ABSTRACT

CAMPBELL, CAROLINE LLOYD. The Effects of Cocoa Polyphenols and Whey Proteins on Biomarkers of Satiety and Lipid Metabolism. (Under the direction of Dr. Keith Harris, Dr. Allen Foegeding, and Dr. Clay Clark).

Low-calorie, hunger suppressing snacks are important in regulating daily calorie intake and in preventing long-term weight gain. Satiety, or the feeling of fullness that occurs between meals, is an important factor in determining total calorie intake. Formulation of appetizing foods with bioactive ingredients is a potential strategy to increase satiety and promote weight management. Possible ingredients include plant and dairy proteins to induce long-lasting satiety as well as fruit, vegetable, and whole grain phytonutrients for disease prevention. Whey protein has been demonstrated to decrease subjective ratings of hunger and *ad libitum* food intake as well as to increase levels of satiation and satiety hormones. Cocoa polyphenolics may moderate glucose levels and increase physiological and psychological markers of satiety. While flavored whey protein beverages are popular supplements for muscle recovery, the combined effects of whey protein and cocoa upon satiety and lipid metabolism are unknown.

This study investigated the bioactive effects of cocoa and whey protein *in vitro* and in a human clinical trial. *In vitro*, 3T3-L1 pre-adipocytes and differentiated adipocytes were treated with 0.5-100 μg/ml cocoa polyphenolic extract (CPE) and/or 1-15 mM leucine, a major whey protein amino acid. Extent of differentiation and lipid accumulation were quantified by Oil-Red-O staining and leptin production was measured by leptin ELISA. 10, 50, and 100 μg/ml CPE treatments inhibited pre-adipocyte lipid accumulation by 17, 35, and 50%, respectively. CPE treatment of 0.5 μg/ml increased leptin production but was not statistically significant. Leucine treatment had no effect upon lipid accumulation but

increased leptin production by 26-37%. In combination, CPE and 15 mM leucine inhibited lipid accumulation by 10-36% and slightly increased leptin production.

In a six-week clinical trial, nine healthy panelists (age: 22.6 ± 1.7; BMI: 22.3 ± 2.1) consumed chocolate-protein beverages once per week. Isocaloric beverage formulations included: placebo, whey protein isolate (WPI), low polyphenolic cocoa (LP), high polyphenolic cocoa (HP), LP-WPI, and HP-WPI. Panelists responded to a hunger questionnaire, measured blood glucose levels, and provided blood samples after fasting and 0.5, 1, 2, and 4 hours following beverage consumption. Hunger ratings were measured using a 100 mm visual analogue scale (VAS), blood glucose levels were measured using a commercial blood sugar meter, and serum leptin and adiponectin levels were assayed with appropriate ELISA kits. While not statistically significant, the WPI beverage produced the greatest decreases in hunger ratings while HP cocoa suppressed hunger better than LP cocoa. 30 minutes post-consumption, the WPI, HP-WPI, and LP-WPI beverages increased blood glucose 8, 12, and 16% above baseline levels, respectively, compared to increases of 27, 39, and 58% in HP, LP, and placebo beverages, respectively. The moderation of blood glucose by all beverages was statistically significant as compared to the placebo and WPI and HP cocoa-containing beverages were the most effective. Serum leptin decreased after consumption of all beverages while WPI and cocoa-containing beverages significantly increased adiponectin levels above that of the placebo. These studies indicate that WPI and high polyphenolic cocoa may have beneficial effects upon lipid metabolism, feelings of satiety, and blood glucose and adiponectin levels. The combination of these bioactive ingredients in foods formulated for health may provide substantial benefits in promoting satiety and weight maintenance and in regulating lipid and glucose metabolism.

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# The Effects of Cocoa Polyphenols and Whey Proteins on Biomarkers of Satiety and Lipid Metabolism

by
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## BIOGRAPHY

Caroline Campbell was born and raised in the idyllic town of Moscow, Idaho. Though Idaho is widely renowned for its superior potatoes, Moscow and the surrounding region is actually the pea and lentil capitol of the world. Caroline enjoyed a busy childhood, including eleven years of competitive swimming and playing piano, and eventually decided to remain in Moscow to attend the University of Idaho. She spent four years as a Vandal-Cougar hybrid, pursuing her B.S. in Food Science in the University of Idaho/Washington State University Bi-State School of Food Science. During her years at the UI, Caroline was an active member of the Student Alumni Relations Board, served as the president and vice-president of the UI Food Science Club, and participated in three alternative service break volunteer trips. Caroline graduated with her B.S. in 2011 and promptly took a year off. During this time, she studied abroad in Chile and completed two internships, one at Cargill and one at Smucker's, exploring the subtleties of modified starches and coffee flavorings.

Though Caroline enjoyed a happy twenty-two years in Idaho, her family roots in the Tarheel State (or the familiar draw of a parallel state potato industry) fated her to move to the Northernmost Carolina. Following in her parents' footsteps, Caroline began her Master's degree at North Carolina State University in Fall 2012. At NCSU, Caroline was an active member of the NCSU Food Science Club, twice serving as co-chair for the club's Dairy Bar fundraiser at the NC State Fair. Caroline also minored in Biochemistry while completing her Master's. Upon completion of her Master's degree, Caroline will pursue a PhD in Food Science under the direction of Dr. Allen Foegeding studying the structure and breakdown patterns of model foods and how these properties impact satiety.

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## LIST OF ABBREVIATIONS

Akt ---------- protein kinase B
AMPK-α------ AMP-activated protein kinase-α
BCAA--------- branched chain amino acids
BMI ---------- body mass index
CCK---------- cholecystokinin
C/EBP--------- CCAAT/enhancer binding protein-α
CPE ---------- cocoa polyphenolic extract
DMAC -------- 4-dimethylaminocinnamaldehyde assay for total flavanols
DMEM-------- Dulbecco's modified eagle medium
EGCG--------- epigallocatechin gallate
ELISA -------- enzyme linked immuno-absorbent assay
ERK ---------- extracellular signal-regulated kinase
FAS ---------- fatty acid synthase
FBS---------- fetal bovine serum
GIP ---------- gastric inhibitory polypeptide
GLP-1 --------- glucagon-like peptide
GLUT --------- glucose transporter
GMP ---------- glycomacropeptide
HP ---------- high polyphenolic cocoa
Leu ---------- leucine
LP ---------- low polyphenolic cocoa
mTOR--------- mammalian target of rapamycin
MTT---------- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
OB---------- obesity gene
ORO---------- Oil-Red-O
OXM ---------- oxyntomodulin
PAC 1-10----- Proanthocyanidin 1-10 (assay and descriptor for polymers of 1-10 monomers)
PPAR --------- peroxisome proliferator-activated receptor
PYY ---------- peptide YY
SCD-1--------- stearoyl-CoA desaturase-1
SGLT --------- sodium-dependent glucose transporter
SREBP-------- sterol regulatory element-binding protein-1c
VAS ---------- visual analogue scale
WPC ---------- whey protein concentrate
WPI ---------- whey protein isolate

# CHAPTER 1: LITERATURE REVIEW

## 1.1 INTRODUCTION

Within the last two decades, scientists have observed a significant upsurge in both childhood and adult U.S. obesity rates ("Nutritional Benefits of Dairy Ingredients" 2010). Though obesity has myriad causes, the growing abundance, affordability, and convenience of high calorie foods is widely accepted as a major contributing factor. While the appeal of convenience and packaged foods is not likely to diminish, the nutritional profiles of these foods can be enhanced to support healthier lifestyles. This presents a demand for products that support weight management and control hunger at meals and snack times: products that increase satiety and are enjoyable, affordable, and convenient.

Obesity is a complex issue, encompassing nutritional content, availability, and affordability of foods as well as consumer consumption and exercise habits (Ruhm 2012). While most adults are well aware of proper nutrition and its benefits, many either do not have access to healthy foods or simply do not have the means to purchase these foods (Asp 1999). Areas devoid of grocery stores or fresh foods, called food deserts, are widespread in large metropolitan and economically depressed areas. People residing in these food deserts may have to travel miles to the nearest grocery stores, limiting their access to fresh, nutritious foods with short shelf-lives (fruits and vegetables). Fresh foods also demand a premium price and often force consumers on limited budgets to choose cheap, high calorie processed foods over foods with higher nutrient densities (Almiron-Roig, Flores, and Drewnowski 2004). An increasingly sedentary, time-strapped population combined with a reliance upon fast, convenient foods has also contributed to the increasing obesity rate. Many processed foods

and restaurant meals also provide too-large serving sizes and high levels of fat, starch, and sugar, leading to overeating.

Excess body weight is correlated with higher morbidity rates in both children and adults as well as increased risk of numerous diseases, including heart disease, stroke, some cancers, Type II diabetes, and osteoarthritis (Finkelstein, Ruhm, and Kosa 2005). Body mass index, or BMI, is a simplified, widely used indicator of obesity and takes into account both weight and height. Obesity is also often accompanied by hyperinsulinemia (high plasma insulin levels) and leptin resistance: an acquired dysfunction in which the body does not register hormonal leptin production that signals fullness between meals (Lustig 2006). Between 1999 and 2012, the percentage of obese adults in the U.S. rose from 30.3% to 35.9% and the percentage of obese children increased from 11% to 18% ("Health, United States, 2011" 2012). In 2010, one in five children five years or older was obese and adults with grade 1 (BMI 30.0-34.9), 2 (BMI 35.0-39.9), and 3 obesity (BMI 40 or greater) increased from 14% to 20%, 5% to 9%, and 3% to 6%, respectively ("Health, United States, 2011" 2012).

Finkelstein et al. (2012) applied a nonlinear prediction model to obesity statistics and estimated that by 2030, 42% of the U.S. population will be obese and 11% will be severely obese. The costs of obesity to the economy are also significant: estimates indicate that 9% of medical expenses can be attributed to obesity, totaling $147 billion each year, and will only increase as the obesity rate rises ("Health, United States, 2011" 2012). In addition, it is estimated that $549.5 billion in health care costs could be saved in the next 20 years if the obesity level remained at its current level (Finkelstein et al. 2012).

Though obesity is influenced by diet, exercise, genetics, and environment, the increased obesity rate over the last four decades appears to be most correlated with changes in diet and exercise (Finkelstein, Ruhm, and Kosa 2005). In addition to growing portion sizes and an increase in meals eaten outside of the home, the price disparity between whole foods and processed, high caloric foods with low nutrient densities deeply impacts consumer-purchasing decisions (Asp 1999). Food scientists and the food industry have limited impact upon consumer access to healthy foods and consumption and exercise habits, but they can improve the nutritional quality of processed foods. Herein lies a niche for macro- and micronutrient fortified foods that control hunger and promote weight management. To formulate a product with these characteristics, food scientists can find methods to capture the nutrients of whole foods and package them in a convenient, shelf-stable, affordable, and widely available form. Meeting these specifications involves innovation and ingenuity at the interface of product development, food engineering, and food processing.

Preventing obesity through food choices involves formulating foods that both suppress long-term hunger and limit fat accumulation. Three major sectors of nutrient-fortified products exist within the food industry: 1. athletic recovery products, 2. meal replacement and filling low calorie snack products and, 3. products that provide comprehensive nutrition to the elderly or those with no appetite. The segment most concerned with and prone to obesity is the second category and includes a target market of consumers of ages 18 to 60 (Food Product Design 2011). These consumers use meal replacement products and snacks to limit their calorie consumption with an end goal of losing weight or maintaining their current weight (Food Product Design 2011). In general, these

products contain 50-60% carbohydrates, 10-15% protein, and 25-40% fat and between 150 to 300 calories per serving (Food Product Design 2011). Meal-replacement sales were $2.3 billion in 2010 and have experienced an increase of 34% since 2008, indicating a growing interest in and need for more filling and convenient food products (Food Product Design 2011).

Within the meal replacement and low-calorie snack market, product composition is key. In order to effectively provide weight maintenance support to a broad segment of the population, these products must meet a variety of requirements at each stage of formulation. The first goal is to support health, which involves the incorporation of ingredients that induce satiety and deliver basic nutrition (Benelam 2009). Secondly is the consideration of flavor and taste: consumers will generally not continue to purchase foods that they do not enjoy eating, even if they know the food is healthy. Thirdly, all product ingredients must be affordable and widely available to consumers, regardless of regional location and grocery environment (Asp 1999).

In the selection of ingredients that induce satiety and are affordable and enjoyable, protein is an obvious choice. Protein has been shown to have a greater positive effect upon after-meal fullness when compared to fats and carbohydrates (Veldhorst et al. 2008). For this reason, high-protein diets are often recommended for weight loss. Of the many types of available proteins, whey protein is a dairy by-product with a range of biological and physiochemical health benefits (Pal, Ellis, and Dhaliwal 2010).

A smaller number of studies have also indicated that polyphenolic compounds exhibit beneficial effects in weight loss and maintenance (Panickar 2013). Availability, affordability,

and shelf-life of polyphenolic-rich whole fruits and vegetables is limited but can be extended through processing. Cocoa beans contain high levels of polyphenolic compounds, have a familiar, enjoyable flavor, and are an affordable, fat-free ingredient when processed into cocoa powder. The combination of whey proteins and cocoa has the potential to meet all of the above requirements for a targeted weight maintenance food product.

## 1.2 SATIETY AND ITS EFFECTORS

Increasing both physiological and psychological satiety, or the feeling of fullness after eating, is vital to limiting calorie consumption and weight gain. While often used interchangeably, satiation and satiety refer to different mechanisms and are defined by different time frames. Satiation refers to the feeling of fullness during food consumption that leads to termination of eating; satiety is the long-term feeling of fullness between meals before hunger returns (Benelam 2009). Therefore, satiation controls total calories consumed in one meal and satiety affects the length of time between meals and impacts the number of calories consumed in between meals and at the following meal. Figure 1.1 depicts idealized hunger levels over 24 hours. Feelings of hunger and fullness are clearly subjective and vary between subjects. In addition, indications of appetite do not always correlate with food intake, as hunger is both a physical and psychological sensation. Though increased control of appetite and food intake is possible by satiety-inducing foods, the reality remains that our physical and psychological desires are not always the same: we often fail to eat when hungry or continue to eat when full (De Graaf et al. 2004).

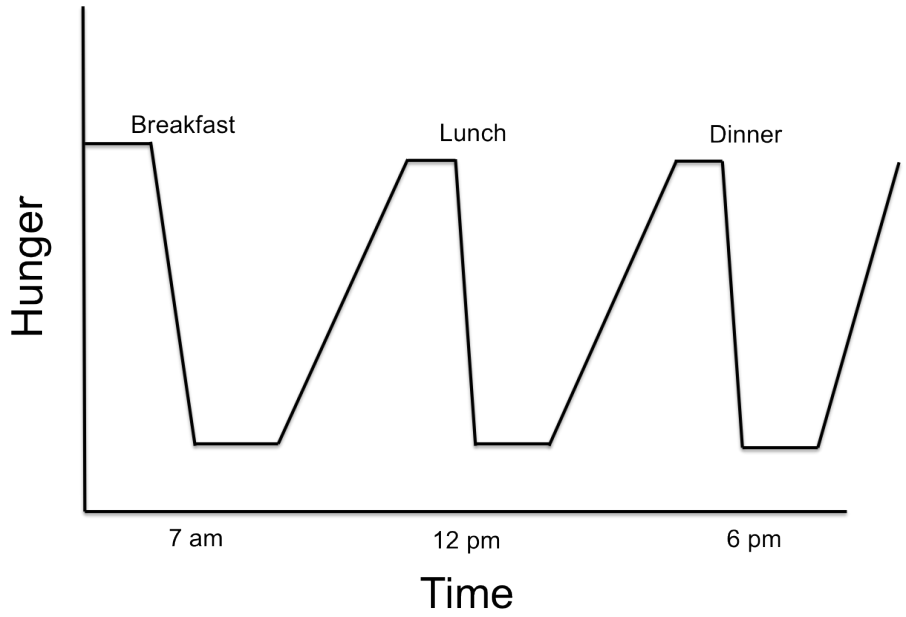


**Figure 1.1.** A depiction of hunger level versus time over 24 hours.

Triggers of psychological satiety vary, but physiological satiety is controlled by peptide hormones, including ghrelin, leptin, insulin, peptide $YY_{3-36}$ (PYY), glucagon-like peptide (GLP-1), oxyntomodulin (OXM) and cholecystokinin (CCK) (Beglinger and Degen 2006, Lustig 2006). Each of these is secreted from different sites and at different time points during and after digestion. Endocrine signaling then proceeds through the bloodstream and targets the brain to regulate energy homeostasis. Of the above signaling molecules, ghrelin is the only compound associated with appetite stimulation, while the remaining molecules act to inhibit appetite.

Ghrelin is a peptide hormone that is secreted by X/A-like cells in the stomach and is found at lower concentrations in the small intestine, pancreas, gallbladder, liver, spleen and immune cells (Zwirska-Korczala et al. 2007). Ghrelin activates neuropeptide Y and agouti-

related peptide, neuropeptides that act on the hypothalamus to indicate hunger and increase appetite, and is the only known hormone regulator of feeding and weight gain (Castañeda et al. 2010).

$PYY_{3-36}$, GLP-1, OXM, and CCK are gut peptides secreted from L-cells in the gastrointestinal tract during digestion (Beglinger and Degen 2006). GLP-1 stimulates insulin production and inhibits glucagon secretion, actions that lower blood sugar and prepare the body to store glucose in the form of glycogen (liver and skeletal muscle) or triglycerides (adipocytes). Administration of OXM has been shown to decrease food intake and to increase weight loss in humans (Benelam 2009). GLP-1, OXM, and peptide $YY_{3-36}$ delay gastric emptying and decrease appetite (Beglinger and Degen 2006). CCK production is stimulated by ingestion of lipids and protein and also delays gastric emptying, increases pancreatic insulin secretion, and may act in coordination with leptin, a satiety hormone secreted by adipose tissue (Benelam 2009). Though all are released from the small intestine, it is suggested that CCK affects satiation while PYY and GLP-1 affect satiety (De Graaf et al. 2004). Table 1.1 gives an overview of peptide regulation of satiation and satiety.

**Table 1.1.** Biomarkers of satiation and satiety.

| Biomarker | Production site | Effect on appetite | Additional effects |
|---|---|---|---|
| Ghrelin | Stomach | Increases hunger | |
| CCK | Small intestine | Increases satiation | Delays gastric emptying, increases insulin secretion |
| GLP-1 | Intestine | Increases satiation and short-term satiety | Delays gastric emptying, stimulates insulin production and inhibits glucagon secretion, moderates gastric acid secretion |
| OXM | Intestine | Increases satiety | Delays gastric emptying, increases energy expenditure |
| PYY | Small intestine | Increases satiety | Delays gastric emptying, reduces gastric secretions, increases intestinal transit time |
| PP | Pancreas | Increases satiety | |
| Leptin | Adipose tissue | Increases long-term satiety | Maintains homeostasis in thyroid, moderates energy intake and expenditure |

CCK: cholecystokinin, GLP-1: glucagon-like peptide, OXM: oxyntomodulin, PYY: peptide $YY_{3-36}$, PP: pancreatic polypeptide
(De Graaf et al. 2004, Lustig 2006, P. G. Cammisotto and Bendayan 2007, Benelam 2009)

In contrast to the aforementioned appetite-regulating signals, leptin is produced and secreted by white adipose tissue and at very low levels by gastrointestinal cells. Like the biomarkers discussed above, leptin also decreases food intake and increases energy expenditure. However, its signaling mechanism and time frame are different due to its mechanism of secretion and signaling (Lustig 2006, Zwirska-Korczala et al. 2007). Leptin production by the gastric mucosa regulates short-term nutrient absorption while adipose tissue production influences long-term satiety and energy storage (Cammisotto and Bendayan 2007). The gene encoding for leptin was identified and named the obesity (OB) gene due to its apparent connection with obesity in OB-deficient mice (Zhang et al. 2005). Due to their lack of leptin expression, OB-deficient mice exhibit increased appetite, increased rates of

diabetes and infertility, and decreased basal metabolic rate and physical activity (Lustig 2006). Adipocytes are the only cell type that expresses leptin through the OB gene.

A 16-kDa, 167 amino acid hormonal signaling protein, leptin is an endocrine hormone produced by white adipose tissue (Cammisotto and Bendayan 2007). Leptin regulates appetite and metabolism within the endocrine system and maintains homeostasis in the thyroid and immune system via cytokine action (Zhang et al. 2005). Plasma leptin concentrations also affect the development of diabetes and cardiovascular and gastrointestinal diseases. Production of leptin varies by species: subcutaneous adipose tissue has been shown to secrete the highest levels of leptin in humans while epididymal tissue has the highest production in rats (Cammisotto and Bendayan 2007).

Under non-stimulated conditions, leptin synthesis and secretion remains constant. Stimulation by exogenous or endogenous agents increases leptin levels but it appears that several hours of leptin production are required to reach the leptin levels necessary to induce satiety (Cammisotto et al. 2006). Regardless, increased body fat decreases neuropeptide Y levels (hunger) *in vivo* and increases leptin levels (satiety) due to the presence of more leptin-secreting fat cells (Gregoire, Smas, and Sul 1998). In addition, individual food components and their metabolites may have different effects upon leptin secretion. Figure 1.2 demonstrates the changes in plasma leptin levels over a 24-hour period.

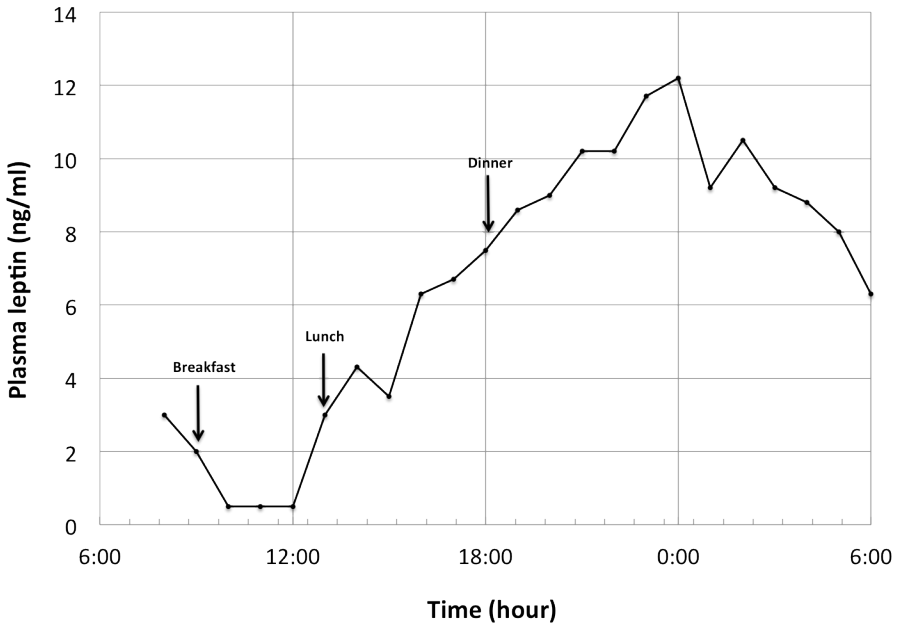


**Figure 1.2.** An idealized representation of serum leptin levels over 24-hours.
Based upon research by (Teff et al. 2004). Panelists were provided with high glucose beverages in addition to each meal.

Leptin is produced in proportion to the amount of fat stores in the body, so obese individuals usually exhibit increased serum levels of leptin (Fried et al. 2000). It has also been shown that leptin mRNA is overexpressed in subcutaneous and visceral adipose tissue of obese individuals (Cammisotto and Bendayan 2007). This is explained not only by an increase in fat stores, but also by an increase in fat cell size in obese patients when compared to lean patients; this 2-4 time increase in adipose cell size appears to increase leptin production up to seven times that of lean patients (Fried et al. 2000). Increases in leptin secretion during weight gain and decreases with weight loss have also been observed but it is still unclear whether leptin is the major determining signal of energy expenditure (Maffei et al. 1995, Rosenbaum et al. 1997).

Leptin resistance, a recently recognized condition, occurs when the body does not recognize or respond to leptin production at normal or even elevated levels (Lustig 2006). This is similar to, but less dangerous than, Type II diabetes in which the body does not respond adequately to insulin production after food consumption. Individuals that display leptin resistance require higher levels of circulating leptin to generate the same satiety response as non-leptin resistant individuals. It is suggested that leptin resistance is a hallmark of obesity: decreasing food intake further decreases leptin, coordinating a starvation response that lowers resting energy expenditure, increases appetite, and slows weight loss (Lustig 2006). Leptin resistance may also be due to a reduction in leptin transport across the blood-brain barrier, explaining why obese patients with high plasma leptin levels do not register appropriate satiety signals (Myers, Cowley, and Münzberg 2008).

A few studies have shown that leptin administration can enhance weight loss (Heymsfield et al. 1999, Rosenbaum et al. 1997). Because the insulin and leptin signaling pathways overlap, it is also possible that insulin resistance may potentiate leptin resistance (Lustig 2006). Increased insulin levels can thus interfere with cellular leptin signaling. Insulin also plays a significant role in energy intake by signaling short-term changes in available energy.

Though the physiology of satiation and satiety play a large role in energy intake, the impact of hedonic reward during eating cannot be overlooked. During eating, these signals dictate the pleasurable aspects of food and how much a specific food is liked or disliked. Hedonic signals often overpower physiological signals of fullness (Lustig 2006). Leptin is believed to play a role in hedonic reward regulation but the fact remains that the hedonic

contribution to food intake is psychological, individually specific, and difficult to control through clinical means (Myers, Cowley, and Münzberg 2008).

Within the body, satiety is induced by physical and biochemical means. Physically, food consumption increases body temperature as macronutrients are broken down and oxidized in the intestinal tract (De Graaf et al. 2004). Increased body temperature, called diet-induced thermogenesis, has been found to be casually associated with increased satiety. A study by Westerterp-Plantenga et al. (1999) indicated that meals high in protein cause a greater increase in thermogenesis than meals high in carbohydrate or fat. Body temperature, however, has not been found to be a reliable method for quantifying satiety. In satiety studies, three measurements predominate: patient hunger scaling, quantification of *ad libitum* intake following consumption of a test food, and analysis of satiety biomarkers from plasma. The first measure is subjective, based upon the individuals tested, but gives a representation of satiety without biochemical analyses.

## 1.3 WHEY PROTEIN

Milk was the first, and may remain the only, food explicitly intended for human consumption. Milk's unique nutritional composition of protein, fat, lactose, and minerals was biologically designed as the ideal food for infant growth. Casein and whey proteins make up the protein fraction and serve different roles in structure and function. Caseins are defined as those proteins precipitated during cheese-making, forming cheese curds. This class of proteins, originally defined by macroscopic properties, is a group of gene products that are found in milk associated into a supra-molecular micelle structure, which also contains

colloidal calcium and phosphate (Smithers 2008). The so-called whey proteins are those that remain soluble in the milk serum that is expelled in the "whey" during cheese production. Whey proteins account for about 20% of the total protein in bovine milk and as a cheese by-product, can be purified and used as a nutritional supplements and/or functional ingredients (Dunshea et al. 2007).

Liquid whey has a protein concentration of about 65% and is most often purified to produce whey protein concentrate (WPC) and whey protein isolate (WPI), which contain 34-80% and greater than 90% protein, respectively (Luhovyy, Akhavan, and Anderson 2007). Once simply a waste-stream from cheese production, whey ingredient production has grown 1-2% annually (Smithers 2008). β-lactoglobulin and α-lactalbumin are the two major protein constituents of whey, with serum albumin and immunoglobulins present at lower levels. In addition, fresh whey often contains glycomacropeptide, or GMP, which is a bioactive peptide formed by the cleavage of κ-casein by chymosin during cheese-making (Dunshea et al. 2007). GMP is believed to be a stimulator of CCK production, thereby increasing satiety (Burton-Freeman 2008). A variety of other bioactive peptides are formed during whey digestion and act via receptors in the intestinal lumen and after bloodstream circulation. These peptides are believed to play beneficial roles in blood pressure, anti-inflammatory pathways, hyperglycemia, and satiety (Luhovyy, Akhavan, and Anderson 2007).

Whey protein has found its functional niche in nutritional supplements, supporting muscle growth, post-workout recovery, and satiety. This is due to the speed of protein digestion: in comparison to casein, whey is metabolized and available for protein synthesis in a much shorter time period (Luhovyy, Akhavan, and Anderson 2007). The biological value

of whey protein, which describes the body's ability to utilize dietary protein, exceeds that of other proteins: surpassing egg by 15% and meat and soy by 35% (Smithers 2008). The satiety and muscle-building capacity of whey protein is due to short-term increases in blood amino acid levels, which are higher than casein within two hours of consumption (Boirie et al. 1997). The increase in amino acid circulation signals satiety to the brain and also increases amino acid availability for protein synthesis. Whey protein contains all of the essential amino acids and has a high protein efficiency ratio, a description of protein quality. However, its nutritional and bioactive benefits may be attributable to its unique amino acid composition. In comparison to others, whey proteins are much higher in the branched chain amino acids (BCAA) valine, leucine, and isoleucine. Of particular interest is L-leucine, which enters the blood stream faster than other amino acids and activates the mammalian target of rapamycin receptor (mTOR) signaling pathway. This receptor regulates protein synthesis and maintains lean body mass during times of energy deficit (Layman and Walker 2006). A comparison of BCAAs in dietary proteins is shown in Table 1.2.

**Table 1.2.** Comparison of leucine and total branched chain amino acid (BCAA) content of various dietary proteins. Expressed in g amino acids/100 g total protein.

| | **Amino acid content (g amino acids/100 g protein)** | |
|---|---|---|
| **Protein** | Leucine | BCAA |
| Whey protein isolate | 14% | 26% |
| Milk protein isolate | 10% | 21% |
| Casein | 9% | 20% |
| Egg protein | 8.5% | 20% |
| Muscle protein | 8% | 18% |
| Soy protein isolate | 8% | 18% |

(Hulmi, Lockwood, and Stout 2010, "Nutritional Benefits of Dairy Ingredients" 2010)

Katsanos et al. (2006) investigated the effects of leucine supplementation upon muscle protein synthesis in both young and elderly populations. Rates of muscle synthesis increased significantly in elderly patients after consumption of a 41% leucine amino acid mixture and in young patients after both 26% and 41% leucine supplementation. Leucine has also been found to regulate leptin secretion *in vitro* through the mTOR-signaling pathway (Lynch et al. 2006). Oral gavage of rats with leucine or norleucine increased plasma leptin concentrations, but not to the same extent as a meal feeding. A meal lacking leucine reduced leptin synthesis by 40% in comparison to a control meal; no reductions in leptin were observed in subsequent meals lacking branched-chain amino acids or all amino acids (Lynch et al. 2006). Adipose tissue also plays a major role in BCAA metabolism, indicated by a faster amino acid oxidation rate than in skeletal muscle (Herman et al. 2010). Obese and insulin-resistant individuals show reduced BCAA adipose metabolism, attributed to overexpression of the insulin-mediated glucose transporter-4 (GLUT-4). Leptin is only one indicator of satiety, so it remains unclear whether or not individual amino acids play a significant role in determining satiety.

In addition to its contribution to muscle anabolism and satiety, whey protein may also decrease plasma lipid levels and improve body composition. Pal et al. (2010) found that when compared to casein or glucose, whey protein supplementation lowered total cholesterol and LDL cholesterol levels after 12 weeks of treatment. Whey protein consumption also improved insulin resistance but did not significantly change body composition or glucose levels in obese patients (Pal, Ellis, and Dhaliwal 2010). Whey protein has also been shown to counteract the effects of high fat diets. Whey supplementation reduced fat mass and

prevented increases in leptin and insulin expression when compared to casein (McAllan et al. 2013). However, Noatsch et al. (2011) found no significant differences in body weight or body composition in mice fed whey protein or leucine-supplemented diets but did observe decreases in plasma triglycerides in the protein-supplemented diets.

Whey protein has also been shown to control or mitigate symptoms of metabolic syndrome. Nilsson et al. (2007) showed that in addition to its impact upon satiety, the specific amino acid composition of whey protein may be responsible for its insulinotropic benefits. Drinks fortified with varying ratios of amino acids had different effects upon insulin and glucose levels. Those drinks containing the BCAAs leucine, isoleucine, and valine, as well as lysine and threonine, decreased plasma glucose by 44% and increased insulin production by 31%. This amino acid mixture showed similarities to an identical drink containing whey protein, which decreased glucose levels by 56% and increased insulin levels by 60% (Nilsson, Holst, and Björck 2007). However, this study did not find that the BCAA mixture stimulated satiety to the same extent as whey protein. This may be explained by the faster absorption of BCAA into the blood stream or to the activity of bioactive peptides found in whey proteins.

Because whey proteins isolates can be formulated with and without GMP, numerous studies have investigated the effects of GMP on satiety. Royle et al. (2008) found that a GMP-supplemented whey protein diet slightly decreased weight gain in rats when compared with whey alone. GMP-whey intake also decreased insulin and triacylglyceride serum levels (Royle, McIntosh, and Clifton 2007). The researchers postulated that 70% of the weight control benefits could be attributed to whey protein while GMP was responsible for the

remaining 30%. Burton-Freeman (2008) and Lam et al. (2009) found that whey proteins induced slightly more satiety than GMP-supplemented whey proteins. *Ad libitum* intakes were decreased in the study by Burton-Freeman (2008) while Lam et al. (2009) demonstrated no differences in total caloric intake. Research by Veldhorst et al. (2009b) contradicted these findings, showing that whey proteins without GMP increased insulin levels and *ad libitum* intake. While the effects of whey protein and GMP-supplementation are promising, study inconsistencies suggest that more research is necessary to determine the exact impact and mechanism of whey proteins upon satiety.

## 1.4 COCOA

For centuries, mankind has relied upon the natural health benefits of foods containing polyphenols. Whether the presence of these compounds was known or simply associated with improved health, entire civilizations have showed reliance upon foods such as green tea in Asia, fruits and vegetables in the Mediterranean, and chocolate in South and Latin America. Until very recently in our modern culture, chocolate would never have been considered a "health food." Chocolate is generally treated as a novelty or indulgence and is not usually consumed for its disease-fighting properties. However, within the last decade, cocoa has been correlated with a decrease in the susceptibility and development of certain diseases, including cardiovascular disease, insulin resistance, and neurological diseases (Katz, Doughty, and Ali 2011).

Cocoa and chocolate are derived from beans of the tree *Theobroma cacao*. After harvesting, the beans are fermented (enzymatic process) in the field, roasted, crushed, and

milled into cocoa liquor, which is then separated into cocoa powder and cocoa butter (Perego et al. 2004). Cocoa butter contains most of the cocoa lipids while cocoa powder contains most of the fiber, polyphenols, vitamins, and minerals. Commercial chocolate is made by combining cocoa liquor with cocoa butter and sugar to make a solid product while cocoa is simply the ground and dried powder after removal of the cocoa butter (Katz, Doughty, and Ali 2011). The final polyphenolic content of cocoa depends upon the source of the beans as well as the processing conditions. Dutch-processed cocoa has been shown to substantially decrease the flavonoid content of cocoa powder due to is high alkalinity and high temperatures (Payne et al. 2010).

Though chocolate is considered to have little nutritional value, phenolic compounds in chocolate have been shown to improve multiple health-related conditions (Katz, Doughty, and Ali 2011). The major polyphenols in chocolate are flavanols, including (+)-catechin, (-)-epicatechin, and the polymers of these compounds, procyanidins. These flavanols are present in cocoa at higher concentrations than in apples, cranberries, red wine, or black tea (Lee et al. 2003, Steinberg, Bearden, and Keen 2003). Lee et al. (2003) showed that the flavonoid content and antioxidant activity of cocoa are 4-5 times greater than black tea, 2-3 times greater than green tea, and 2 times greater than red wine (Lee et al. 2003). The polyphenol content of cocoa is about 52.4 mg/g while 88% dark chocolate contains 32 mg/g (Steinberg, Bearden, and Keen 2003, Belščak et al. 2009). Cocoa-containing foods have also been shown to retain their oxygen radical absorbance capacity (free radical quenching) much longer than other food products. A sample polyphenolic analysis of a natural cocoa powder is given in Table 1.3.

**Table 1.3.** Proanthocyanidin content of a standard natural cocoa powder.

| Compound | Polyphenolic content (mg per g) |
|---|---|
| Catechin | 1.1 |
| Epicatechin | 4.5 |
| **Total Flavanols (DMAC)** | **49.0** |
| **Proanthocyanidins 1-10 (PAC 1-10)** | **29.5** |
| PACs 1 mers | 5.3 |
| PACs 2 mers | 3.5 |
| PACs 3 mers | 3.2 |
| PACs 4 mers | 3.2 |
| PACs 5 mers | 2.6 |
| PACs 6 mers | 3.2 |
| PACs 7 mers | 3.3 |
| PACs 8 mers | 1.2 |
| PACs 9 mers | 2.8 |
| PACs 10 mers | 1.2 |

Total proanthocyanidins expressed via 4-dimethylaminocinnamaldehyde (DMAC) analysis or proanthocyanidin 1-10 analysis. DMAC measures total flavanols (all polymer lengths) while PAC 1-10 describes only catechin or epicatechin polymers between 1 and 10 units in length.
Analysis courtesy of The Hershey Company

The health benefits of cocoa are most often associated with its polyphenolic antioxidant activity. The structure of catechins and procyanidins chelate metals and bind free radicals, preventing oxidative aging reactions *in vivo* (Jalil and Ismail 2008). Cocoa treatment in clinical studies has mainly focused on cardiovascular diseases, including its effects upon low-density lipoproteins, blood pressure, arterial dilation, and platelet aggregation. Multiple studies, however, have indicated that cocoa consumption can help to diminish the symptoms of metabolic syndrome, which encompasses diabetes, dyslipidemia, and hypertension (Gu and Lambert 2013).

*In vitro*, 1-10 μM epicatechin and 1-10 μg/ml cocoa polyphenolic extract treatment

increased insulin receptor activity, insulin signaling, and glucose transport while decreasing glucose production in HepG2 cells (Cordero-Herrera et al. 2013). *In vivo*, 0.5% epicatechin consumption in drinking water (equivalent to 250 g of dark chocolate consumption per day) decreased the incidence of diabetes by 50% and increased insulin levels in mice (Fu, Yuskavage, and Liu 2013).

Cocoa extract treatments of 10-30 mg per 100 g body weight and 1-3% of total diet was shown to lower the plasma glucose, total cholesterol, LDL cholesterol, and triglyceride levels and to increase HDL cholesterol profiles in diabetic obese mice (Ruzaidi et al. 2008). Effects were dose-dependent between 1-3% cocoa extract treatments. A four-week cocoa extract treatment (containing 4 mg total cocoa polyphenols) of obese-diabetic rats lowered plasma levels of total cholesterol, triglyceride, low-density lipoprotein cholesterol, and glucose levels (Jalil et al. 2009). No significant changes in insulin levels or insulin sensitivity were observed.

From the perspective of energy regulation, cocoa consumption has been shown to regulate body weight and to increase insulin production and sensitivity. In mouse and human clinical studies, flavonol-rich cocoa or chocolate consumption decreased visceral adipose accumulation and fatty acid synthesis, increased insulin sensitivity, and lowered short-term blood glucose and free fatty acid levels (Matsui et al. 2005, Grassi et al. 2008, A. M. Jalil et al. 2009). 8% cocoa supplementation of a high fat diet was also found to prevent weight gain and inflammation biomarkers in obese mice (Gu and Lambert 2013). Even in the absence of substantial cocoa polyphenols, a variety of chocolate flavored desserts increased plasma

insulin levels more than the same vanilla or strawberry-flavored foods (Brand-Miller et al. 2003).

Flavan-3-ols vary in their stability and bioavailability throughout digestion. Monomeric flavan-3-ols and procyanidins are stable through the oral and gastric tracts, with recovery rates of 85-102% and 97-125%, respectively (Neilson and Ferruzzi 2011). Procyanidins are often broken into catechin monomers during digestion, accounting for the increase in recovery. This hydrolysis to monomers also increases their bioavailability in the intestine (Spencer et al. 2000). However, flavan-3-ols are less resistant to intestinal breakdown, with green tea flavanol recovery ranging from 1-71% (Record and Lane 2001). Intestinal absorption of these compounds is also low, with bioavailability estimates ranging from 0.1-10% (Neilson and Ferruzzi 2011).

Food matrix composition has a significant effect upon the bioavailability and bioactivity of cocoa. Neilsen et al. (2010) and Rodriguez-Mateos (2012) found that the incorporation of sucrose in chocolate products increases flavanol bioavailability *in vivo*, perhaps by increasing their solubility within the intestine. Chocolate confections with high milk protein contents were shown to decrease epicatechin bioavailability (Serafini et al., 2003). Chocolate beverage studies, however, have shown no significant differences in bioavailability between aqueous and milk-based matrices, though the presence of milk protein does appear to change flavanol excretion profiles over time (Keogh, McInerney, and Clifton 2007, Roura et al. 2007, Roura et al. 2008). The presence of lipids also appears to protect catechins during digestion, decreasing flavanol absorption (Neilson et al. 2009).

In regards to food form, research suggests that chocolate beverages may offer increased flavanol bioavailability over solid chocolate. Neilson et al. (2009) found that beverages containing sucrose and milk protein or a non-nutritive sweetener and milk protein significantly increased plasma epicatechin levels when compared to solid chocolate with high milk protein. This observation could be due to the speed at which beverages travel through the intestine and gastric emptying time. In addition to formulation, high temperature processing of cocoa-containing products can decrease bioactive content through isomerization and auto-oxidation of flavan-3-ols (Neilson and Ferruzzi 2011). Gossai and Lau-Cam (2009) found an inverse relationship between milk fat content and catechin absorption when consumed in combination *in vivo*. Plasma concentrations of catechins post-consumption were greater when consumed with no or low fat dairy (water or skim milk) as compared with whole milk and heavy cream (Gossai and Lau-Cam 2009).

In addition to matrix, the binding affinity between proteins and polyphenols can also impact nutrient bioavailability and product appearance and texture. Binding can initiate precipitation of these compounds in liquids and gels, with phenolic acids exhibiting the strongest affinity for proteins. Non-covalent bonds formed with β-lactoglobulin have been shown to remain stable at pH values found in the gastrointestinal tract (Stojadinovic et al. 2013). This binding can also slow protein digestion by pepsin and pancreatin, with polyphenols protecting protein degradation down to pH 1.2. The anti-oxidant activity of the polyphenols also decreased when associated with proteins (Stojadinovic et al. 2013). Proline-rich proteins, such as those found in saliva, tend to bind polyphenols strongest due to their loose tertiary protein structure (Ferruzzi, Bordenave, and Hamaker 2012). These interactions

have the capacity to delay increases in plasma amino acids and to further limit the anti-oxidant activity of bioactive foods.

## 1.5 FACTORS THAT IMPACT SATIETY

### 1.5.1 *PHYSICAL PROPERTIES OF FOOD*

Physical properties of foods, including composition, form, and texture are determinants have been shown to have a significant impact upon satiety. The time necessary to chew and swallow food during oral processing is determined by variations in texture. This combination of chewing intensity and time sends satiety signals sent to the brain, though it is estimated that after eating, it takes 20 minutes before the stomach and brain register feelings of fullness (Stewart 2011). Foods that require longer chewing slow the consumption of food and may help to limit the number of calories consumed before a feeling of fullness is recognized. A study of oral and gastric stimulation found that both chewing without ingestion of food and intestinal infusion with liquid decreased *ad libitum* food intake (Wijlens et al. 2012). Studies comparing the satiation and satiety-inducing effects of specific foods have found conflicting results. Though it is generally accepted that solid foods provide higher satiety than liquid foods when total calories and macronutrients are equal, many studies also indicate that the stomach-filling sensation induced by liquid foods may compensate for the lack of mastication time (Stull et al. 2008).

The effects of liquid and solid foods upon satiety have been extensively researched. A comparison of liquid (shake) and solid (bar) meal replacement products found increased

hunger, desire to eat, and a 13.4% increase in ad libitum intake after 120 minutes in subjects who consumed the liquid form (Stull et al. 2008). A second study also found increased hunger and desire to eat, lower fullness, and increased levels of the hunger hormone ghrelin after liquid consumption in comparison to its solid counterpart (Leidy, Bales-Voelker, and Harris 2011). However, food form was not found to impact satiety hormone levels including glucagon-like peptide (GLP-1), CCK, or leptin (Tieken et al. 2007).

A high-protein solid breakfast was also found to lower appetite and to decrease *ad libitum* intake over a high-protein liquid breakfast (Leidy, Bales-Voelker, and Harris 2011). The consumption of orange or grape juice decreased satiety and increased insulin production when compared to whole oranges or grapes (Bolton et al. 1981). Research by Mattes et al (2009) also found that whole apples induced satiety better than applesauce, which in turn was better than apple juice. Mourau et al. (2007) investigated the changes in appetite and calorie consumption after solid and liquid foods high in carbohydrates (watermelon and watermelon juice), protein (cheese and milk), and fat (coconut meat and coconut milk). In all categories, the solid food form increased satiety more than the liquid form. Consumption of the liquid form increased total daily caloric intake increased by 12.4, 15, 19% for the carbohydrate, protein, and fat categories, respectively.

A number of studies of food-induced satiety and hormone production have contradicted the above studies. Consumption of matched-calorie samples of regular cola and fat-free cookies found no differences in satiety or *ad libitum* food intake (Almiron-Roig, Flores, and Drewnowski 2004). However, cola consumption significantly reduced thirst at an *ad libitum* meal. Ingestion of a solid or homogenized form of the same salad components

(vegetables, cheese, croutons, olive oil) showed that the homogenized form increased fullness and satiety, decreased desire to eat, and slowed gastric emptying time and peak production of cholecystokinin, a gastric satiety peptide (Santangelo et al. 1998). A subsequent study of the same samples observed that the homogenized form increased insulin production, glucose-dependent insulinotropic peptide (an endocrine hormone), and body temperature after consumption over the solid food form (Peracchi et al. 2000). Orange juice or sweetened fruit drink consumption was also found to have stronger effects upon satiety suppression compared to rice or spaghetti (Ranawana and Henry 2011). In regard to beverage composition, Maersk and others (2012) showed that a skim milk-based beverage induced greater satiety and increased plasma GLP-1 and GIP by 31% and 45%, respectively, compared to a sugar-sweetened soft drink.

The conflicting results of liquid versus solid satiety studies indicate that the volume of food consumed as well as its macronutrient composition may influence satiety more than food form. Bolton et al. (1981) attributed differences in insulin and glucose production to differences in fiber content between whole fruit and fruit juice. It is also possible that the effects of food form upon satiety may vary between different nutrients. Solid gelatin and sweet whey samples induced greater satiety than their liquid counterparts while no differences were observed in sugar or acid whey samples (Akhavan, Luhovyy, and Anderson 2010).

### 1.5.2 *MACROMOLECULAR COMPOSITION OF FOOD*

Regarding food composition, specific macronutrients have been shown to have a more significant impact in increasing satiety and decreasing overall calorie intake. Protein and fiber in particular seems to be determining factors of post-meal satiety (Veldhorst et al. 2008, Benelam 2009). The USDA recommends that healthy diets provide 0.66 g/kg/day for adults, or approximately 46 g/day for women and 56 g/day for men (Food and Nutrition Board 2013). These encompass about 10-35% of total daily caloric intake. However, extensive research indicates that protein consumption at levels higher than these recommendations can induce greater satiety and limit overall daily calorie intake.

When meals are varied in protein, carbohydrate, and fat percentage, most studies show that meals higher in protein induce greater satiety and reduce *ad libitum* intake. A 12-week comparison of 15 and 30% protein diets (keeping carbohydrate constant and varying lipid content) showed higher satiety ratings, decreased *ad libitum* intake, and average weight loss of 4.9 kg for subjects on the high protein diet (Weigle et al. 2005). A 10 and 30% protein comparison showed increased satiety and decreased hunger, though the total calories consumed were identical (Lejeune et al. 2006). Veldhorst et al. (2009a) also found that breakfasts containing 25% calories from protein from casein, whey, or soy induced greater satiety than their 10% protein counterparts. In general, it has been shown that diets consisting of greater than 30% calories from protein induce satiety and decrease food intake better than lower protein diets (M. Veldhorst et al. 2008). Pasiakos et al. (2013) showed that variations in protein consumption did not affect weight loss but that higher protein consumption (2-3

times the recommended dietary allowance) increased overall fat loss and improved body composition.

Protein metabolism has been shown to increase satiety by diet-induced thermogenesis, to improve body composition, and to decrease energy efficiency (Westerterp-Plantenga et al. 2006). The satiety induction provided by protein requires fewer grams and calories than that obtained from carbohydrates or lipids, thereby reducing total calorie intake and speeding weight loss and maintenance. This satiety may be attributed to an increase in plasma levels of the satiation hormone GLP-1 (Westerterp-Plantenga et al. 1999, Lejeune et al. 2006). Protein consumption also appears to increase fat-free mass during weight gain or maintenance (Westerterp-Plantenga et al. 2006). While it is apparent that protein induces greater satiety than lipids or carbohydrates, there is no uniform agreement on the percent of daily protein calories necessary for significant protein-induced satiety (Benelam 2009).

Comparisons between protein sources are also generally inconclusive. Various metabolic evaluations of animal and plant proteins have shown both large differences and no difference in satiety-inducing capacity. Clinical trials by Lang et al. (1998) found no differences between egg albumin, casein, gelatin, soy protein, pea protein, and wheat gluten on satiety. Aldrich et al. (2011) also found no significant differences in weight loss, fat loss, or satiety ratings between subjects fed low protein, whey protein, or mixed protein diets. However, the whey protein diet induced greater regional fat loss and lowered blood pressure (Aldrich et al. 2011).

Of those studies that indicate differences between specific proteins, whey, pea, and soy may be the most promising in increasing satiety. Anderson et al. (2004) found that whey

and soy proteins increased satiety and decreased food intake over egg albumen and sucrose. In a clinical study of protein-fortified shakes, whey or pea protein hydrolysate increased satiety and fullness more than milk or a combination of whey and pea proteins (Diepvens, Häberer, and Westerterp-Plantenga 2007). Milk protein treatment had the greatest effect upon satiety hormones GLP-1 and CCK production while the whey/pea combination increased PYY to the greatest extent. Overall, pea protein showed the greatest satiety, decreasing both hunger scores and ghrelin production (Diepvens, Häberer, and Westerterp-Plantenga 2007).

However, pea and casein beverage preloads decreased food intake more than whey or egg proteins when consumed 30 minutes before a meal but no differences in caloric intake were observed when the same preloads were eaten as an appetizer before a meal (Abou-Samra et al. 2011). Pal and Ellis (2010) found that a whey protein meal increased satiety and plasma insulin response and decreased *ad libitum* intake at a subsequent meal when compared to tuna, turkey, and egg albumin. Though whey, pea, and soy proteins may have greater effects upon overall satiety, it is clear that all proteins influence satiety ratings, total caloric intake, plasma amino acid levels, and hunger hormones to different degrees.

Direct comparisons of milk-specific proteins have found that whey protein decreases *ad libitum* food intake by 19% and increases satiety ratings when compared to casein (Hall et al. 2007). Whey also increased postprandial plasma amino acid, CCK, and GLP-1 levels by 28%, 60%, and 65%, respectively, above that of casein (Hall et al. 2007). 10% whey-based breakfast custards were more satiating than those prepared with casein or soy, but no differences in satiety or energy intake were observed at a 25% protein level (Veldhorst et al.

2009). The 25% whey protein custard showed the largest increase in GLP-1 and insulin levels. Pichon et al. (2007) showed that whey proteins were more effective than whole milk protein in limiting weight gain and increasing insulin and leptin levels in rats fed high-protein diets. β-lactoglobulin in particular decreased adiposity more than when part of a mixture of whey protein or casein (Pichon et al. 2007).

Mellinkoff et al. (1997) postulated that increases in plasma amino acid levels are associated with higher satiety and reduction in appetite. If true, this provides a distinction between macronutrients in regards to weight loss: fewer protein calories could initiate the same level of satiety as greater quantities of carbohydrates or lipids calories. In mice, increases in plasma amino acids that were not destined for protein synthesis increased satiety, while an increase in appetite was accompanied by decreases in plasma amino acids. Morrison et al. (2007) found that intracerebroventricular injections of a mixture of amino acids or leucine alone reduced caloric intake in mice over 24 hours. This effect was attributed to amino acid stimulation of the mammalian target of rapamycin signaling (mTOR) pathway, which regulates insulin signaling and muscle synthesis (Morrison et al. 2007).

Accordingly, researchers have suggested that specific protein sources and their distinctive amino acid ratios may play different roles in satiety. Ingestion of whey protein increased plasma leucine, lysine, tryptophan, isoleucine, and threonine more than casein or soy (Veldhorst et al. 2009). A clinical comparison of whey protein consumption found no differences in plasma amino acid levels after WPI or β-lactoglobulin-enriched WPI, but found that both these WPI forms increased plasma leucine and branched chain amino acid levels above that of hydrolyzed WPI (Farnfield et al. 2009). Another study showed that

inclusion of GMP in a WPI-based breakfast increased plasma serine, threonine, alanine, alpha-aminobutyric acid and isoleucine, indicating that GMP could play a role in amino acid metabolism and satiety (Veldhorst et al. 2009).

### 1.5.3 *MICROMOLECULAR COMPOSITION OF FOOD*

A limited amount of research has indicated that micronutrients such as polyphenolic compounds can also contribute to satiety and fat metabolism. Polyphenols bind strongly to proteins and have been shown to bind digestive enzymes *in vitro*, demonstrating their potential to slow digestion and nutrient metabolism. Tannins in cocoa, pomegranate, cranberry, and grape extracts inhibited both α-amylase and glucoamylase while cocoa extracts and cocoa procyanidins inhibited α-amylase, pancreatic lipase, and secreted phospholipase $A_2$ activity (Gu et al. 2011, Barrett et al., 2013). *In vivo*, green tea catechins were also shown to increase satiety and fullness and to limit lipid nutrient absorption by inhibiting pancreatic phospholipase $A_2$ (Wang, Noh, and Koo 2006, Josic et al. 2010). Extensively polymerized polyphenols such as cranberry and pomegranate appear to more effectively bind these digestive enzymes and decrease their activity (Barrett et al. 2013).

Chocolate-specific human clinical studies show that in a comparison of dark and milk chocolate, consumption of polyphenol-rich dark chocolate decreased hunger ratings and *ad libitum* intake by 17% and increased satiety more than milk chocolate (Sørensen and Astrup 2011). Eating or simply smelling chocolate also increased satiety, though smelling had no effect upon biomarkers of hunger and satiety (Massolt et al. 2010). The comparison of chocolate and yogurt snacks before a meal found increased fullness ratings after eating

yogurt, which is higher in protein (Chapelot and Payen 2009). However, the duration of satiety was similar between both foods and there was no difference in *ad libitum* intake at the next meal. In obese-diabetic rats, treatment with cocoa extract was found to significantly reduce plasma glucose, free fatty acid, and oxidative stress biomarker levels as well as to increase the activity of the antioxidant enzyme superoxide dismutase in the hours following treatment (Jalil et al. 2008). No differences in fasting glucose or insulin levels were observed after four-months of treatment.

## 1.6 3T3-L1 PRE-ADIPOCYTES AS A MODEL FOR SATIETY AND WEIGHT MANAGEMENT

Preventing obesity involves both a reduction in food consumption (impacted by satiety) and an increase in energy expenditure, both of which serve to decrease overall body fat mass. White adipose tissue is the major energy reserve within the human body, in which energy is stored as triacylglycerol. These tissues grow and shrink with changing caloric intake and influence the lipid metabolism of surrounding cells as well as the endocrine satiety and hunger signals sent to the central nervous system (Hwang et al. 1997). An over abundance of energy signals adipocytes to increase their storage of triacylglycerol and when a storage limit is reached, these mature fat cells signal the conversion of pre-adipocytes into mature adipocytes (Harmon and Harp 2001). 50% of mature fat cells are replaced every eight years (Moreno-Navarrete and Fernández-Real 2012).

This communication between undeveloped and mature adipocytes accommodates the demand for excess energy storage. New adipocytes form and change size throughout our

lifetimes and adipocytes have been found to be larger and more prevalent in obese individuals (Harmon and Harp 2001). Mature adipocytes account for only one-third of body fat mass. The remaining two-thirds are small mesenchymal stem cells, T regulatory cells, endothelial precursor cells, macrophages, and preadipocytes (Moreno-Navarrete and Fernández-Real 2012).

*In vitro*, the effects of dietary molecules upon fat accumulation, lipid metabolism, and biomarkers of satiety are studied using pre-adipocyte cell lines including 3T3-L1, 3T3-F442A, and Ob17. A common model is the 3T3-L1 pre-adipocyte, derived from a subclone of Swiss 3T3a mice (ATCC 2013). These cells are considered pre-adipocytes because they have undergone commitment to the adipocyte cell type but do not express adipocyte genes. In the pre-adipocyte state, they are adherent to growth flasks and plates and exhibit a fibroblast morphology (ATCC 2013). The transformation from pre-adipocyte to adipocyte, called differentiation or adipogenesis, can be spontaneous or induced and significantly affects cell morphology and gene expression. After differentiation, cells become circular and monovacuolar, acquire a large, central lipid droplet, and begin to express adipocyte-specific genes. Because only adipocytes produce leptin, these cell lines are also powerful models for understanding satiety and energy balance. However, *in vitro*, these cells lack the paracrine and endocrine signaling abilities that exist *in vivo*. Due to this, it is believed that adipocytes *in vitro* express only 1% of the total leptin expressed *in vivo*, somewhat limiting their use as a model of fat mass *in vivo* (MacDougald et al. 1995). However, conclusions can be extrapolated from *in vitro* observations and these cell lines serve as an adequate initial indication of adipocyte response to specific environmental conditions.

Induced differentiation of 3T3-L1 cells *in vitro* is accomplished by treatment of pre-adipocytes with specific compounds added to their growth media. After growing past confluence for two days, cells are treated with 3-isobutyl-1-methylxanthine, dexamethasone, and insulin for 48 hours followed by treatment with insulin for 48 hours. As previously mentioned, the insulin and leptin pathways are parallel, so cellular treatment with insulin effectively upregulates leptin gene expression, further encouraging differentiation. This is followed by growth in regular growth medium (Dulbecco's modified eagle medium, 10% fetal bovine serum, 1% penicillin/streptomycin) for 48 hours until 70-80% of cells exhibit mature adipocyte morphologies. The differentiation cocktail inhibits production of preadipocyte factor 1, a transmembrane protein that inhibits adipogenesis and that is only produced by pre-adipocytes (Moreno-Navarrete and Fernández-Real 2012). The differentiation process also increases intracellular cAMP levels, activating adipocyte gene expression and converting pre-adipocytes to mature adipocytes with increased paracrine (cell to cell) and endocrine (long distance cell to environment) communication *in vivo* (Gregoire, Smas, and Sul 1998, Moreno-Navarrete and Fernández-Real 2012).

Differentiation occurs in three steps: 1. growth arrest, 2. clonal expansion, and 3. expression of adipocyte genes and terminal differentiation (Hwang et al. 1997, Gregoire, Smas, and Sul 1998). Growth arrest occurs as cells grow to confluence, or until an even layer of cells and cell-to-cell contact is achieved. 3T3-L1 cells then undergo one to two rounds of division. It is suggested that the unwinding of DNA during division allows transcription factors to both silence and activate more than 2,000 genes that regulate pre-adipocyte and adipocyte functions (Moreno-Navarrete and Fernández-Real 2012). Changes in morphology

and adipocyte-specific gene expression are activated by the transcription factors peroxisome proliferator-activated receptor-γ (PPAR- γ), CCAAT/enhancer binding protein-α (C/EBP- α), and sterol regulatory element-binding protein-1c (SREBP-1c) (Gregoire, Smas, and Sul 1998).

During terminal differentiation, enzymatic activity involved in fatty acid synthesis and degradation increases by 10-100 times (Gregoire, Smas, and Sul 1998). Adipocytes also become increasingly more sensitive to insulin and begin to secrete peptides including leptin, adipsin, adiponectin, and resistin, all of which have effects upon energy metabolism and storage (Moreno-Navarrete and Fernández-Real 2012). This increase in enzymatic activity, protein synthesis and peptide secretion converts adipocytes into an endocrine organ with increased sensitivity and control of energy storage and metabolism. Figure 1.2 depicts the stages of pre-adipocyte differentiation and the changes occurring at each step.

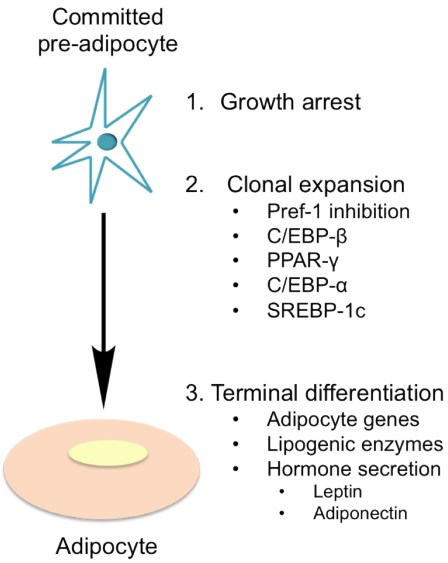


**Figure 1.3.** Stages of pre-adipocyte differentiation.
(Hwang et al. 1997, Gregoire, Smas, and Sul 1998, Moreno-Navarrete and Fernández-Real 2012)

Inhibiting the process of adipogenesis with bioactive compounds found in food could have profound effects upon overall body fat mass, health, and quality of life. Various polyphenolic compounds, including catechins, anthocyanins, and isoflavones, have been shown to inhibit fat cell growth and to increase hunger hormone secretion *in vitro* and *in vivo*. Treatment of 3T3-L1 pre-adipocytes with curcumin, a polyphenol found in turmeric, inhibited lipid accumulation by 50-80% at concentrations from 10-16.1 μM and 100% at 25-30 μM (Ahn et al. 2010, Kim et al. 2011). Expression of C/EBP-β, PPAR-γ, C/EBP-α, leptin, adiponectin, and resistin were also lower in cells treated with curcumin throughout the differentiation. Fatty acid metabolism in curcumin-treated cells also increased in a dose dependent manner between 5-20 μM. *In vivo*, mice fed high fat diets supplemented with curcumin for 12 weeks acquired less body weight and fat and exhibited lowered free fatty acid, cholesterol, and triglyceride plasma levels (Ejaz et al. 2009).

Polyphenolic compounds found in tea and fruits, mainly catechins, anthocyanins, and their derivatives, have recently gained attention due to their anti-oxidant, anti-inflammatory, and cancer-fighting properties. Green tea in particular contains high levels of epigallocatechin and has been linked with these benefits. (-)-Epigallocatechin-3-gallate (EGCG) treatment of 3T3-L1 cells decreased cell growth by 40.5% at 10 μM and inhibited lipid accumulation by 43.3%, 57%, and 55% when cells were treated during the initial growth phase, during differentiation, and post-differentiation, respectively (Chan et al. 2011). C/EBP-α, PPAR-γ, and forkhead transcription factor class O1 (FoxO1) expression are also suppressed by EGCG concentrations between 1-100 μM (Chan et al. 2011, Kim and

Sakamoto 2012). Kim et al. (2010) also found that EGCG treatment reduced glycerol-3-phosphate dehydrogenase activity, an enzyme involved in lipid synthesis, and SREBP-1c.

Beyond treatment with pure polyphenolic compounds, Söhle et al. (2009) found that treating human pre-adipocytes *in vitro* with 0.1%, 0.25%, 0.5% or 0.75% white tea extract decreased lipid accumulation by up to 70%. Tea treatment also decreased mRNA levels of C/EBP-α, C/EBP-δ, and PPAR-γ as well as mRNA and protein levels of ADD1/SREBP-1c (Söhle et al. 2009). *In vivo*, EGCG supplementation of a high fat diet in mice limited body fat gain and decreased leptin, SREBP-1c, fatty acid synthase (FAS), and stearoyl-CoA desaturase-1 (SCD-1) gene expression in white fat (Klaus et al. 2005, Park et al. 2011). This is indicative of a lower fat mass (lower leptin expression) and decreased fatty acid synthesis by FAS and SCD-1.

3T3-L1 treatment with 250-400 μg/ml grape skin extract produced 50% and 77% decreases in pre-adipocyte and adipocyte lipid accumulation (Jeong et al. 2012, Zhang et al. 2012). Grape skin extract also reduced glycerol-3-phosphate dehydrogenase activity, levels of transcription factors C/EBP-α, PPAR-γ, and SREBP1, adiponectin secretion, and lipolysis enzymes. Cell treatment with 10 μM resveratrol, the major polyphenol in grapes, and its metabolites inhibited lipid accumulation by 32-60% and lowered C/EBP-α, C/EBP- β, PPAR-γ, lipoprotein lipase (LPL), and GLUT-4 expression (Mercader, Palou, and Bonet 2011, Lasa et al. 2012). Resveratrol metabolites showed similar effects in pre-adipocytes. Blueberry polyphenolic extract also inhibited lipid accumulation in 3T3-F442A pre-adipocytes by 27%, 63%, and 74% at 150, 200, and 250 μg/ml, respectively (Moghe et al. 2012). *In vivo*, grape seed extract decreased abdominal fat accumulation by 29% in hamsters

fed a high fat diet as compared to high fat diet controls (Décordé et al. 2009). The grape seed extract also slightly mitigated increases in plasma glucose levels and insulin resistance observed in the controls.

While the general effect of polyphenolic compounds upon adipogenesis *in vitro* and *in vivo* is clear, little specific research exists on cocoa polyphenols. High in catechin, epicatechin, and procyanidins, these compounds have similar anti-oxidant activity of those previously tested. The only existing study found that cocoa polyphenol extract treatment of 3T3-L1 cells at 100 or 200 μg/ml inhibited adipogenesis by 30% and 72%, respectively (Min et al. 2012). This correlated with reduced expression of C/EBP-α, PPAR-γ, and FAS and inhibition of the insulin receptor by polyphenolic binding. Reduced clonal expansion was shown by a reduction in extracellular signal-regulated kinase (ERK) and protein kinase B (Akt), critical in signaling cascades for proliferation (Min et al. 2012). In mice, the same authors found that cocoa polyphenol extract supplementation of a high fat diet reduces fat accumulation by 5.8% and 12.6% at 40 and 200 mg/kg, respectively, when compared to a high fat diet control. Treatment of HepG2 and Caco2 cells with cocoa polyphenols increased apolipoprotein (a major constituent of high density lipoprotein), SREBP levels, and mRNA expression (Yasuda et al. 2011). This indicates that cocoa polyphenols may help to upregulate HDL, or good cholesterol, levels within the body.

Leptin production is dependent upon many paracrine and endocrine factors *in vivo* but is believed to be under partial control of the mammalian target of rapamycin receptor (mTOR) (Szkudelski 2007). This receptor is found in a variety of cell types and is believed to play a role in obesity, diabetes, and cancer (Dann, Selvaraj, and Thomas 2007). mTOR is a

major regulator of cellular growth and homeostasis, including anabolic pathways and leptin secretion. mTOR consists of two complexes, only one of which is activated by nutrients and dietary components. This nutritionally-relevant complex is inhibited by rapamycin and activated by insulin through the secondary messenger phosphatidylinositol 3-kinase. This partially explains the induction of adipogenesis and lipogenesis in adipocytes by insulin (Cammisotto et al. 2006, Szkudelski 2007, Zeigerer et al. 2008). Increases in ATP and malonyl-CoA are known to increase leptin secretion while increases in intracellular cAMP and fatty acid concentrations may inhibit secretion (Szkudelski, Nowicka, and Szkudelska 2005, Szkudelski 2007).

Roh et al. (2003) suggests that adipocytes pre-synthesize and store leptin and that secretion is then controlled by a variety of dietary and signaling factors that act to maintain energy homeostasis. However, insulin treatment of adipocytes *in vitro* does not change leptin mRNA levels, suggesting that insulin is not the sole regulator of leptin production or secretion (Bradley and Cheatham 1999, Roh et al. 2003). In addition to insulin, the mTOR pathway is sensitive to certain nutrients, but nutrient stimulation varies between cell types (Lynch 2001, Roh et al. 2003, Lynch et al. 2006).

Free L-leucine appears to play an important role in adipocyte mTOR activation and its regulation of protein synthesis (Avruch et al. 2008). These effects are independent of insulin and act through a different mTOR mechanism but both compounds are believed to act synergistically: insulin promotes leucine binding and leucine increases insulin secretion (Avruch et al. 2008). At much higher concentrations, amino acids with structural similarities to leucine also activate mTOR. Norleucine has shown the highest effectiveness, followed by

isoleucine, threo-L-β-hydroxyleucine, methionine and valine (Lynch 2001). Though leptin secretion and the mTOR pathway have many effectors, this mechanism may provide an explanation for the influence of dietary compounds and plasma leptin levels on satiety (Roh et al. 2003).

Roh et al. (2003) found that treatment of adipocytes with 5 mM leucine increased leptin secretion 4-5 fold within four hours of treatment. Cammisotto et al. (2006) showed that leucine and glutamate increases leptin secretion by almost 2-fold and slightly increased intracellular leptin content. Leucine treatment also increased fatty acid oxidation in muscle cells and lowered fatty acid synthesis (Sun and Zemel 2007). In obese rats, serum adiponectin, which regulates fatty acid breakdown, was increased while total cholesterol was reduced after leucine supplementation (Torres-Leal et al. 2011). Leucine treatments of rats in meal form found increases in plasma leptin that peaked three hours post-meal. Leptin secretion decreased by 40% in rats fed leucine-deficient meals (Lynch et al. 2006). Oral gavage of leucine and norleucine also increased plasma leptin concentrations, but not to the same extent as when incorporated into a meal.

The presence of other macromolecular nutrients also influences leptin production. Cammisotto et al. (2005) showed that in adipocytes *in vitro*, the presence of glucose was correlated with the maintenance of basal leptin and slight increases in leptin secretion. However, high glucose levels did not increase leptin production beyond that of low glucose treatment (Cammisotto et al. 2005). Treatment with insulin or amino acids strongly increased leptin synthesis: insulin doubled leptin production in the presence of glucose while the effects of individual amino acids varied (Cammisotto et al., 2005, Cammisotto and Bendayan

2007). L-glycine and L-alanine maintained basal leptin production but did not contribute to increased leptin secretion in the presence of insulin. L-aspartate, L-valine, L-methionine, L-phenylalanine, and L-leucine increased leptin production in the presence of both insulin and glucose and all but L-leucine increased leptin levels in the presence of only insulin (Cammisotto et al. 2005). L-glutamate increased leptin production without insulin or glucose. In addition, it was found that these dietary substrates must be broken down in order to induce leptin secretion.

The same group also demonstrated that the presence of free fatty acids in the blood has no effect on leptin secretion. They did find, however, that the intracellular lipolysis of fat stores due to stress or lack of food intake inhibits leptin secretion (Cammisotto and Bendayan 2007). It follows that this lack of leptin production in times of low food intake correlates with low satiety and an increase in feelings of hunger.

Research indicates that a variety of polyphenolic compounds have significant effects upon adipogenesis and adipocyte gene expression. Specifically, Min et al. (2012) has shown that cocoa polyphenols inhibit *in vitro* cellular lipid accumulation by reducing expression of the transcription factors PPAR-γ and CEBP-α and by directly binding to the insulin receptor. Insulin appears to play a key role in the regulation of leptin secretion. Thus, it is possible that cocoa polyphenols could act as a ligand to influence the mTOR signaling pathway: upregulating leptin secretion and increasing satiety.

In addition, leucine appears to play a role in mTOR regulation of leptin secretion and contributing to overall satiety (Lynch et al. 2006, Torres-Leal et al. 2011). However, *in vitro* studies investigating the effects of BCAAs upon leptin secretion have only been conducted in

the absence of other major nutrients. This does not adequately examine the effects of BCAAs, as cells may react differently if deprived of an essential nutrient such as glucose or specific amino acids. In *in vivo* and clinical studies, protein and amino acids make up only a portion of the experimental diet. Thus, the effects of increases in dietary protein are tested as a supplement to overall food intake.

Accordingly, research is lacking in two major areas surrounding the effects of cocoa and BCAAs upon satiety. Currently, no research exists on the effects of cocoa polyphenolics upon leptin production *in vitro* or *in vivo*. Also, no *in vitro* studies have investigated the effects of BCAA supplementation of standard growth media upon leptin secretion. The combinatorial effects of whey protein and cocoa upon lipid accumulation and leptin secretion are also unknown. This project aims to provide insight into the following areas while simulating real-world conditions as accurately as possible. This will be done by determining:

1. The *in vitro* effects of cocoa polyphenolics on lipid accumulation and leptin secretion of pre-adipocytes and adipocytes
2. The *in vitro* effects of branched chain amino acid supplementation on leptin secretion of adipocytes
3. The effects of a chocolate whey protein-based beverage on satiety ratings, blood glucose levels, and plasma leptin and adiponectin levels in a human clinical trial

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# CHAPTER 2: EFFECTS OF COCOA POLYPHENOLS AND LEUCINE ON DIFFERENTIATION, LIPID ACCUMULATION, AND LEPTIN PRODUCTION IN 3T3-L1 PRE-ADIPOCYTES

## 2.1 ABSTRACT

Bioactive food ingredients exhibit the potential to counteract obesity and facilitate weight loss. Whey protein intake has been demonstrated to decrease total body fat while increasing satiety. Cocoa phenolics have been reported to prevent lipid accumulation, improve insulin signaling, and slow digestion. Little research exists on the effects of cocoa upon satiety. This study aimed to determine the effects of cocoa and leucine, a major whey protein amino acid, upon differentiation, lipid accumulation, and leptin production in murine 3T3-L1 pre-adipocytes. 3T3-L1 cells were treated with 0.5-100 ug/ml cocoa phenolic extract (CPE) and/or 1-15 mM leucine during and after differentiation. Extent of differentiation and lipid accumulation were quantified by Oil-Red-O staining and leptin production was measured by leptin enzyme-linked immunosorbent assay (ELISA). Results showed that 10, 50, and 100 ug/ml CPE treatments inhibited pre-adipocyte lipid accumulation by 17, 35, and 50%, respectively. CPE treatments of 0.5 ug/ml increased leptin production by 66%. Leucine treatment had no effect upon lipid accumulation but increased leptin production by 26-37%. In combination, CPE and 15 mM leucine inhibited lipid accumulation by 10-40% and increased leptin production by 14%. This study indicates that the consumption of cocoa and whey protein, individually and in combination, may increase lipid metabolism and satiety.

## 2.2 INTRODUCTION

Polyphenolic compounds found in cocoa, fruits, and tea have been shown to increase satiety, insulin sensitivity, and fat metabolism (Jalil et al. 2009, Panickar 2013). Cocoa tannins, catechins, and procyanidins effectively slowed digestion *in vitro* by binding α-

amylase, glucoamylase, and pancreatic lipase (Gu et al. 2011, Barrett et al., 2013). In mouse and human clinical studies, flavanol-rich cocoa or chocolate consumption decreased fat accumulation and fatty acid synthesis, increased insulin sensitivity, and lowered short-term blood glucose and free fatty acid levels (Matsui et al. 2005, Grassi et al. 2008, Jalil et al. 2009, Min et al. 2012). Epicatechin consumption in drinking water (equivalent to 250 g of dark chocolate per day) decreased the incidence of diabetes by 50% and increased insulin levels in mice (Fu, Yuskavage, and Liu 2013). Polyphenol-rich dark chocolate consumption decreased hunger ratings and ad libitum intake by 17% and increased satiety more than milk chocolate (Sørensen and Astrup 2011, Fu, Yuskavage, and Liu 2013). Even in the absence of substantial cocoa polyphenols, a variety of chocolate flavored desserts increased plasma insulin levels more than the same vanilla or strawberry-flavored foods (Brand-Miller et al. 2003).

Of the major food macromolecules, whey proteins have been shown to increase satiety, quantified both by subjective satiety scaling and hormonal markers. Whey protein decreased *ad libitum* food intake by 19% and increased levels of satiation hormones by 60% in comparison to casein (Hall et al. 2007). Pichon et al. (2007) showed that whey proteins were more effective than whole milk protein in limiting weight gain and increasing insulin and leptin levels in rats fed high-protein diets. The satiety-inducing benefits of whey proteins are attributed to their high branched chain amino acid content (Layman and Walker 2006). Valine, leucine, and isoleucine are more quickly metabolized by the body; the subsequent increase in plasma amino acids appears to trigger the release of satiety hormones and to induce greater feelings of satiety (Boirie et al. 1997). Leucine supplementation *in vivo* and in

clinical trials has been found to increase muscle synthesis, increase plasma leptin, decrease plasma lipid levels, and improve body composition (Katsanos et al. 2006, Lynch et al. 2006, Pal, Ellis, and Dhaliwal 2010).

Adipose tissue is the major energy reserve within the human body and influences both lipid metabolism and satiety signals sent to the brain (Hwang et al. 1997). The satiety hormone leptin is only secreted by white adipose tissue and its appetite-suppressing action influences the length of time between meals, snacking habits, and calories consumed at the following meal (De Graaf et al. 2004, Benelam 2009). The 3T3-L1 murine pre-adipocyte cell line is a common model used to evaluate the *in vitro* effectors of adipocyte lipid metabolism and hormonal secretion. *In vitro*, adipocytes have been demonstrated to express only 1% of the total leptin expressed *in vivo*, somewhat limiting their use as a model of fat mass *in vivo* (MacDougald et al. 1995). However, conclusions can be extrapolated from *in vitro* observations and these cell lines serve as an adequate initial indication of adipocyte response to specific environmental conditions.

Polyphenolics from tea and fruits have been shown to decrease adipogenesis and lipid accumulation but less research exists on cocoa polyphenolics (Kim and Sakamoto 2012, Zhang et al. 2012). In 3T3-L1 cells, 100 or 200 μg/ml cocoa polyphenol extract inhibited adipogenesis by 30% and 72%, respectively (Min et al. 2012). To mimic whey protein consumption *in vitro*, treatment of adipocytes with 5 mM leucine increased leptin secretion 2-5 fold, increased fatty acid oxidation, and lowered fatty acid synthesis (Cammisotto et al. 2006, Roh et al. 2003). Cammisotto et al. (2006) indicated that leucine and glutamate increased leptin secretion by almost 2-fold and slightly increased intracellular leptin content.

Leucine treatment also increased fatty acid oxidation in muscle cells and lowered fatty acid synthesis (Sun and Zemel 2007).

Beverages designed for weight control or nutritional supplementation can contain whey proteins and cocoa. No research exists on the effects of cocoa polyphenols on leptin levels *in vitro* or *in vivo*. In addition, the combined effects of cocoa and whey proteins on parameters contributing to obesity have not been investigated. This study aimed to examine the *in vitro* effects of cocoa polyphenols and leucine on 3T3-L1 differentiation, lipid accumulation, and leptin secretion. Existing *in vitro* studies have treated cells with leucine in the absence of basic growth media, implying that the cells were potentially starved of essential nutrients. The treatments in this study intended to mimic *in vivo* conditions as closely as possible: leucine concentrations were similar to those used in clinical protein supplementation studies and cells were treated with leucine in the presence of all other basic growth nutrients. The results of this study assess the individual and combined effects of cocoa and whey protein ingredients on *in vitro* indicators of satiety and weight management.

## 2.3 MATERIALS AND METHODS

### 2.3.1 *MATERIALS AND REAGENTS*

Tissue culture plates and flasks were purchased from BD Biosciences (San Jose, CA, USA). All reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) or Fisher Scientific (Waltham, MA, USA) and were of the highest purity available. Dulbecco's Modified Eagle's Medium and streptomycin-penicillin-glutamine were purchased from

Invitrogen-Life Technologies (Carlsbad, CA, USA) and fetal bovine serum was purchased from Atlanta Biologicals (Flowery Branch, GA, USA). Hershey's cocoa powder was purchased from a local grocery store (Raleigh, NC, USA). 1-methyl-3-isobutylxanthine and dexamethasone were purchased from Sigma-Aldrich (St. Louis MO, USA) and insulin from Santa Cruz Biotechnology (Dallas, TX, USA). MTT was purchased from Molecular Probes (Eugene, OR, USA) and Oil-Red-O from Sigma-Aldrich (St. Louis, MO, USA). L-leucine was purchased from Sigma-Aldrich (St. Louis, MO, USA).

### 2.3.2 *PREPARATION OF COCOA PHENOLIC EXTRACT (CPE)*

Extraction of cocoa polyphenols was performed as by (Adamson et al. 1999). Briefly, 4 g of cocoa powder was defatted by three extractions with 25 ml hexanes. The remaining cocoa pellet was then dried with nitrogen in an analytical rotary evaporator (Organomation, Berlin, MA, USA). Polyphenolics were then extracted by three extractions of 1 g of dried cocoa solids with 5 ml of 70% acetone, 29.5% water, and 0.5% glacial acetic acid. Extractions were performed by sonication for 10 minutes and centrifugation for 10 minutes at 1500 x *g* and the extract from each extraction was decanted and combined to yield 15 ml total extract. Extract was centrifuged at 3300 x *g* for 35 minutes in 25 ml conical tubes to remove fine cocoa solids, filter sterilized through a 0.22 μm filter (Genesee Scientific, San Diego, CA, USA), and stored at -20°C until use. Phenolic content of the cocoa extract was determined by the Folin-Ciocalteu Total Phenolics assay as by (Magalhães et al. 2010) and read at 690 nm. Cocoa polyphenolic treatments were based on the total phenolics assay and expressed in μg/ml gallic acid equivalents. A more specific HPLC analysis of CPE content

was performed by The Hershey Company. The results of these analyses, including epicatechin polymer and methylxanthine content, are shown in Tables A.1 and A.2 in the Appendix.

### 2.3.3 *CELL CULTURE*

3T3-L1 pre-adipocytes were obtained from ATCC (Manassas, VA, USA). Cells were grown in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% streptomycin-penicillin in 75 $cm^3$ Falcon flasks at 37°C with 5% $CO_2$. Cell differentiation to adipocytes was performed as by (Phrakonkham et al. 2008). Briefly, at 90% confluency, 5,000 cells/well were seeded into 96-well cell culture plates, grown to confluency, and then maintained for 48 hours post-confluency in the same growth media (day 0). At day 0, cells were treated with DMEM containing 0.5 mM 1-methyl-3-isobutylxanthine, 0.25 μM dexamethasone, and 170 nM insulin for 48 hours. Cells were then treated with DMEM/10% FBS with 166 nM insulin for 48 hours. At day 4, media was replaced every two days with DMEM/10% FBS until cells were 80-90% differentiated. To test the effects of cocoa and leucine upon pre-adipocyte differentiation, wells were treated in triplicate with 0.5-100 μg/ml cocoa phenolic extract or 1-15 mM leucine from days 0-2. To determine adipocyte lipid accumulation and leptin secretion, cells were treated with 0.5-100 μg/ml cocoa extract or 1-15 mM leucine for 48 hours after complete differentiation was achieved (between days 6-10). For lipid accumulation experiments, DMEM/10% FBS/1% streptomycin-penicillin served as a negative control. A second negative control + solvent consisted of 100 μg/ml acetone/water/acetic acid in order to control for the effects of the CPE

extraction solvent. For leptin secretion experiments, 0.5 mM insulin treatment served as a positive control.

### 2.3.4 *CELL VIABILITY*

Cell viability was assessed using an MTT cell proliferation assay. Following differentiation and cocoa/leucine treatment, growth media was replaced with DMEM/10% FBS containing 0.5 mg/ml MTT. Cells were incubated at 37°C with 5% $CO_2$ for 3.5 hours until MTT was reduced to form purple formazan crystals at the bottom of each well. Media was removed, replaced with dimethyl sulfoxide to dissolve the crystals, and the contents of each well were transferred to corresponding wells of a clear 96-well plate. Absorbance was read at 570 nm. Triplicate negative control readings were averaged and assigned a value of 100% viability; triplicate experimental absorbance values were converted to an average percent of the negative control.

### 2.3.5 *CELLULAR LIPID ACCUMULATION*

3T3-L1 cellular lipid accumulation was determined using an Oil-Red-O staining assay. Following differentiation and cocoa extract or leucine treatment, growth media was removed and cells were fixed with formalin. Cells were then treated with 60% isopropanol for 5 minutes and stained with 0.6 mg/ml Oil-Red-O in isopropanol for 30 minutes. Wells were washed three times with DI water, photographed, and the water was replaced with isopropanol to dissolve the Oil-Red-O dye. The contents of each well were transferred to a clear 96-well plate and absorbance was read at 520 nm. Triplicate negative control readings

were averaged and assigned a value of 100%; triplicate experimental absorbance values were converted to an average percent of the negative control.

### 2.3.6 *LEPTIN SECRETION*

3T3-L1 pre-adipocytes were differentiated as above and treated with cocoa extract, leucine, or a combination of CPE and leucine for 48 hours post-differentiation. After 48 hours of treatment, growth media was removed from each well and assayed for leptin content with a Mouse Leptin ELISA kit (Sigma-Aldrich, St. Louis, MO) according to manufacturer instructions.

### 2.3.7 *STATISTICAL ANALYSIS*

A one-way ANOVA for Tukey's honestly significant differences was applied using SAS software to make all pairwise comparisons among treatment means at an experiment-wise error rate of 0.05.

## 2.4 RESULTS AND DISCUSSION

### 2.4.1 *EFFECTS OF COCOA EXTRACT AND LEUCINE ON 3T3-L1 CELL VIABILITY*

A MTT cell viability assay was used to determine the effects of CPE and leucine on cell growth. As shown below in Figures 2.1 to 2.4, the treatment of cells with CPE and/or leucine during (days 0-2) or after differentiation (days 6-8) did not significantly affect viability. All treatments exhibited cell viability values above 82% of the control.

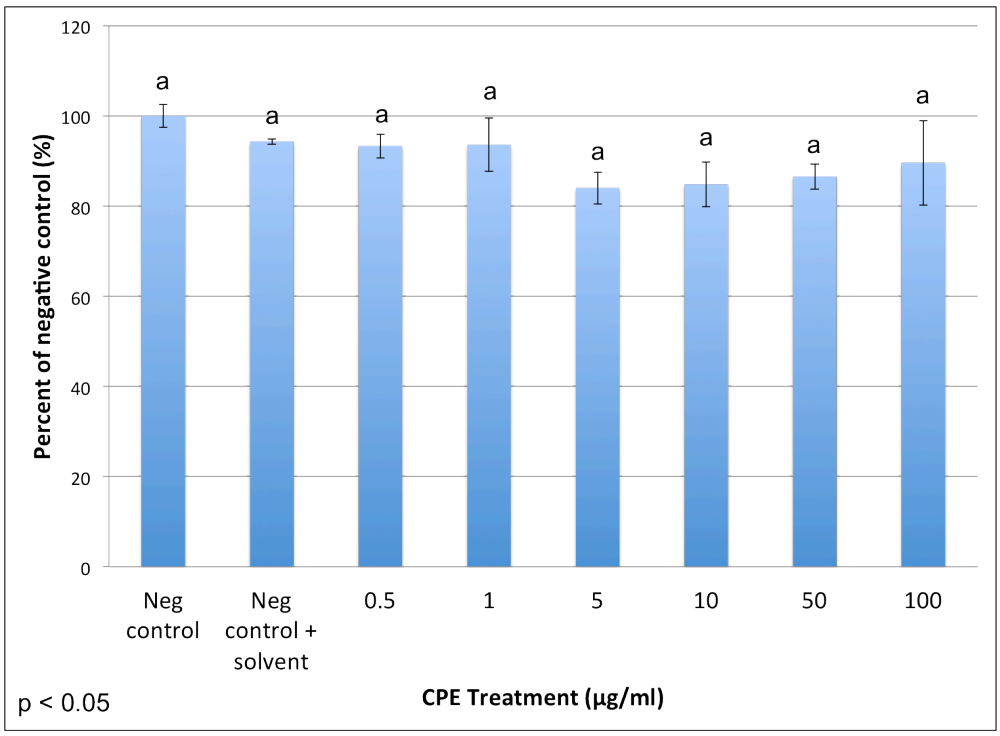


**Figure 2.1.** Effects of CPE treatment on pre-adipocyte cell viability during differentiation (day 0-2).

Treatments with same letter are not statistically different. Data expressed as percent of negative control as mean ± S.E.

Neg control: complete growth media, Neg control + solvent: complete growth media + 100 µg/ml acetone solvent

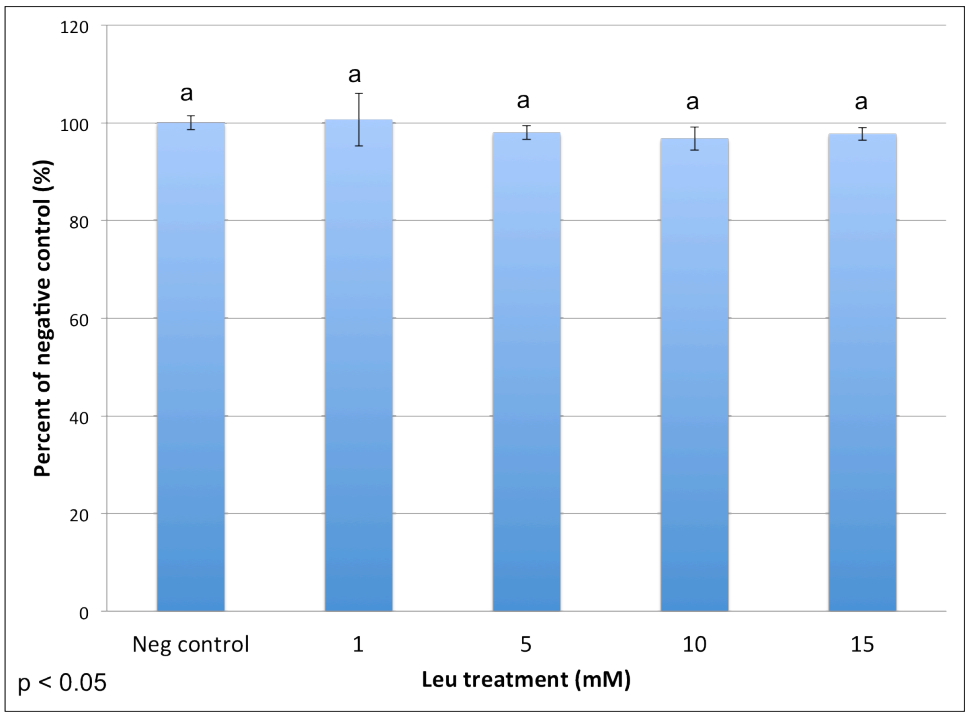


**Figure 2.2.** Effects of leucine treatment on pre-adipocyte cell viability during differentiation. Treatments with same letter are not statistically different. Data expressed as percent of negative control as mean ± S.E.
Neg control: complete growth media

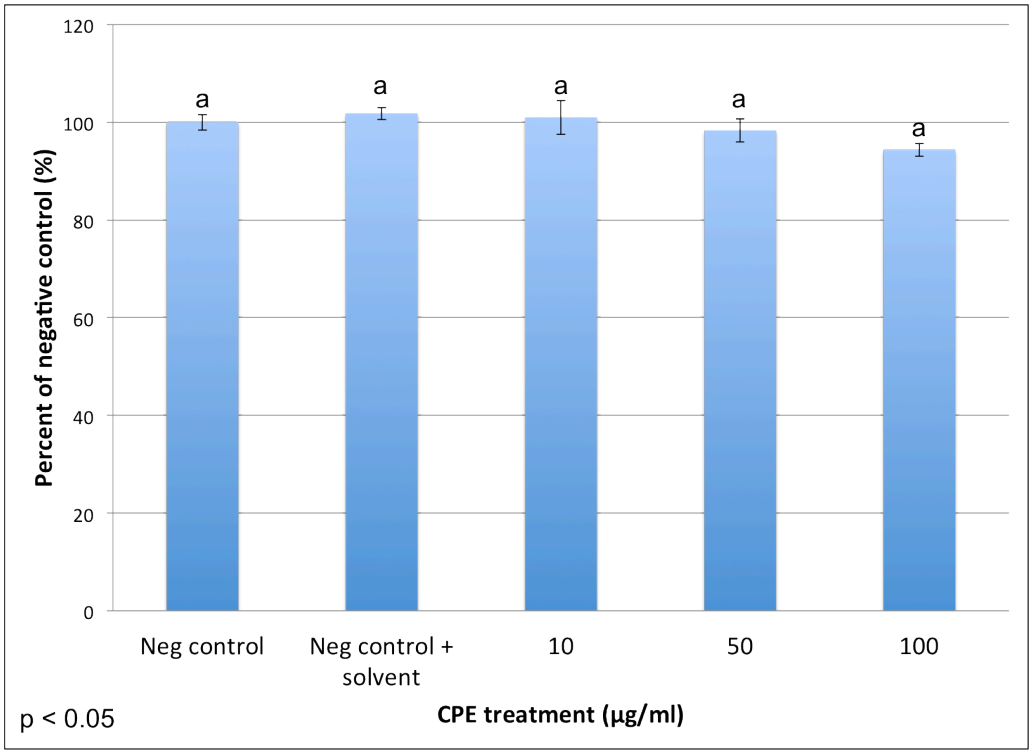


**Figure 2.3.** Effects of CPE and 15 mM Leu treatment on pre-adipocyte cell viability. Treatments with same letter are not statistically different. Data expressed as percent of negative control as mean ± S.E.

Neg control: complete growth media, Neg control + solvent: complete growth media + 100 µg/ml acetone solvent

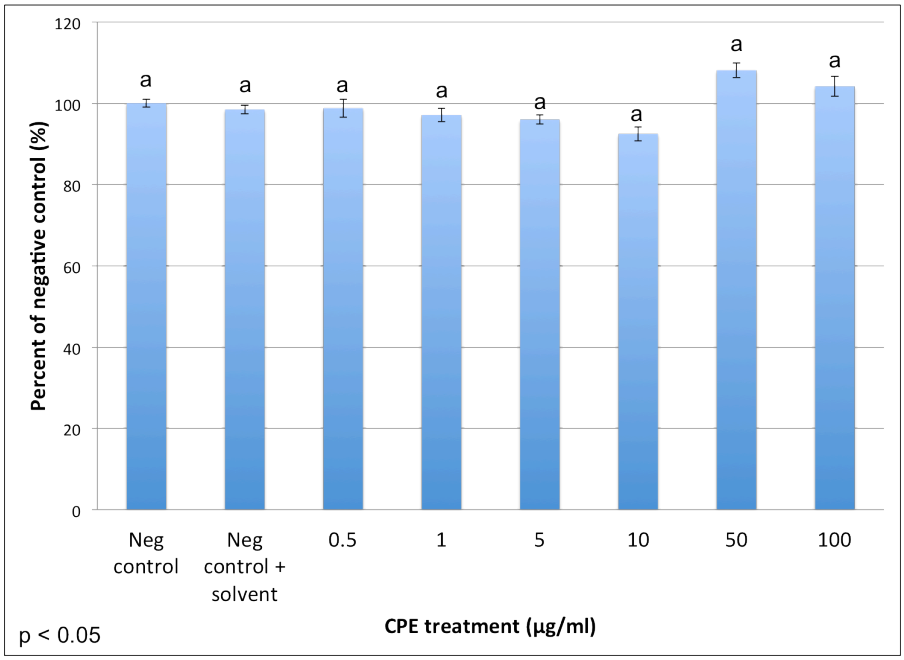


**Figure 2.4.** Effects of CPE treatment on adipocyte cell viability. Treatments with same letter are not statistically different. Data expressed as percent of negative control as mean ± S.E. Neg control: complete growth media, Neg control + solvent: complete growth media + 100 µg/ml acetone solvent

### 2.4.2 *EFFECTS OF COCOA EXTRACT AND LEUCINE ON 3T3-L1 DIFFERENTIATION AND LIPID ACCUMLATION*

To isolate the effects of CPE and leucine upon pre-adipocyte differentiation and adipocyte lipid metabolism, 3T3-L1 cells were treated either during differentiation (days 0-2) or after differentiation (days 6-8), respectively. Following treatment, cells were stained with Oil-Red-O to determine the effect of each treatment on cellular lipid content. In addition to a standard control treatment (DMEM growth medium), a second set of control wells were treated with DMEM growth medium supplemented with 100 µg/ml of the CPE extraction solvent (70% acetone, 29.5% water, and 0.5% acetic acid) in order to differentiate between the effects of the CPE solvent and the cocoa polyphenolic treatment. In addition, all

treatments, regardless of CPE concentration, were standardized to 100 μg/ml total extraction solvent so as to mitigate any concentration-based cellular effects of the acetone mixture.

As shown in Figure 2.5, treatment of 3T3-L1 cells with CPE during differentiation significantly decreased the final lipid content of the cells. As compared to the control treatment, CPE treatment during differentiation decreased cellular lipid content by 17, 35, and 50% at CPE treatments of 10, 50, and 100 μg/ml, respectively. Lipid decreases at 50 and 100 μg/ml were found to be statistically significant. Figure 2.6 shows microscopy images of the (a) control treatment, (b) 50 μg/ml CPE treatment, and (c) 100 μg/ml CPE treatment after Oil-Red-O staining. Uptake of the red Oil-Red-O stain within cells represents the differences upon differentiation and lipid accumulation. In Figure 2.6a, 90% of pre-adipocytes successfully differentiated into mature adipocytes after treatment with standard growth media and the differentiation cocktail. Figures 2.6b and c show the 35 and 50% decrease in lipid accumulation after 50 and 100 μg/ml CPE treatment, respectively, during differentiation. While some cells differentiated to mature adipocytes during 50 μg/ml CPE treatment, Figure 2.6c shows that 100 μg/ml CPE treatment prevented major differentiation. At 100 μg/ml CPE treatment, cells retained their pre-adipocyte morphology and fewer cells accumulated lipids.

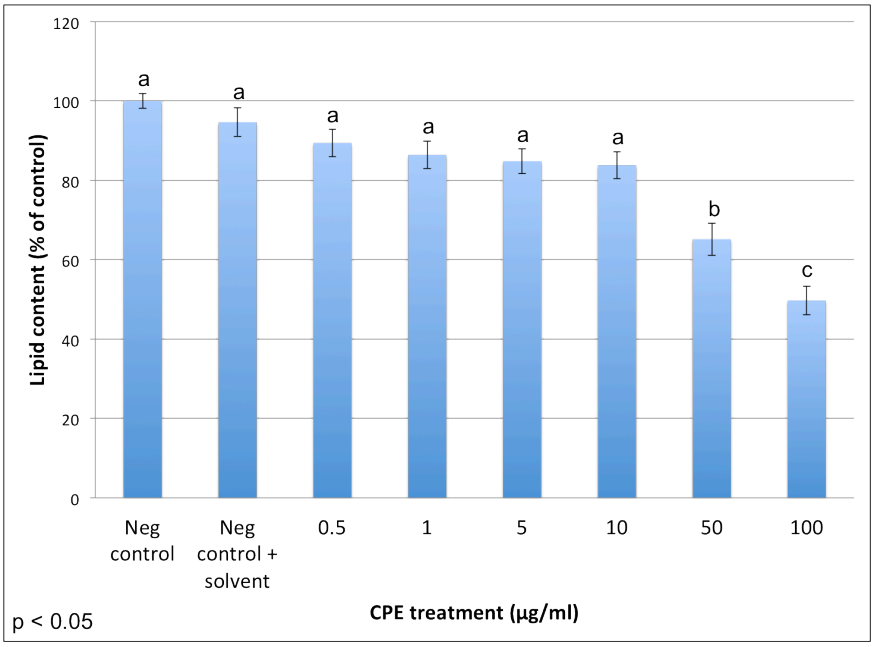


**Figure 2.5.** Effects of CPE treatment on pre-adipocyte lipid accumulation during differentiation. Treatments with same letter are not statistically different. Data expressed as percent of negative control as mean ± S.E.
Neg control: complete growth media, Neg control + solvent: complete growth media + 100 µg/ml acetone solvent

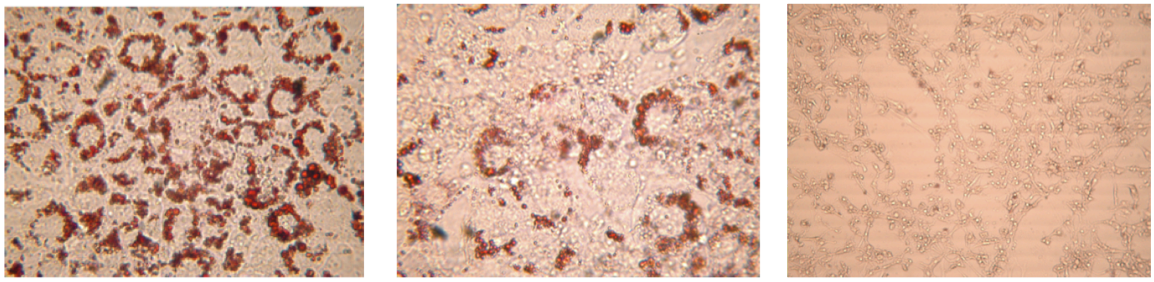


**Figure 2.6.** Effects of CPE on lipid accumulation and extent of cell differentiation as viewed by microscopy after ORO lipid staining. Red color indicates presence of lipids within mature adipocytes. (a) negative control, (b) 50 µg/ml CPE, and (c) 100 µg/ml CPE treatments.

These results are concurrent with (Min et al. 2012) who showed that 100 and 200 μg/ml CPE treatments during differentiation decreased cellular lipids by 30 and 72%, respectively. Min and others (2012) also reported a decrease in fatty acid synthase with CPE treatment, attributed to CPE treatment. Our results showed an additional 20% decrease in lipid accumulation at 100 μg/ml CPE, likely due to differences in cocoa source and extraction preparation. Similar levels of inhibition were observed with treatments of epigallocatechin gallate and resveratrol-amplified grape skin extract (Chan et al. 2011, Lasa et al. 2012, Zhang et al. 2012).

While pre-adipocytes possess the necessary genes for lipid accumulation, these genes are not expressed until stimulated by extracellular factors (Rosen and Spiegelman 2000). Thus, the observed decreases in lipid accumulation are due to an inhibition of the genes necessary for pre-adipocyte differentiation. Inhibition of pre-adipocyte differentiation and the accompanying decrease in lipid accumulation is attributed to CPE inhibition of the transcription factors peroxisome proliferator-activated receptor-γ and -α (PPAR-γ, PPAR-α) and CCAAT/enhancer binding protein-α (C/EBP-α), two proteins involved in transcription of genes important to cell growth (Rayalam et al., 2008, Min et al. 2012). These factors increase expression of adipocyte-specific genes, including those that regulate lipid synthesis (Rosen 2002). Min et al. (2012) also showed that compounds in CPE bind directly to the insulin receptor and reduce phosphorylation of extracellular signal-regulated kinase (ERK) and protein kinase b (Akt), critical downstream signals for cell proliferation.

Contradictory research on fat metabolism indicates that activation of PPAR by polyphenolic compounds in mature cells may actually increase fatty acid oxidation. Genistein

isoflavones and grape flavonoids were found to increase PPAR mRNA and protein expression *in vitro*, inducing fatty acid lipolysis (S. Kim et al. 2004, Goldwasser et al. 2010). It must be mentioned that these studies were done with mature liver and kidney cells. This research provides a comparison for the effects of polyphenolics in undifferentiated v. mature cells, as well as between different cell types. It is possible that phenolic treatment accomplishes the same goal across cell types, but through different mechanisms. As shown in the present study and by Min et al. (2012), cocoa polyphenolics inhibit lipid accumulation in immature adipocytes by inhibiting expression of PPAR-α. Conversely, in mature cells, or potentially simply other cell types, phenolic treatment *activates* PPAR expression and subsequent lipolysis, thus inhibiting lipid accumulation. More research is needed to determine which specific polyphenolics downregulate lipid accumulation or upregulate lipolysis in certain cell types. Figure 2.7 demonstrates the hypothetical regulatory effects of polyphenolics upon PPAR in pre-adipocytes and adipocytes.

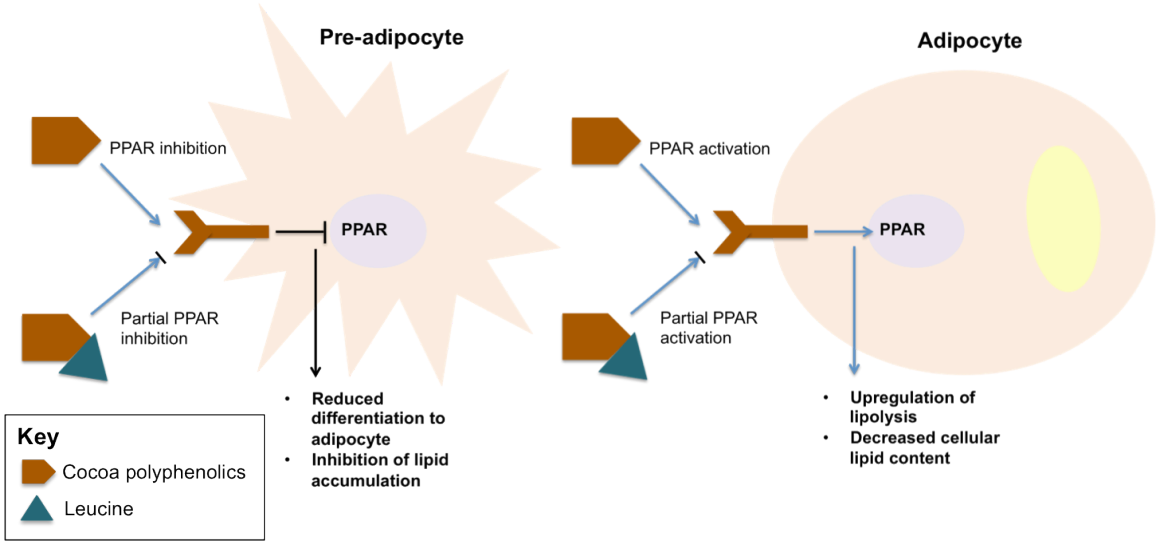


**Figure 2.7.** The hypothetical mechanistic effects of cocoa polyphenolics, alone and in combination with leucine, on pre-adipocytes and adipocytes. Polyphenolic treatment of adipocytes inhibits PPAR while treatment of adipocytes activates PPAR expression. Both effects may result in reduced cellular lipid content.

As shown in Figure 2.8, treatment of mature adipocytes with 10, 50, and 100 µg/ml CPE decreased cellular lipid contents by 19, 12, and 16%, respectively. Decreases were only statistically significant at treatment levels of 10 and 100 µg/ml. This treatment demonstrated the effects of CPE upon lipid accumulation in mature adipocytes as compared to pre-adipocyte differentiation during treatment days 0-2. CPE treatment of mature adipocytes did not inhibit lipid accumulation to the same extent as in pre-adipocytes, nor did it follow a significant dose-dependent trend.

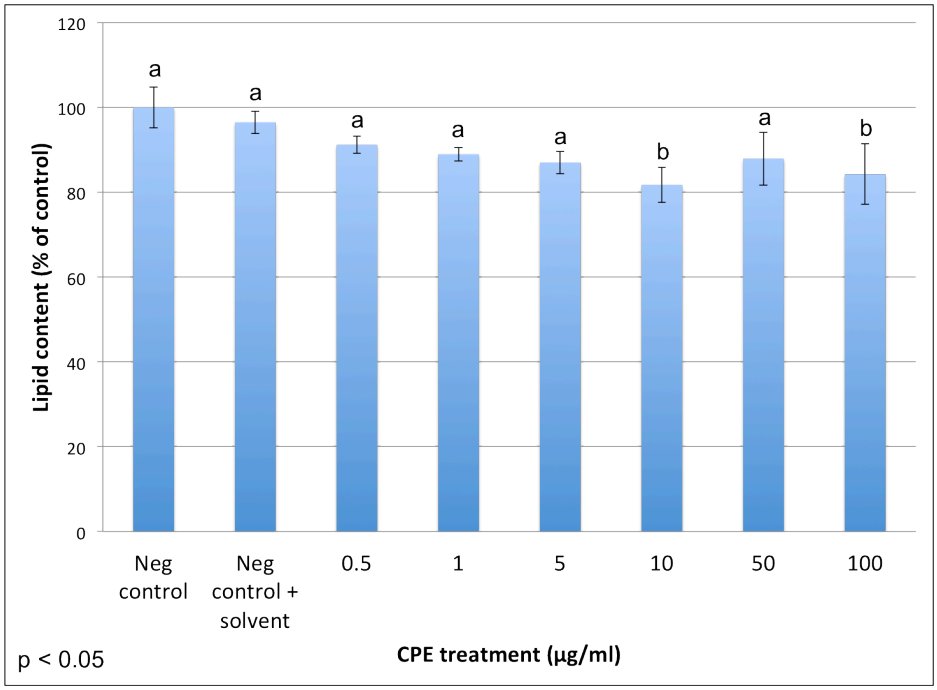


**Figure 2.8.** Effects of CPE treatment on adipocyte lipid accumulation. Treatments with same letter are not statistically different. Data expressed as percent of negative control as mean ± S.E.
Neg control: complete growth media, Neg control + solvent: complete growth media + 100 µg/ml acetone solvent

This observation corresponds with research conducted by (Kim et al. 2011), who treated 3T3-L1 pre-adipocytes with curcumin during varying stages of differentiation. Their results show that polyphenolic treatment during the earliest stage of differentiation (first two days) have the most pronounced impact upon final cellular lipid content (Kim et al. 2011). Curcumin treatment during early differentiation decreased lipid accumulation and differentiation by 80-90% while treatment during intermediate and terminal differentiation only decreased lipid accumulation by 20-40%. This indicates that CPE may have a greater inhibitory effect upon PPAR-γ and C/EBP-α during the early stages of genetic transcription

and translation. After these adipocyte-specific genes have been activated, CPE only exerts a limited effect upon inhibition of differentiation and lipid accumulation (Lasa et al. 2012).

1-15 mM leucine treatment of pre-adipocytes during or after differentiation did not significantly affect lipid content, indicating that leucine alone does not affect the pre-adipocyte growth cycle or lipid metabolism (see Figure A.1 in Appendix). The results of combined CPE and leucine treatment during differentiation are shown in Figure 2.9. 15 mM leucine and 10, 50, and 100 μg/ml CPE treatment during differentiation decreased cellular lipids by 10, 22, and 36%, respectively and were significantly different from both controls at 50 and 100 μg/ml. These decreases mirrored the dose-dependent trend in the isolated CPE treatments but combined CPE and leucine inhibition of lipid accumulation was not as extensive as that found with CPE treatments alone.

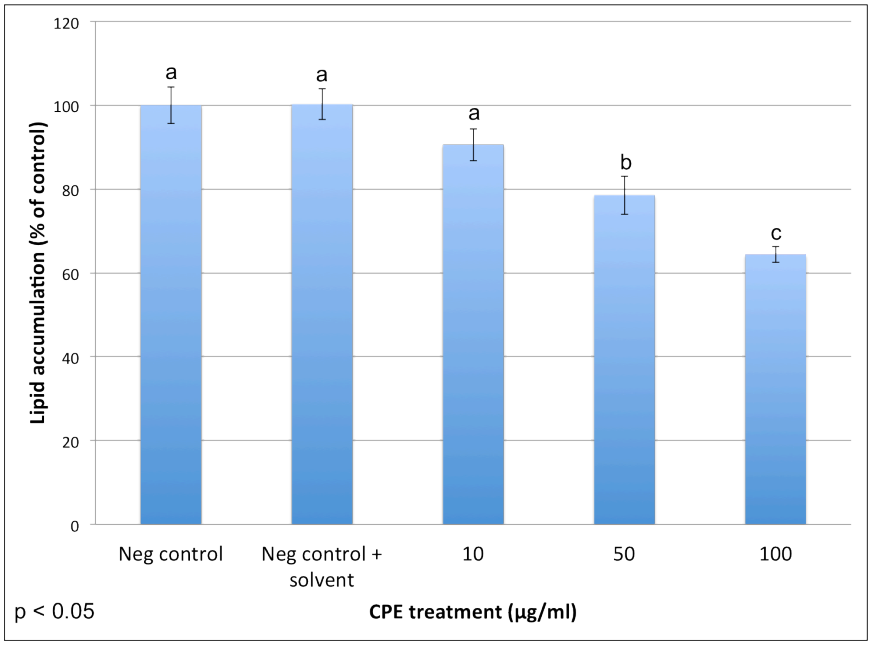


**Figure 2.9.** Effects of CPE + 15 mM Leu treatment on pre-adipocyte lipid accumulation during differentiation. Treatments with same letter are not statistically different. Data expressed as percent of negative control as mean ± S.E.
Neg control: complete growth media, Neg control + solvent: complete growth media + 100 µg/ml acetone solvent

It is possible that cocoa polyphenols and leucine competed for similar cellular binding sites or that polyphenols and leucine may affect cell growth and metabolism through conflicting mechanisms. It is also possible that the presence of leucine prevented or changed the binding affinity of polyphenols to cellular receptors. The strong affinity between proteins and polyphenols has been established in the literature; compound binding affinities are dependent upon number and strength of charges. In a study of polyphenol-protein binding, (Nagy et al. 2012) found that catechin-like polyphenols bind well to highly charged protein sequences. Leucine has a partial positive and partial negative charge in neutral cell culture media and exhibits surfactant, or emulsifying, properties. However, Kanakis et al. (2011)

reported that catechins interact with β-lactoglobulin through both hydrophilic and hydrophobic contacts. While catechin and β-lactoglobulin bound weakly in buffered solution, leucine and isoleucine accounted for four of the thirteen amino acid residues involved in stable catechin binding (Kanakis et al. 2011). Though leucine was present as a single amino acid in this study, rather than in a protein, it is possible that it may competitively inhibit polyphenolic cocoa compounds from interacting with cell surface receptor binding. In the present study, leucine appears to dampen the effects of CPE upon adipocyte lipid accumulation. However, it is still unclear how well cocoa polyphenolic compounds may bind to leucine in solution and how this may impact CPE bioactivity.

Treatment of 3T3-L1 cells with leucine was intended to model the effects of whey protein metabolism on pre-adipocytes and adipocytes. When compared to other food-derived proteins, whey proteins contain a significantly higher proportion of the branched chain amino acids leucine, isoleucine, and valine (Luhovyy, Akhavan, and Anderson 2007). Leucine is of particular interest in muscle anabolism and lipid metabolism because it has been shown to enter the blood stream faster than other amino acids. *In vitro* and *in vivo*, leucine and whey protein has been shown to decrease expression of fatty acid synthase in adipocytes, to increase fatty acid oxidation in muscle cells, to improve body composition, and to limit weight gain (Pichon et al. 2007, Sun and Zemel 2007, Pal, Ellis, and Dhaliwal 2010). In the case of leucine, the insignificant changes in lipid metabolism observed in this study could be explained by cellular signaling of the mammalian target of rapamycin (mTOR) (Dann, Selvaraj, and Thomas 2007). This protein kinase integrates signals from insulin, growth factors, and amino acids and detects cellular nutrient and energy levels (Roh et al. 2003). In

addition, mTOR regulates cell growth and protein synthesis and maintains lean body mass during times of energy deficit (Layman and Walker 2006, Dann, Selvaraj, and Thomas 2007). Amino acids, and leucine in particular, have been suggested to act as potent ligands of the mTOR signaling cascade and its subsequent upregulation of protein synthesis (Kimball and Jefferson 2006). One would then predict that leucine treatment in this study may have stimulated protein synthesis, controlled by mTOR signaling, while having little effect upon cellular lipid accumulation. In addition, because the pre-adipocytes and mature adipocytes were isolated *in vitro*, the effects of endocrine signaling between different types of cells upon lipid metabolism in the presence of leucine and CPE cannot be determined.

50% of mature fat cells are replaced only every eight years, indicating that the regulation of adipocyte lipid metabolism *in vivo* may have a greater impact upon controlling obesity than the regulation of pre-adipocyte differentiation into mature fat cells (Moreno-Navarrete and Fernández-Real 2012). While CPE demonstrated greater effects in pre-adipocyte lipid accumulation, the smaller decreases observed in adipocyte lipid accumulation indicate that cocoa could still be beneficial in controlling fat accumulation over time.

### 2.4.3 *EFFECTS OF COCOA EXTRACT AND LEUCINE ON 3T3-L1 LEPTIN SECRETION*

To determine the effects of CPE and leucine on leptin secretion, differentiated adipocytes were treated with 0.5-100 μg/ml CPE or 1-15 mM leucine. Adipocyte leptin secretion is partially controlled by the mTOR pathway, which is upregulated by insulin ( Szkudelski 2007). Thus, 0.5 μM insulin treatment (with acetone solvent) served as a positive control for leptin production. As shown in Figure 2.10, CPE treatment elicited a reversed

dose-dependent trend: the lowest CPE treatments (0.5, 1, and 5 μg/ml) increased leptin production above that of the negative control by 66, 17, and 10%, respectively. Only the 0.5 μg/ml CPE treatment was significantly different from the negative control. However, this upregulation was not significantly different than that of the positive control or the negative control containing acetone solvent. Higher CPE treatments of 50 and 100 μg/ml significantly decreased leptin production, 78% below that of the negative control.

These concentration-based results provide an interesting contrast to the inhibition of lipid accumulation by CPE. While high CPE treatments (10-100 μg/ml) were most effective in inhibiting lipid accumulation in pre-adipocytes, the lowest CPE treatments (0.5-5 μg/ml) were most effective in increasing cellular leptin secretion in mature adipocytes. This suggests that polyphenolic compounds may exhibit hormesis-like effects upon pre-adipocyte and adipocyte metabolism; inhibition of lipid accumulation or upregulation of leptin production appears to be concentration specific.

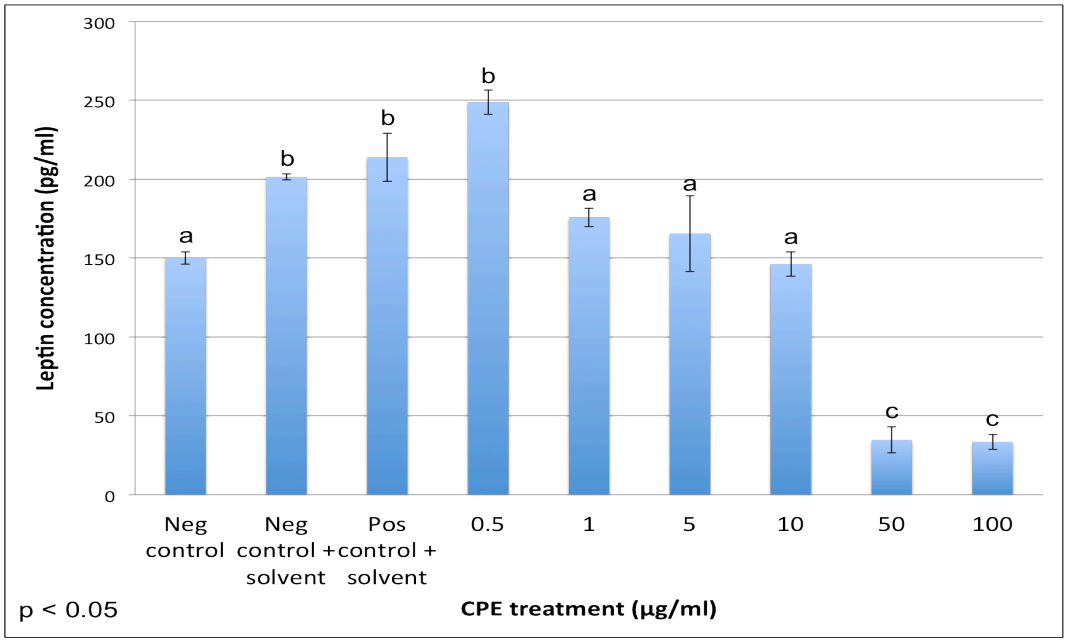


**Figure 2.10.** Leptin secretion in adipocytes treated with CPE. Treatments with same letter are not statistically different. Data expressed as mean ± S.E.
Neg control: complete growth media, Neg control + solvent: complete growth media + 100 µg/ml acetone solvent, Pos control + solvent: complete growth media + 0.5 µM insulin + 100 µg/ml acetone solvent

To test the effects of leucine upon leptin secretion, leucine treatments were administered as a supplementation of regular growth media. All treatment groups were provided with basic DMEM growth medium supplemented with FBS and provided 32 mM total protein in the form of amino acids. Leucine treatments of 1, 5, 10, and 15 mM represented growth media amino acid supplementations of 3.5, 17, 30, and 50%, respectively. These treatments were meant to be representative of protein supplementation treatments commonly found in *in vivo* and clinical trial studies. With this treatment method, the cells were provided with a surplus, and variety, of amino acids in addition to all essential nutrients.

Leucine supplementation in this manner was designed to prevent cellular nutrient starvation and subsequent misleading results.

As shown in Figure 2.11, all leucine treatments were effective in upregulating leptin secretion by 37, 36, 26, and 26% above that of the negative control (at 1, 5, 10, and 15 mM leucine treatments, respectively). All leucine treatments were significantly higher than the control. The combined effects of CPE and leucine upon leptin secretion were less clear (Figure 2.12). Because the most significant increases in leptin secretion were observed at the lowest CPE treatment, all leucine treatment concentrations were combined with 0.5 μg/ml CPE. 1, 5, and 10 mM leucine treatments combined with 0.5 μg/ml CPE showed similar leptin secretion to the negative control. Only the 15 mM leucine + 0.5 μg/ml CPE treatment significantly increased leptin secretion above that of the negative control, by 14%, but was still significantly lower than the negative control with acetone solvent and the positive control.

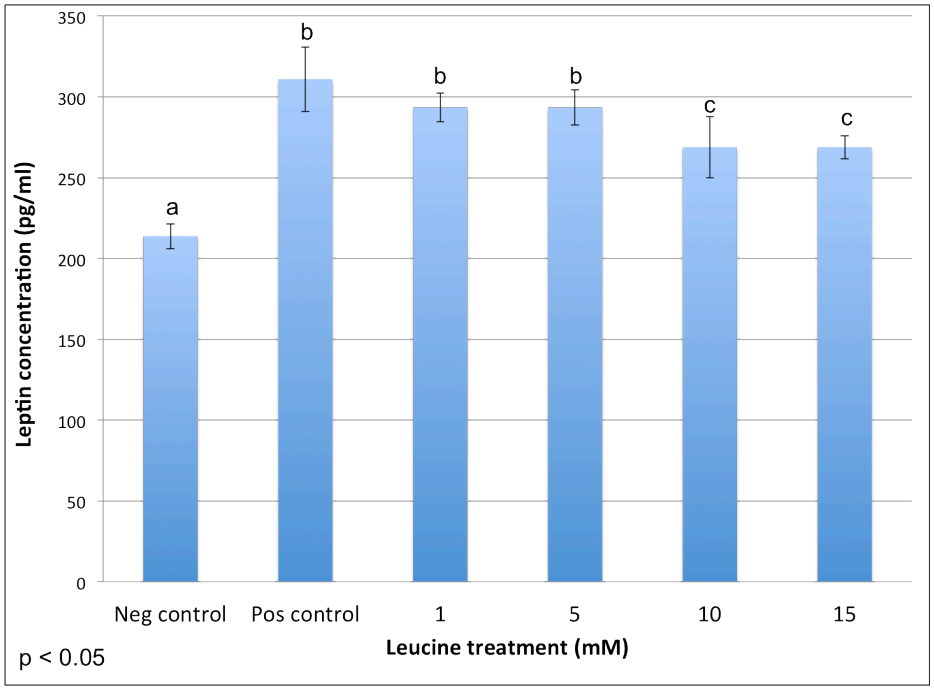


**Figure 2.11.** Leptin secretion in adipocytes treated with leucine. Treatments with same letter are not statistically different. Data expressed as mean ± S.E.
Neg control: complete growth media, Pos control: complete growth media + 0.5 µM insulin

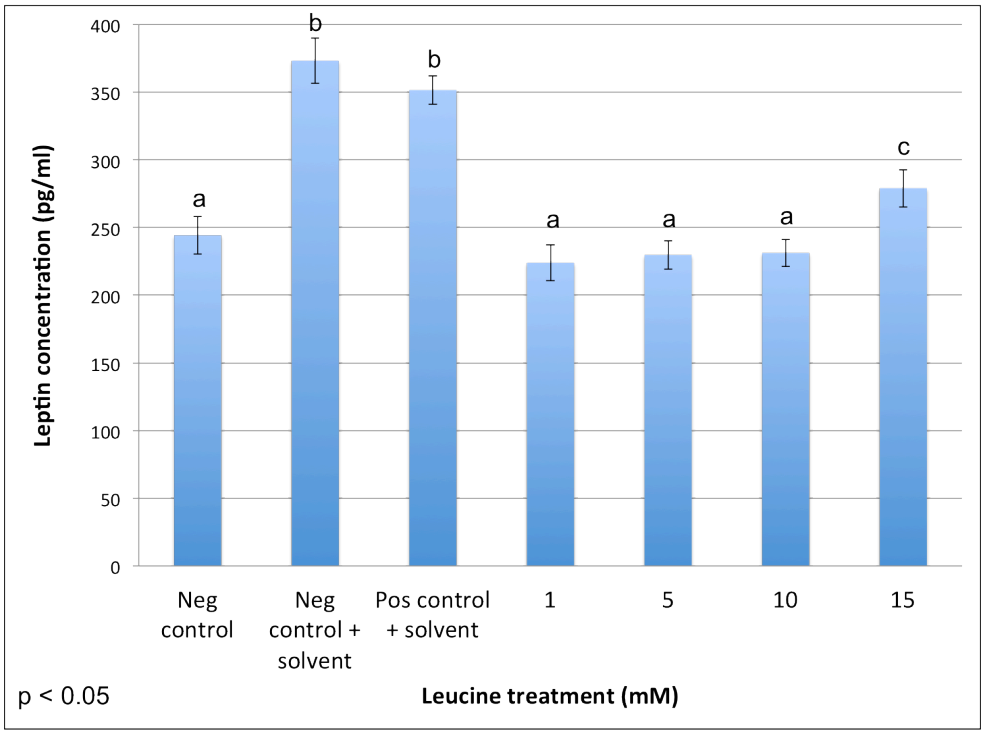


**Figure 2.12.** Leptin secretion in adipocytes treated with leucine and 0.5 µg/ml CPE. Treatments with same letter are not statistically different. Data expressed as mean ± S.E.
Neg control: complete growth media, Neg control + solvent: complete growth media + 100 µg/ml acetone solvent, Pos control + solvent: complete growth media + 0.5 µM insulin + 100 µg/ml acetone solvent

While this study found increases in leptin secretion after CPE and leucine treatment, the increases observed here were not as pronounced as those in similar studies. *In vitro* induction of adipocyte leptin secretion by leucine was demonstrated by Roh et al. (2003). Treatment of adipocytes with 5 mM leucine increased leptin secretion 4-5 fold within four hours of treatment (Roh et al. 2003). Cammisotto et al. (2006) showed that leucine and glutamate increased leptin secretion by almost 2-fold and slightly increased intracellular leptin content. *In vivo*, a high leucine meal increased plasma leptin in rats by 3 fold, peaking three hours post-feeding (Lynch et al. 2006). The same authors found that *in vivo* leptin

secretion decreased by 40% in rats fed leucine-deficient meals (Lynch et al. 2006). Oral gavage of leucine and norleucine also increased plasma leptin concentrations, but not to the same extent as when incorporated into a meal.

The differences in magnitude of leptin secretion observed in this study may be explained by the treatment method. This study examined the effects of leucine supplementation of basic growth media while studies by (Roh et al. 2003, Cammisotto et al. 2005) treated adipocytes with leucine in phosphate buffer, in the absence of other energy substrates. The availability of all necessary energy substrates in the present study may have diminished the expected increases in leptin secretion.

Cammisotto et al. (2005) showed that the presence of glucose was correlated with the maintenance of basal leptin and slight increases in leptin secretion in adipocytes. However, high glucose levels did not increase leptin production beyond that of low glucose treatment (Cammisotto et al. 2005). As aforementioned, insulin and leucine have both been found to be positive regulators of leptin secretion through the mTOR-signaling pathway (Lynch et al. 2006). Though insulin increases mTOR signaling and leptin secretion and served as the positive control in this study, it is important to note that insulin and leucine regulate mTOR through different mechanisms, an important, but subtle, distinction in signaling that could account for the complexities observed in leptin secretion studies *in vitro* (Avruch et al. 2008).

Treatment with insulin or amino acids have been shown to strongly increase leptin synthesis: insulin doubled leptin production in the presence of glucose while the effects of individual amino acids varied (Cammisotto et al., 2005, Cammisotto and Bendayan 2007). L-glycine and L-alanine maintained basal leptin production but did not contribute to increased

leptin secretion in the presence of insulin. L-aspartate, L-valine, L-methionine, and L-phenylalanine increased leptin secretion in the presence of both insulin and glucose (Cammisotto et al. 2005). L-leucine and L-glutamate increased leptin levels in the presence and absence of insulin or glucose. In addition, it was found that these dietary substrates must be broken down in order to induce leptin secretion (Cammisotto et al. 2005). The basic DMEM + FBS growth media used to culture pre-adipocytes contained all of these basic amino acids as well as glucose. Thus, interacting effects of the other energy substrates may have masked the effects of leucine.

The amino acid supplementation method may also explain the decrease in leptin secretion with increasing leucine treatment concentration. Above 10 mM, the leucine content may have saturated cellular receptors so that the presence of additional amino acids had no further effect upon mTOR signaling. Though more complicated to interpret, the media supplementation method may provide a more realistic observation of the effects of leucine upon adipocytes *in vitro* and thus, may serve as a better model and predictor for higher animal studies.

Few studies have examined the effects of polyphenolic compounds on leptin and signaling peptide secretion in adipocytes. (−)-Catechin and green tea polyphenolic treatment of 3T3-L1 adipocytes increased secretion of adiponectin, a protein that regulates glucose levels and fatty acid metabolism (Cho et al. 2006). It was observed that (−)-catechin suppressed expression of Kruppel-like factor 7, increasing adiponectin levels as well as leptin, PPAR-γ, and C/EBP-α expression. *In vivo*, Décordé et al (2009) supplemented high fat hamster diets with chardonnay grape polyphenols, which prevented weight gain,

increased adiponectin production, and decreased insulin resistance. However, leptin production was also decreased. Treatment of insulin-resistant 3T3-L1 adipocytes with 4-100 μg/ml polyphenolic extracts from *Hibiscus sabdariffa* was found to significantly decrease secretion of eight adipokines, including leptin secretion by 20-50% (Herranz-López et al. 2012). The results from Herranz-López et al (2012) and the present study suggest that the positive or negative effects of polyphenols upon biomarkers of obesity may be concentration-specific. Polyphenolic treatment *in vitro* may follow a J-shaped curve, in which lower concentrations of polyphenolics decrease obesity risk factors while higher treatments increase risk.

Any *in vitro* cell culture study must mention the inherent variability of cells grown outside of the body. Adipocytes *in vitro* have been shown to express only 1% of the total leptin expressed by adipocytes *in vivo*, somewhat limiting their use as a model of fat mass *in vivo* (MacDougald et al. 1995). It follows that the picogram level leptin concentrations quantified by ELISA assays *in vitro* require the investigator to make conclusions from slim margins in leptin secretion. However, the conclusions extrapolated from *in vitro* observations serve as an adequate initial indication of adipocyte response to specific and controlled environmental conditions.

## 2.5 CONCLUSIONS

These cell culture experiments evaluated the effects of CPE and leucine on three aspects of obesity and weight management: pre-adipocyte differentiation, adipocyte lipid accumulation, and adipocyte leptin secretion. The results indicate that both cocoa

polyphenols and whey protein metabolites have the potential to positively affect lipid metabolism and satiety signaling. However, these compounds appear to have greater impacts upon specific aspects of physiological obesity and satiety.

Cocoa polyphenolic treatment had the greatest effect upon limiting lipid accumulation in pre-adipocytes during differentiation. Significant decreases in pre-adipocyte differentiation and lipid accumulation of 35 and 50% were found at CPE concentrations of 50 and 100 μg/ml, respectively. CPE treatment of adipocytes post-differentiation significantly decreased lipid accumulation by 19 and 16% at 10 and 100 μg/ml, respectively. This indicates that cocoa polyphenols are more effective in preventing formation of new fat cells than preventing lipid storage and expansion of existing fat cells. Leucine treatment had no effect upon adipocyte lipid metabolism but combinations of 15 mM leucine and 50 and 100 μg/ml CPE treatment during differentiation decreased cellular lipids by 22 and 36%, respectively. Thus, CPE treatment was slightly less effective in the presence of a leucine.

0.5 μg/ml CPE increased leptin secretion by 66% over the negative control but was not significantly higher than the positive control. 50 and 100 μg/ml CPE treatment almost completely inhibited leptin secretion. 1-15 mM leucine increased leptin by 26-37% and the combination of 0.5 μg/ml CPE and 15 mM leucine increased leptin secretion by 14% above the negative control but was significantly lower than the positive control. CPE was observed to have opposing concentration-dependent effects upon both lipid accumulation and leptin secretion. Of the outputs measured, leucine had a much greater impact upon leptin secretion than lipid accumulation.

This study indicates that cocoa polyphenols may be beneficial in decreasing body fat and that both cocoa polyphenols and leucine may increase satiety by upregulating adipocyte leptin secretion. Cocoa and whey ingredients, individually and in combination, show promise in the formulation of foods that increase satiety and fat metabolism and aid in weight management and the mitigation of hunger. The cocoa extract tested in this study contained cocoa polyphenolics as they exist in plants, rather than their *in vivo* metabolites. To better model the effects of cocoa consumption *in vivo*, *f*urther research must be conducted to determine the effects of catechin and proanthocyanidin metabolites upon pre-adipocyte lipid accumulation and adipocyte leptin production.

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# CHAPTER 3: THE EFFECTS OF COCOA POLYPHENOLICS AND WHEY PROTEIN ON BIOMARKERS OF SATIETY AND GLUCOSE METABOLISM IN A HUMAN CLINICAL TRIAL

## 3.1 ABSTRACT

Low-calorie, hunger-suppressing snacks are important in regulating daily calorie intake and in preventing long-term weight gain. While flavored whey protein beverages are popular supplements for muscle recovery, the combined effects of whey protein and cocoa upon satiety are unknown. Whey protein has been demonstrated to decrease subjective ratings of hunger and *ad libitum* calorie intake as well as to increase levels of satiation and satiety hormones. Cocoa polyphenolics may moderate glucose levels and increase physiological and psychological markers of satiety. This study was a six-week clinical trial in which nine healthy panelists (age: 22.6 ± 1.7; BMI: 22.3 ± 2.1) consumed chocolate-protein beverages once per week. Isocaloric beverage formulations included: placebo, whey protein isolate (WPI), low polyphenolic cocoa (LP), high polyphenolic cocoa (HP), LP-WPI, and HP-WPI. Panelists responded to a hunger questionnaire, measured blood glucose levels, and provided blood samples in a fasted state and 0.5, 1, 2, and 4 hours following beverage consumption. Hunger ratings were measured using a 100 mm visual analogue scale (VAS), blood glucose levels were measured using a commercial blood sugar meter, and serum leptin and adiponectin levels were assayed with appropriate ELISA kits. Beverage formulation with WPI decreased ratings of hunger while HP cocoa suppressed hunger more than LP cocoa. At 30 minutes post-consumption, the WPI, HP-WPI, and LP-WPI beverages increased blood glucose 8, 12, and 16% above baseline levels, respectively, compared to increases of 27, 39, and 58% in HP, LP, and placebo beverages, respectively. The moderation of blood glucose levels by WPI and HP cococa was statistically significant as compared to the placebo. Serum leptin decreased after consumption of all beverages while all WPI and cocoa-containing

beverages significantly increased adiponectin levels above that of the placebo. This study indicates that WPI and high polyphenolic cocoa may have beneficial effects, alone and in combination, upon feelings of satiety and blood glucose and adiponectin levels.

## 3.2 INTRODUCTION

Obesity is an epidemic with a multitude of causes, including the availability, affordability, and convenience of high calorie foods as well as an increase in sedentary lifestyles (Asp 1999, Finkelstein, Ruhm, and Kosa 2005). The prevailing treatment of obesity advises a reduction in calorie consumption and an increase in energy expenditure. However, this ideology is based solely upon physiological factors that impact food intake and does not consider more subjective, psychological influences including social and emotional factors. From a scientific perspective, these factors are difficult, perhaps impossible, to control. In addition, research suggests that individual-specific physiological conditions, such as leptin resistance, may hinder or prevent weight loss (Lustig 2006, Myers, Cowley, and Münzberg 2008). These sometimes conflicting and entangled factors only increase the difficulty in identifying effective approaches to weight management.

Food structure and texture, as well as macro and micromolecular composition, have physiological and psychological effects upon food consumption and fullness between meals. Altering the formulation, oral perception, and breakdown of food products may increase feelings of satiety and decrease calorie intake. Specifically, foods that require extensive chewing action or that provide a large volume with low calorie density, such as soups and beverages, may slow food consumption and signal additional fullness to the brain (Mattes

and Campbell 2009). Generally, subjective rating and *ad libitum* feeding studies have indicated that solid foods induce greater satiety than liquids (Tieken et al. 2007, Leidy, Bales-Voelker, and Harris 2011). A study of oral and gastric stimulation found that both chewing without ingestion of food and intestinal infusion with liquid decreased *ad libitum* food intake (Wijlens et al. 2012). Though it is generally accepted that solid foods provide higher satiety than liquid foods when total calories and macronutrients are equal, many studies also indicate that the stomach-filling sensation induced by liquid foods may compensate for the lack of mastication time (Stull et al. 2008). However, these studies have not found differences in satiety hormone profiles between solid and liquid foods.

Selection of specific ingredients may also aid in controlling fat accumulation and satiety, or the feeling of fullness between meals (Cammisotto et al. 2005, Sun and Zemel 2007, Min et al. 2012). Protein and fiber in particular appear to be determining factors of post- and between-meal satiety (Veldhorst et al. 2008, Benelam 2009). Pasiakos et al. (2013) showed that variations in protein consumption did not affect total weight loss but that higher protein consumption (2-3 times the recommended dietary intake) increased overall fat loss and improved body composition. In general, diets consisting of greater than 30% calories from protein are more effective in inducing satiety and reducing food intake (Weigle et al. 2005, Lejeune et al. 2006, Veldhorst et al. 2008). It is suggested that the feeling of satiety provided by protein requires fewer grams and calories than that obtained from carbohydrates or lipids, thereby reducing total calorie intake and speeding weight loss. This satiety may be attributed to an increase in plasma levels of the satiation and satiety hormones GLP-1, CCK,

PYY and leptin or an increase in plasma amino acid levels (Westerterp-Plantenga et al. 1999, Lejeune et al. 2006).

While it is apparent that protein induces greater satiety than lipids or carbohydrates, there is no uniform agreement on a specific type or percent of daily protein calories necessary for increasing satiety (Benelam 2009). Clinical trials by Lang et al. (1998) found no differences between egg albumin, casein, gelatin, soy protein, pea protein, or wheat gluten on satiety. Aldrich et al. (2011) also found no significant differences in weight loss, fat loss, or satiety ratings among subjects fed low protein, whey protein, or mixed protein diets. However, the whey protein diet induced greater regional fat loss and lowered blood pressure (Aldrich et al. 2011). Of those studies that indicate differences between specific proteins, whey, pea, and soy appear most promising in increasing satiety (Anderson et al. 2004, Diepvens, Häberer, and Westerterp-Plantenga 2007).

In comparison to other proteins, whey has a higher proportion of branched chain amino acids and is more rapidly absorbed, quickly increasing plasma amino acids levels (Boirie et al. 1997, Farnfield et al. 2009). Pal and Ellis (2010) found that a whey protein meal increased satiety and plasma insulin response and decreased *ad libitum* intake at a subsequent meal when compared to tuna, turkey, and egg albumin. Direct comparisons of milk proteins found that whey protein decreases *ad libitum* food intake by 19% and increases satiety ratings when compared to casein (Hall et al. 2007). Whey proteins also increased postprandial plasma amino acids, CCK, and GLP-1 levels by 28%, 60%, and 65%, respectively, above that of casein (Hall et al. 2007). This effect may be attributed to amino

acid stimulation of the mammalian target of rapamycin signaling (mTOR) pathway, which regulates insulin signaling and muscle synthesis (Morrison et al. 2007).

A limited amount of research has indicated that micronutrients such as polyphenolic compounds may also contribute to satiety and fat metabolism. Polyphenols bind strongly to proteins and have been shown to bind digestive enzymes *in vitro*, demonstrating their potential to slow digestion and nutrient metabolism (Gu et al. 2011, Barrett et al., 2013). *In vivo*, green tea catechins were also shown to increase satiety and fullness and to limit lipid nutrient absorption by inhibiting pancreatic phospholipase $A_2$ (Wang, Noh, and Koo 2006, Josic et al. 2010). Chocolate-specific human clinical studies show that consumption of polyphenol-rich dark chocolate decreased hunger ratings and *ad libitum* intake by 17% and increased satiety more than milk chocolate (Sørensen and Astrup 2011). In obese-diabetic rats, treatment with cocoa extract was found to significantly reduce plasma glucose, free fatty acid, and oxidative stress biomarker levels as well as to increase the activity of the antioxidant enzyme superoxide dismutase in the hours following treatment (Jalil et al. 2008).

The available literature and results of cell culture studies reported in Chapter 2 suggest that cocoa and whey protein ingredients, individually and in combination, may increase satiety and prevent lipid accumulation. The present study is the first to examine the effects of high cocoa polyphenolic doses on satiety and the first to combine these polyphenolics with whey protein in a human clinical trial. The aim of the study was to isolate the effects of cocoa polyphenolics and whey proteins upon physiological satiety and glucose metabolism. We hypothesized that combinations of cocoa and whey protein would exhibit

additive or synergistic modulatory effects upon satiety hormone levels, subjective ratings of satiety, and plasma glucose levels.

## 3.3 MATERIALS AND METHODS

### 3.3.1 *SUBJECTS*

All procedures used in this study were approved through the North Carolina State University Institutional Review Board. Panelists were recruited via email advertisements and announcements made to the North Carolina State University Food, Nutrition, and Bioprocessing department and undergraduate classes. Panelist criteria were based on the following: men and women between 18 and 35 years old, healthy weight (body mass index between 18 and 25 and/or waist to hip circumference ratio of ≤0.95 for men and ≤0.8 for women), not dieting, no loss or gain of significant weight (defined as 10% of body weight) in the last year, hadn't recently started or ceased a regular exercise program, no dietary allergies or intolerances to dairy or cocoa ingredients, and no history of Type I or Type II diabetes. A total of 9 panelists were selected based upon the above requirements and an initial screening which involved body mass index and hip to waist ratio measurements as well as surveys of medical history, eating behavior, and physical activity. Baseline panelist information is listed in Table 3.1. All panelists were informed of the procedures, outcomes, potential risks, and benefits of study participation and gave written informed consent prior to beginning the trial. Monetary compensation was also provided for trial participation.

**Table 3.1.** Panelist measurements at beginning of trial.

| Panelist attribute | |
|---|---|
| **Age (years)** | 22.6 ± 1.7 |
| **Weight (kg)** | 65.5 ± 8.1 |
| **Height (m)** | 1.71 ± 0.05 |
| **BMI (kg/m$^{-2}$)** | 22.3 ± 2.1 |
| **Waist (cm)** | 79.9 ± 5.7 |
| **Hip (cm)** | 98.8 ± 5.0 |
| **Waist/hip ratio** | 0.81 ± 0.03 |

n = 9, 4 females and 5 males
Abbreviations: BMI: body mass index

### 3.3.2 *ANTHROPOMETRY*

Panelist weight was measured to the nearest kilogram using a Taylor lithium electronic digital scale accurate to 0.1 kg (Oak Brook, IL, USA) and height was measured to the nearest centimeter using a wall-mounted ruler. Hip and waist measurements were measured to the nearest centimeter via a tape measure (Stanley, New Britain, CT, USA) with panelists in standing position. Hip measurement was defined by the widest circumference between the waist and thighs, while waist measurement was the narrowest circumference near the belly button.

### 3.3.3 *EXPERIMENTAL DESIGN*

This was a randomized study including 6, 4-hour sessions per participant. For each session, panelists fasted overnight (no food, caloric beverages, or smoking for 12 hours) and arrived at the laboratory at 7 am. Panelists were also required to refrain from vigorous physical activity for 24 hours prior to a scheduled session. In addition, panelists were asked to refrain from consuming high polyphenolic foods the night before a session, including

cooca/chocolate products, tea, wine, berries, and peanuts. Upon arrival, panelists filled out a baseline hunger questionnaire (Figure A.3), sterilized their fingertip with an alcohol wipe, and used a Fisherbrand sterile high flow safety lancet (Waltham, MA, USA) to prick their finger and draw blood. Each panelist used a Nova Max Plus glucose/ketone monitor and Nova Max blood glucose test strips (Waltham, MA, USA) to determine their baseline blood glucose level and then provided 200 μl of blood into a BD microtainer serum separator blood collection tube (Waltham, MA, USA). The panelist instructions used in each session is shown in Figure A.2.

After initial blood glucose readings and blood collection, panelists consumed a 340 gram (12 oz) beverage. Following beverage consumption, the beverage container was washed and shaken with 200 ml of water, which was then consumed by the panelist to ensure ingestion of the entire dosage. The test beverage consumed by panelists at each session was randomized and each beverage was consumed within 10 minutes. Panelists ranked their hedonic liking of the beverage immediately following consumption (Figure A.3). Panelists provided blood samples, tested blood glucose levels, and ranked their appetite at 30, 60, 120, and 240 minutes following beverage consumption.

### 3.3.4 *QUESTIONNAIRE*

During each session, panelists recorded their blood glucose readings and feelings of satiety at each time point. The questionnaire asked the panelists to rate their feelings of satiety on a 100 mm horizontal visual analogue scale (VAS), anchored at both ends with qualifying statements. The statement “not at all hungry” was labeled at 0 mm while 100 mm

was labeled with the statement “extremely hungry.” This measurement method was validated by (Flint et al. 2000) for single test meal studies. Panelist ratings were measured by ruler and quantified in millimeter values. Panelists also scored their liking of the overall beverage, beverage flavor, and beverage texture/mouthfeel post-consumption on a 9-point hedonic scale. The complete form used to record panelist responses and blood glucose levels is shown in Figure A.2 and A.3.

### 3.3.5 *MATERIALS*

Regular (high polyphenolic) and Dutched (low polyphenolic) cocoa powder was provided by The Hershey Company (Hershey, PA, USA). BiPRO whey protein isolate was provided by Davisco, Inc (Le Sueur, MN, USA). Clintose maltodextrin CR10 (DE 10), Fibersol-2 resistant maltodextrin, and crystalline fructose were provided by ADM (Decatur, IL, USA). A carrageenan-based hydrocolloid stabilizer was provided by TIC Gums (White Marsh, MD, USA).

### 3.3.6 *BEVERAGE FORMULATION AND PREPARATION*

The beverages tested are outlined in Table 3.2. Beverage formulations included:

1. Placebo
2. Whey protein isolate (WPI)
3. Low polyphenolic cocoa (LP)
4. High polyphenolic cocoa (HP)
5. LP + WPI
6. HP + WPI

The WPI-containing beverages were designed to deliver 10 g total protein (20% of DRI, an excellent source of protein). The HP beverages delivered 1,580 mg of total flavanols as

determined by the DMAC (4-dimethylaminocinnamaldehyde) assay or 870 mg of total cocoa proanthocyanidins, as measured by the proanthocyanidin 1-10 (PAC 1-10) assay, both performed by The Hershey Company. The PAC 1-10 assay measured all present proanthocyanidins composed of 1 to 10 monomer units. The LP served as a control for the effect of polyphenolic compounds by providing the fiber and other nutrients regularly found in cocoa but very low levels of flavanols and proanthocyanidins (6.7 mg by DMAC and 1.8 mg by PAC 1-10). The comparison of DMAC and PAC 1-10 analyses of HP and LP are found in Table A.4. It must be stated that the LP cocoa still contained flavanols but at a much lower concentration. Maltodextrin was used as a direct replacement for WPI in the placebo, LP, and HP samples. Fibersol-2, a digestion-resistant maltodextrin, was used to replace cocoa in the placebo and WPI-only sample. All beverages were isocaloric and provided 150 calories.

**Table 3.2.** Formulation of the six test beverages.

| Ingredients (g) | Placebo | WPI | LP | HP | LP-WPI | HP-WPI |
|---|---|---|---|---|---|---|
| Water | 283.8 | 283.8 | 283.8 | 283.8 | 283.8 | 283.8 |
| HP cocoa | - | - | - | 36 | - | 36 |
| LP cocoa | - | - | 36 | - | 36 | - |
| WPI | - | 10.8 | - | - | 10.8 | 10.8 |
| Fibersol-2 | 36 | 36 | - | - | - | - |
| Maltodextrin | 10.8 | - | 10.8 | 10.8 | - | - |
| Fructose | 8.6 | 8.6 | 8.6 | 8.6 | 8.6 | 8.6 |
| Stabilizer | 0.75 | 0.75 | 0.75 | 0.75 | 0.75 | 0.75 |

Fibersol-2 and maltodextrin served as direct replacements for cocoa and WPI, respectively.
Abbreviations: WPI: whey protein isolate; LP: low polyphenolic cocoa; HP: high polyphenolic cocoa

Bulk dry mixes of each beverage treatment were prepared by combining the dry ingredients and mixing in a KitchenAid stand mixer (St. Joseph, MI, USA) for three minutes on speed two followed by hand mixing for two minutes. This mix was then divided into single beverage portions and stored until use. Twelve hours prior to panelist consumption, each portion was hydrated with deionized water to 340 grams in a BlenderBottle SportMixer shaker bottle (Lehi, UT, USA) by shaking vigorously for three minutes. All beverages were refrigerated overnight at 4°C and shaken again immediately before panelist consumption.

### 3.3.7 *SERUM PEPTIDE ANALYSIS/BLOOD PARAMTERS*

Blood samples were collected in BD Microtainer serum separator blood collection tubes (Waltham, MA, USA) at five time points (0, 30, 60, 120, and 240 minutes) for leptin and adiponectin analysis. Serum was prepared by allowing the blood samples to clot at room temperature for 30 minutes followed by centrifugation for 2.5 minutes at 6,000 g. The serum layer was transferred to 600 μL tubes and stored at -80C. Analysis for serum leptin and adiponectin levels were performed using human leptin ELISA kits and human ADIPOQ/Adiponectin ELISA kits, respectively, all purchased from Sigma-Aldrich (St. Louis, MO, USA). All analyses were performed per the manufacturer's instructions. Leptin and adiponectin levels were expressed as percent changes from baseline (fasted, time point 1).

### 3.3.8 *STATISTICAL ANALYSIS*

Randomization of sample order for each panelist was established using a randomized block design using JMP software (Cary, NC, USA). Due to the inherent variability between

panelists, hunger ratings, blood glucose, leptin, and adiponectin data were calculated using the percent change from baseline (fasted, time 0) for each panelist. The experimental design was a randomized complete block split plot in time, where panelists served as complete blocks and beverages as the whole plot blocks, measured repeatedly in time. For each of four responses (hunger rating, blood glucose level, leptin, and adiponectin), the linear mixed effects model, appropriate to the design, was fit with all factorial effects: beverage, time, and all possible interactions and random effects for panelist and panelist-by-beverage. The levels of "beverage" are combinations of two crossed factors, cocoa and whey, and were coded as such in all analyses of variance. Statistical analysis utilized SAS software (Cary, NC, USA). The data are presented as mean ± S.E.

## 3.4 RESULTS AND DISCUSSION

### 3.4.1. *EFFECTS OF COCOA POLYPHENOLS AND WHEY PROTEIN ON SUBJECTIVE RATINGS OF HUNGER*

Panelist hunger ratings were quantified by marking a 100 mm visual analogue scale (VAS) at each time point. The distance of this mark from 0 ("not at all hungry") was recorded in millimeters. Figure 3.1 shows the changes in average panelist hunger ratings following beverage consumption. All beverages similarly decreased feelings of hunger 30 minutes following beverage consumption and hunger was significantly decreased at the 30 and 60-minute time point. The two-hour time point was not significantly different from baseline (time 0) hunger ratings but the four-hour rating was significantly higher than all

previous time points. No significant differences were found in overall hunger ratings (hours 0-4) between beverage treatments, nor between any individual cocoa or whey variables.

One to four hours post-consumption, the WPI and HP-WPI beverages were most effective in controlling hunger ratings. The LP beverage was the least effective at controlling hunger. After 30 minutes, hunger levels increased quickly for the LP cocoa beverage and slightly more slowly for the placebo, WPI, HP, and LP-WPI samples. Cocoa-only beverages (HP or LP) elicited the highest final hunger ratings. The remaining beverages showed similar final hunger ratings four hours post-consumption. The HP-WPI beverage most effectively controlled hunger between hours one and four. This beverage showed the slowest increases in hunger over time but reached a similar final hunger rating as the WPI and LP-WPI beverages.

Because panelist satiety ratings may have been impacted by beverage hedonic factors, panelists also rated their overall, flavor, and texture liking of each beverage. The results of the hedonic questionnaire are provided in Table A.4. Overall liking was shown to be more highly correlated with flavor than texture. From panelist comments, the bitterness and astringency of the high dose cocoa polyphenolics appeared to reduce panelist liking while the addition of WPI to HP and LP beverages appeared to increase beverage liking. This may be due to a change or masking of cocoa flavor and gritty texture.

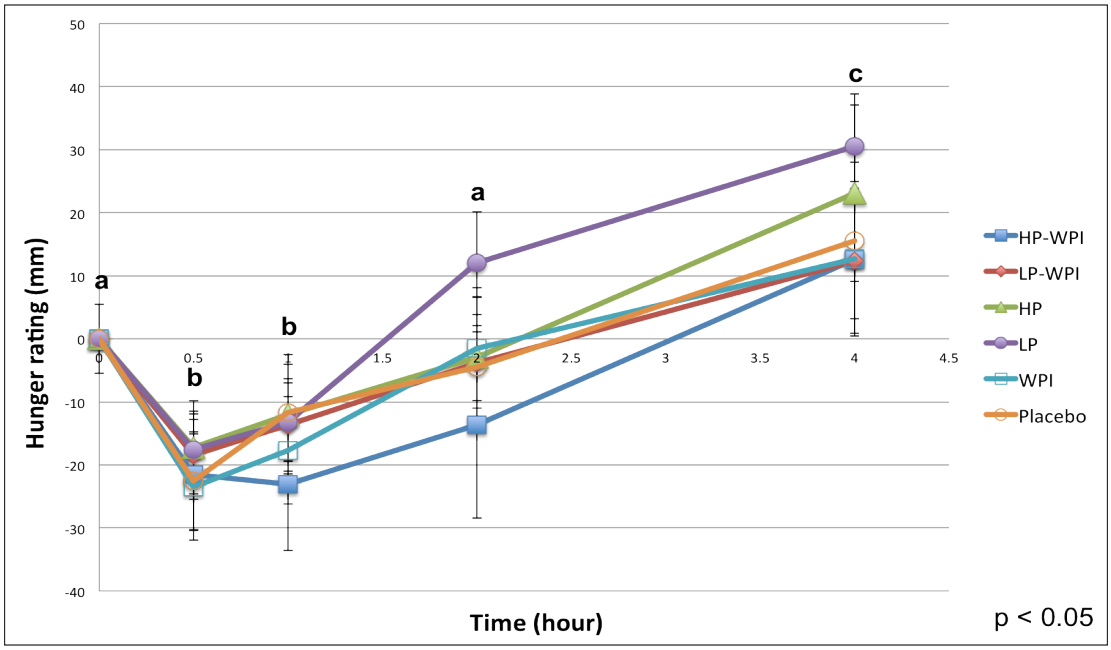


**Figure 3.1.** Changes in hunger ratings before and after beverage consumption. Values expressed as changes from baseline in mean ± S.E. from a 100 mm visual analogue scale. Letters refer to significant differences; time points with same letter are not statistically different.
n = 9
Abbreviations: LP: low polyphenolic cocoa, HP: high polyphenolic cocoa, WPI: whey protein isolate, LP-WPI: low polyphenolic cocoa + whey protein isolate, HP-WPI: high polyphenolic cocoa + whey protein isolate.

The WPI-containing beverages contained 10 g of WPI, representing 29% of the total calories. This dose was selected to provide an "excellent" source of protein as defined by the USDA Dietary Reference Intake for protein and is comparable to chocolate protein beverages on the market (Food and Nutrition Board, 2013). In addition, it has been shown that diets consisting of greater than 30% calories from protein induce satiety and decrease food intake better than lower protein diets (Veldhorst et al. 2008). In test beverages without WPI, WPI was replaced isocalorically with maltodextrin.

Two test beverages containing whey protein slowed hunger response two hours (WPI) and three hours (HP-WPI) post-consumption. These ingredient-based changes in satiety ratings are supported by the literature. Weigle et al. (2005) and Lejeune et al. (2006) found that when compared to a 10 or 15% protein diet, a 30% protein diet increased satiety ratings, decreased *ad libitum* food intake, and increased average weight loss in separate clinical trials. Veldhorst et al. (2009) also found that breakfasts containing 25% calories from protein (casein, whey, or soy) induced greater satiety than their 10% protein counterparts.

Mellinkoff et al. (1997) postulated that increases in plasma amino acid levels are associated with higher satiety and reduction in appetite. If true, this provides a distinction between macronutrients in regards to weight loss: fewer protein calories could initiate the same level of satiety as greater calorie intakes of carbohydrates or lipids. In mice, increases in plasma amino acids that were not destined for protein synthesis increased satiety, while an increase in appetite was accompanied by decreases in plasma amino acids. Morrison et al. (2007) found that intracerebroventricular injections of amino acids or leucine alone reduced caloric intake in mice over 24 hours. In the present study, it appears that the presence of amino acids in the form of whey protein decreased hunger as compared to the carbohydrate control. This satiety may be attributed to an increase in plasma levels of the satiation hormones GLP-1, CCK or PYY, which will be monitored in future studies (Westerterp-Plantenga et al. 1999, Lejeune et al. 2006, Diepvens, Häberer, and Westerterp-Plantenga 2007).

The combination of HP cocoa and WPI in this study had the greatest effect upon suppressing hunger ratings. While the HP-only beverage did not exhibit the same hunger

suppressing effects as HP-WPI, the HP-WPI beverage suppressed hunger ratings over the LP-WPI beverage. This indicates that the cocoa polyphenols may have played a role in appetite suppression. While WPI increases plasma amino acid levels to suppress hunger, cocoa polyphenols may bind digestive enzymes and slow digestion and nutrient metabolism. *In vitro*, tannins and procyanidins in cocoa, pomegranate, cranberry, and grape extracts have been shown to inhibit α-amylase, glucoamylase, pancreatic lipase, and secreted phospholipase $A_2$ activity (Gu et al. 2011, Barrett et al., 2013). *In vivo*, green tea catechins were also shown to increase satiety and fullness and to limit lipid nutrient absorption by inhibiting pancreatic phospholipase $A_2$ (Wang, Noh, and Koo 2006, Josic et al. 2010).

Chocolate-specific human clinical studies have shown that consumption of polyphenol-rich dark chocolate decreased hunger ratings and *ad libitum* intake by 17% and increased satiety more than milk chocolate (Sørensen and Astrup 2011). Eating or simply smelling chocolate also increased satiety ratings, though smelling had no effect upon known physiological biomarkers of hunger and satiety (Massolt et al. 2010). The comparison of chocolate and yogurt snacks before a meal found increased fullness ratings after eating yogurt, which is higher in protein (Chapelot and Payen 2009). However, the duration of satiety was similar between both foods and there was no difference in *ad libitum* intake at the next meal. The hunger ratings reported in the present study indicate that whey protein was the major factor in determining satiety four hours post-consumption. Cocoa polyphenolics were a secondary, and minimized, determining factor. Due to wide variances in individual panelist hunger ratings, further studies with much larger sample sizes are necessary to

determine the psychological and physical effects of WPI and cocoa upon feelings hunger and satiety.

### 3.4.2. *EFFECTS OF COCOA POLYPHENOLS AND WHEY PROTEIN ON BLOOD GLUCOSE LEVELS*

Blood glucose levels before and following beverage consumption were determined via commercial glucose meters and recorded in mg/dL. Figure 3.2 shows the percent changes in average blood glucose levels four hours following beverage consumption. All changes were measured as percent changes from baseline at time 0 (fasted). The most significant difference among beverage formulations occurred 30 minutes following beverage consumption. At this time point, the WPI, HP-WPI, and LP-WPI beverages increased blood glucose 8, 12, and 16% above baseline levels, respectively, compared to increases of 27, 39, and 58% in HP, LP, and placebo beverages, respectively. Although all beverages were formulated isocalorically, those with less protein contained more carbohydrates. Predictably, those beverages containing the most high glycemic glucose-based carbohydrates (Placebo, LP, HP) produced the largest spikes in blood glucose. The glucose spikes observed in the placebo, LP, and HP beverages were 7.3, 4.9, and 3.4 times greater than that of the WPI-only beverage. The presence of WPI in the remaining three beverages had a significant moderating effect upon glucose levels over the hours following consumption. A similar statistically significant trend was observed in those beverages containing LP and HP cocoa: the HP cocoa moderated glucose levels better than LP cocoa, whether present alone or combined with WPI. Though all beverages elicited similar glucose levels four hours post-

consumption, the placebo and LP cocoa beverages exhibited more erratic fluctuations than the HP and WPI beverages.

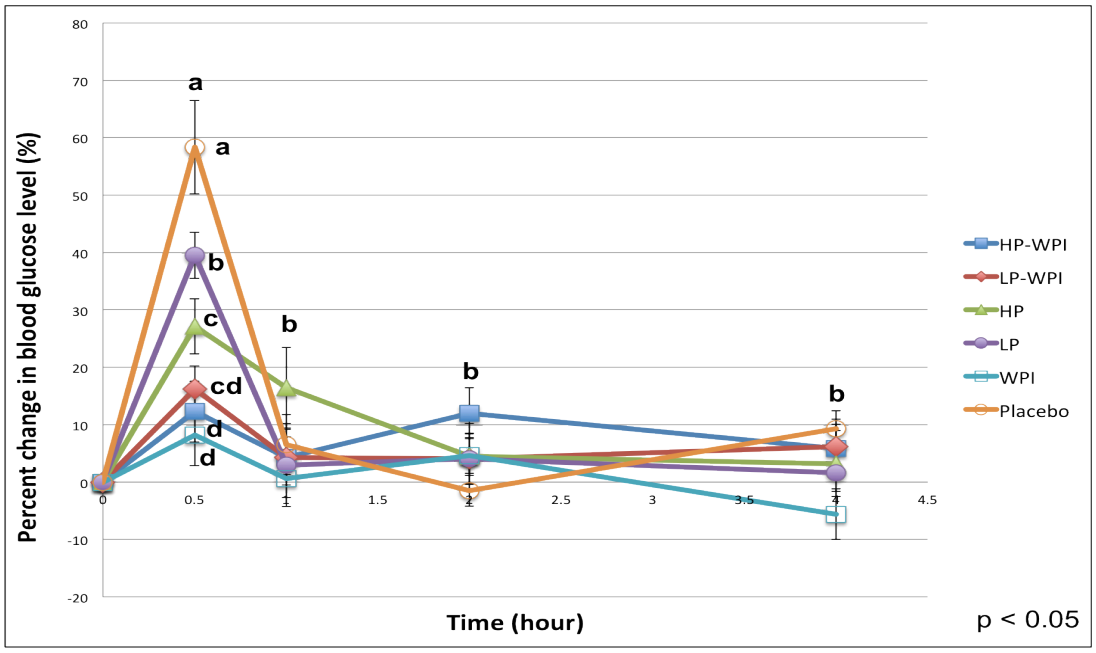


**Figure 3.2.** Percent changes in blood glucose levels before and after beverage consumption. Values expressed as percent changes from baseline in mean ± S.E. Letters refer to significant differences between time points or beverages at the 30-minute time point; variables with the same letter are not statistically different.
n = 9
Abbreviations: LP: low polyphenolic cocoa, HP: high polyphenolic cocoa, WPI: whey protein isolate, LP-WPI: low polyphenolic cocoa + whey protein isolate, HP-WPI: high polyphenolic cocoa + whey protein isolate.

In this study, blood glucose responses to each beverage formulation were indicative of the total glucose or carbohydrate content. Those beverages formulated with maltodextrin to replace WPI (thus increasing glucose content and increasing glycemic index) elicited larger spikes in blood glucose. Fibersol, a resistant starch, was used to replace cocoa in the placebo and WPI beverages. Though not an identical replacement, Fibersol mimicked the

high fiber content of cocoa powder. However, the placebo and LP cocoa beverages elicited large spikes in glucose levels, up to 58% of baseline. Blood glucose levels dropped almost completely to baseline levels one hour post-consumption. While all beverages decreased to similar glucose levels one hour post-consumption, the magnitude of these short-term glucose spikes is indicative of energy level and glucose metabolism. The non-significant increases in blood glucose levels observed between hours two and four after the placebo may be explained by the induction of gluconeogenesis and subsequent glucose transport to the brain.

While proteins alone do not contribute to blood glucose levels, Nilsson, Holst, and Björck (2007) showed that specific protein amino acid compositions may stimulate insulin production. Drinks containing the branched chain amino acids leucine, isoleucine, and valine decreased plasma glucose by 44% and increased insulin production by 31%. A similar drink containing whey protein decreased glucose levels by 56% and increased insulin levels by 60% (Nilsson, Holst, and Björck 2007). Similar effects of WPI on blood glucose levels were observed in the current study, in which the WPI, HP-WPI, and LP-WPI lowered blood glucose levels by 85, 78, and 71%, respectively, as compared to the placebo at the 30-minute time point. The WPI beverage only elicited an 8% increase in blood glucose levels at 30 minutes compared to a 58% increase by the placebo. This is partially explained by the lower carbohydrate content of these beverages, which provide less glucose-based polymers for digestion and circulation. However, it is possible that an increase in plasma amino acids or bioactive peptides found in whey proteins increased the production of insulin, and thus the cellular absorption of plasma glucose.

With or without WPI, beverages containing HP cocoa better moderated changes in blood glucose levels. The LP cocoa beverage produced a 39% increase in blood glucose as compared to a 27% increase following the HP cocoa beverage. A similar, but blunted, trend was observed when cocoa was combined with WPI: the LP-WPI and HP-WPI beverages elicited blood glucose increases of 16% and 12%, respectively. Because the HP and LP beverage formulations only varied in their polyphenolic content, the differences between the initial spikes in blood glucose may be attributed to the polyphenolic content of the HP cocoa.

Plant polyphenolics have also demonstrated effects on blood glucose levels and insulin sensitivity. *In vitro*, 1-10 μM epicatechin and 1-10 μg/ml cocoa polyphenolic treatment increased insulin receptor activity, insulin signaling, and glucose transport while decreasing glucose production in HepG2 cells (Cordero-Herrera et al. 2013). *In vivo*, 0.5% epicatechin consumption in drinking water (equivalent to 250 g of dark chocolate consumption per day) increased insulin levels in mice (Fu, Yuskavage, and Liu 2013). In mouse and human clinical studies, flavanol-rich cocoa or chocolate consumption increased insulin sensitivity, and lowered short-term blood glucose and free fatty acid levels (Matsui et al. 2005, Grassi et al. 2008, Jalil et al. 2009). In obese-diabetic rats, treatment with 1-3% cocoa extract was found to significantly reduce plasma glucose by 60-75% (Ruzaidi et al. 2005). Brand-Miller et al. (2003) even showed that chocolate flavors may have psychological effects upon physiological biomarkers: in the absence of substantial cocoa polyphenols, a variety of chocolate flavored desserts increased plasma insulin levels more than the same vanilla or strawberry-flavored foods.

The mechanisms by which polyphenols moderate changes in plasma glucose levels have been demonstrated *in vitro*. Johnston et al. (2005) and Kobayashi et al. (2000) found that dietary polyphenolics, including catechin, epicatechin, and their derivatives inhibit cellular glucose uptake via steric hindrance of the sodium-dependent glucose transporter (SGLT1) and the facilitated di*ff*usion glucose transporter (GLUT1) in lipid membranes. This would slow cellular glucose uptake and inhibit rapid declines in glood glucose. Alternatively, spikes in blood glucose levels may be moderated by polyphenolic upregulation of GLUT1 and GLUT4 protein synthesis and activation of phosphatidylinositol 3-kinase, a signaling enzyme involved in muscle cell growth (Purintrapiban, Suttajit, and Forsberg 2006). These effects are mediated by polyphenolic upregulation of PPAR-γ, which in turn upregulates genetic expression of insulin and glucose transporters (Ali, Ismail, and Kersten 2014). Jalil et al. (2009) reported that caffeine consumption can also reduce insulin-stimulated glucose uptake, moderating and slowing decreases in blood glucose levels. Dutching, or alkali treatment, of cocoa powder lowers caffeine content by approximately one third (Li et al. 2012). These reported effects of cocoa polyphenolics and caffeine may explain the differences observed in the HP v. LP cocoa beverages in moderating blood glucose levels.

### 3.4.3. *EFFECTS OF COCOA POLYPHENOLS AND WHEY PROTEIN ON SERUM LEPTIN LEVELS*

Figure 3.3 shows the percent changes in mean serum leptin levels following consumption of each beverage. Average panelist leptin levels fell below baseline levels 30-minutes post consumption and remained below baseline at all subsequent time points. Leptin levels reached lowest levels at one or two hours post-consumption. At 30-minutes post-

consumption, leptin levels decreased most in the LP cocoa beverage, followed by the WPI beverage. LP cocoa leptin levels remained the lowest of all treatments until experiencing a steep increase at the four-hour time point. LP-WPI and HP beverages followed similar trends, with leptin levels increasing between the two and four-hour time points. The leptin levels of the remaining beverages, placebo, HP-WPI, and WPI, remained constant between the two and four-hour time points. The LP-WPI and LP cocoa beverages had the highest final leptin levels, with decreases from baseline of 17%.

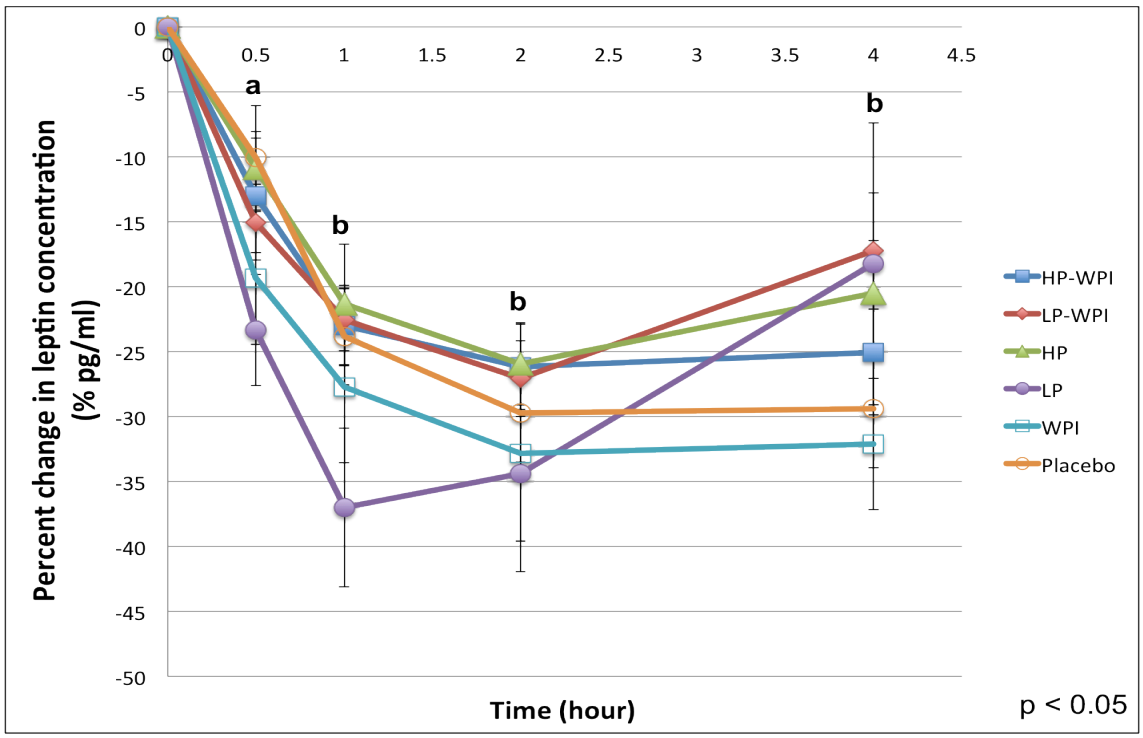


**Figure 3.3.** Percent changes in serum leptin levels before and after beverage consumption. Values expressed as percent changes from baseline in mean ± S.E. Letters refer to significant differences between time points or beverages; variables with the same letter are not statistically different.
n = 9
Abbreviations: LP: low polyphenolic cocoa, HP: high polyphenolic cocoa, WPI: whey protein isolate, LP-WPI: low polyphenolic cocoa + whey protein isolate, HP-WPI: high polyphenolic cocoa + whey protein isolate.

Serum leptin levels unexpectedly decreased, and remained below baseline, following consumption of all beverage treatments. Levels decreased most quickly during the first hour and then slightly increased (LP, LP-WPI, and HP) or remained constant (placebo, WPI, and HP-WPI) between hours two and four. LP cocoa ingredients were associated with higher final leptin levels while WPI-containing beverages were not correlated with a specific pattern in serum leptin.

Based upon previous research, WPI beverages high in leucine were expected to have the greatest impact upon leptin levels. Leucine has been found to regulate leptin secretion *in vitro* through the mTOR-signaling pathway (Lynch et al. 2006). Oral gavage of rats with leucine or norleucine doubled plasma leptin concentrations, but not to the same extent as a regular meal feeding. A meal lacking leucine reduced leptin synthesis by 40% in comparison to a control meal; no reductions in leptin were observed in subsequent meals lacking branched-chain amino acids or all amino acids (Lynch et al. 2006).

It is possible that no increases in leptin were observed because the beverages did not provide enough calories to induce an increase in leptin above baseline. Liu, Askari, and Dagogo-Jack (1999) found that fasting plasma leptin concentrations are reproducible in both lean and obese adults over 26 days, indicating that the below baseline leptin levels observed in this study should be reliable representations across panelists and weekly sessions. The question remains whether the low calorie beverage provided enough energy to stimulate a measureable physiological response. The beverages formulated for this study were intended to mimic the calorie and macronutrient content of a supplementary snack rather than a meal replacement product. These 150-calorie beverages were meant to represent commercial

chocolate-protein beverages that would provide longer and more satisfying hunger control than other higher calorie snack options. The beverages may have been so low in calories that, after a 12-hour fast, the body continued to decrease leptin production until finally sensing the small calorie intake. This is demonstrated by the slight increases in leptin levels between hours two and four in three of the treatments.

Consumption of these beverages appeared to blunt declining leptin concentrations and partially returned the declining levels back to baseline. These observations are supported by Wadikar and Premavalli (2011), who showed that appetizers of 59-94 calories actually decreased plasma leptin levels by 6-16% 30-minutes post consumption, effectively increasing appetite. The control appetizer treatment elicited the smallest decreases in leptin levels. It is possible that if the test beverages in the present study had provided a more substantial calorie content, such as a 300-400 calorie meal equivalent, panelist physiological responses would have been observed faster or may have followed clearer trends. Previous research has also found that *in vitro* and *in vivo*, plasma leptin levels peak three to four hours following meal consumption (Roh et al. 2003, Diepvens, Häberer, and Westerterp-Plantenga 2007). It is possible that in the present study, panelist serum levels were not monitored for enough hours following beverage consumption.

Multiple studies have demonstrated that circulating leptin levels are highly correlated with insulin induction, and thus with glucose uptake. Insulin and glucose infusion studies in humans provide evidence that serum leptin and insulin levels are inversely correlated over 24-hour cycles (Utriainen et al. 1996, Boden et al. 1997, Saad et al. 1998). Serum leptin levels were not associated with serum glucose or free fatty acid levels and increases of at

least 200 pM serum insulin are necessary to significantly increase serum leptin (Boden et al. 1997). Koopmans et al. (1998) showed that increases in serum leptin levels in rats are dose and time dependent: noticeable changes in serum leptin levels were not observed until over four hours of insulin infusion. During a six-hour prolonged fast in lean children, Levitt Katz et al. (2006) also found that steady declines in leptin levels during fasting are associated with decreased insulin secretion.

The beverages used in this study were formulated with fructose, rather than glucose. This was done consciously to avoid misleading glucose-mediated changes in blood biomarkers that were unrelated to experimental beverage ingredients. The previously referenced studies indicate that substantial and prolonged increases in insulin production upregulate leptin levels *in vivo*. A study by Teff et al. (2004) monitored serum glucose, insulin, and leptin levels during a 24-hour, controlled diet study. When fructose constituted the carbohydrate portion of meals, leptin levels declined by 33 and 21% after 12 and 24 hours, respectively. Glucose consumption also suppressed the hunger hormone ghrelin by 30% as compared to fructose (Teff et al. 2004). Test beverage formulation with fructose may explain the lack of significant increases in serum leptin levels observed in the present study. Additionally, leptin is only one indicator of satiety so it remains unclear whether the null results observed in this study can be extrapolated to other physiological biomarkers of satiation and satiety.

### 3.4.4. *EFFECTS OF COCOA POLYPHENOLS AND WHEY PROTEINS ON SERUM ADIPONECTIN LEVELS*

Figure 3.4 shows the percent changes in mean serum adiponectin levels following

consumption of each beverage. Much smaller percent changes were observed in adiponectin levels as compared to those of leptin. All WPI and cocoa beverages increased serum adiponectin levels above that of the placebo while the placebo lowered levels below baseline for the four hours following consumption. The WPI beverage elicited the highest increases in adiponectin levels, significantly higher than the placebo at 30 minutes and one and two hours post-consumption. The HP beverage significantly increased adiponectin levels above the placebo at the one hour time point. However, there were no significant differences between cocoa and WPI variables. HP-WPI and LP-WPI beverages had the smallest serum adiponectin increases from baseline while the LP and HP beverages increased adiponectin to a greater degree. The WPI and LP beverages followed very similar trends with adiponectin levels peaking at the 30-minute time point. The HP, HP-WPI, and LP-WPI beverages reached peak adiponectin levels at the one-hour time point.

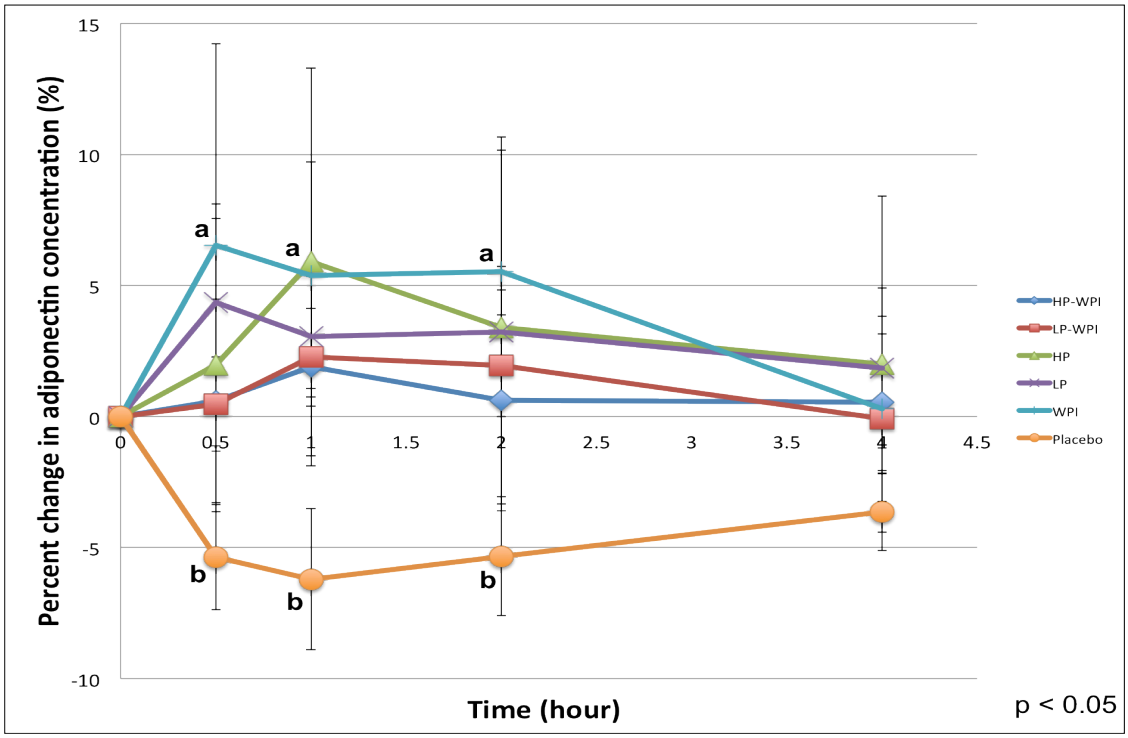


**Figure 3.4.** Percent changes in serum adiponectin levels before and after beverage consumption. Values expressed as percent changes from baseline in mean ± S.E. Letters refer to significant differences between time points or beverages; variables with the same letter are not statistically different.
n = 9
Abbreviations: LP: low polyphenolic cocoa, HP: high polyphenolic cocoa, WPI: whey protein isolate, LP-WPI: low polyphenolic cocoa + whey protein isolate, HP-WPI: high polyphenolic cocoa + whey protein isolate.

Adiponectin is a hormone-like peptide secreted by fat cells *in vivo*. This adipocytokine increases fatty acid oxidation and glucose uptake in muscle and liver cells (Huang et al. 2008). Unlike leptin, adiponectin levels are lower in obese subjects and higher in healthy people with less body fat (Yamashita et al. 2012). Physiological doses of adiponectin have been shown to decrease insulin resistance (Yamauchi et al. 2001, Tschritter et al. 2003). Most studies of diet-induced changes upon adiponectin have investigated the

effects of dietary protein and lipid content. Torres-Leal et al. (2011) found that leucine supplementation increased serum adiponectin levels and reduced total cholesterol in obese rats. Huang et al. (2008) reported that whey protein intake increased adiponectin production in mice on a high-fat diet. This stimulation of adiponectin was associated with decreased appetite and moderation of weight gain. Dietary supplementation with α-lactalbumin has also been shown to increase adiponectin levels as well as to decrease blood glucose in diabetic mice after glucose loading (Yamaguchi and Takai 2014). Conversely, no significant differences in adiponectin levels were found between low fat/high carbohydrate or low fat/high protein diets in a human clinical trial (Claessens et al. 2009). The results of the current study support the observation that whey protein significantly increased adiponectin levels *in vivo*.

Polyphenolic upregulation of adiponectin expression is less studied but has also been reported. Ølholm et al. (2010) showed that after induction of inflammation *in vitro*, resveratrol treatment increased adiponectin mRNA expression. These changes in adiponectin expression are attributed to PPAR-γ regulation by polyphenolic ligands. Maeda et al. (2001) found that treatment of diabetic humans and rodents with thiazolidinediones, synthetic polyphenolic PPAR-γ ligands, increased plasma adiponectin levels without changing body weight. Yamashita et al. (2012) reported that a high-fat diet supplemented with cocoa procyanidins significantly increased plasma adiponectin levels in mice. Cocoa procyanidins were shown to activate AMP-activated protein kinase-α (AMPK- α) and to increase GLUT4 levels in the plasma membrane of liver and adipose tissue. Adiponectin increases AMPK-α expression, which in turn downregulates fatty acid synthesis and upregulates fatty acid

oxidation. These studies indicate that through PPAR-γ and AMPK-α activation, polyphenols may reverse the effects of metabolic syndrome by increasing both blood glucose uptake and lipolysis. The present study showed that both high and low polyphenolic cocoa significantly increases adiponectin levels above that of a placebo. In the cocoa-only beverages, HP cocoa induced a greater increase than LP cocoa. However, when combined with WPI, adiponectin levels decreased slightly. Because the percent changes in adiponectin levels were so small, additional studies are necessary to better elucidate the effects of combining cocoa polyphenolics and whey protein.

This study investigated the differences between cocoa and whey protein ingredients upon three physiological markers of satiety (blood glucose, leptin, and adiponectin levels) and one subjective marker of satiety (hunger ratings). It must be stated that it is often difficult to isolate physiological and psychological effects on satiety: both factors influence each other and, consciously and subconsciously, determine eating habits. While the beverage formulations used in this study were controlled for calorie content, the placebo and WPI beverages had a slightly different textures and distinctly different appearances when compared to the cocoa-containing beverages. These differences may have subconsciously impacted panelist hunger ratings throughout the study.

In addition, though the panelists were aware that each beverage was isocaloric, the color differences between the cocoa and non-cocoa beverages (brown v. clear) may also have influenced the panelists to feel, and give responses, that matched the type of beverage they believed they had consumed (i.e. lower ratings of satiety for clear beverages that were perceived as the control). A study by Crum et al. (2011) investigated the physiological effects

of food labeling and panelist preconceptions of two identical milkshakes. On separate days, panelists consumed two identical milkshakes, one labeled a 620 calorie "indulgent" shake and the other labeled a 140 calorie "sensible" shake. Crum et al. (2011) found significant decreases in panelist ghrelin levels following the indulgent milkshake while consumption of the sensible milkshake elicited no changes in ghrelin levels. This study indicates that more research is necessary to better understand the interactions between psychological and physiological parameters that may influence total calorie intake, food selection, and eating habits.

## 3.5 CONCLUSIONS

This study investigated the acute effects of cocoa polyphenolics and whey protein upon markers of satiety and glucose and lipid metabolism in humans. These measures encompassed both psychological and physiological indicators, including subjective ratings of satiety, blood glucose levels, as well as serum leptin and adiponectin levels. Beverages containing whey protein increased satiety in the hours following beverage consumption. The presence of cocoa polyphenolics in the HP and HP-WPI beverages also increased satiety over that of the LP cocoa beverages. Based on the literature, these effects may be due to increases in plasma amino acids and polyphenolic partial inhibition of digestive enzymes.

WPI and HP beverages were also found to significantly moderate spikes in blood glucose following beverage consumption. 30 minutes post-consumption, the WPI, HP-WPI, and LP-WPI beverages increased blood glucose 8, 12, and 16% above baseline levels, respectively, compared to increases of 27, 39, and 58% in HP, LP, and placebo beverages,

respectively. In addition to higher initial levels, the placebo and LP beverage also exhibited more erratic fluctuations in blood glucose over time. These results may indicate that an increase in plasma amino acids or bioactive compounds found in whey proteins and cocoa can increase the production of insulin and cellular glucose transporters, and thus the cellular absorption of plasma glucose.

Average panelist leptin levels fell below baseline levels 30-minutes post consumption and remained below baseline at all subsequent time points. Levels decreased most quickly during the first hour and then slightly increased (LP, LP-WPI, and HP) or remained constant (placebo, WPI, and HP-WPI) between hours two and four. LP cocoa ingredients were associated with higher final leptin levels while WPI-containing beverages were not correlated with a specific pattern in serum leptin. A lack of increases in leptin may be due to the low calorie content (150 kcal) of the test beverages or to a lack of insulin induction. In addition, leptin levels may not have been monitored for enough hours following beverage consumption. Leptin is only one indicator of satiety, and is often difficult to correlate with individual meals, so it remains unclear whether the null results observed in this study can be extrapolated to other physiological biomarkers of satiation and satiety.

All test beverages increased adiponectin levels above that of the placebo. The WPI beverage significantly increased adiponectin levels compared to the placebo at 30 minutes and one and two hours post-consumption while the HP beverage reached significantly higher adiponectin levels one hour post-consumption. Adiponectin levels were not significantly different among WPI or cocoa variables. In the absence of WPI, HP cocoa elicited higher adiponectin levels than LP; the combination of WPI and cocoa decreased adiponectin levels

in comparison to cocoa alone. The results of this clinical trial indicate that WPI suppresses hunger, significantly moderates blood glucose, and significantly increases adiponectin levels above that of the placebo. Cocoa polyphenolic consumption moderated blood glucose levels and increased adiponectin levels better than a low polyphenolic control and the placebo. Expanded human clinical trials are necessary to better elucidate the acute and chronic effects of cocoa polyphenolics and whey protein upon biomarkers of satiety, weight gain, and metabolic syndrome. However, this study indicates that cocoa and whey protein consumption, alone and in combination, may be potential tools in the formulation of low calorie, satiety-inducing foods.

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# APPENDICES

# APPENDIX A: CELL CULTURE SUPPLEMENTARY MATERIAL

**Table A.1.** Epicatechin polymer (1-10) content of cocoa polyphenolic extract used in 3T3-L1 cell culture treatments.

| Epicatechin degree of polymerization | Concentration (ppm) |
|---|---|
| 1 | 504 |
| 2 | 324 |
| 3 | 216 |
| 4 | 207 |
| 5 | 132 |
| 6 | 108 |
| 7 | 124 |
| 8 | 142 |
| 9 | 71 |
| 10 | 52 |
| **Total** | **1878** |

**Table A.2.** Methylxanthine content of cocoa polyphenolic extract used in 3T3-L1 cell culture treatments.

| Methylxanthine | Concentration (mg/ml) |
|---|---|
| Proanthocyanidins (1-10 monomers) | 1.88 |
| Theobromine | 1.73 |
| Caffeine | 0.15 |

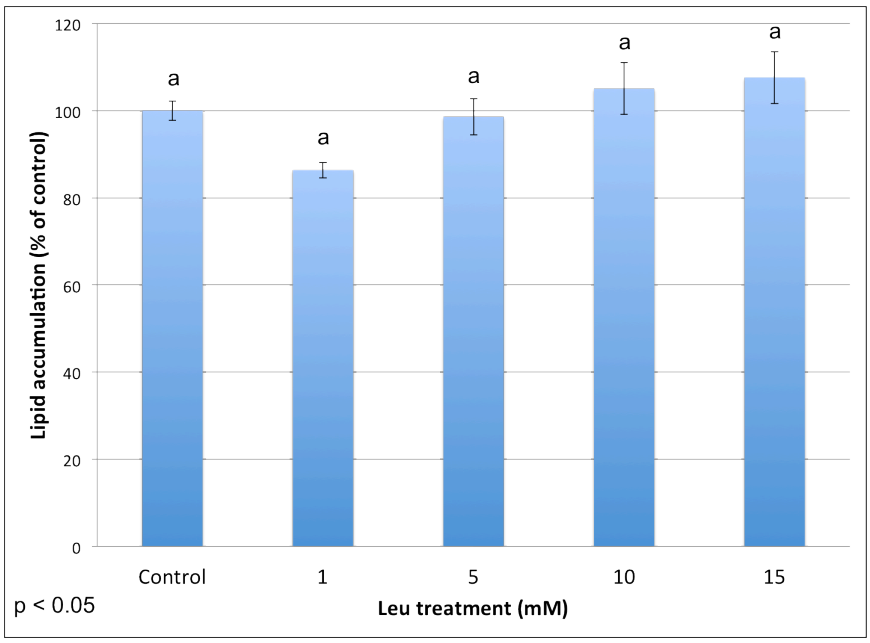


**Figure A.1.** Effects of leucine treatment on pre-adipocyte lipid accumulation during differentiation

# APPENDIX B: CLINICAL TRIAL SUPPLEMENTARY MATERIAL

**Table A.3.** Compositional, DMAC, and PAC 1-10 analysis results for regular high polyphenolic and Dutched low polyphenolic cocoa.

| **Analysis** | **Content (per g of cocoa)** | |
|---|---|---|
| | **Regular, high polyphenolic cocoa** | **Dutched, low polyphenolic cocoa** |
| Dose, g | 1 | |
| | | |
| Calories | 2 | |
| Fat, g (calculated) | 0.11 | |
| Sat fat, g | 0.066 | |
| Trans fat, g | 0 | |
| Cholesterol, mg | 0 | |
| Sodium, mg | 0.2 | |
| Carbohydrates, g | 0.58 | |
| Dietary fiber, g | 0.332 | |
| Sugar, g | 0.0175 | |
| Protein, g | 0.196 | |
| Vitamin A, IU | 0 | |
| Vitamin C, mg | 0 | |
| Calcium, mg | 1.28 | |
| Iron, mg | 0.4 | |
| Magnesium, mg | 5 | |
| Caffeine, mg | 0.23 | |
| Theobromine, mg | 2.2 | |
| | | |
| Total Polyphenols, mg | 62 | |
| Catechin, mg | 1.5 | |
| Epicatechin, mg | 3.5 | |
| | | |
| **Total Flavanols (DMAC), mg** | 44 | 6.7 |
| | | |
| **Proanthocyanidins (PACs)** | | |
| PAC 1-10, mg on whole product | 24.16 | 1.82 |
| PACs 1 mers | 5.6 | 0.6 |
| PACs 2 mers | 3.5 | 0.3 |
| PACs 3 mers | 2.5 | 0.2 |
| PACs 4 mers | 2.9 | 0.2 |
| PACs 5 mers | 2.4 | 0.1 |
| PACs 6 mers | 2.4 | 0.1 |
| PACs 7 mers | 2.2 | 0.2 |
| PACs 8 mers | 0.72 | 0.1 |
| PACs 9 mers | 1.4 | 0.1 |
| PACs 10 mers | 0.45 | 0.1 |

All analyses performed courtesy of The Hershey Co.
Abbreviations: DMAC: 4-dimethylaminocinnamaldehyde, PAC 1-10: proanthocyanidin 1-10. Both representative of proanthocyanidin content; DMAC measures total flavanols (all polymer lengths) while PAC 1-10 describes only catechin or epicatechin polymers between 1 and 10 units in length.

**Figure A.2.** Clinical trial panelist instructions for each session.

## Instructions

**Please read all guidelines carefully before beginning.**
You will collect a total of 5 blood glucose readings. The first reading will be done before consuming any sample. The 4 subsequent readings will be done 30, 60, 120, and 240 minutes after consuming the sample. You will provide 100 microliters of blood before, and 30, 60, 120, and 240 minutes after consuming a sample. **Please ask if you have any questions.**

1. Make sure that you have the following items:
   - 1 blood glucose meter
   - 5 lancets
   - 5 blood glucose test strips
   - 5 blood collection vials
   - 1 drink sample
   - 1 record sheet
   - 1 timer
   - 5 bandaids
   - 5 disinfecting wipes
   - 200 ml water

2. Write your **panelist code** and **today's date** on the record sheet.
   **Rank your appetite** for time 0.

3. **Measure and record 1st blood glucose reading** (fasting blood glucose). If meter shows a reading **above 110 mg/dl**, please alert one of the researchers. Follow these instructions to obtain a blood glucose reading:

   a. Insert top end of test strip into blood glucose meter.

   b. Rub fingertip vigorously for a few seconds until you feel it getting warm.
   c. Disinfect fingertip and nail bed with alcohol wipe.
   d. With the lancet needle end toward your nail bed, press the yellow button and lance finger.

e. Gently touch one edge of test strip to blood sample until you see a countdown. Wait for number to appear. If E-4 appears on the screen, there was not enough blood on the test strip to obtain the reading. Replace the test strip with a clean one and repeat steps 5-7.

For best results hold meter this way. Blood will be suctioned into the test strip.

f. On the record sheet, record the number displayed on the screen and time at which you took the reading.
g. Dispose of lancet and test strip in **designated waste container** after each reading.

Collect 100 microliters of blood into designated sterile tube (see picture below). **Make sure the tube is labeled with YOUR CODE, TIME 0, and WEEK #.** Press fingertip to allow blood to drip into vial. If you have trouble getting enough blood, use a new lancing device to lance the side or tip of a different finger.

4. Close vial, invert 5 times, and give to researcher. Disinfect fingertip and apply bandage if needed**.**

**Gently scrape blood on finger along inside edge of tube and tap tube to get blood to the bottom of tube.**

Fill to this level

5. **Shake contents of drink sample,** then open and consume entire contents of sample in **less then 10 minutes.** Add 200 ml of water to shaker bottled, shake, and consume remaining sample/water. Immediately, **set timer to 30 minutes and begin timing.** Drink water throughout remaining session as needed.

6. Rank and comment on your **liking of the sample** on the record sheet form.

7. *30 minutes after drinking sample:*

    a. When timer goes off, take **2nd blood glucose reading.** Record the number and time.
    b. Set timer for 30 minutes and begin timing.
    c. Immediately **collect 100 microliters of blood** into designated sterile tube labeled **with YOUR CODE, TIME 30, and WEEK #.** Disinfect fingertip and apply a bandage if necessary. Invert sample tube 5 times.
    d. **Rank you appetite** for 30 minute time point.

3. *60 minutes after drinking sample:*
    a. When timer goes off, take **3rd blood glucose reading.** Record the number and time.
    b. Set timer for 60 minutes and begin timing.
    c. Immediately **collect 100 microliters of blood** into designated sterile tube labeled **with YOUR CODE, TIME 60, and WEEK #.** Disinfect fingertip and apply a bandage if necessary. Invert sample tube 5 times.
    d. **Rank you appetite** for 60 minute time point.

4. *120 minutes after drinking sample:*
    a. When timer goes off, take **4th blood glucose reading.** Record the number and time.
    b. Set timer for 120 minutes and begin timing.
    c. Immediately **collect 100 microliters of blood** into designated sterile tube labeled **with YOUR CODE, TIME 120, and WEEK #.** Disinfect fingertip and apply a bandage if necessary. Invert sample tube 5 times.
    d. **Rank you appetite** for 120 minute time point.

5. *240 minutes after drinking sample:*
    a. When timer goes off, **take 5th blood glucose reading.** Record the number and time.
    b. Immediately **collect 100 microliters of blood** into designated sterile tube labeled **with YOUR CODE, TIME 240, and WEEK #.** Disinfect fingertip and apply a bandage if necessary. Invert sample tube 5 times.
    c. **Rank you appetite** for 240 minute time point.

6. Please place unused lancets, bandages, alcohol wipes, and test strips on your panelist mat. Dispose of used lancets, disinfecting wipes, and bandages in designated biohazard waste bin. **Hand in record sheet and leave everything else at the table.**

## Thank you for your help!

**Figure A.3.** Panelist session record form.

Panelist code:
Sample code:

## Panelist Record Sheet

Date: _____

**Time 0:**

Please record your **blood glucose reading** directly from the instrument: _____

**How hungry do you feel?** Please mark a vertical line with your response on the scale below.

Not at all hungry _____ Extremely hungry

**30 minutes:**

Please record your **blood glucose reading** directly from the instrument: _____

**How hungry do you feel?** Please mark a vertical line with your response on the scale below.

Not at all hungry _____ Extremely hungry

**60 minutes:**

Please record your **blood glucose reading** directly from the instrument: _____

**How hungry do you feel?** Please mark a vertical line with your response on the scale below.

Not at all hungry _____ Extremely hungry

**120 minutes:**

Please record your **blood glucose reading** directly from the instrument: _____

**How hungry do you feel?** Please mark a vertical line with your response on the scale below.

Not at all hungry _____ Extremely hungry

**240 minutes:**

Please record your **blood glucose reading** directly from the instrument: _____

**How hungry do you feel?** Please mark a vertical line with your response on the scale below.

Not at all hungry _____ Extremely hungry

Panelist code:
Sample code:

## Sample Liking

Please circle the number that corresponds with your like or dislike of the **overall sample:**

| | |
|---|---|
| 9 | Like extremely |
| 8 | Like very much |
| 7 | Like moderately |
| 6 | Like slightly |
| 5 | Neither like or dislike |
| 4 | Dislike slightly |
| 3 | Dislike moderately |
| 2 | Dislike very much |
| 1 | Dislike extremely |

Please circle the number that corresponds with your like or dislike of the **flavor**:

| | |
|---|---|
| 9 | Like extremely |
| 8 | Like very much |
| 7 | Like moderately |
| 6 | Like slightly |
| 5 | Neither like or dislike |
| 4 | Dislike slightly |
| 3 | Dislike moderately |
| 2 | Dislike very much |
| 1 | Dislike extremely |

Please circle the number that corresponds with your like or dislike of the **texture/mouthfeel**:

| | |
|---|---|
| 9 | Like extremely |
| 8 | Like very much |
| 7 | Like moderately |
| 6 | Like slightly |
| 5 | Neither like or dislike |
| 4 | Dislike slightly |
| 3 | Dislike moderately |
| 2 | Dislike very much |
| 1 | Dislike extremely |

**Comments:**

_____

_____

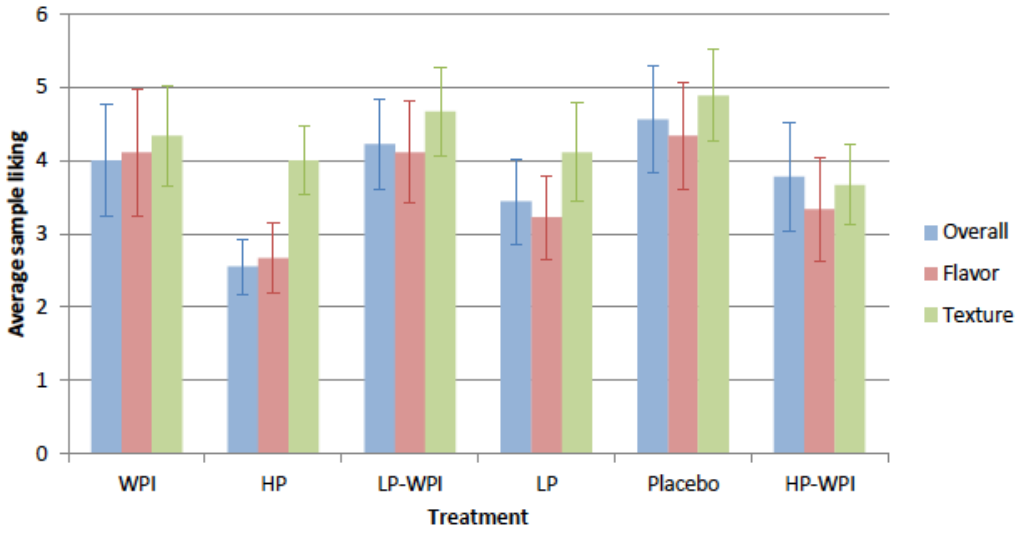


**Figure A.4.** Averaged hedonic liking results for overall beverage, flavor, and texture. Ranking on a 9-point scale, with 0 labeled as "extremely dislike" and 9 as "like extremely." Values shown in mean ± S.E.