. The HC diet has 2.4% Ca, which would translate into 12g of Ca per day for humans based on a 2000 kcal diet. This is an excessive dose that would not be applicable to humans; however, the dose was selected based on efficacy and dietary requirements in animals. Studies in humans have shown that 1500mg of Ca and/or three or more servings of low-fat dairy foods per day are effective in regulating metabolic parameters [60, 99, 156]. Moreover, a meta-analysis by Christensen *et al* [72], found that for every 1241mg of Ca consumed, there was 5.2 g of FF per day (range of 1.6-8.8 g) suggesting that an amount between 1241-1500 mg/day would likely be sufficient to see health effects. This level of Ca intake is safe for human health as the tolerable upper limit defined by the Institute of Medicine (Health Canada) for adults is 2500 mg/day.

Although it has been suggested that HC diets cause a taste aversion (TA) in rats, there is little empirical evidence to support this notion. The fear is a TA would reduce food intake and result in weight loss [69, 109]. For each of the studies presented, detailed food intake records were collected and no difference in food consumption between the HC and LC diets was found. As well, prior to undertaking the three main studies in this

thesis, a pilot study investigating the role of the experimental proteins and Ca levels in TA and satiety was undertaken (Chapter 3). The pilot study was completed to determine if Sprague-Dawley rats have a TA to HC and if satiety responses varied in response to diets containing casein, soy, complete dairy, and whey protein. We found that there was no TA with 2.4% Ca (HC) and that when protein taste was masked with banana flavouring, *ad libitum* fed rats consumed equal amounts of diets. This precluded any influence of the various protein sources on satiety.

7.2.3 Experimental Design

Our full factorial experimental design in Chapters 5 and 6 allowed us to examine potential protein – Ca interactions during *ad libitum* feeding and ER. In Chapter 5 there were eight experimental diets examining four levels of protein and two levels of Ca during ER (Figure 5.1); and in Chapter 6 three levels of protein with two levels of Ca during HFHS *ad libitum* feeding (Figure 6.1). This design allowed for the meaningful analysis of the main effects of protein and Ca, as well as the interaction effects of protein and Ca. One of the most pressing questions in this field is the differential effect of dairy foods and/or Ca and their potential interactions. This is the first study to date that provides a full factorial design examining the role of complete dairy, individual dairy proteins, and Ca.

The only deviation from a full factorial design was in Chapter 4. In this study we had eight experimental diets that examined three levels of protein, two levels of Ca, and two levels of energy density; however, within energy density we did not have a complete set of treatments (only casein/LC and casein/HC for the NE diets) (Figure 4.1). Inclusion of the normal and high energy densities in this study was in response to previous work

that had shown Ca is only effective in individuals with lower energy intake. Given that we did not find an effect of energy density on the prevention of weight gain, we eliminated this variable for our second *ad libitum* study (Chapter 6).

7.2.4 Measurements of Insulin Sensitivity and Glucose Homeostasis

Throughout this thesis, various techniques were used to determine measurements of insulin sensitivity and glucose homeostasis. In Chapters 4 and 5, we used a meal tolerance test (MTT) to examine post-prandial glucose and hormonal responses. For the MTT, a liquefied portion of respective diet was administered by oral gavage (3g emulsified to 3 mL in water). Providing each animal with their respective meal allowed us to examine the acute changes resulting from the diets. It did not, however, allow us to rule out the confounding effect of the meal composition on metabolic responses during the MTT. In any study where the experimental diets are considered to produce both long-term adaptation and acute meal effects, the debate continues as to which design is the most meaningful representation of dietary response. To strictly examine the long-term adaptive response to the diets, in the final study we utilized an OGTT which effectively eliminates any influence of meal composition as all animals are administered a dose of glucose based on body weight. We found very consistent data between the MTT and the OGTT when comparing the four diet treatments that occurred in both the first study (Chapter 4) and the final study (Chapter 6). The MTT and OGTT are appropriate and simple tests to determine the influence of ingested food or glucose on whole body postprandial glucose responses. Although it would seem obvious that elevated plasma glucose during a MTT or OGTT corresponds well to insulin resistance, care must be taken in interpreting this data. In some individuals with impaired glucose tolerance,

there can be notable differences in insulin secretion. For example, if the subject has excessive insulin secretion, this may result in the appearance of a normal glucose response; however because more insulin is required to achieve the same glucose response, they are, in fact, less insulin sensitive. Furthermore, a MTT or OGTT may not be sufficient to cause adequate amounts of insulin secretion to impair hepatic glucose production. This results in increased blood glucose and the appearance of insulin resistance or impaired glucose uptake. As well, the role of gut hormones, gastric emptying, and splanchnic glucose uptake are not adequately considered and can affect insulin secretion. Nonetheless, the MTT and OGTT are practical, easy to perform measurements of glucose homeostasis. In the final study (Chapter 6), we also utilized the insulin tolerance test (ITT) to determine the physiological response to a known amount of insulin.

To estimate insulin resistance we used the homeostatic model of assessment (HOMA-IR) [baseline glucose (mmol/L) x baseline insulin uU/ml)]/22.5]. This method provides a simple estimate of insulin sensitivity based on baseline glucose and insulin levels and correlates well with data obtained via the euglycemic-hyperinsulinemic clamp [134, 157]. A lower HOMA-IR value indicates better insulin sensitivity. A limitation of HOMA-IR is that in normoglycemic subjects, baseline insulin becomes the most significant contributor and HOMA-IR values may correlate better to baseline insulin concentrations than to insulin resistance.

The methods described above for measuring or estimating glucose-insulin homeostasis provide only an indirect measurement of insulin sensitivity based on the relationship between plasma levels of glucose and insulin. However, neither variable

(glucose or insulin) is held constant during a MTT, OGTT, or ITT. The majority of data in Chapter 4 and 5 provide results that suggest an improvement of insulin sensitivity with complete dairy; however, there are some discrepancies that suggest otherwise (Tables 4.2, 5.1, 5.2, and 5.3). Therefore, to eliminate the feedback regulation of the glucose-insulin system, we used a euglycemic-hyperinsulinemic clamp for the final study (Chapter 6). The euglycemic-hyperinsulinemic clamp is the only test for measuring the rate of glucose uptake at a consistent level of insulin [158-160]. This method assumes that at a steady rate of glucose infusion (mg/min), if blood glucose remains constant, there is an equal amount of glucose being taken up by tissues. However, this is only true if hepatic glucose production is suspended – to stop hepatic glucose production, high circulating insulin levels are required which justifies the relatively high dose of insulin in this protocol (40mU/kg/min). The limitation of our euglycemic-hyperinsulinemic clamp was that we did not use a radioactive isotope to trace the pathway of glucose disposal. Therefore we do not know where in the body the glucose was utilized and whether it entered oxidative or non-oxidative pathways [161]. Our rationale for not including a tracer was to accommodate our secondary goal of examining gene expression via microarray. The use of radioactive tissue in the University of Calgary Affymetrix Core Facility is not permitted. However, this limited our findings to whole body glucose uptake instead of tissue specific glucose uptake. Finally, the euglycemic-hyperinsulinemic clamp does not distinguish between insulin-dependent and insulin-independent glucose uptake, as it reflects whole body glucose disposal.

7.2.5 Plasma Biochemistry

We measured satiety-related hormones because it has been widely hypothesized that a change in satiety hormones and appetite response is responsible for alterations in metabolic profiles seen with dairy food and Ca consumption [132]. Our first two main studies (Chapters 4 and 5) examined both plasma levels of satiety signals (ghrelin, GLP-1, amylin, insulin, leptin, and glucagon) as well as respective gene expression. We found no differences in these hormones and drew the conclusion from our data that in diets with normal protein levels (10-12% kcal), satiety hormone response is not a factor. As well, our detailed food intake measurements showed no difference in *ad libitum* food intake for any study and corresponded to the lack of change in plasma satiety hormones.

For analysis of plasma levels of satiety signals, two different types of assays were used. Ghrelin and active GLP-1 were quantified using enzyme-linked immunosorbant assays (ELISA). The remainder of the hormones were measured via LINCOplex immunoassay. These multiplex assays use a traditional sandwich principle, but instead of traditional antibodies, utilize fluorescently labelled microsphere beads. Each sphere contains an analyte specific antibody. The beads are incubated with the sample and then bound to detection antibody. The final step is incubation with streptavidin-phycoerythrin, which amplifies the fluorescence into meaningful data. The advantage of this technique is that very small amounts of sample can be used and many different analytes measured collectively in one well. Therefore, with 10 μl of plasma, simultaneous detection of up to 100 different analytes is possible. The reliability and validity of these assays have been independently demonstrated elsewhere [162].

7.2.6 Gene Expression via Real-Time Polymerase Chain Reaction (rt-PCR)

Immediately upon killing rats, tissues were excised and flash frozen in liquid nitrogen. Tissue was stored at -80˚C until analysis. Total RNA was extracted from tissues using Trizol Reagent (Invitrogen, Carlsbad, CA) and quality assured via agarose gel electrophoresis using ethidium bromide as a stain. RNA was then quantified with Ribogreen and reverse transcription (RT) performed using the Omniscript RT Kit as per manufacturers' directions (Qiagen, Mississauga, ON, Canada). All RT reactions began with 1 μg of total RNA. Resultant cDNA was amplified via real time reverse transcription polymerase chain reaction (rt-PCR). Primers for each gene of interest were designed to include an intron surrounded by two partial exons and were obtained from the University of Calgary Core Services (Calgary, AB, Canada). All primers used for this thesis are presented in Appendix C. Gene expression analysis was performed in triplicate and quantified using a Bio-Rad Thermal iCycler (Bio-Rad, Mississauga, ON, Canada) using SYBR Green as a fluorescent dye. SYBR Green only binds to double stranded DNA (dsDNA). As rt-PCR continues through each cycle, more dsDNA is produced and the resultant fluorescence also increases. Measurement of relative gene expression was quantified via the $2^{-\Delta Ct}$ method. The gene of interest was compared to either GAPDH or β-actin as an internal, housekeeping control. To ensure good normalization procedures were followed, sample sizes of tissue for RNA extraction were similar between samples. As well, for RT, 1μg of RNA was used, which ensured similar RNA concentrations prior to generating cDNA. Furthermore, with the use of the $2^{-\Delta Ct}$ method, one is able to reduce between sample variations. All data is presented in arbitrary units. Units are specific for each figure and are based upon comparison of the

housekeeping gene to the gene of interest. Arbitrary units were used to show the relative up and down regulation of each gene.

Although rt-PCR is a well established, sensitive, and reliable measurement of gene expression, this type of data is limited in scope [163]. Data obtained with rt-PCR solely provides a measurement of gene expression. Any sort of processing of the gene product, such as downstream regulation, translation, or post-translational expression is not considered. Additional techniques such as Western blotting or protein assays are useful to interpret changes downstream of gene transcription. Determination of liver triglyceride and cholesterol content also provided further biochemical support for rt-PCR data generated in regards to hepatic lipid metabolism. Furthermore, plasma levels of satiety hormones were determined which matched the respective mRNA expression patterns.

7.2.7 Microarray

The high-throughput ability of the microarray facilitated a complete examination of hepatic genes related to metabolism. The microarray analysis allowed for expression profiling of hepatic genes up or down regulated due to a change in dietary protein (complete dairy or leucine). Although this microarray study (Chapter 6) was not hypothesis based, the lack of progress in the field in determining the mechanisms of action of complete dairy on metabolic parameters justified this technique. This will permit future hypothesis driven studies to focus on pathways that were altered by the complete dairy treatment. Although only liver tissue was examined at this stage, other tissues, including skeletal muscle and white adipose were collected for future studies. The first two main studies (Chapters 4 and 5) provide justification for using only the

liver, as adipose and gut tissue did not have any alterations of candidate genes selected for rt-PCR analysis. Examination of the skeletal muscle should provide interesting findings as skeletal muscle preferentially metabolizes BCAA.

7.3 Overall Summary and Interpretation of Results

The primary findings of this thesis were (1) complete dairy, but not individual dairy-protein fractions, improve body weight regulation during ER and *ad libitum* HFHS feeding in DIO rats and that supplementation with HC further enhances this effect; (2) levels of glucose, insulin, and glucagon were altered by both protein source and Ca level; and (3) measurements of insulin sensitivity suggest that complete dairy and leucine have varying roles in whole body glucose homeostasis and hepatic gene expression data may explain these variations (Figures 7.1 and 7.2). This final summary will interpret the findings from Chapters 3-6 in regards to body composition, parameters of glucose homeostasis, and hepatic gene expression data.

7.3.1 Body Composition

The novel finding of this thesis is that complete dairy food – but not a specific protein fraction therein (i.e., casein, whey, or leucine) – prevents the accumulation of FM during *ad libitum* HFHS feeding (Tables 4.1 and 6.1, Figure 4.2), and accelerates fat loss during ER (Table 5.1). Additionally, consumption of complete dairy maintained LBM during 70% ER more than casein, whey, or soy protein (Table 5.2). This demonstrates that the protein quality of skim milk powder is high and there was no retardation of normal protein synthesis. The promotion of a healthy body weight with complete dairy was seen unanimously in all studies.

Consistent effects on weight were also seen within and across all experiments with Ca consumption. All rats that received the HC treatment showed less weight gain than LC treated rats. Our work was consistent with previous literature, as almost every study examining the role of Ca in weight management has found an improvement with Ca, which is most likely due to FF. Unexpectedly, however, we saw greater Ca bioavailability in rats consuming the HC treatment as measured by BMD (Tables 4.1 and 6.1). This suggests that the increased Ca has more of an effect than simply binding to intestinal fat and bile acids to increase energy loss via FF. This is in contrast to many authors who have suggested that the extra Ca is not bioavailable and additional Ca supplementation (>0.4% of diet) does not result in any physiological manifestation [34, 69, 72, 99]. However, the current data support findings by Zemel *et al* who suggest dietary Ca has the ability to alter intracellular adipocyte Ca concentrations [156]. Until now no study examining dairy foods or Ca in weight management has provided a measure of BMD. This is a major shortcoming because much of the consumed Ca was potentially not accounted for (ie. no measurements of serum Ca, FF, or BMD) resulting in an incorrect assumption that all extra Ca was excreted as FF.

In addition to the individual effects of protein and Ca on body composition, we found significant protein – Ca interactions during ER. Because the ER study had a factorial design (Figure 5.1), we could effectively determine the influence of protein and Ca, as well as the effects of their interaction. No other study to date has had a complete factorial design and consequently their conclusions are less rigorous. For example many studies have used a low dose of supplemental Ca, a low dose of dairy Ca, and then a high dose of dairy Ca [39, 50, 58, 65]. Although this provides interesting data, the study

design would be enhanced with one other group examining high supplemental Ca intake to determine protein – Ca interactions. During ER, we found that the complete dairy with HC significantly accelerated FM loss more so than casein, soy, or whey supplemented with HC (Figure 5.2). This is in contrast to the *ad libitum* studies (Chapters 4 and 6), where there was no protein – Ca interaction. This finding supports a synergistic interaction between Ca and dairy foods affecting increased weight loss during periods of ER. This could also support why in humans, the most notable and significant role of dairy foods is during ER and not free-feeding conditions [24, 65, 156].

The food consumption data, in addition to the data measuring satiety response, provides evidence that changes in body composition are not attributable to altered food intake or appetite signalling. The current paradigm within the literature is that complete dairy or whey might only be affecting appetite regulation [132]. We found no difference in GLP-1, amylin, or ghrelin within each study. Expression levels of associated genes also were not altered by protein or Ca treatments. Consistent with this finding is that *ad libitum* rats did not have significantly different energy intake during the HFHS protocols outlined in Chapters 4 and 6. Therefore in this study, satiety hormone secretion and associated feeding responses do not play a role in the change in body composition.

It is likely that these changes in body composition are attributable to alterations of genes related to glucose and lipid metabolism and nutrient partitioning. Results seen for body composition could be in part due to alterations in hepatic genes related to lipogenesis [164, 165](Appendix E). With complete dairy (Chapter 4) there was down regulation of hepatic SREBP1c and PPAR γ (Figure 4.5). Additionally, the soy treatment with HC treatment resulted in up regulation of PPARγ (Chapter 5); complete dairy, whey,

and casein had PPARγ expression that was less than soy (Figure 5.5A). The complete dairy treatment also resulted in lower PPARγ when compared to casein or leucine (Figure 6.2).

SREBP1c and PPARγ have been shown to be important genes involved in hepatic lipid metabolism. Both of these genes are responsible for the up regulation of lipogenic genes such as FAS and ACC, and in obesity, there is usually an upregulation of both SREBP1c and PPARγ [140] (Appendix E). PPARγ stimulates the uptake of glucose and lipid into peripheral tissues [164, 165]; it is possible that down regulation of PPARγ contributes to the improved body composition seen with complete dairy treatment. These data, therefore, provide strong support for the consumption of complete dairy and HC for helping with the maintenance of healthy body composition, both during *ad libitum* feeding and ER. The mechanistic response associated with consuming complete dairy and Ca appears to be unrelated to food intake. It is likely that treatment with complete dairy results in a unique metabolic environment with nutrient partitioning that promotes the maintenance of LBM, not FM. Although the gene data provide some insight into potential mechanisms (ie. reduction of PPARγ), future work needs to focus on the fate of consumed nutrients. Furthermore, the role of EE in the form of physical activity, behavioural changes, and adaptive thermogenesis must be quantified. In addition to changes in UCP activity or concentrations, it is plausible that animals consuming complete dairy have alterations in spontaneous physical activity levels or increased futile cycling of substrates (Ca, fat, and protein). Cold-water fish have a unique organ solely for the production of heat where Ca passively enters a network of sarcoplasmic reticulum and T-tubules and is pumped out by an ATP-driven Ca pump. The cycling of Ca results

in heat being produced and increased EE. Currently there is some evidence of a similar mechanism in rats and humans; however, the role of dietary Ca in futile Ca cycling in mammals is currently unknown. A further possible mechanism for increased adaptive thermogenesis is via the futile cycling of various substrates. Substrate cycling occurs when there is increased turnover within a tissue. Fatty acid cycling occurs usually upon cold exposure and is when there is an increased rate of cycling between de novo lipogenesis and lipid oxidation. Protein turnover, on the other hand occurs usually during over feeding and results in an increased rate of both muscle anabolism and catabolism [166]. Further mechanistic studies examining regulation of adaptive thermogenesis via the above mentioned mechanisms and UCP are warranted.

7.3.2 Measurements of Glucose Homeostasis and Insulin Sensitivity

Type 2 diabetes is typically indicated by elevated fasting glucose, insulin, and glucagon; impaired response to a MTT, OGTT, and ITT; and insulin resistance. The three main studies outlined in this thesis (Chapters 4, 5, and 6) demonstrate variations in both baseline and post-prandial levels of glucose, insulin, and glucagon related to protein source and Ca level. In Chapter 4, baseline concentrations of glucose and insulin were both decreased with complete dairy treatment. Postprandial insulin responses were blunted and glucose responses increased with the complete dairy treatment (Table 4.2, Figure 4.4). In the ER study (Chapter 5), complete dairy resulted in decreased baseline insulin and glucagon and a blunted insulin meal response (Table 5.2). Treatment with complete dairy also resulted in an improved HOMA-IR but conversely, a significantly greater glucose AUC in response to a meal (Table 5.2). In Chapter 6, there were no significant differences in baseline insulin or glucose between protein treatments, however

the response to the ITT was greatest (best) for the complete dairy treatment (Table 6.2) when compared to both the casein and the leucine treatments. In addition to the direct measurements of glucose and insulin, we calculated HOMA-IR for an indirect estimate of insulin resistance [134]. The rats with complete dairy treatment consistently had a lower (improved) HOMA-IR when compared to other protein treatments (Chapters 4, 5, and 6). These studies were not designed to sufficiently determine the mechanisms related to this improvement; however, the improved ratio of LBM to FM in these rats likely contributed. This improvement of HOMA-IR predicts improved insulin sensitivity. It is plausible that this improvement in insulin sensitivity is due to alterations in plasma concentrations of cytokines such as adiponectin. The dairy proteins have naturally occurring angiotensin inhibitors. In previous work with a synthetic angiotensin receptor blocker, various metabolic parameters improved in rats: fasting glucose decreased, adiponectin levels increased, and insulin resistance decreased. Adipocytes had a phenotypic change with an increase in small, newly differentiated fat cells which could be an effect of the increased adiponectin [83]. Moreover, the remodelling of adipocytes may assist in the explanation of some leptin data that is not correlated with body fat percentage (Tables 4.1 and 4.2). Furthermore, the discrepancy in insulin – glucose homeostasis data may be due in part to stress levels of the animals. During the energy restriction study (Chapter 5) baseline insulin levels were significantly higher than during the *ad libitum* study (Chapter 4). We did not measure glucocorticoids, catecholamines, or any other markers of stress and this could have influenced our data. Additionally, although rats were food deprived over night prior to all testing (Chapters 4, 5, and 6),

some rats consumed their feces which cold have also contributed to variations in fasting levels of glucose, insulin, and glucagon.

In addition to the protein effects on insulin and glucose, we found that HC significantly decreased glucagon AUC during ER (Chapter 5) (Table 5.3). Glucagon is normally secreted into the bloodstream in response to low glucose. This in turn stimulates the hepatic production of glucose and provides the major counter regulatory effect on insulin [167]. In type 2 diabetes, there is often an increase in glucagon secretion during fasting and postprandial states; lack of suppression of postprandial glucagon also plays an important role in postprandial hyperglycaemia [168]. In Chapter 5, during ER, we found that HC diets reduced the glucagon to insulin ratio, which would suggest an improvement in glucose homeostasis. This is plausible since other cations, such as zinc, are known to regulate the secretion of glucagon [142]. In the two *ad libitum* studies, there appeared to be minimal effect of protein or Ca on glucagon. This improvement in glucagon with Ca supplementation may be one mechanism by which dairy foods and Ca exert greater effects during ER as opposed to *ad libitum* feeding.

Based on the data from Chapters 4 and 5 – specifically, the inconsistencies between parameters of glucose homeostasis – we examined insulin sensitivity via the euglycemic-hyperinsulinemic clamp technique in Chapter 6 [158, 169]. The data from this final study demonstrate that the complete dairy rats did not have improved insulin sensitivity as suggested by the previous data (Tables 4.2 and 5.1). This suggests that the complete dairy rats had improved *hepatic* insulin sensitivity, yet poor *whole body* insulin sensitivity [158, 169, 170]. Indeed, HOMA-IR, although highly correlated with whole body insulin sensitivity, is a better indicator of liver and splanchnic glucose uptake [170,

171]. The leucine treatment improved whole-body insulin sensitivity even though rats had significantly greater FM. This is consistent with a study by Zhang *et al* who provided obese rats supplementary leucine in their drinking water. They showed that leucine improved glucose metabolism and reduced diet-induced insulin resistance [172]. Although no mechanisms of action were determined in this study, it is hypothesized that skeletal muscle or central mTOR signalling improved glucose disposal and insulin sensitivity. In our study, we found no alterations in the hepatic mTOR pathway that would explain this (Table 6.5); however, we cannot rule out the role of central or skeletal muscle mTOR activation in improving whole body insulin sensitivity since they were not directly measured. This body of work solely examined peripheral responses to dietary interventions; however, central responses arguably play an equal role in the promotion and/or prevention of obesity.

Alterations in glucose homeostasis by complete dairy can possibly be explained by analysis of hepatic gene expression. A principle constituent of whole body glucose homeostasis is the hepatic production of glucose. Excessive hepatic glucose production is a hallmark feature of type 2 diabetes. Hepatic glucose production can occur via the breakdown of glycogen (glycogenolysis) or by gluconeogenesis from a variety of substrates (e.g., amino acids, glycerol, and/or lactate) [167]. PEPCK, G6Pase, and PGC1α are key enzymes that control the rate of gluconeogenesis (Appendix E) [167]. In Chapters 5 and 6 we found alterations in these three genes with both protein source and Ca level. PEPCK is responsible for the reaction that converts oxaloacetate to phosphoenolpyruvate and G6Pase catalyzes glucose from glucose-6-phosphate. These genes are primarily controlled by insulin and glucagon, however the transcriptional

coactivator, PGC1α, also regulates them [167]. Insulin, glucose, and PGC1α suppress PEPCK whereas only insulin and PGC1α suppress G6Pase. Upregulation of hepatic PGC1α occur with cAMP and during fasting and in type 2 diabetes; increases of hepatic PGC1α have been linked to metabolic conditions such as heart disease and type 2 diabetes [167]. In our study, we saw down regulation of hepatic PGC1α with complete dairy (Chapter 6) and up regulation with soy compared to casein, whey, and complete dairy (Chapter 5). The responses of PGC1α, PEPCK, and G6Pase may help in explaining the baseline glucose values seen with complete dairy. If these genes were down regulated, hepatic glucose production would be reduced during fasting and result in lower baseline glucose. The physiological response to dysregulation of these genes is supported by numerous studies examining the role of over expression of PEPCK and G6PAse [167]. One particular study found that with overexpression of G6Pase, there were a cascade of metabolic abnormalities typically associated with type 2 diabetes, including glucose intolerance and hyperinsulinemia. The over expression of hepatic G6Pase was enough to cause whole body alterations in both glucose and lipid metabolism [173]. Moreover, mice that over express PEPCK show an increase in hepatic glucose production but normal whole-body glucose disposal during a euglycemic-hyperinsulinemic clamp [174]. The variations we observed with PEPCK, PGC1α, and G6Pase may be sufficient to explain the variations in measurements of insulin sensitivity found throughout this thesis. Further supporting the idea that complete dairy treatment improved hepatic insulin sensitivity, the direction of change seen in hepatic genes suggest

a decrease in gluconeogenesis. We also saw a reduction in fat accumulation in the liver that would also result in improved hepatic insulin sensitivity.

7.3.3 Hepatic Gene Expression

In addition to the alteration in genes related to glucose homeostasis, we also identified changes in the expression of genes related more specifically to insulin sensitivity and lipid metabolism. PPARs are members of the nuclear receptor super family of ligand-dependent transcription factors. In addition to the activation of lipogenic genes (ACC and FAS), hepatic PPARγ alters insulin sensitivity [165] (Appendix E). Of interest, many PPARγ agonists, including thiazolidinedione, pioglitazone, and rosiglitazone, are currently used as pharmaceuticals for insulin sensitization [164]. However, the relationship between PPARγ and insulin sensitivity remains very controversial. The controversy stems from the finding that a reduction of hepatic PPARγ activity results in an increase in fatty acid oxidation and a decrease in lipogenesis with a concurrent improvement in insulin resistance; and conversely, the PPARγ agonists also improve insulin sensitivity and decrease fat deposition. It has been suggested that PPARγ is a thrifty gene that has an optimal level of expression, and upward or downward deviations in regulation causes similar effects [165].

Additionally, in Chapter 6 with microarray analysis, we determined other metabolic pathways that may play a significant role in the phenotype seen with complete dairy. The complete dairy diet was associated with down regulation of ERK1, GSK3β, and AMPKα2 (Table 6.6). These are genes responsible for regulation of many metabolic pathways including glucose homeostasis and insulin signalling. Data from our three studies demonstrated that there does appear to be a role of protein source and Ca on

parameters of insulin sensitivity and glucose homeostasis; however the mechanisms of action need further clarification. The current set of data does not provide a clear picture and future work needs to address these limitations. Examination of the role of ERK1 and GSK3β, two insulin receptor substrate (IRS1) serine-kinases that promote hepatic insulin resistance [175], and AMPKα2, a cellular energy sensor should be considered [176].

7.3.4 Conclusions

In conclusion we have identified several potential pathways through which complete dairy may act to influence glucose metabolism and body weight. Interestingly, many of the genes regulated by complete dairy were not influenced by the leucine treatment. Analysis of the microarray data suggests that complete dairy and leucine have very different hepatic effects. Furthermore, the body composition and levels of blood glucose and plasma hormones were quite distinct between these two groups. Overall, it can be concluded that complete dairy consumption with HC supplementation does promote a healthy body weight (increased LBM and less FM) during both ER and *ad libitum* feeding in Sprague-Dawley DIO rats. However, the role of complete dairy on parameters of glucose metabolism and insulin sensitivity need further clarification to determine if the underlying effects are positive or negative (Figures 7.1 and 7.2).

Figure 7.1 Overall Summary of Findings for Complete Dairy and High Calcium Treatments During *Ad Libitum* Feeding

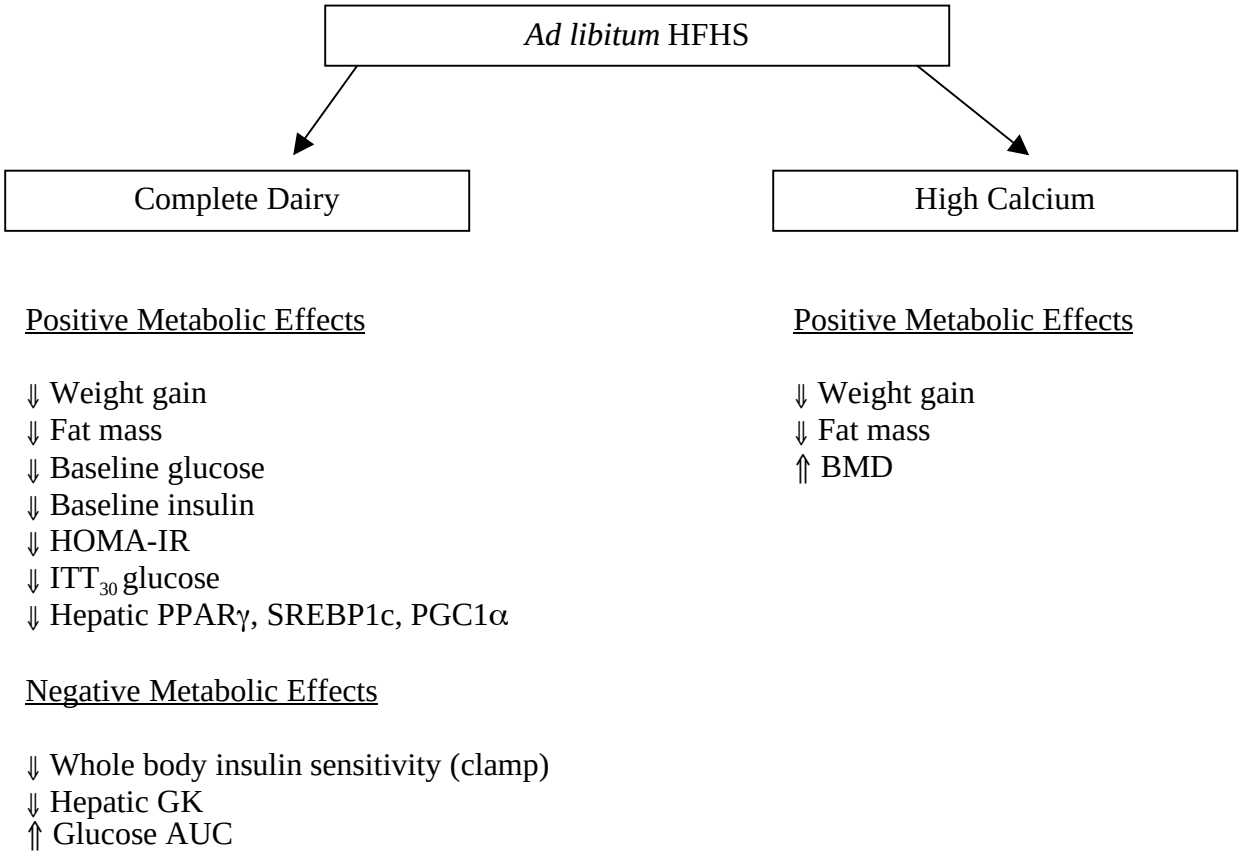

Figure 7.1: An overall summary of the major findings related to consumption of complete dairy protein and high calcium during high fat, high sucrose *ad libitum* feeding. Outcome variables for complete dairy were compared to casein.

Figure 7.2 Overall Summary of Findings for Complete Dairy and High Calcium Treatments During Energy Restriction

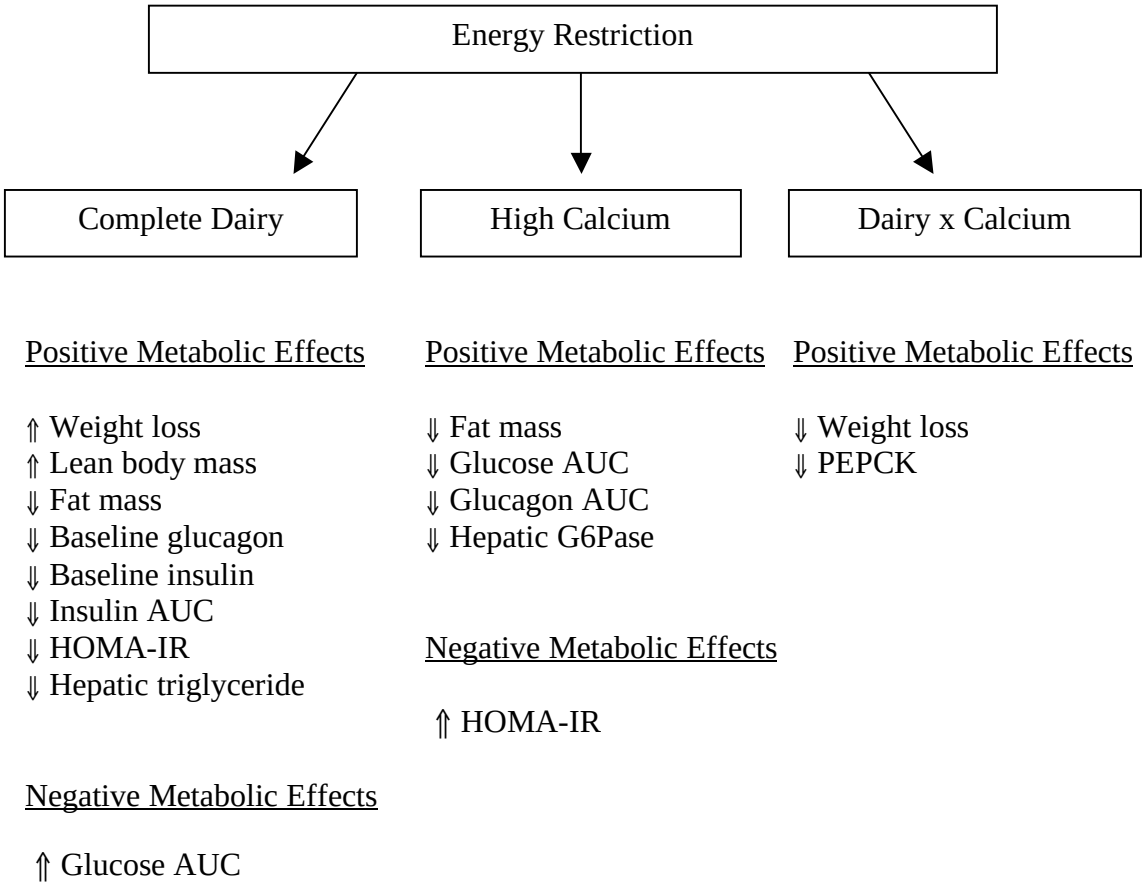

Figure 7.2: An overall summary of the major findings related to consumption of complete dairy protein and high calcium during energy restriction; interactions between complete dairy and calcium are also included. Outcome variables for complete dairy were compared to casein.

7.4 Future Directions

This study helps to elucidate the role of complete dairy, associated proteins, and Ca, in the regulation of metabolic parameters. There are, however, many aspects of this research that require future attention. Most pressing is continued work on delineating the critical bioactive component(s) of complete dairy. This is a serious limitation of all research in this area, as the putative compound remains unknown. Isolation of this specific dairy fraction and subsequent validation in animals is required if functional foods with isolated dairy components are to gain sufficient scientific evidence for promotion.

Furthermore, following the third study (Chapter 6), utilizing the euglycemic-hyperinsulinemic clamp, many questions become apparent. The data demonstrate that the rats consuming leucine, despite greater FM, had improved whole body insulin sensitivity. More research is required to determine why these animals were more insulin sensitive and how leucine elicits improved insulin sensitivity. Examination of skeletal muscle and white adipose tissues should provide further insight. It would also be beneficial to specifically measure hepatic insulin sensitivity to verify if complete dairy alters this outcome. Additionally, the measurement of cytokines such as adiponectin would be beneficial to determining the mechanisms of action for the changes in insulin sensitivity.

The gene expression data (microarray and rt-PCR) provided data that demonstrated differential expression of hepatic genes related to weight control, glucose homeostasis, and insulin signalling. Future work examining particular pathways associated with these changes is justified. For example, experiments with the goal of

examining PPARγ, PGC1α, ERK1, and AMPK responses to dairy are warranted, as these are major genes affiliated with metabolic health.

Though animal models provide the only ethical means of studying many of these pathways, the inherent caveat of all animal work – the questionable applicability to humans – clearly remains. A well-controlled study with complete factorial design examining the effects of complete dairy with LC or HC in individuals with obesity or type 2 diabetes would provide further insight. Currently, there are no human studies that effectively compare complete dairy to non-dairy protein, with HC or LC. It still is not known what the relationship between rising obesity rates and decreased dairy food and Ca consumption is. Dairy food and Ca supplementation may provide a unique and simple way to assist with weight management. If benefits from animal work are to be translated to humans, more studies need to be completed.

Finally, and worthy of mention, is the current controversy over bias surrounding studies involving the role of milk in body weight management. A significant portion of work completed in this area has been done with the financial support of the dairy industry. Future work funded independently of the dairy industry would allow an entire field of literature to either be validated or discredited: but at least these studies could then be evaluated on their scientific merit, as opposed to an anecdotally based supposed bias.

7.5 Conclusions and Significance

Collectively this thesis provides evidence for a role of complete dairy – but not individual dairy-protein fractions – in the management of metabolic factors involved with body weight regulation. Ca appears to be implicitly involved in this process. Sprague

Dawley rats that consumed complete dairy experienced attenuated weight gain during *ad libitum* feeding, and accelerated weight loss during ER; Ca supplementation in conjunction with complete dairy enhanced both these effects. The data also suggest reduced whole-body insulin sensitivity with complete dairy, but increased with leucine supplementation. These phenotypic differences seen with complete dairy, Ca supplemented groups suggests alterations to hepatic pathways responsible for lipid and glucose metabolism, as well as insulin signalling. Myriad questions pertaining to the exact pathways responsible for these effects require further study.

Considering that 65-84% of Canadian adults aged 31 or over, currently consume less than two servings of dairy products per day, the addition of dairy foods may make a marked positive contribution towards metabolic health. Research into the specific putative bioactive components found in complete dairy warrant a more detailed examination.

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APPENDIX A: SUMMARY OF HUMAN RANDOMIZED CONTROL STUDIES

Table A.1 Summary of Randomized Control Trials with Crossover Design

Table A.1: Summary of human randomized control trials with crossover design published from 1994-2009. Studies were included if the primary study objective was to determine the role of dairy foods and/or calcium supplementation on metabolic parameters. Studies were only included if body composition, energy metabolism, metabolic syndrome, or weight regulatory mechanisms were used as an original study outcome variable.

N	Time	Treatments (/day)	Main Findings	Ref
10	5 wk	(a) 800mg Ca (b) Placebo	No difference in fat OX or EE	[43]
11	1 wk	(a) High dairy (2300mg Ca) (b) Low dairy (700mg Ca)	High dairy doubled FF	[35]
58	1 wk	(a) <1 dairy serving (b) >3 dairy servings	No difference in subjective hunger; >3 servings group ate 209 kcal/d more without weight gain	[42]
18	Acute	(a) High dairy (b) Medium dairy (c) Low dairy (d) High Ca supp	Blood lipids decreased after dairy; No different in appetite	[36]

10	1 wk	(a) 400 mg dairy Ca (b) 1200 mg dairy Ca (c) 2500 mg dairy Ca (d) 1200 mg Ca supp	Decrease WAT, FAS, and TG; increase in FF with greater Ca dose	[34]
31	4 wk	(a) 1000mg Ca in bread (b) Placebo	Ca lowered total cholesterol	[44]
10	1 wk	(a) Low Ca/normal protein (b) High Ca/normal protein (c) High Ca/high protein	High Ca/normal protein increased FF by 350KJ per day; no difference in EE or fat OX	[37]
12	1 wk	(a) High Ca/high dairy (b) High Ca/low dairy (c) Low Ca/low dairy	No difference in 24 hr EE, fat OX or related gene expression	[38]
19	1 wk	(a) 3-4 servings dairy (b) 1-2 servings dairy	Dairy foods increased 24 hr EE	[39]
11	Acute	(a) Low dairy breakfast (b) High dairy breakfast	2 hr food intake was lower after high dairy	[40]
10	2 wk	(a) Chocolate supplemented with 900mg of Ca (b) Placebo	LDL and total cholesterol lowered with Ca	[41]

Table A.2 Summary of Randomized Control Trials with Parallel Design

Table A.2: Summary of human randomized control trials with parallel design published from 1994-2009. Studies were included if the primary study objective was to determine the role of dairy foods and/or calcium supplementation on metabolic parameters. Studies were only included if body composition, energy metabolism, metabolic syndrome, or weight regulatory mechanisms were used as an original study outcome variable.

N	Time	Treatments (/day)	Main Findings	Ref
340	2 yr	(a) Placebo (b) 2 doses of 750mg Ca	No difference in body weight	[45]
36,282	7 yr	(a) 1000mg Ca + 400 IU Vit D (b) Placebo	Supplementation had small effect on the prevention of weight gain in women who had low Ca intake prior to study	[46]
100	25 wk	(a) 1000mg Ca (b) Placebo	No difference in body weight	[47]
45	16 wk	(a) Control (b) Milk only (c) Milk+1 food daily	Milk group lost significantly more body weight	[55]

37	1 yr	(a) 1125 mg Ca in drink with kcal (b) Placebo	No difference in weight, but Ca group consumed more calories	[48]
44	48 wk	(a) 400mg Ca (b) 700 mg Ca	Pre-study low Ca consumers lost most weight with 700mg Ca	[49]
90	48 wk	(a) 500 kcal ER +800mg Ca (b) 500 kcal ER +1400mg Ca	No difference in body composition	[50]
155	1 yr	(a) <800mg Ca (b) 1000mg Ca (c) 1400mg Ca/day	No change in body weight or body fat although medium and high Ca groups ate slightly more kcal	[51]
35	8 wk	(a) 3 servings of yogurt (b) 3 servings of non-dairy protein (c) 3 servings of carbohydrate	Yogurt decreased body fat even with increased calorie consumption	[56]
67	3 wk	(a) 600 kcal ER (b) 600 kcal ER + 500mg Ca	Preservation of FFM and lower hunger with Ca	[52]

56	12 wk	Resistance trained 5d/wk and consumed: (a) Skim milk (b) Fat free soy protein (c) Maltodextrin	Milk promoted muscle hypertrophy better than soy or maltodextrin	[57]
29	24 wk	(a) 500kcal ER + 3 dairy servings (b) 500 kcal ER + <1 dairy serving	Dairy group lost 2x as much body weight and fat with maintenance of LBM. Decreased insulin with dairy.	[58]
204	12 wk	(a) 3 cups skim or 1% milk (b) <1.5 servings of milk	Milk group gained 0.6kg, which was less than expected based on calorie intake.	[59]
34	12 wk	(a) 500kcal ER + 3 yogurt servings (b) 500 kcal ER + <1 yogurt serving	Yogurt group lost 22% more body weight, 66% more body fat, and 81% more trunk fat.	[60]

63	15 wk	(a) 700kcal ER +1200 mg Ca, 400 IU vitamin D (b) 700 kcal CR	Improvements in blood lipids with Ca	[53]
72	48 wk	(a) 500 kcal ER + high dairy (1400mg Ca) (b) 500 kcal ER + high dairy/ high fiber/low GI (c) 500 kcal ER + dairy (800mg)	All groups lost same amount of body weight, but high dairy group consumed 100-150 more kcal per day.	[50]
50	16 wk	(a) 1300kcal as high dairy/high Ca (2400mg Ca) (b) 1300 kcal as mixed protein/moderate Ca (500 mg Ca)	No difference in weight, insulin levels, or lipid profile.	[61]
54	1 yr	(a) 500 kcal ER + 1 dairy serving (b) 500 kcal ER + 4 dairy servings	No difference in body composition but dairy group had significantly higher calorie intake	[62]
1471	30 mo	(a) 1000 mg of Ca (b) Placebo	No difference in body weight and a small decrease in blood pressure with Ca	[54]

19	1 yr	(a) 3-4 servings dairy (b) 1-2 servings dairy	Increased fat OX and postprandial EE. Decrease in parathyroid hormone	[63]
32	24 wk	(a) 500kcal ER + 3 dairy servings (1300 mg Ca) (b) 500 kcal ER + 1300 mg Ca	Dairy lost 70% more body weight and 64% more fat than Ca	[65]

APPENDIX B: DIET COMPOSITION

Table A.3 Composition of High Fat/High Sucrose Experimental Diets

Table A.3: Detailed diet composition for the high fat, high sucrose (HFHS) diets utilized in experiments outlined in Chapters 4, 5, and 6. All HFHS diets have an energy density of 4.6 kcal/gram.

g/kg	Casein LC	Casein HC	Whey LC	Whey HC	Dairy LC	Dairy HC	Leucine LC	Leucine HC
Sucrose	514.2	514.2	514.2	514.2	314.7	314.7	514.2	514.2
Lard	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Soybean Oil	100.0	100.0	100.0	100.0	100.0	100.0	100.0	100.0
Protein[1]	140.0	140.0	140.0	140.0	0	0	100.0	100.0
Protein[2]	0	0	0	0	343.0	343.0	40.0	40.0
α-Cellulose	50.0	50.0	50.0	50.0	50.0	50.0	50.0	50.0
Mineral Mix	35.0	35.0	35.0	35.0	35.0	35.0	35.0	35.0
Vitamin Mix	10.0	10.0	10.0	10.0	10.0	10.0	10.0	10.0
L-Cystine	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
$CaCO_3$[3]	4.3	46.5	4.3	46.5	0	43.0	4.3	46.5
Cornstarch	42.2	0	42.2	0	43.0	0	42.2	0
% kcal								
Protein	10.1	10.2	10.1	10.2	10.0	10.1	10.1	10.2
Carbohydrate	48.6	48.2	48.6	48.2	48.2	47.8	48.6	48.2
Fat	41.3	41.7	41.3	41.7	41.8	42.1	41.3	41.7

Diets were prepared in-house based on previously published HFHS diets and meet all AIN-93M requirements [105]. All diet ingedients were obtained from Dyets, Inc. (Bethlehem, PA). [1]Protein source is casein or whey as specified by diet. [2]Protein source is non-fortified skim milk powder or leucine, dependent on diet. [3] Calcium carbonate is 40% elemental Ca.

Table A.4 Composition of Normal Energy Experimental Diets

Table A.4: Detailed diet composition for the normal energy (NE) diets utilized in experiments outlined in Chapters 3, 4, 5 and 6. All NE diets have an energy density of 3.6 kcal/gram.

g/kg	Casein, Soy or Whey LC	Casein, Soy, or Whey HC	Dairy LC	Dairy HC
Sucrose	100.0	100.0	100.0	100.0
Soybean Oil	40.0	40.0	40.0	40.0
Protein[1]	140.0	140.0	0	0
Protein[2]	0	0	345.0	345.0
α-Cellulose	50.0	50.0	50.0	50.0
Mineral Mix	35.0	35.0	35.0	35.0
Vitamin Mix	10.0	10.0	10.0	10.0
L-Cystine	1.8	1.8	1.8	1.8
Choline bitartrate	2.5	2.5	2.5	2.5
$CaCO_3$ [3]	4.3	47.5	0	43.0
Cornstarch	461.4	418.2	260.7	217.7
Dextrinized cornstarch	155.0	155.0	155.0	155.0
% kcal				
Protein	12.3	12.2	12.3	12.2
Carbohydrate	77.7	77.8	77.7	77.8
Fat	10.0	10.0	10.0	10.0

Diets were prepared in-house based on AIN-93M and meet all respective requirements [105]. All diet ingedients were obtained from Dyets, Inc (Bethlehem, PA). [1]Protein

source is casein, soy, or whey as specified by diet. [2]Protein source is non-fortified skim milk powder (dairy). [3]Calcium carbonate is 40% elemental Ca.

APPENDIX C: REAL TIME POLYMERASE CHAIN REACTION PRIMERS

Table A.5 Real-Time Polymerase Chain Reaction Primers

Table A.5: All primers used for rt-PCR in Chapters 4, 5, and 6. Each primer is presented in the 3' to 5' direction.

Gene	Forward Primer	Reverse Primer
ACC	CCTTCTTCTACTGGCGACTGAG	TAAGCCTTCACTGTGCCTTCC
Amylin	CTGCCACTGCCCACTGAAAG	CACTTCCGTTTGTCCACCTGAG
β Actin	TATCGGCAATGAGCGGTTCC	AGCACTGTGTTGGCATAGAGG
CCK	GCCGCCTGCCCTCAAC	ACACACGCCGCACTTCATATC
FAS	GAGGACTTGGGTGCCGATTAC	GCTGTGGATGATGTTGATGATAGAC
GAPDH	CAAGTTCAACGGCACAGTCAAG	ACATACTCAGCACCAGCATCAC
Ghrelin	AGAGGCGCCAGCTAACAAGTAA	GCAGGAGAGTGCTGGGAGTT
Glucokinase	CCGAGTGGCTTACAGTTCTG	ACCTGAGTGTTGGAGATGATTC
PGC1α	AGAGGCAGAAGCAGAAAGC	TGTCTCCATCATCCCGCAG
PPARγ	ATGTCTCACAATGCCATCAGG	CCAGGGCTCGCAGATCAG
Proglucagon	ACCGCCCTGAGATTACTTTTCTG	AGTTCTCTTTCCAGGTTCACCAC
PYY	AGCGGTATGGGAAAAGAGAAGTC	ACCACTGGTCCACACCTTCTG

SREBP-1c	TCATCAACAACCAAGACAGTG	AGAGAAGCAGGAGAAGAGAAG
UCP2	GCAGATCCAAGGAGAGAG	GACCAGCCCATTGTAGAG
G6Pase	GCTGGAGTCTTGTCAGGCATTG	AGTGCGAAACCAAACAGGAAGAA
PEPCK	CGTGGCTGAGACAAGTGATGG	ACGGTTCCTCATCCTGTGGTC

APPENDIX D: SUMMARY OF MICROARRAY DATA

Table A.6 Expression for Genes Altered by Complete Dairy in the KEGG Metabolic Pathway

Table A.6: Expression for those genes altered only by complete dairy treatment in the KEGG Metabolic Pathways at the end of the eight-week HFHS *ad libitum* feeding period in Sprague-Dawley DIO rats fed casein (control) or complete dairy diets. Data is presented as mean ± SEM, n=4/protein treatment. (*) All listed genes, $p<0.05$ between casein (control) and complete dairy treatments.

Casein	Dairy*	Gene ID	Gene Name
4.49±0.11	5.17±0.22	Papss1	3'-phosphoadenosine 5'-phosphosulfate synthase 1
12.53±0.09	11.87±0.12	Hpd	4-hydroxyphenylpyruvate dioxygenase
7.15±0.11	6.84±0.06	Mthfs	5,10-methenyltetrahydrofolate synthetase
6.00±0.27	6.81±0.18	Mthfr	5,10-methylenetetrahydrofolate reductase (NADPH)
6.28±0.10	6.91±0.14	Pgls	6-phosphogluconolactonase
4.72±0.12	5.05±0.15	Abo	ABO blood group transferase A, alpha 1-3-N-acetylgalactosaminyltransferase; transferase α1-3-galactosyltransferase
9.52±0.11	9.01±0.09	Acsl4	Acyl-CoA synthetase long-chain family member 4
5.16±0.06	4.88±0.09	Agk	Acylglycerol kinase
7.27±0.35	5.40±0.49	Adh6	Alcohol dehydrogenase 6 (class V)
6.93±0.13	6.10±0.23	Adh7	Alcohol dehydrogenase 7 (class IV),

			mu or sigma polypeptide
10.69±0.11	10.23±0.12	Aldh7a1	Aldehyde dehydrogenase 7 family, member A1
9.84±0.23	9.01±0.20	Aadat	Aminoadipate aminotransferase
6.06±0.19	5.25±0.08	Apip	APAF1 interacting protein
11.17±0.11	10.57±0.15	Arg1	Arginase, liver
10.38±0.08	9.75±0.20	Asl	Argininosuccinate lyase
12.38±0.15	11.33±0.15	Ass1	Argininosuccinate synthetase 1
9.13±0.18	8.23±0.23	Alg11	Asparagine-linked glycosylation 11, alpha-1,2-mannosyltransferase homolog (yeast)
7.11±0.07	7.69±0.17	Atp5i	ATP synthase, H+ transporting, mitochondrial F0 complex, subunit E
6.89±0.09	6.44±0.02	Atp5c1	ATP synthase, H+ transporting, mitochondrial F1 complex, gamma polypeptide 1
8.12±0.18	7.64±0.13	Atp6v1a	ATPase, H+ transporting, lysosomal V1 subunit A
7.33±0.06	7.70±0.06	B3gat3	Beta-1,3-glucuronyltransferase 3
12.91±0.18	11.86±0.11	Bhmt	Betaine-homocysteine methyltransferase
5.05±0.04	5.26±0.06	Bcat2	Branched chain aminotransferase 2, mitochondrial
12.66±0.11	12.07±0.06	Cps1	Carbamoyl-phosphate synthetase 1
6.06±0.12	5.38±0.22	Cbr1	Carbonyl reductase 1
6.42±0.16	5.84±0.08	Coq7	Coenzyme Q7 homolog, ubiquinone
7.68±0.18	7.08±0.06	Cox15	COX15 homolog, cytochrome c oxidase assembly protein (yeast)
10.57±0.09	11.02±0.07	Cox6a1	Cytochrome c oxidase, subunit VIa,

			polypeptide 1
10.66±0.22	10.38±0.04	Cox6c	Cytochrome c oxidase, subunit VIc
2.69±0.03	2.84±0.04	Cyp11a1	Cytochrome P450, family 11, subfamily a, polypeptide 1
9.23±0.34	7.22±0.42	Cyp2c12	Cytochrome P450, family 2, subfamily c, polypeptide 12
12.26±0.13	11.42±0.23	Cyp2c23	Cytochrome P450, family 2, subfamily c, polypeptide 23
12.20±0.12	11.46±0.26	Cyp2e1	Cytochrome P450, family 2, subfamily e, polypeptide 1
3.74±0.09	3.80±0.04	Cyp2e1	Cytochrome P450, family 2, subfamily e, polypeptide 1
7.53±0.18	6.32±0.41	Cyp2j4	Cytochrome P450, family 2, subfamily J, polypeptide 4
10.32±0.08	9.69±0.12	Cyp27a1	Cytochrome P450, family 27, subfamily a, polypeptide 1
6.25±0.50	4.79±0.23	Cyp3a9	Cytochrome P450, family 3, subfamily a, polypeptide 9
8.71±0.11	8.24±0.07	Dbt	Dihydrolipoamide branched chain transacylase E2
5.49±0.19	6.25±0.10	Dpm3	Dolichyl-phosphate mannosyltransferase polypeptide 3
4.24±0.08	4.76±0.19	Enpp7	Ectonucleotide pyrophosphatase/phosphodiesterase 7
11.37±0.11	10.34±0.27	Ehhadh	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase
9.95±0.12	9.24±0.17	Fh1	Fumarate hydratase 1
7.19±0.11	7.80±0.05	Gcs1	Glucosidase 1
2.31±0.04	2.54±0.05	Gad2	Glutamate decarboxylase 2

9.99±0.17	8.78±0.25	Got1	Glutamic-oxaloacetic transaminase 1, soluble (aspartate aminotransferase 1)
9.88±0.19	8.87±0.34	Gls2	Glutaminase 2 (liver, mitochondrial)
6.78±0.11	6.09±0.22	Hexb	Hexosaminidase B
10.33±0.18	9.32±0.22	Hal	Histidine ammonia lyase
2.69±0.04	2.44±0.06	Hyal5	Hyaluronoglucosaminidase 5
4.45±0.15	5.00±0.13	Impdh1	IMP (inosine monophosphate) dehydrogenase 1
7.63±0.09	6.99±0.07	Idh3a	Isocitrate dehydrogenase 3 (NAD+) alpha
9.42±0.13	8.93±0.05	Ivd	Isovaleryl coenzyme A dehydrogenase
11.08±0.13	10.41±0.15	Lipc	Lipase, hepatic
6.91±0.08	7.17±0.04	Mcat	Malonyl CoA:ACP acyltransferase
12.01±0.10	11.62±0.07	Mat1a	Methionine adenosyltransferase I, alpha
7.75±0.11	7.27±0.15	Mccc1	Methylcrotonoyl-Coenzyme A carboxylase 1 (alpha)
8.11±0.18	7.48±0.11	Mccc2	Methylcrotonoyl-Coenzyme A carboxylase 2 (beta)
9.35±0.19	9.82±0.05	Ndufa11	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex 11
5.83±0.08	6.27±0.14	Ndufa2	NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 2
9.24±0.16	8.63±0.17	Ndufs1	NADH dehydrogenase (ubiquinone) Fe-S protein 1
10.17±0.11	10.61±0.09	Nme2	Non-metastatic cells 2, protein (NM23B)
8.50±0.22	7.74±0.15	Nme3	Non-metastatic cells 3, protein expressed in
5.38±0.06	5.61±0.03	Nme6	Non-metastatic cells 6, protein

			expressed in (nucleoside-diphosphate kinase)
8.51±0.19	7.32±0.21	Oat	Ornithine aminotransferase (gyrate atrophy)
7.98±0.18	7.26±0.22	Ppap2a	Phosphatidic acid phosphatase type 2A
6.20±0.16	5.58±0.15	Pigl	Phosphatidylinositol glycan anchor biosynthesis, class L
5.10±0.09	4.81±0.05	Pigv	Phosphatidylinositol glycan anchor biosynthesis, class V
5.75±0.13	6.68±0.24	Pck2	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)
5.10±0.29	6.57±0.36	Phgdh	Phosphoglycerate dehydrogenase
4.75±0.25	5.43±0.06	Pgam2	Phosphoglycerate mutase 2 (muscle)
5.32±0.12	5.89±0.13	Pla2g2d	Phospholipase A2, group IID
5.38±0.05	5.75±0.10	Pla2g4b	Phospholipase A2, group IVB
6.28±0.03	6.44±0.02	Plcb3	Phospholipase C, beta 3 (phosphatidylinositol-specific)
5.24±0.27	6.41±0.35	Psat1	Phosphoserine aminotransferase 1
4.91±0.06	4.56±0.06	Pola1	Polymerase (DNA directed), alpha 1
2.35±0.09	2.70±0.05	Pole2	Polymerase (DNA directed), epsilon 2
5.43±0.11	4.79±0.14	Polr3f	Polymerase (RNA) III (DNA directed) polypeptide F, 39 kDa
2.88±0.07	3.35±0.10	P4ha3	Procollagen-proline, 2-oxoglutarate 4-dioxygenase (proline 4-hydroxylase), alpha polypeptide III
10.05±0.19	8.69±0.26	Prodh	Proline dehydrogenase
7.75±0.11	7.26±0.09	Pcca	Propionyl-coenzyme A carboxylase, alpha polypeptide
10.38±0.25	9.55±0.18	Qdpr	Quinoid dihydropteridine reductase

8.79±0.02	8.04±0.20	LOC314140	Ribose-phosphate pyrophosphokinase I
7.81±0.39	5.65±0.47	Sds	Serine dehydratase
4.11±0.09	3.73±0.11	RGD1560755	Similar to D8Ertd354e protein
7.90±0.24	7.73±0.16	RGD1560755	Similar to D8Ertd354e protein
2.54±0.13	2.14±0.11	MGC109340	Similar to Microsomal signal peptidase 23 kDa subunit (SPase 22 kDa subunit) (SPC22/23)
3.93±0.11	3.59±0.16	MGC109340	Similar to Microsomal signal peptidase 23 kDa subunit
7.34±0.27	6.11±0.38	LOC681086	Similar to Phosphatidylinositol-glycan biosynthesis, class F protein (PIG-F)
4.91±0.06	5.62±0.20	-	Similar to ubiquinol-cytochrome c
7.77±0.18	7.91±0.25	St3gal3	ST3 beta-galactoside alpha-2,3-sialyltransferase 3
9.42±0.32	8.49±0.12	Sucla2	Succinate-CoA ligase, ADP-forming, beta subunit
9.41±0.23	10.45±0.13	Uqcr	Ubiquinol-cytochrome c reductase
9.66±0.16	10.41±0.11	Uqcrq	Ubiquinol-cytochrome c reductase, complex III subunit VII
8.96±0.42	7.11±0.47	Ugt2b17	UDP glucuronosyltransferase 2 family, polypeptide B17
6.30±0.27	5.48±0.18	B3galt1	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 1
10.20±0.15	9.60±0.36	B3galt1	UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 1
4.36±0.11	4.71±0.03	B3gnt6	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 6
5.64±0.20	4.79±0.09	Uros	Uroporphyrinogen III synthase

Table A.7 Expression for Genes Altered by Leucine in the KEGG Metabolic Pathway

Table A.7: Expression data for genes altered by the leucine treatment in the KEGG Metabolic Pathway at the end of the eight-week high fat, high sucrose ad libitum feeding period in Sprague-Dawley DIO rats fed casein (control) and leucine diets. Data is presented as mean ± SEM, n=4/protein treatment. (*) All listed genes, $p<0.05$ between casein (control) and leucine treatments.

Casein	Leucine*	Gene ID	Gene Name
5.71±0.05	5.23±0.13	Agpat1	1-acylglycerol-3-phosphate O-acyltransferase 1
4.77±0.09	3.92±0.04	Nt5m	5',3'-nucleotidase, mitochondrial
8.13±0.28	6.85±0.28	Acsm2	Acyl-CoA synthetase medium-chain family mem. 2
8.71±0.25	6.66±0.26	Acsm5	Acyl-CoA synthetase medium-chain family mem 5
9.69±0.12	10.41±0.09	Acox2	Acyl-Coenzyme A oxidase 2, branched chain
8.05±0.07	7.66±0.06	Alg3	Asparagine-linked glycosylation 3, alpha-1,3- mannosyltransferase homolog
7.05±0.07	6.51±0.03	Chkb	Choline kinase beta
4.44±0.03	4.11±0.03	Gmds	GDP-mannose 4, 6-dehydratase
5.85±0.13	4.11±0.25	Inpp4b	Inositol polyphosphate-4-phosphatase, II
6.16±0.08	6.68±0.04	Inpp5a	Inositol polyphosphate-5-phosphatase A
10.25±0.10	10.02±0.06	Lap3	Leucine aminopeptidase 3
5.94±0.14	6.51±0.10	Nmnat1	Nicotinamide nucleotide

			adenylyltransferase 1
7.22±0.10	6.64±0.15	Ppt2	Palmitoyl-protein thioesterase 2
8.64±0.09	8.28±0.03	Polr2e	Polymerase (RNA) II (DNA directed) polypeptide E,
5.70±0.08	5.28±0.11	-	Similar to 5(3)-deoxyribonucleotidase, cytosolic type
6.77±0.14	6.55±0.04	RGD1306404	Similar to mKIAA1402 protein
3.32±0.02	3.54±0.05	RGD1565316	Similar to sphingomyelin phosphodiesterase 3, neutral membrane
5.68±0.08	5.27±0.11	Smpd4	Sphingomyelin phosphodiesterase 4
5.00±0.08	5.35±0.05	St3gal2	ST3 beta-galactoside alpha-2,3-sialyltransferase 2
7.68±0.14	7.08±0.11	Tcirg1	T-cell, immune regulator 1, ATPase, H+ transporting, lysosomal V0 subunit A3
5.07±0.13	4.36±0.15	Tbxas1	Thromboxane A synthase 1, platelet
3.12±0.07	3.08±0.11	Tpo	Thyroid peroxidase

APPENDIX E: HEPATIC GENES

Figure A.1 Schematic of Hepatic Genes Altered by Protein and Calcium Treatments

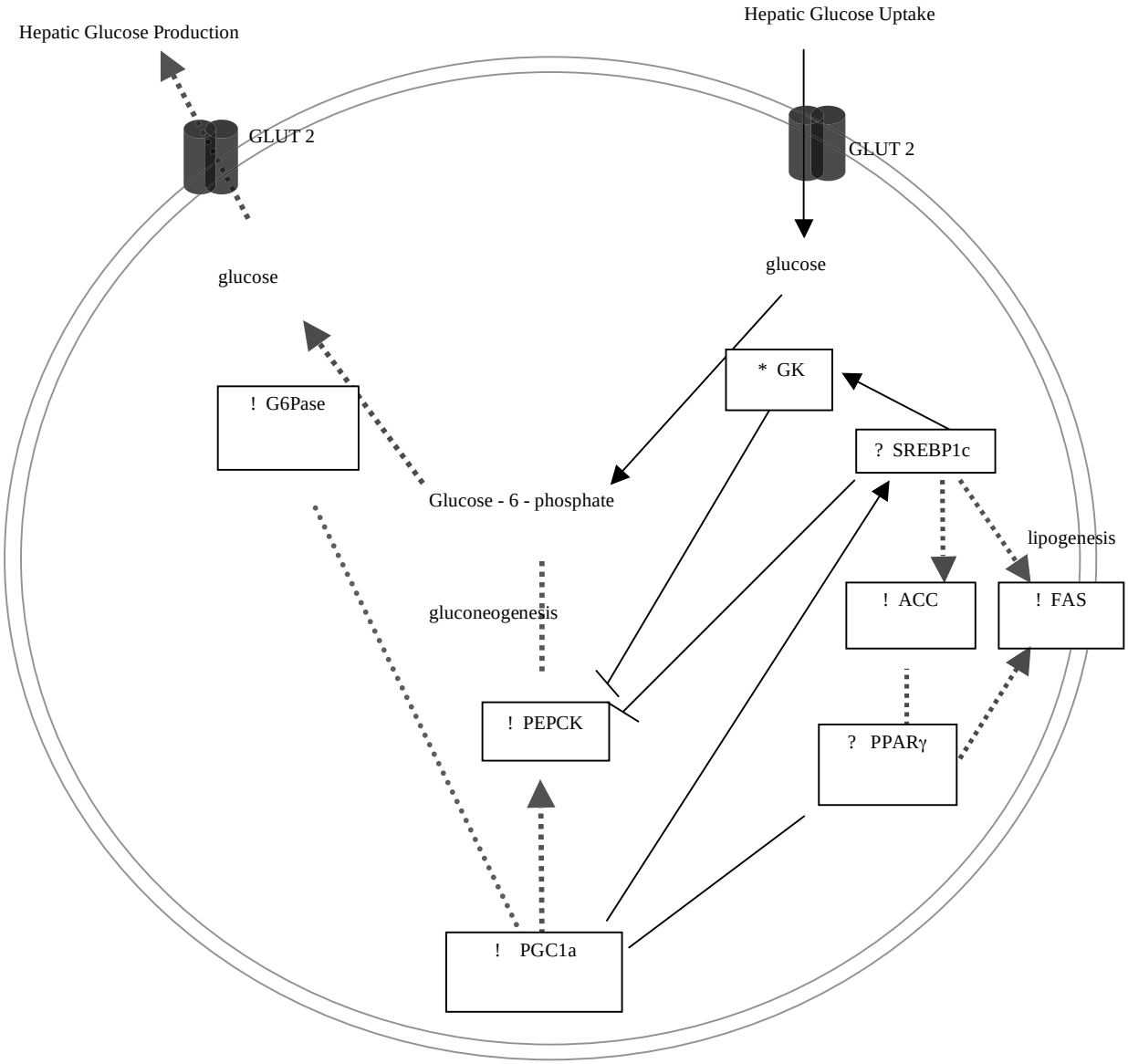

Figure A.1: A schematic representing genes that were altered by dietary treatments. Flux through the various hepatic pathways results in regulation of blood glucose concentrations. The exclamation mark (!) and dotted lines (…..) indicate that upregulation leads to a phenotype that is often found with obesity or type 2 diabetes. The asterisk (*) and solid lines (___) represent pathways that reduce metabolic dysfunction when up regulated. The question mark (?) indicates that it is unknown what the result of upregulation or down regulation is.

Glucokinase (GK) is a rate-limiting enzyme responsible for regulating hepatic glucose uptake. Peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1α), peroxisome proliferator-activated receptor gamma (PPARγ), and sterol regulatory element binding protein 1c (SREBP1c) are transcription factors or coactivators that are critical in the presentation of diabetic phenotypes. However, the role of SREBP1c is controversial and whether up or down regulation is beneficial is not entirely elucidated. For example, SREBP1c is insulin mediated and is responsible for control of hepatic lipogenesis [acetyl CoA carboxylase (ACC) and fatty acid synthase (FAS)], yet reduces hepatic glucose output by upregulation of GK. PGC1α upregulates hepatic glucose production via phosphoenolpyruvate carboxylase (PEPCK) and glucose-6-phosphatase (G6Pase); however, PGC1α also up regulates both PPARγ and SREBP1c, which results in down regulation of hepatic glucose production.

APPENDIX F: OBESE RAT SELECTION

Figure A.2 Diet Induced Obese (Obesity Prone) Rat Selection

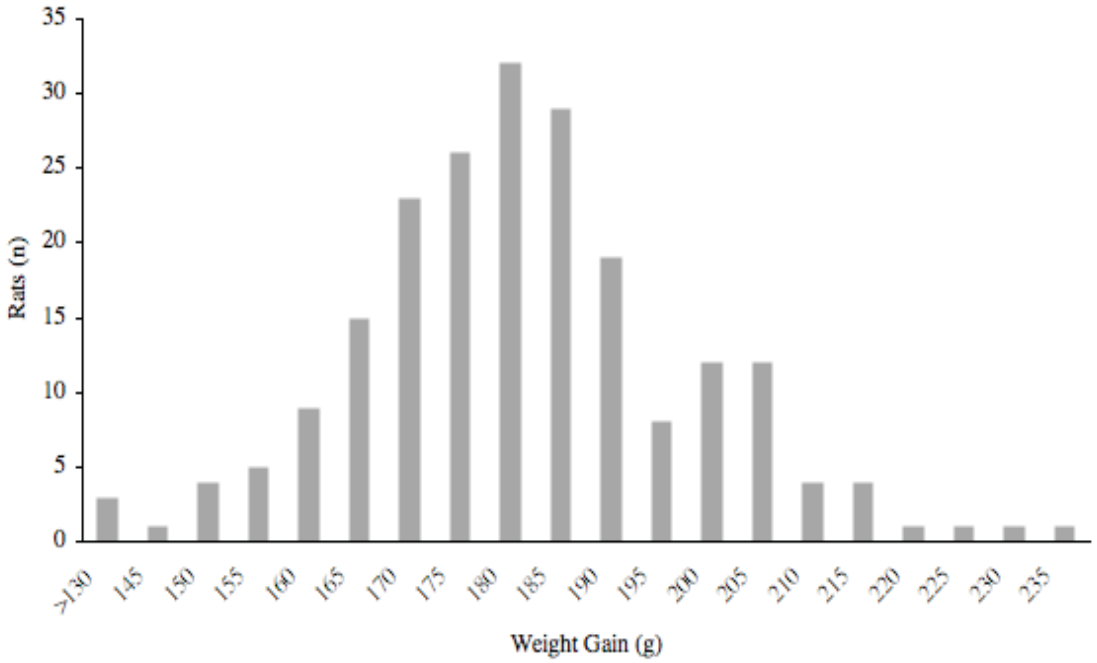

Figure A.2: The distribution of weight gain (g) over six weeks of *ad libitum* feeding in Sprague-Dawley rats. Rats were selected for participation in studies outlined in Chapters 4 and 5 if they weighed greater than 183g as they were determined to be more obesity prone.