DO PROTEIN CONTENT AND PROTEIN QUALITY INFLUENCE HUMAN FOOD INTAKE? TESTING THE PROTEIN LEVERAGE HYPOTHESIS.

by

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Abstract

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Do protein content and protein quality influence human food intake?

Testing the Protein Leverage Hypothesis.

Thesis directed by Professor Darna Dufour

Why do people eat what they eat? An important goals of nutritional anthropologists is to seek answers to this deceptively simple question. In this research, we tested the Protein Leverage Hypothesis (PLH), an explanatory framework that my help us understand how broad-scale dietary changes influence individual human food intake. The PLH suggests that all animals, including humans, prioritize the intake of protein over total energy intake (EI). This means that if a diet is high in protein, people will eat less food overall, since they can easily meet their protein requirements without having to consume much food. On the other hand, if a diet is low in protein, people will tend to eat more food overall as they attempt to consume enough protein. The PLH has important implications for contemporary human nutrition: as our diets are becoming increasingly dominated by processed, high-carbohydrate, low-protein foods, people will tend to overeat, and this will contribute to the global obesity epidemic.

We tested the PLH in three ways. First, we analyzed population-level data on dietary intake and anthropometry for USA adults from the time period 2005-20006 through 2015-2016 to uncover any trends supportive of the PLH. Second, we conducted an *ad libitum* feeding experiment to further test the proposed link between the protein characteristics of the diet and individual EI. This experiment was an improvement over previous tests of the PLH, allowing for a clearer analysis of how protein affects food intake. Third, we conducted an acute hormone and satiety study to investigate how dietary protein characteristics affected not EI, but a related

phenomenon: satiety. Our results demonstrated clear evidence for only one component of the PLH: the consistency of absolute protein intake over time. On the other hand, our data did not support another fundamental component of PLH: an inverse relationship between the protein content of the diet and total energy intake. Overall, the data collected in this research study failed to provide consistent evidence for the PLH. Future research is needed to explore other physiological, evolutionary, ecological, and sociocultural mechanisms that help us to understand why people eat what they eat.

DEDICATION

I dedicate this dissertation to Annette Merritt, my beloved *tita* and godmother.

The last time I heard your voice, I told you the good news that I had finally received funding and support for this project, and that I could begin my dissertation work at last.

I hope the result would make you proud.

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My advisor, Dr. Darna Dufour, has been a superlative mentor to me for many years. Through her guidance, I have learned not only the science of nutritional anthropology, but also the discipline and foresight required to be a researcher, and the thoughtfulness and empathy required to be an effective communicator and ambassador of science. Even into her retirement, Dr. Dufour has continued to provide me with invaluable support and input into my research. I thank her for her unending encouragement and patience over the course of my lengthy graduate career.

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through the most difficult periods of discouragement and self-doubt. I can never thank them enough for a lifetime of love and support, and I hope that this work will make them proud.

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CHAPTER 1. INTRODUCTION

In this dissertation, I explore the Protein Leverage Hypothesis (PLH), a theoretical framework that may aid our understanding of the relationship between population-level changes in dietary characteristics and individual behavior, nutrition, and health (Simpson and Raubenheimer, 2005). According to the PLH, food intake in animals, including humans, is constrained to prioritize dietary protein adequacy. That is, animals will alter their food intake behavior to ensure the adequate consumption of protein, even if this results in an under- or overconsumption of carbohydrates or fat. Hence, if the protein density of a diet decreases, then individuals will over-consume the diet in order to meet their protein requirements. Protein intake remains constant in this scenario, but individuals will consume more energy due to the increased overall food intake. Conversely, a shift to a higher-protein diet leads to a decrease in energy intake, since individuals are able to meet their protein requirements with less total food consumption; again, according to the PLH, protein intake would still remain constant in this case.

The goal of this research is to test the PLH in humans. Utilizing data from national health and nutrition surveys, we assess whether the quantity of protein in the diet is associated with total energy intake among adults in the USA. Next, in two experimental studies conducted in Boulder, CO, we directly test the effect of dietary protein quantity on human energy intake and satiety. Additionally, in an extension of the PLH, in these experiments we test whether the quality of dietary protein, i.e., plant-source *vs*. animal-source, independently exerts an effect on energy intake and satiety.

The dissertation is organized into 6 chapters and 4 appendices. Following this brief introduction, Chapter 2 provides a general background to the study, including a survey of

theoretical perspectives in nutritional anthropology and a review of the current research and evidence regarding the PLH.

Chapters 3, 4, and 5 present the quantitative results of the research project. Chapter 3 analyzes national-level health and nutritional survey data for the USA population. Chapter 4 describes an *ad libitum* feeding experiment, in which the effects of diets varying in protein quantity and protein quality on total energy intake are analyzed. Chapter 5 presents results from a second experiment, in which the effects of the same experimental diets on a biomarker of satiety are measured.

Chapters 3, 4, and 5 are each prepared in the format of research papers to subsequently be submitted to refereed journals. Since these three chapters are written as stand-alone papers, there is necessarily some degree of overlap in their literature reviews and references. Final manuscript preparation will be done with the collaborators who helped design and execute this research project. Authorship of the paper resulting from Chapter 3 will be:

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Authorship of the papers resulting from Chapters 4 and 5 will be:

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Chapter 6 summarizes the results of the three studies, provides a broader discussion of the research project overall, and suggests further theoretical implications of the work. Detailed methods for the two experimental studies (Chapters 4 & 5) are described in the protocol documents contained in Appendix A and Appendix B. Appendix C contains the University of Colorado Boulder Institutional Review Board (IRB) consent form, while Appendix D contains the experimental instruments used in the implementation of the experimental studies.

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CHAPTER 2. BACKGROUND

Why do people eat what they eat? A fundamental goal of nutritional anthropology is to seek answers to this deceptively simple question, and the aim of our study is to contribute a useful piece to this important puzzle. We do so by testing an explanatory framework that may link population-level shifts in dietary composition to changes in individual food intake: the Protein Leverage Hypothesis (PLH). This research will not only help us to understand the variability in diets within and between extant human populations but will also engage with contemporary debates in paleoanthropology and primatology over the relative roles of energy and macronutrient (protein, carbohydrate, fat) availability in shaping feeding behaviors. In this way, our research can help us to understand one important aspect of why we eat what we eat.

THEORETICAL PERSPECTIVES IN NUTRITIONAL ANTHROPOLOGY

Diverse theoretical perspectives exist within nutritional anthropology. Some of these perspectives have grown from within the discipline of anthropology, while others draw extensively from the ecology, physiology, and epidemiology literatures. What these perspectives all share in common is an ultimate (though not necessarily proximate) grounding in evolutionary theory, as well as a focus on inter- and intrapopulation variability. For instance, is feeding behavior shaped by evolution to maximize energy intake?

This idea is exemplified by optimal foraging theory (OFT), a theoretical framework that has historically been a prevalent tool within anthropology. OFT considers animal feeding behavior from the standpoint of a mathematical optimization function. Animals are modeled as seeking optimal intake of a particular nutritional currency, such as intake per unit time. The ability to optimize currency is limited and shaped by constraints, such as search time. Finally,

decision rules define the animal's behavioral strategy in optimizing currency within a particular context of constraints (Lambert & Rothman, 2015).

In anthropology, OFT has been used to model dietary decision-making in contemporary human populations (Lieberman, 2006), extant hunter-gatherer and horticultural populations (Hawkes et al., 1982; Koster, 2008), in archaeological human populations (Raab, 1992; Byers & Ugan, 2005) and in earlier hominins (Kurland & Beckerman, 1985; Sorensen & Leonard, 2001). More recently, a number of critiques of OFT have been presented. Zeder (2012), for example, argues that OFT situates human populations in a one-way adaptive framework, and hence disregards niche construction behaviors, while Lambert & Rothman (2015) suggest that certain factors clasically regarded as constraints (e.g., digestive biology) might be better regarded as variables potentially under the organism's control.

Nonetheless, OFT is still applied in contemporary anthropological research, particularly in archaeology (Arroyo, 2009; Dusseldorp, 2012; Jones & Hurley, 2017; Piperno et al., 2017). If our research finds support for the PLH, it would contradict a central principle of OFT, and other models of feeding behavior based on the optimization or maximalization of energy intake. The PLH predicts that individuals do not maximize energy intake, but rather will under- or over-consume energy as a function of the protein content of the diet. OFT is, however, but one of the numerous theoretical perspectives that have driven research in nutritional anthropology over the past 50 years.

What might define a superior theoretical perspective, or even a good or useful one? In one sense, the answer to this question will be dependent on the particular research question at hand. Some theoretical frameworks are intended to explain broad-scale global trends, while others are structured to deal with more localized, contextualized phenomena. On the other hand,

one universal characteristic of good theories is that they are parsimonious: they reduce the complexity of the world of our experience into more readily interpretable components. A good theory is simple, as opposed to simplistic, in that it allows researchers to efficiently and validly organize data within an explanatory framework. Less is more.

It can be tempting to introduce complexity into theory merely for complexity's sake, as this can provide a superficial sense of insightfulness or profundity. This would be misguided; as Dufour & Bender (2013:38) argue, "all models are simplifications of reality…No single model can explain everything, so any model should be evaluated in terms of what it was meant to do rather than what we wish it would do." Here, the authors follow the theoretical ecologist R Levins, who in 1966 proposed that model-building in the biological sciences involved a necessary tradeoff among generality, realism, and precision. This influential article (Levins, 1966) has generated a great deal of discussion, both supportive (Odenbaugh, 2003, 2006; Weisberg, 2006) and critical (Orzack & Sober, 1993; Orzack, 2005), within the biology and ecology literatures. Whether Levins's particular formulation is correct or not, the central idea remains: the predictions and explanations provided by theoretical models are always simple when compared to the real world; indeed, that is the whole point. Theories should be judged not according to their complexity, but to their ability to aid our understanding.

What are the current foci of theoretical perspectives within nutritional anthropology? One primary goal is to understand recent shifts and transitions in diets and physical activity patterns, at both local and regional levels. The commoditization and globalization of the world food system, as well as the emergence of the global obesity epidemic (Lobstein, 2011) within the last 30-40 years, fuel this focus. Additionally, anthropologists are interested in the drivers of nutritional variability within populations, particularly in the context of socioeconomic

differences. To that end, a major focus of current research effort and theoretical framing is the global poverty-obesity paradox (Dinour et al., 2007; Tanumihardjo et al., 2007).

The fact that high rates of obesity are increasingly found in low-income developing countries (Monteiro et al., 2004; Prentice, 2006), and in the low-income segments of developed countries (Drewnowski & Specter, 2004), presents an apparent paradox. At its simplest, the presence of obesity implies an excessive consumption of food energy or a low level of physical activity energy expenditure or both. Since food costs money, and physically-demanding jobs such as agricultural work tend to be low-paying, it would seem that socioeconomic status should be positively associated with obesity: compared to wealthier people, poorer people have fewer financial resources to purchase food and are more likely to work in physically-active jobs. Thus, obesity has been characterized as a disorder of convenience (Ulijaszek, 2007), in the sense that the convenient or luxurious aspects of high socioeconomic status – higher income, less physically-demanding work – actually fuel the proliferation of obesity and its many associated health consequences. So, it seems paradoxical that obesity is in fact *negatively* associated with socioeconomic status in different populations throughout the world (Sobal & Stunkard, 1989; McLaren, 2007). Again, this observation has been a primary target, but not the sole target, of current research and theory development within nutritional anthropology.

Adaptation

Before turning to a closer examination of prominent theoretical perspectives in nutritional anthropology, it is useful to review the concept of *adaptation*. Strictly speaking, an adaptation can be defined within modern evolutionary theory as a trait that has been shaped by natural selection to play a functional role in the life history of an organism (e.g., Bock, 1980; Reeve & Sherman, 1993). More generally, an adaptation can be conceived as any characteristic,

physiological trait, or behavior pattern that increases an individual's survivability and health status within a particular natural and/or sociocultural environment. Importantly, adaptations in this broad conception need not be limited to the genetic adaptations that form the basis of classical evolutionary theory. Instead, other forms of adaptation, including developmental, physiological, and cultural/behavioral, can be represented as existing along a continuum of flexibility and reversibility (Figure 2-1).

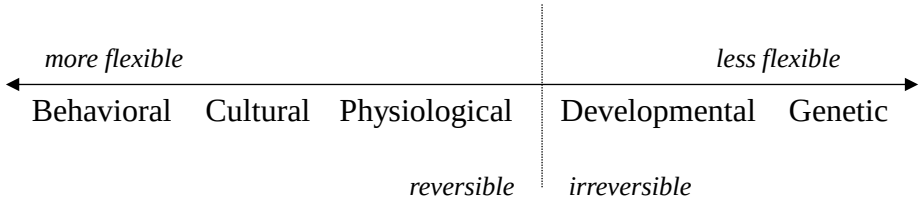

Figure 2-1 Types of adaptations in humans. Adaptations are arranged along a continuum from most to least flexible, with a division between potentially reversible and irreversible.

Genetic adaptations are the adaptations that are classically under consideration in evolutionary theory. They are genetically-linked phenotypic traits that an individual either possesses or does not possess; they are not attained or lost during an individual lifetime, and in this sense they are irreversible. An example of a genetic adaptation in humans is variation in skin pigmentation in response to ultraviolet radiation intensity (Jablonski, 2004).

Developmental adaptations are phenotypic traits that require a genetic potential but may or may not be expressed in the phenotype depending on the environmental context of the organism's growth. For developmental adaptations, there is a defined life history window in which environmental factors can cause the adaptation to be expressed or not. Outside of this window, the organism either possesses or does not possess the adaptation, and therefore developmental adaptations are also irreversible. One example of a developmental adaptation is

the increased chest circumference, and hence increased lung volume, of Quechua individuals who grew up in high-altitude areas of Peru (Frisancho & Baker, 1970).

Physiological adaptations, on the other hand, are reversible throughout the individual lifespan. All individuals within the species are considered to possess the ability to physiologically adapt, although the rapidity, efficiency, and effectiveness of the adaptive response may vary. Shivering as a thermogenic response to cold temperature (Hemingway, 1963) is a classic example of a physiological adaptation to environmental stress. Physiological adaptation to changes in environmental conditions in particular have also been referred to as acclimatization (e.g., acclimatization to high altitude (Levine & Stray-Gundersen, 1997)).

Finally, cultural and behavioral adaptations are reversible and highly flexible responses to the environment. Again, all individuals are considered to possess the capacity to adapt in this way, with the adaptations themselves being driven by either individual innovation or the transmission of shared knowledge; the distinction between cultural and behavioral adaptation is diffuse and reflects a differing emphasis on group- or individual-level behavior. These adaptations can be as simple as seeking shade from the sun and as complex as the construction of thermally-efficient clothing by arctic hunter-gatherers (Stenton, 1991).

Note that an organism's *capacity* to adapt in developmental, physiological, and/or cultural/behavioral ways is itself likely to be a genetic adaptation (e.g., Lasker, 1969; Price et al., 2003; Bateson et al., 2004; Feinberg, 2007). However, the important point here is that thinking about adaptations on more proximal levels, rather than on a strictly ultimate level, allows for a more parsimonious and practical understanding of human dietary variability. Two examples of nutritional adaptation will help to clarify the view of an adaptive continuum.

One well-known example of a genetic adaptation to diet is the ability of some human populations to continue to produce the enzyme lactase in adulthood, and thus to digest lactose (i.e., the main carbohydrate in fluid milk and other dairy products). Early in their life history, all mammals, including humans, produce lactase and are able to digest the lactose in their mothers' milk. However, after weaning, mammals cease to produce lactase, as they will never again encounter dietary lactose. Therefore, the ability of certain adult humans to digest lactose is an evolutionary novelty. Aside from these few lactase persistent humans, no other adult mammal consumes milk (Swallow, 2003).

The anthropological explanation for the lactase persistence phenomenon is that particular populations in Europe, Africa, the Middle East, and South Asia have a long history of herding domesticated animals such as cattle. Since fluid milk and dairy products can be rich in energy, essential amino acids and fatty acids, and micronutrients, herding populations living in close association with domesticated mammals could derive a substantial nutritional benefit if they were able to digest their animals' milk. Thus, a natural selection scenario emerges in which individuals who happen to possess a mutation for adult lactase production are better able to take nutritional advantage of milk (Itan et al., 2009), an evolutionarily novel food source that other animals do not (and cannot) compete for. At the same time, it can be noted that lactase impersistent humans may have already been consuming milk for its protein, fat, and micronutrient content, even if they were unable to digest the lactose (Holden & Mace, 1997). Due to the agency of certain human populations in choosing to consume milk as a nutritional strategy, the lactase persistence adaptation can be seen as an example of niche construction (Gerbault et al., 2011). Milk consumption can allow herding populations to survive in environments characterized by low-quality plant foods, such as grasslands, steppes, and tundra,

in that the domesticated animals are able to convert low-quality plant matter into foods accessible by humans (i.e., milk, blood, and meat).

The idea of lactase persistence as a genetic adaptation to a particular nutritional environment is strengthened by the observation that this trait has apparently evolved independently multiple times over the past ~7,000 years. Although there is a clear geographic pattern to the worldwide distribution of lactase persistence (Itan et al., 2010), it is inaccurate to think of this trait as a so-called racial or ethnic characteristic, because it is so strongly linked to a history of herding (Beja-Pereira et al., 2003). Additionally, there is substantial diversity in lactase-persistent genotypes within Old World milk-consuming populations (Hollox et al., 2001; Tishkoff et al., 2006; Ingram et al., 2007). In other words, lactase persistence is not a single trait, but rather a complex genetic trait with a phenotypic range that appears to be adaptively linked to population history.

In contrast to lactase persistence, a genetic adaption, a good example of a cultural adaptation to diet is the maize processing technique known as nixtamalization. As a food crop, maize is a highly productive source of calories, but it is deficient in essential amino acids and free niacin (Vitamin B_3). Because of this, populations depending on maize as a staple crop may be able to obtain adequate food energy, but can suffer from protein or vitamin deficiency, specifically the niacin-deficiency disease pellagra (Sydenstricker, 1958; Kumaravel, 2000). In fact, during the early 20th century, the incidence of pellagra reached epidemic proportions in several growing maize-dependent populations, such as rural populations in the southern United States (Roe, 1973; Kumaravel, 2000). However, historically maize-dependent populations in Central America did not suffer from this disease (Katz et al., 1974).

The anthropological explanation of this apparent contradiction is that the Central American populations, in contrast to the United States populations, did not consume maize whole. Rather, they first processed the maize by treating the kernels in an alkaline solution, grinding the treated kernels into flour. This process, called nixtamalization, has the biochemical effect of freeing bound niacin. Thus, populations that consume nixtamalized maize are protected from the niacin deficiency that leads to pellagra (Katz et al., 1974; Ellwood et al., 2013), and also reap the caloric benefits of maize as a food crop.

In this way, nixtamalization can be viewed as a cultural adaptation to a nutritional environment rich in maize but deficient in micronutrients. While this adaptation is population-specific, there is no indication that it is biologically-linked in any way. Also of importance is the fact that this interpretation of the nixtamalization technique is etic, not emic. That is, the populations that practice this technique almost certainly do not think of themselves as improving the niacin bioavailability of their maize. Rather, they likely think of themselves as improving the flavor, texture, and usability of their food, or they do not think consciously of their behavior at all. The point is that cultural or behavioral adaptations need not be recognized as such by internal observers.

Theoretical frameworks: extrinsic and intrinsic

The many theoretical frameworks employed by anthropologists to understand and explain individual dietary and physical activity behaviors can be conceived in two broad categories. Extrinsic perspectives emphasize environmental factors, such as food availability, economic constraints, and sociocultural drivers and constraints, as the primary forces behind inter- and intra-population variability in nutrition and health. Intrinsic perspectives, on the other hand, emphasize biological factors, such as population genetic history and developmental adaptation,

as the main drivers of nutrition-related health outcomes worldwide. This broad conceptual distinction among extrinsic and intrinsic theoretical perspectives mirrors a similar distinction between extrinsic characteristics of foods, e.g., their availability or cost, and intrinsic characteristics of foods, e.g., their inherent nutritional, physical, or chemical composition (Lambert, 2007).

Figure 2-2 represents some of the major environmental (extrinsic) and biological (intrinsic) factors that may influence individual dietary behavior and physical activity patterns, and thereby anthropometric and health outcomes. It is essential to clarify that the distinction between extrinsic and intrinsic factors is intended here as merely a heuristic tool for organizing the extensive body of nutritional anthropological theory; extrinsic and intrinsic factors are capable of interacting in complex ways. Indeed, many of the theoretical perspectives discussed below depend precisely on such interactions. Specifically, a mismatch between modern human biology, shaped by evolution, and contemporary food environments, shaped by socioeconomic forces, is identified as a central driver of current global trends in nutrition and health (although, again, different theoretical frameworks emphasize different aspects of this interaction). This broad perspective is so prevalent that it is worth examining in closer detail.

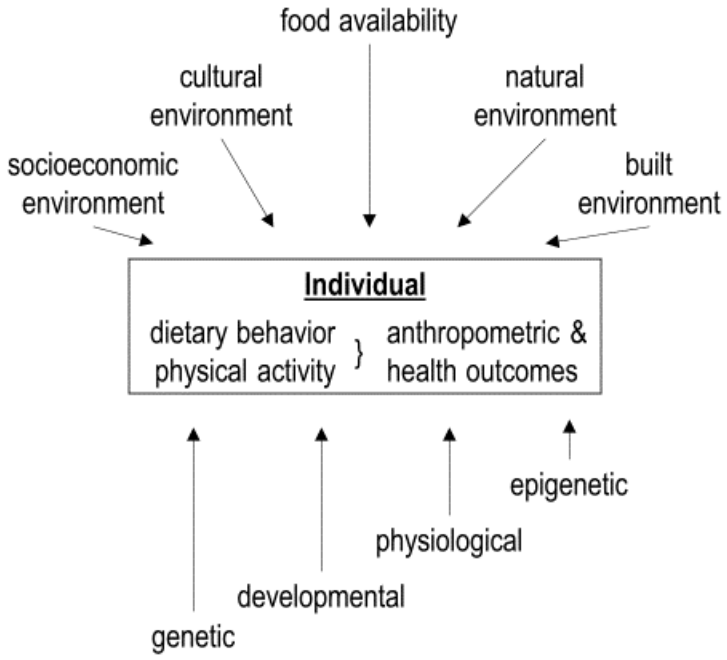

Figure 2-2 Broad representation of extrinsic (top) and intrinsic (bottom) factors that may influence individual dietary behavior and physical activity patterns, and thereby anthropometric and health outcomes.

The mismatch perspective

"The evolutionary collision of our ancient genome with the nutritional qualities of recently introduced foods may underlie many of the chronic diseases of Western civilization."
Cordain et al., 2005:341

Many theoretical approaches within nutritional anthropology emphasize a mismatch between environment and biology. These approaches view modern human physiology and dietary behavior as maladaptive in a dietary environment that has changed too quickly in the past 50-100 years for evolution to keep pace. The mismatch between biology and environment is the primary underlying cause of so-called Western or industrialized disorders and chronic diseases such as obesity, type II diabetes mellitus, cardiovascular disease, and so on.

Perhaps the most widely-known mismatch approach in nutritional anthropology is the "Paleolithic nutrition" concept of Eaton & Konner (1985), along with its many subsequent

retrospectives (e.g., Eaton et al., 1997; Eaton, 2006; Konner and Eaton, 2010) and related approaches (e.g., Cordain et al., 2005; Lindeberg et al., 2007; Frassetto et al., 2009). The central idea is that the diet of Paleolithic peoples – researchers disagree over what exactly this diet was – represents the ideal diet for humans, in the sense that human physiology has been shaped by evolution to function best with the Paleolithic diet. Modern departures from the Paleolithic dietary pattern are therefore evolutionarily novel to human physiology, resulting in maladaptation and poor health outcomes. Many other theoretical perspectives within nutritional anthropology also invoke a mismatch concept to a greater or lesser extent; e.g., optimal foraging in obesogenic environments (Lieberman, 2006, 2016; Brunstrom & Cheon, 2018) or obesity as a disorder of convenience (Ulijaszek and Lofink, 2006; Ulijaszek, 2007; Hruschka, 2012; Kirchengast, 2017). These mismatch approaches all share five fundamental assumptions, which may not all be valid.

1. Past populations provide a valid context for human evolution: The mismatch perspective depends on the idea that modern humans currently live in a nutritional environment that is different from their evolutionary environment. This evolutionary environment is defined as the environment of past humans, or, in some approaches, past nonhuman primates (e.g., baboons (Jolly, 2001)). So, if we know what the environment of past populations was like, we are in a position to understand the evolutionary context of modern populations. Past populations were matched to their environment, unlike modern populations.

But, there is a great deal of variability in past environments and lifeways. Humans have long been characterized by a great variety of subsistence strategies and dietary ecologies, even within the hunting-gathering or foraging economy (Kelly, 1995). There is no single past

environment that is characteristic of all human populations (e.g., Dean et al., 1985; Meltzer, 1988; Moore & Hillman, 1992).

2. Modern traditional populations are like past populations: According to the mismatch perspective, the evolutionary environment is substantially different from the modern environment. Modern humans that engage in traditional subsistence strategies, e.g., hunting and gathering, are viewed as unchanged holdovers from the past environmental context (Eaton et al., 2010; Konner & Eaton, 2010). Therefore, the observation of modern traditional societies provides a window into the ecology, nutrition, and behavior of the past human populations that were better matched to their environment. For example, in discussing the importance of dietary data from contemporary hunter-gatherer populations in East Africa, Eaton et al. (2010:295) argue that "conditions on humanity's mother continent most nearly match the ancestral paradigm and therefore accord best with our underlying genetic and epigenetic makeup."

But, there are complex interactions and influences among modern populations. Societies with different subsistence economies do not exist in isolated bubbles. Instead, they are powerfully shaped by complex environmental, sociocultural, political, and economic forces. Dufour & Bender (2013:374), in discussing traditional subsistence strategies, point out that

> there are no pure strategies: hunter-gatherers often trade with cultivators, cultivators hunt for meat and gather wild plant foods, many pastoralists cultivate grains. Furthermore, these characteristics are not set in stone; populations can and do modify their subsistence strategies in response to changing environmental, economic, or political factors.

Thus, there is no particular reason to assume that modern hunter-gatherers (or pastoralists, small-scale horticulturalists, etc.) are the same as past hunter-gatherers (Ember, 1978; Nestle, 2001). On the contrary, the nutritional ecology of modern traditional populations is best understood in the context of the *modern* environment, not an idealized past environment (Crittenden & Schnorr, 2016).

3. Genetic adaptation to the nutritional environment occurs slowly: For an evolutionary mismatch to exist, changes in the human nutritional environment must have happened too rapidly for genetic adaptation to keep pace. This implies that genetic adaptation occurs at a relatively slow pace compared to the rate at which the nutritional environment can change and has changed. The inability to genetically adapt quickly enough to a rapidly changing nutritional environment allows a mismatch to develop.

But, some genetic adaptations to the nutritional environment may occur quite rapidly. For example, lactase persistence, the ability of some humans to digest lactose into adulthood, is a genetic adaptation that appears to have evolved several times independently within the last 3,000 – 7,000 years (Enattah et al., 2008; Romero et al., 2012). Granted, many of the nutritional-environmental changes under consideration in the current literature, such as the recent increase in the consumption of vegetable oils and sugars worldwide (Popkin & Gordon-Larsen, 2004), are posited to have occurred very rapidly, within the last 100 or even 50 years. Still, it is not enough to simply assume that human genetic adaptation cannot occur on these timescales; more fine-grained evaluations are needed.

4. Non-genetic adaptations to the nutritional environment are less important: The mismatch perspective focuses on the inability of human genetic adaptation to keep pace with a rapidly-changing nutritional environment. This implies that other forms of adaptation, including developmental, physiological, and cultural/behavioral adaptation, are incapable of mediating the deleterious effects of the mismatch between genetic adaptations and the nutritional environment, and are therefore of lesser importance in understanding modern human nutritional ecology.

But, developmental, physiological, and/or cultural/behavioral factors can be highly important adaptations to the nutritional environment. For instance, the maize processing

technique known as nixtamalization is a crucial cultural adaptation with profound nutritional and health implications. Indeed, a broad spectrum of non-genetic adaptations, ranging from food storage and preparation techniques to fetal programming, are likely to mediate the relationship between population genetic backgrounds and nutritional environments.

5. An evolutionary mismatch exists for most people: Finally, the mismatch perspective requires that most people, regardless of genetic background, developmental circumstances, and current natural, sociocultural, and economic environment, are evolutionarily mismatched (or will soon become mismatched if current global trends continue). This is particularly true for those populations or segments of populations currently experiencing an increase in obesity and associated metabolic disorders.

But, this may not be true for the populations of all non-industrialized nations, or even for all segments of the population within industrialized nations. As populations worldwide become less isolated and more genetically heterogeneous (Tishkoff & Kidd, 2004; Weir et al., 2005; Barreiro et al., 2008), as local economies diversify (Frieden, 1991; Denis et al., 2002; Helmsing, 2003), and as the food system becomes more globalized (Sobal, 1999; Raynolds, 2004; Phillips, 2006), it becomes increasingly unlikely that population-level evolutionary mismatches can be defined in any straightforward way. Instead, mismatches must be conceived in a more nuanced, localized, or even individualized way.

To conclude, the mismatch perspective has been and continues to be a cornerstone of nutritional-anthropological and -epidemiological theory, despite its many (potentially invalid) assumptions. Indeed, perhaps the most fundamental assumption of the mismatch perspective is the very concept of an evolutionary mismatch as a novel driver of current global trends. Consider, however, that no organism exists in a completely static environment, and that non-

selective forces of evolution (i.e., mutation, gene flow, genetic drift) are continually in effect. This means that natural selection is always operating, and therefore *all* organisms are to some extent evolutionarily mismatched to their environments. If they were matched, then natural selection would cease. So, if an evolutionary mismatch is a general characteristic of any organism in a dynamic environment, how much explanatory power is carried by the mismatch idea itself in explaining current human health and nutritional trends?

The human dietary niche

The diets of both past and contemporary human populations are notable for their great diversity. Indeed, human diets exhibit such breadth that it would be a monumental, if not impossible, task to attempt to enumerate and define them all in either a past or present context. Nonetheless, anthropologists have uncovered several important themes that describe the general characteristics of the human dietary niche, in addition to the aforementioned diversity and felxibility of human diets (Turner & Thompson, 2012). Much of this work has depended on the use of contemporary hunter-gatherer populations as proxies for human dietary history and evolution (Crittenden & Schnorr, 2017), although there is increasing recognition that such populations tend to be environmentally and economically marginalized and are hence problematic models (Marlowe, 2005).

First, despite the recognition that animal-source foods were an important component of the diet of later hominins (particularly beginning with *Homo erectus*) and some modern human populations, plant-source foods continue to form the cornerstone of most human diets. In fact, Hardy et al. (2015) propose that starchy plants were essential for the evolution of the Pleistocene human phenotype, and Power et al. (2018) present evidence that even Neanderthals, a recent hominin generally regarded as highly carnivorous, regularly consumed plant-source foods.

Second, the controlled use of fire to cook food items appears to be a crucial human dietary adaptation. Wrangham & Carmody (2010) show that the net caloric value and digestibility of many important hominin foods, including protein-rich animal tissue and the starchy underground storage organs of plants, is improved by cooking. Thus, cooking through the controlled use of fire has allowed humans to efficiently access nutrients from the environment that would otherwise be unavailable from their digestive morphology and physiology alone. More recently, Carmody et al. (2016) have argued that the human genome bears signals of adaptation to a cooked-food diet.

Third, human dietary ecology seems best-understood from the perspective of niche construction; that is, the ability of organisms to shape and modify their environments, rather than be unilaterally constrained by their environments. Wollstonecroft (2011:141) considers humans and their hominin ancestors to be "the ultimate nich constructors due to [their] ability to modify selection pressures through diverse culturally generated and transmitted cultural means, i.e. cultural niche construction." Specifically, human food processing methods and agricultural practices represent a powerful means for humans to modify their own evolutionary selection pressures (Wollstonecroft, 2011), generating a scenario of tightly linked gene-culture coevolution (O'Brien & Laland, 2012).

Finally, the recent emergence of obesity as a worldwide phenomenon has generated a new phase of research interest into the evolution of human adiposity. Wells (2006) argues that human susceptibility to obesity is unusual among mammals, as is our relatively high level of adiposity in adulthood, and these traits may be linked to adaption to a more seasonal environment concurrent with the evolution of an energetically-expensive brain. Thus,

contemporary human obesity may be the result of energetic adaptations to protect the expensive brain within a modern environment of heightened energy availability (Wells, 2012).

THE PROTEIN LEVERAGE HYPOTHESIS

A promising theoretical development in nutritional ecology, and the focus of this research project, is the "protein leverage hypothesis" (PLH) proposed by Simpson and Raubenheimer (2005). According to this hypothesis, energy intake in animals is constrained to prioritize dietary protein adequacy. That is, animals will alter their food intake behavior to ensure the adequate consumption of protein, even if this results in an under- or overconsumption of carbohydrates or fat. Thus, if the protein density of a diet decreases, then individuals are predicted to over-consume the diet in order to meet their protein requirements. Consequently, total energy intake would increase in this scenario. Conversely, a shift to a higher-protein diet should lead to a decrease in energy intake, since individuals are able to meet their protein requirements with less total food consumption (Figure 2-3).

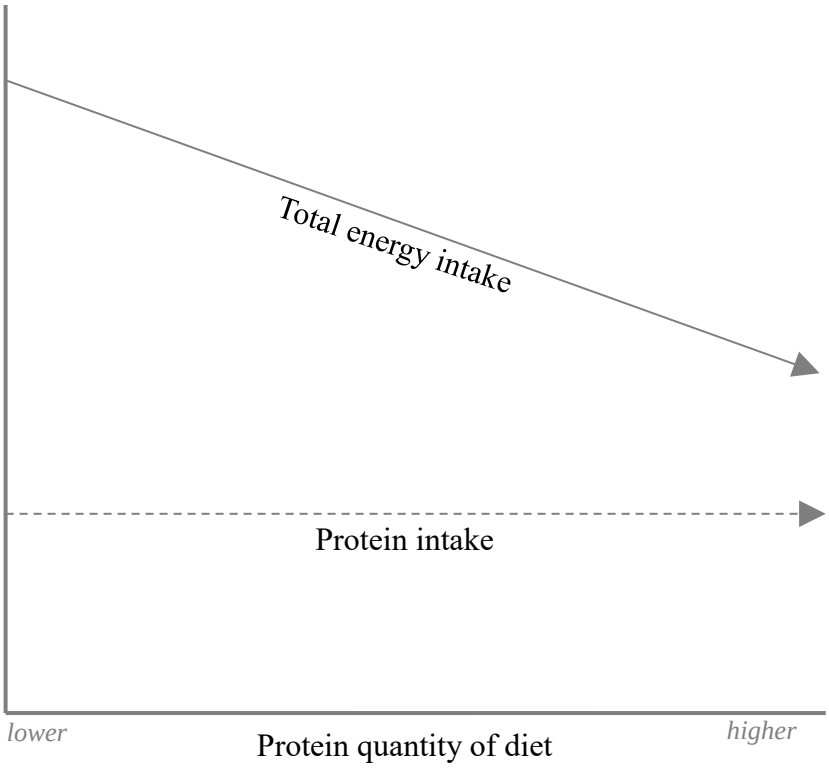

Figure 2-3 Schematic representation of the Protein Leverage Hypothesis

This "leverage" effect of protein is taken to be generally adaptive, since it drives a behavioral shift in the face of a dietary deficit. However, protein leverage can have adverse effects in certain nutritional environments if it leads to inadequate or excessive energy intake. In terms of human nutritional epidemiology, the practical consequences of protein leverage are argued as follows. Highly processed foods are becoming increasingly prevalent in diets worldwide, perhaps due to Nutrition Transition-type processes (e.g., Popkin, 1993, 2006). These processed foods are calorie-dense and rich in simple carbohydrates, but deficient in dietary fiber, protein, and micronutrients (Cordain et al., 2005). They may also contain high amounts of added sugars, added sodium, and fats implicated in undesirable health outcomes (e.g., *trans* fatty acids, saturated fats). Since highly-processed foods tend also to be protein-deficient (Mauron, 1990), individuals are physiologically driven to over-consume these foods in order to meet protein

requirements (Simpson & Raubenheimer, 2005). The result of this over-consumption is an excess intake of total calories, and possibly an excess intake of added sugars, added sodium, and unhealthy fats as well. In socioeconomic terms, the situation is compounded by the overall higher monetary cost of high-protein foods; the lower cost of carbohydrate-dense foods "may bias consumers towards diets high in carbohydrate energy, leading them to consume excessive energy to meet their dietary protein needs" (Brooks et al., 2010:887).

How plausible is the protein leverage hypothesis? In principle, it makes sense that animal physiology could be shaped by evolution to regulate the intake of one or more of the main macronutrients, i.e., protein, carbohydrate, and fat, because all of these macronutrients are essential for various reasons. Dietary protein is necessary for supplying essential amino acids, as well as nitrogen and other constituents for the production of non-essential amino acids (Visek, 1984; Wu, 2009). Secondarily, like all macronutrients, protein can also be metabolized for energy, although this requires deamination of amino acids (i.e., removal of the nitrogen component of the amino acid).

The determination of dietary protein requirements in humans has been of great concern in nutritional research, not only because protein requirements can vary substantially by age, sex, and body mass (e.g., Pellett, 1990), but also because inadequate protein intake and protein-energy malnutrition remain major public health concerns worldwide (de Onís et al., 1993; Millward and Jackson, 2003; Ghosh et al., 2012), particularly for children (Kar et al., 2008) and the elderly (Constans et al., 1992).

Currently, total protein requirements are determined through nitrogen balance, i.e., the difference between nitrogen intake and nitrogen excretion through urine, feces, sweat, and other pathways (Pellett, 1990; see also Rand et al., 2003). More recently, a number of different

methods have been employed to determine daily requirements for specific amino acids, including plasma amino acid response, direct amino acid oxidation, and indicator amino acid oxidation (IOM, 2005). However, these methods have produced disparate results and each suffers from various technical limitations; therefore, amino acid requirements are known with much less confidence than total protein requirements.

Despite the public-health focus on protein, it is not the only essential macronutrient, and therefore not the only macronutrient whose intake may be prioritized by some physiological mechanism. Carbohydrate, for example, is necessary for supplying glucose, a metabolic fuel required by the nervous system (Levin et al., 1999; Pellerin & Magistretti, 2003). Fat, in addition to providing a dense source of energy and contributing to thermoregulation, endocrine function, and organ protection through adipose tissues, is necessary for supplying essential fatty acids (Sinclair, 1984; Simopoulos, 1999). Thus, it is plausible that any or all of the macronutrients could "leverage" dietary intake. Hence, it is not necessarily clear that protein intake should be prioritized over the intake of the other macronutrients.

On the other hand, several lines of evidence indicate that protein does apparently exert the greatest "leverage." The current evidence in favor of the PLH derives primarily from animal models and short-term human clinical studies. In locusts, Raubenheimer & Simpson (1993) showed that protein and carbohydrate intake were tightly maintained when the diet was nutritionally diluted with foods containing non-digestible bulk; that is, the locusts over-consumed the low-energy-density foods in order to reach adequate levels of protein and carbohydrate intake. Chambers et al. (1995) also demonstrated protein regulation in locusts who were offered a range of foods containing different macronutrient proportions. The animals self-selected "complimentary" food pairings, consuming different quantities of the various foods such

that protein intake was maintained at baseline levels. Behmer et al. (2001, 2003) noted similar effects in locusts when the environmental frequency of complementary foods or the physical distance between complementary foods was altered. Nutrient-regulating feeding behaviors have been observed in mammals as well, i.e., in rats. Theal et al. (1984) and Tews et al. (1992) found that rats presented with mixed diets self-selected food items to maintain protein adequacy, over-consuming other nutrients if necessary. Simpson & Raubenheimer (1997) also describe protein regulation in the feeding behavior of rats, and attempt to unify these studies of nutrient regulation within a "geometric analysis." The so-called "geometric framework" is a mathematical construct that "unifies within a single model an organism and its multidimensional nutritional environment" (Simpson et al., 2003:123) and "provides a powerful set of methodologies" (Simpson et al., 2003:124) for assessing the regulation of nutrient intake.

As a brief aside, it is worth pointing out that this "geometric framework" is not nearly as mathematically complex as Simpson & Raubenheimer (1993, 1995, 1996, 1999) make it out to be. For instance, in describing his "right-angled mixture triangle," part of the "geometric framework," Raubenheimer (2011:409) explains that

> the components of a mixture are constrained to add to unity, and therefore the *n*th component can be deduced as (100% – [1 + 2 + … + *n* – 1]) where 1, 2…*n* represents the full set of components of the mixture. […] Arithmetically this follows from the fact that knowing either *X* or *Y* enables the equation *Y* = 100% – *X* to be solved for the other.

The author is not describing a complex mathematical structure here. He is merely pointing out, in a rather convoluted way, that percentages add up to 100. Furthermore, the geometric construct of the "right-angled mixture triangle" is only necessary – and only useful – because there happen to be three principal macronutrients in animal physiology (ethanol is generally ignored). If there were only two macronutrients, a simple bivariate scatterplot would suffice to illustrate nutrient mixtures. If there are four or more nutrients under consideration, then a triangle lacks sufficient

axes and the nutrient mixture cannot be easily portrayed on a two-dimensional plane. (Four-nutrient mixtures are, on the other hand, readily analyzable within a simple *algebraic* framework.)

Similarly, when Simpson et al. (2003:124) expound that "…an animal's nutritional relations with its environment is constructed as an *n*-dimensional state-space…foods are represented as linear trajectories (*nutritional rails*) that pass from the origin through nutrient space at an angle…", the authors' language obscures their relatively simple point: that all foods contain varied mixtures of carbohydrate, fat, protein, and other nutrients, that different animals have different nutrient goals, and that animals will adjust their eating behavior to reach these goals. Expressed in simple language, the point is quite clear, and attention can then be turned to the more interesting questions of how the nutritional goals of different animals can be assessed, and how physiology drives eating behavior in order for these goals to be met.

Returning to the currently-available evidence for the PLH: have nutrient-regulating effects been observed in humans? Simpson et al. (2003) present results from a short-term clinical study involving ten British adults. In this study, the dietary choices of the subjects were assessed when they were offered a varied, nutritionally-mixed diet, and also when the diet was restricted to either high-protein, low-carbohydrate/fat or low-protein, high-carbohydrate/fat food items. Simpson et al. (2003) found that the subjects on the high-protein diet tended to under-consume carbohydrate and fat, thereby avoiding excessive protein intake; conversely, subjects on the low-protein diet tended to over-consume carbohydrate and fat in order to maintain adequate protein intake. The authors interpret these findings as evidence that "protein ingestion is more strongly regulated than carbohydrate + fat" (Simpson et al., 2003:123).

A number of other clinical studies have also demonstrated the impact of protein intake on subsequent satiety, hunger sensation, and energy intake. Poppitt et al. (1998) provided 12 lean female subjects with isoenergetic meal preloads rich in either fat, carbohydrate, protein, or alcohol. They found that when the subjects were offered an *ad libitum* meal 90 minutes after the preload, only the high-protein treatment had exerted a significant effect on satiety and eating behavior, with subjects feeling less hungry and consuming less energy. Marmonier et al. (2000) observed a similar effect in 11 young male subjects who were offered a high-fat, high-protein, or high-carbohydrate snack 240 minutes after the lunch-time meal: the high-protein snack caused the greatest delay in the *ad libitum* request for the dinner meal, indicating a greater satiating and hunger-suppressing effect of protein. However, Marmonier et al. (2000) found no effect of the different snack food compositions on subsequent energy or macronutrient intake during the dinner meal. Gosby et al. (2011), rather than offering subjects preloads or snacks before a meal, directly manipulated the macronutrient composition of 28 food items offered to 22 lean subjects under *ad libitum* feeding conditions. These researchers observed that lowering the protein content of the diet from a 15% baseline to 10% resulted in a significantly higher total energy intake, while increasing the protein content from 15% to 25% had no effect on energy intake. These findings are partially consistent with the PLH, which predicts that individuals will over-consume low-protein foods (and thus over-consume total energy) in order to maintain adequate protein intake. On the other hand, Gosby et al. (2011) did not find evidence for the converse effect of a high-protein diet suppressing energy intake.

Many of these clinical studies share the limitation of assessing eating behavior only over very short time periods, e.g., minutes or hours. It is not yet clear whether the anorexic effects of high-protein foods, for example, would persist or diminish over time. Stubbs et al. (1996) found

that high-protein, high-carbohydrate, or high-fat breakfast meals all led to detectable differences in subjective hunger (with the high-protein meal suppressing hunger to the greatest extent), but these differences were not of sufficient magnitude to influence lunch-time food intake 5 hours after the breakfast meal, or overall energy intake for the rest of the day. These researchers conclude that "a single positive balance of each macronutrient can be buffered by oxidation and storage capacity, without leading to changes in meal-to-meal [energy intake]…" (Stubbs et al., 1996:409). Raben et al. (2003:91) also found "no significant differences in hunger or satiety sensations or in *ad libitum* energy intake" over 5 hours following breakfast meals rich in either fat, carbohydrate, protein, or alcohol. Thus, the leveraging effects of protein or other macronutrient intake on energy intake may be relatively short-term phenomena.

On the other hand, some researchers have found the opposite to be true. Martens et al. (2013, 2014), for instance, conducted two similar 12-day crossover studies in which subjects were fed whole-food meals supplemented by protein isolates or beef protein to produce three daily "menus" with 5%, 15%, or 30% energy from protein. The authors found partial support for the PLH: subjects under-consumed energy on the highest-protein diets, but they did not over-consume energy on the lowest-protein diets. In a longer-term study, Weigle et al. (2005) assessed the daily satiety, energy intake, and weight status of subjects placed sequentially on a weight-maintaining diet, an isocaloric high-protein diet, and an *ad libitum* high-protein diet over the course of 16 weeks. These authors found that a high-protein diet "produces a sustained decrease in *ad libitum* caloric intake…and results in significant weight loss" (Weigle et al., 2005:41), indicating a much longer-term effect of protein on energy intake. However, in the high-protein diets provided by both Weigle et al. (2005) and Martens et al. (2013, 2014), protein provided 30% of total energy, much higher than the ~15% of total energy postulated to be the

physiologically-regulated intake target in the protein leverage hypothesis (Simpson & Raubenheimer, 2005). Additional research is required to clarify the temporal extent of protein leverage effects in diets with protein contents within a more typical range for free-living human populations.

Also missing from the PLH is a proposed mechanism for *how* individual dietary behavior is shaped to prioritize protein adequacy. As Morrison et al. (2012) point out, the observed homeostatic regulation of protein consumption lacks a plausible physiological pathway. Presumably, a number of pathways are involved, including hypothalamic and hepatic signaling and other neuroendocrine systems (e.g., Kalra et al., 1991; Kuo et al., 2007; Magni et al., 2009). Also, taste and satiety effects may confound other mechanisms that drive protein intake.

For example, in a clinical study, Griffioen-Roose et al. (2011) fed subjects isoenergetic preloads of varying tastes and protein contents, then presented the subjects with an *ad libitum* lunch buffet also containing foods of varying tastes and protein contents. The authors found that the protein content of the preloads had no effect on subsequent food choice, but there was a taste effect: subjects who consumed a savory-tasting preload had a higher ensuing intake of sweet foods. Griffioen-Roose et al. (2011:779) conclude that "within one eating episode, within-meal protein content in these quantities seems not to have an effect on subsequent food choice." The results of this experiment therefore seem to contradict the PLH, which predicts an inverse relationship between the protein contents of the preload and the subsequent *ad libitum* meal.

Unexpectedly, however, Griffioen-Roose et al. (2011:785) interpret their results as *supportive* of the PLH: "[Considering] that in general savoury products contain higher protein levels than sweet products, [this finding] does seem to be in concordance with the protein-leverage hypothesis." This argument is contradictory and spurious, for several reasons. First,

Griffioen-Roose et al. (2011) unequivocally reported *no effect of protein content* of the preloads on subsequent food choice, meaning that the influence of savory-tasting foods on food choice cannot be explained by their supposed higher protein content, at least not within the context of this particular study.

Second, the claim that the supposed higher protein content of savory-tasting foods accounts for the effect of such foods on eating behavior is a *non sequitur*, since any number of other substances associated with savory taste could be driving the observed differences in food choice. For example, savory-tasting foods may contain greater quantities of sodium or fat, two substances known to be linked to satiety and eating behavior (e.g., Ayya & Beauchamp, 1992; Blundell et al., 1993). In a previous study, Vandewater & Vickers (1996) used a similar experimental design to argue for greater sensory-specific satiety in high-protein foods, but their results may also be confounded by taste differences in the treatments. Additionally, the association between the protein content of foods and their taste profiles, savory or otherwise, is likely to vary widely among foods, and indeed among the typical cuisines of different populations.

Third, even if the association between savory taste and protein content is valid, then the behavior-modifying effects of savory foods observed by Griffioen-Roose et al. (2011) would demonstrate a protein-*limiting* effect, rather than a stimulation of protein intake to satisfy protein adequacy. If the latter were the case, then the sweet-tasting (i.e., low-protein) preload should have been associated with a greater intake of savory-tasting (i.e., high-protein) foods, but no effect of the sweet preload was found. If anything, the findings could be interpreted as evidence of a *carbohydrate*-leveraging effect: if carbohydrate content is generally associated with sweet taste, then it would appear that the subjects observed by Griffioen-Roose et al. (2011) were

driven to seek carbohydrate-rich foods after a savory-tasting (i.e., low-carbohydrate) preload. Conversely, after consuming a sweet-tasting (i.e., carbohydrate-rich) preload, the subjects' need for carbohydrate was satisfied and no further effect on subsequent food choice was noted.

The purpose here is not to impose an undue level of criticism upon Griffioen-Roose et al. (2011) specifically, which after all represents only one of many attempts to investigate the effects of dietary protein on satiety (e.g., Hill & Blundell, 1986; Halton & Hu, 2004; see review in Veldhorst et al., 2008). Instead, the purpose is to emphasize that taste and satiety effects may confound other potential physiological mechanisms driving protein intake. This problem has implications for the conclusions of Simpson et al. (2003), whose short-term clinical study of food choice in humans forms one of the key pieces of evidence in support of the PLH. These authors found that subjects who were fed a high-protein, low-carbohydrate/fat diet under-consumed carbohydrate and fat rather than over-consume protein, whereas subjects who were fed a low-protein, high-carbohydrate/fat diet over-consumed carbohydrate and fat rather than under-consume protein. Simpson et al. (2003:136) interpreted these results as suggesting that "humans balance macronutrient intake, with protein intake being more strongly regulated than carbohydrate + fat intake." In other words, they interpreted the results as evidence of protein leverage in humans. However, the authors did not account for the possible effects of taste-related satiety in their treatment groups.

For example, subjects who were fed the high-protein diet were offered many strong-tasting foods such as ham, Emmental cheese, smoked fish, roast chicken, etc., while subjects on the low-protein diet were mostly offered less strong-tasting foods such as bread, cous cous, pasta, baked potato, etc. The examples of Vandewater & Vickers (1996) and of Griffioen-Roose et al. (2011) suggests that these (admittedly subjective) differences in taste intensity could have

driven the differences in food intake between the two groups. The high-protein group may have consumed less of the strong-tasting foods because they quickly reached satiety, while the low-protein group may have taken longer to reach satiety due to the more neutral-tasting foods.

Altogether, the clinical tests of the PLH have produced mixed results. Some studies have found support for the PLH (or at least, the authors claim that their results support the PLH), some have found evidence against the PLH, and some have produced mixed results. Also, most of the clinical tests have employed one of two basic types of experimental design: 1) a "preload" design, in which subjects are given a macronutrient stimulus shortly before consuming an *ad libitum* meal or before voluntarily initiating a meal, and 2) a "buffet" design, in which subjects consume whole foods *ad libitum* from distinct menus differing in overall macronutrient composition. Neither of these designs is ideal, since the first may not provide enough stimulus or time for a protein-leverage effect to manifest, and the second may be confounded by the different sensory qualities of whole food items.

What about tests of the PLH in free-living human populations? Martinez-Cordero et al. (2012) present such a test. The authors analyzed longitudinal data from adult Filipino women participating in the Cebu Longitudinal Health and Nutrition Survey, 1986-2005. To analyze change over time, they employed linear regression models that included the survey year as the independent variable and the logarithm of daily caloric intake of carbohydrate, protein, and fat as the dependent variables. (The transformed kcal values are presumably $\log_{10}$ values, but the authors do not state this). A positive linear regression slope was interpreted as an increase in macronutrient intake over time, while a negative slope was interpreted as a decrease in intake. Martinez-Cordero et al. (2012:314) found that "although calories of dietary protein increased

slightly over time, the increase was at a slower rate than that for fat, while carbohydrates decreased slightly." Accordingly, the authors conclude that their findings

> indicate that energy from protein intake remained more constant than that from carbohydrates or fat intake…This is consistent with the idea that recent changes in the protein density of the human diet have played a causal role in the developing obesity epidemic – the Protein Leverage Hypothesis (Martinez-Cordero et al., 2012:314).

Unfortunately, there are several conceptual problems with the analysis and conclusions of Martinez-Cordero et al. (2012). First, the authors cite the experimental study by Griffioen-Roose et al. (2011) as support for the statement that "protein is the most satiating and tightly regulated" (Martinez-Cordero et al., 2012:312) of the macronutrients. As explained above, Griffioen-Roose et al. (2011) clearly found *no effect* of protein on satiety or food choice. It is therefore unclear why Martinez-Cordero et al. (2012) would cite their study as evidence for the PLH.

Next, the line of reasoning of Martinez-Cordero et al. (2012) regarding changes or lack of changes in macronutrient intake over time is based on linear regression slopes, but such slopes are only useful analytical tools if the relationship in question is actually linear. Consider, for example, the argument that fat intake increased over time in this sample. This argument depends on the fact that the linear regression slope of log kcal of fat/day on survey year was positive. However, upon examining the authors' Figure 1 (Martinez-Cordero et al., 2012:314), reproduced in Figure 2-4 below, it is clear that fat intake increased from 1986 to 1998, but then *decreased* from 1998 to 2002, and remained essentially unchanged from 2002 to 2005. Thus, the idea of a linear increase in fat intake over time does not usefully describe the observed pattern. In fact, the coefficient of determination (r^2) of the relationship between log kcal of fat/day and survey year, not reported by the authors, is only 0.423; the associated correlation coefficient (r), 0.650, also not reported by the authors, is not significantly different from 0 (two-tailed p-value = 0.235). So, in this data set, the linear relationship between time and fat intake is not a particularly interesting

descriptor of changes in food intake, yet the authors claim that fat intake increased *more* than protein intake over time, merely because a regression slope of questionable validity was more positive.

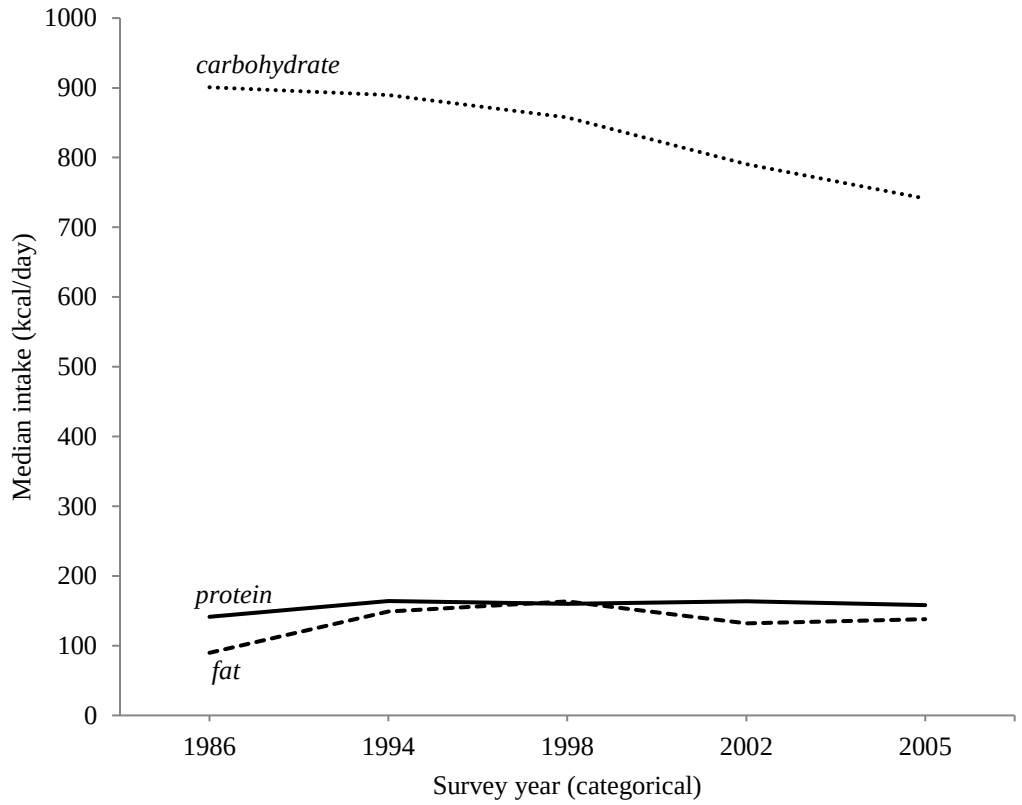

Figure 2-4 Median daily macronutrient intakes of adult Filipino women participating in the Cebu Longitudinal Health and Nutrition Survey, 1986-2005. Reproduced from Martinez-Cordero et al. (2012).

There are two additional problems with the graphical presentation of data by Martinez-Cordero et al. (2012:314). First, the *x*-axis is incorrectly scaled. The survey years are spaced equally along the axis, implying that the time elapsed between survey years was equal. In fact, the time periods between the surveys were 8, 4, 4, and 3 years. Second, the *y*-axis is also improperly scaled. The figure displays macronutrient intake in raw scores of median kcal/day, but Martinez-Cordero et al. (2012) perform all analyses and tests of statistical significance on the *logarithms* of median kcal/day scores. A graphical representation of data should employ the

same units and scaling as the analysis; otherwise, the graphical representation is misleading. Indeed, in the caption of their figure – which uses raw scores – Martinez-Cordero et al. (2012:314) report linear regression slope values, which were calculated from logged scores, and which therefore have no bearing on the figure as presented. Figure 2-5 below, generated from the raw data reported by Martinez-Cordero et al. (2012:313), displays changes in macronutrient intake over time with correctly-scaled axes. When the data are presented with correct scaling, the visual interpretation changes. The supposed linear increase in fat intake, the decrease in carbohydrate intake, and the constancy of protein intake all become less clear than implied by Martinez-Cordero et al. (2012).

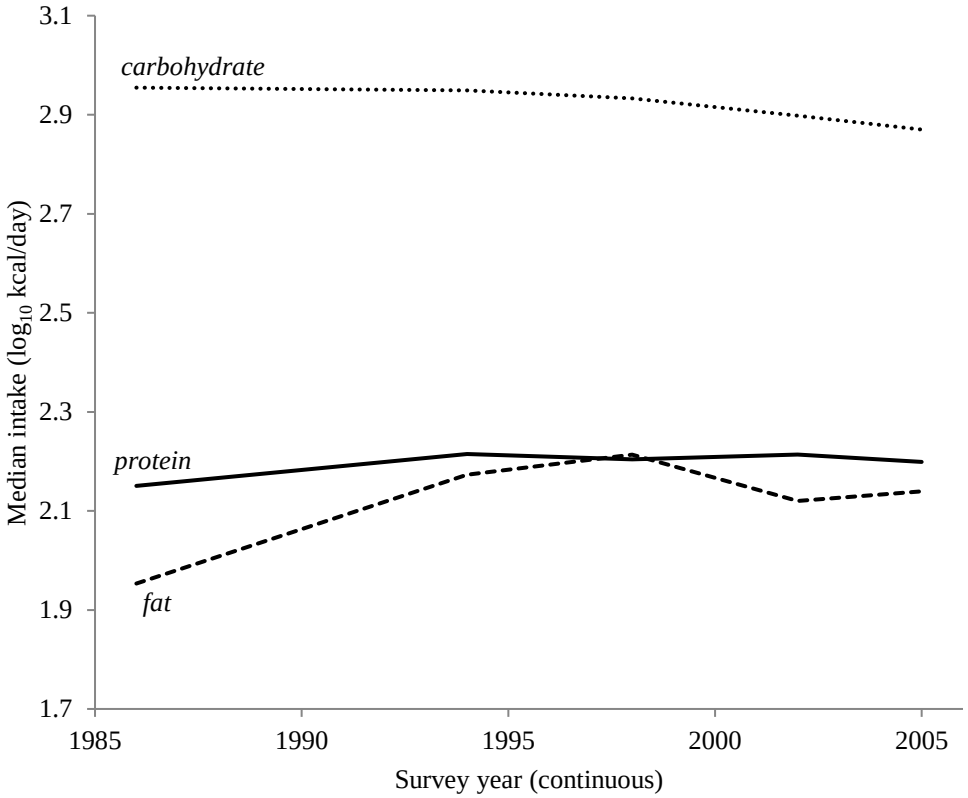

Figure 2-5 Median daily macronutrient intakes of adult Filipino women participating in the Cebu Longitudinal Health and Nutrition Survey, 1986-2005. Data from Martinez-Cordero et al. (2012) with axes corrected for scaling.

In any case, the logarithmic transformation of the energy intake data by Martinez-Cordero et al. (2012), while perhaps useful from a statistical standpoint, does not aid in the interpretation of the data as a test of the PLH. The best graphical representation of these data should portray actual macronutrient intake, in kcal/day, against survey year scaled continuously on the *x*-axis. Furthermore, the graphic should depict total energy intake – data which are presented by the authors in a table, but not included in their figure – since an increase in total energy intake is central to the PLH, as Martinez-Cordero et al. (2012:312; emphasis added) themselves point out: "…the Protein Leverage Hypothesis…postulates that…humans adjust their food intake to maintain a relatively constant dietary protein intake and consequently will have *higher energy intakes* on diets with low protein density."

Figure 2-6 shows the median daily macronutrient and total energy intakes of adult Filipino women from 1986-2005, as reported by Martinez-Cordero et al. (2012:313). Clearly, the temporal trend of total daily energy intake in this sample of Filipino women does not match the steady increase predicted by the protein leverage hypothesis. On the contrary, median total energy intake was lower in 2005, the last survey year (1088 kcal/day), than it was in 1986, the first survey year (1206 kcal/day). Thus, the data are not, as Martinez-Cordero et al. (2012:314) claim, "consistent" with the PLH.

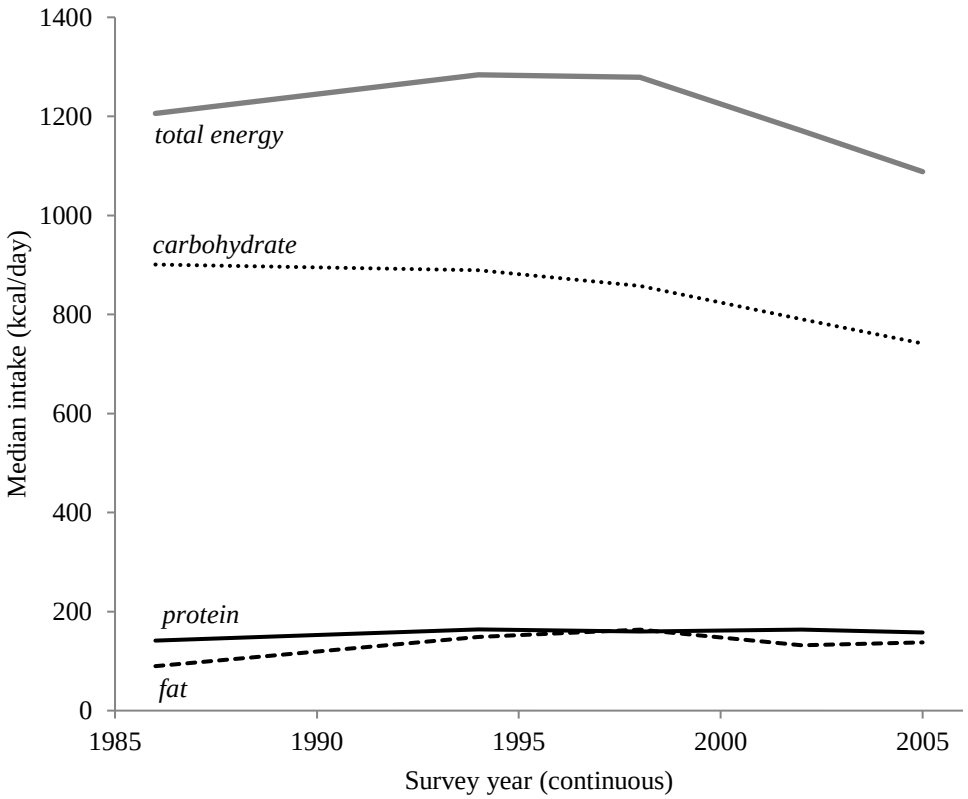

Figure 2-6 Median daily macronutrient and total energy intakes of adult Filipino women participating in the Cebu Longitudinal Health and Nutrition Survey, 1986-2005. Data from Martinez-Cordero et al. (2012).

One final, important flaw must be noted in the analysis of Martinez-Cordero et al. (2012). While the authors note that the Cebu Longitudinal Health and Nutrition Survey "follows a cohort of women" (Martinez-Cordero et al., 2012:312), they do not explicitly state whether the women interviewed during each of the five survey years from 1986 to 2005 are the same individuals. The reader is left to surmise that the women are, in fact, the same individuals, since the median age of the sample increases from 29 to 48 years between 1986 and 2005, and because the total sample size if the women were different in each survey would be $N = 8778$, rather than the stated sample size of $N = 2031$ (Martinez-Cordero et al, 2012:313).

The fact that the same individuals were surveyed over time presents a problem for the authors' analysis and conclusions. The data are interpreted as if they reflect changes (or lack of

changes) in the diet of a *population* over time: "Our findings indicate that energy from protein intake remained more constant than that from carbohydrates or fat intake *in a human population* undergoing shifts in diet and lifestyle…" (Martinez-Cordero et al., 2012:314; emphasis added). However, the data also reflect changes (or lack of changes) in the diets of individual women *as they age*. The PLH makes no explicit predictions regarding the impact of age on food intake. The data are thus confounded, and the authors do not attempt to disentangle the effects of age-related changes in dietary behavior from possible population-level nutritional shifts. In light of this analytical problem, as well as the other issues discussed above, the conclusions of Martinez-Cordero et al. (2012) remain unconvincing.

Data from other free-living populations may not support the protein leverage hypothesis either, or at least not all components of the hypothesis. For example, Dufour et al. (2015) compared food intake patterns and anthropometry among urban Colombian women between 1990-95 and 2008. They found ample evidence for an increase in the prevalence of obesity during this time, but little evidence for changes in the diet. In particular, they found no significant changes between 1990-95 and 2008 in mean daily caloric intake, or in the relative contributions of carbohydrate and protein to total energy intake; there was a small increase in the contribution of fat, from 19% to 23% of total daily energy (Dufour et al., 2015).

On the other hand, Simpson & Raubenheimer (2005:133) state that protein "typically comprises only ~15% of dietary energy, and…protein intake has remained near constant within and across populations throughout the development of the obesity epidemic." Among urban Colombian women, protein intake remained not at 15% of dietary energy, but at 11.2-11.4% (Dufour et al., 2015), perhaps suggesting that protein requirements were lower than implied by Simpson & Raubenheimer (2005). Alternatively, it is possible that urban Colombian women

have failed to meet their 15% protein requirement over time, but this seems highly unlikely because there is almost no evidence of protein malnutrition in this population. Additionally, the PLH would predict that the observed increase in obesity in urban Colombian women over time was driven by an increase in food energy intake, as low-protein processed foods became more prevalent in the diet and individuals over-consumed these foods in order to maintain adequate protein consumption. Again, though, the evidence from urban Colombia suggests that food energy intake did not increase between 1990-95 and 2008, and that low-protein processed foods did not become more prevalent in the diet (Dufour et al., 2015).

To date, the only study designed to test the PLH in a free-living human population is Bekelman et al.'s (2017) analysis of diet, obesity, and socioeconomic status (SES) among urban women in Costa Rica. Consistent with the PLH, Bekelman et al. (2017) found that absolute protein intake (g/day) was similar across low-, middle- and high-SES groups. On the other hand, the protein density of the diet (i.e., protein energy as a proportion of total dietary energy) was inversely correlated with total energy intake in middle- and high-SES women, but not low-SES women, partially consistent with the PLH. Additional prospective research is needed to test the PLH in free-living human populations, particularly in contexts where SES or other sociocultural factors may have a powerful impact on individual diets and nutritional status.

An additional challenge for the PLH is to explain the nutrient intake and nutritional status of voluntary vegetarians (and/or vegans), specifically in high-income nations such as the USA and UK. Vegetarian individuals choose not to consume meat, and they may also avoid other animal-source foods such as eggs or dairy products. Perhaps unsurprisingly, these diets tend to be low in total protein compared to diets that contain meat (Davey et al., 2003; Key et al., 2006), although this does not necessarily mean that vegetarian diets are inadequate in protein.

According to the PLH, vegetarian individuals should be over-consuming the low-protein foods in their diet to ensure adequate protein intake, and therefore they should be over-consuming total energy. Due to this overconsumption of energy, vegetarians should have greater adiposity and a higher prevalence of obesity than non-vegetarians. However, the current evidence suggests that the opposite is true: vegetarians have a consistently *lower* BMI than comparable non-vegetarians (by about 1 kg/m^2; Key et al., 1999), and the prevalence of obesity is also lower among vegetarians (Key & Davey, 1996), contrary to the predictions of the PLH. While the lower obesity prevalence among vegetarians is confounded by their typically higher self-reported health consciousness (e.g., Fox & Ward, 2008), at least in high-income nations, it is unclear why low dietary protein density does not appear to drive higher total energy intake in vegetarians.

Overall, the important contribution of the recent PLH is that it provides a physiological perspective on why individuals worldwide may be driven to over-consume highly-processed, calorie- and carbohydrate-dense foods, beyond the fact that such foods have merely become cheaper and more widely available. However, additional data are required to test this hypothesis in different free-living populations around the world. Also needed is a physiological mechanism that explains the observed protein leveraging effect.

PROTEIN IN ANTHROPOLOGICAL PERSPECTIVE

A long-standing interest in protein consumption exists within anthropology. Cultural anthropologists, primatologists, human biologists, and paleoanthropologists have all approached the question of protein consumption from different perspectives and with different research goals in mind. For example, during the 1970s-1980s, a classic debate in cultural anthropology developed concerning the availability of protein in the Amazon in limiting the size, density, and permanence of indigenous settlements (Gross, 1975; Ross, 1978; Beckerman, 1979; Chagnon &

Hames, 1979; Milton, 1984). If our research finds evidence for the PLH, it will support the idea that Amazonian population sizes may have been limited by protein availability (i.e., Gross, 1975; Ross, 1978), and make it useful to revisit the question.

Another well-developed line of anthropological research into protein consumption is the study of meat-eating in hominin evolution. Many archaeologists and paleoanthropologists agree that meat became an important part of hominin diets at some point during our evolution (Stanford & Bunn, 2001). Much of this work has centered on the problem of how hominins were able to meet the energetic demands of an ever-increasing brain size (Fish & Lockwood, 2003), with proposed mechanisms for the energetic support of the brain including a trade-off between brain size and gut size (Aiello & Wheeler, 1995) and a trade-off between muscle mass and fat mass (Leonard et al., 2007).

In addition to such tradeoffs, Snodgrass et al. (2009) have proposed that an increase in the quality of the diet, e.g., an increase in meat consumption, may have been necessary to support the evolution of the large hominin brain, with a common assumption being that meat increased the energy density of the diet. This is a problematic assumption, however, because meat, i.e., muscle tissue, is not necessarily a rich energy source for humans if it is low in fat (Speth & Spielmann, 1983; Mann, 2000); other parts of an animal carcass, such as marrow, may be superior energy sources (Blumenschine & Madrigal, 1993; Madrigal & Holt, 2002). Meat, on the other hand, is by definition a source of high-quality protein (Milton, 1999). In turn, the PLH predicts that a high-meat hominin diet would be *lower* in total energy, not higher. Thus, if our study finds evidence for the PLH, it will contradict the idea that the primary role of meat in hominin evolution was to provide energy for increasing brain size, and make it useful to reassess these arguments.

Additionally, the PLH engages with the debate over protein as a limiting factor in primate diets. For example, Oftedal (1991) argued that both human and non-human primates have relatively low protein requirements due to their slow growth rates; this suggests that protein availability is not a major limiting factor in primate diets. On the other hand, Chapman et al. (2004, 2015), building on previous work by Milton (1979), have argued that the protein-to-fiber ratio of the nutritional environment is a powerful predictor of primate population abundance; this suggests that protein availability is a limiting factor driving primate feeding behavior. The protein-to-fiber model has subsequently been questioned (Gogarten et al., 2012; Johnson et al., 2017), but other work has continued to explore and refine this concept (Wallis et al., 2012; Ganzhorn et al., 2017). If our experiment supports the PLH, it would lend support to the protein-to-fiber perspective.

Finally, anthropologists also have an ongoing interest in the global obesity epidemic, with particular emphasis on the evolutionary and environmental drivers of this phenomenon (Brown & Konner, 1987; Thompson & Gordon-Larsen, 2011). One perspective is that the human craving for energy-dense foods, and the ability to efficiently store dietary energy, are formerly-adaptive traits that have become maladaptive in modern obesogenic environments (Egger & Swinburn, 1997) characterized by cheap, readily available, high-calorie foods (Lieberman, 2003, 2006; Drewnowski & Specter, 2004). The PLH, on the other hand, proposes that individuals are physiologically driven to satisfy their protein requirements, not to maximize energy intake. Our research, if it supports the PLH, would offer an alternative explanation for this phenomenon: individuals are over-consuming energy not because energy-dense foods are cheap and widely available, but because contemporary processed foods are low in protein (Mauron, 1990).

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CHAPTER 3. USING THE PROTEIN LEVERAGE HYPOTHESIS TO UNDERSTAND ENERGY INTAKE AND BMI AMONG USA ADULTS, NHANES 2005-06 TO 2015-16

INTRODUCTION

The substantial increase in obesity prevalence worldwide, and in the USA specifically, over the past ~30 years (Flegal et al., 2002; 2010, 2012) is a phenomenon of great interest to epidemiologists, nutritional anthropologists, and public health researchers (Sturm, 2007; Wang et al., 2008, 2011; Imes & Burke, 2014). The rapidity of the increase in obesity prevalence suggests an equally profound shift in population energy balance, resulting from changes in diet, physical activity, or a combination of these, over the same time period. Additionally, the observed link between obesity and chronic diseases, such as cardiovascular disease and Type II diabetes, suggests that this so-called obesity "epidemic" (James et al., 2001; Stein & Colditz, 2004; Caballero, 2007) has serious implications for population health and wellbeing. Thus, from not only the standpoint of epidemiology, but also from an anthropological perspective, an explanatory mechanism is required that can link population-level shifts in total dietary intake, diet composition, and/or energy expenditure to changes in the prevalence of obesity and its concurrent negative health and social outcomes (Brown & Konner, 1987; Ulijaszek & Lofink, 2006; Agguire, 2009; Thompson & Gordon-Larsen, 2011; Brewis & Wutich, 2014).

One such framework is the Protein Leverage Hypothesis (PLH), as formulated by Simpson & Raubenheimer (2005). The PLH proposes that protein intake is under tighter physiological regulation than energy intake. Thus, if the protein density of a diet decreases (for example, if carbohydrate-rich foods become more prevalent), then total energy intake (EI) is predicted to increase as individuals over-consume energy in order to meet their constant protein requirements, and therefore body mass index (BMI) should increase as well. Conversely, a shift to a higher-protein diet should lead to a decrease in EI (and a decrease in BMI), since individuals

can meet their protein requirements with less total food consumption. Thus, at the population level, the PLH predicts that an increase in BMI, if driven by an increase in EI, should be associated with a decrease in the protein density of the diet (i.e., a lower percentage of energy from protein), while the consumption of protein in absolute terms remains steady.

The dietary and behavioral predictions of the PLH are portrayed schematically in Figure 3-1, specifically for the scenario in which a decrease in dietary protein density drive an increase in EI. The individual human body carries a daily requirement for protein, at a stable proportion of total body size. For USA adults, for example, the requirement is currently set at 0.8 grams of protein per kilogram of total body weight per day (Institute of Medicine, 2005). An increase in body size (e.g., due to growth), or a decrease in the protein density of the diet, drives an increase in protein demand to meet the requirement. To meet this greater protein demand, the body increases its total food intake. The increased food intake continues until enough protein has been consumed to meet the fundamental goal of the system: meeting the body's absolute protein requirement. A secondary, but important, consequence of the increased food intake is greater overall EI. All else being equal, increased EI will lead to an increase in body size, thus generating positive feedback within the system.

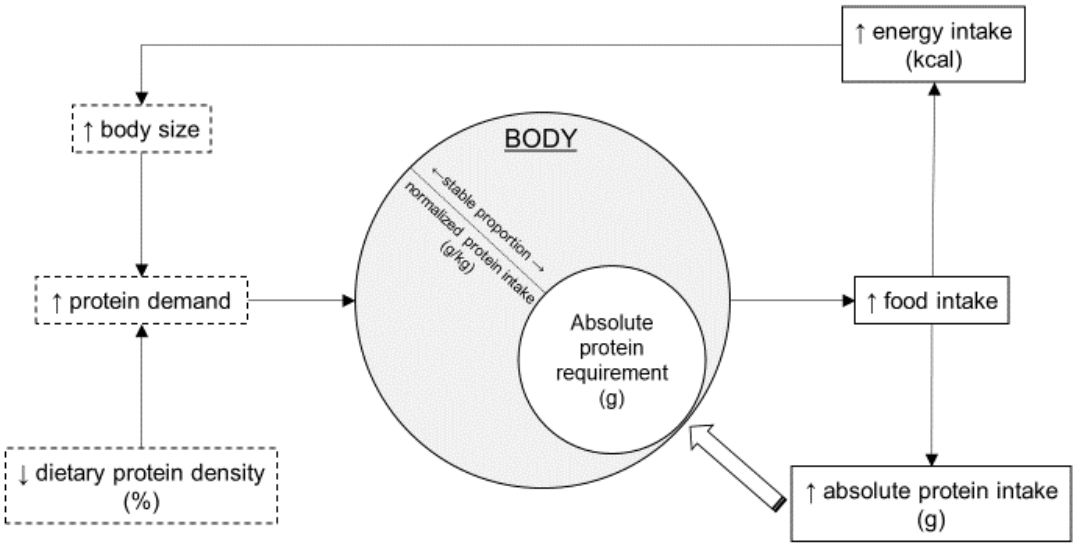

Figure 3-1 Schematic representation of the predictions of the PLH.

While the PLH has been examined in multiple clinical studies (e.g., Gosby et al., 2011; Griffioen-Roose et al., 2011; Simpson et al., 2003), there have been few tests of the hypothesis in free-living populations (but see Martinez-Cordero et al., 2012 and Bekelman et al., 2017). Here, we present an examination of the PLH in the USA, using national-level survey data to evaluate changes in protein intake, total EI, and BMI among adult USA women and men over a 10-year period. We ask whether protein and/or energy intakes among USA adults changed from 2005-2006 to 2015-2016, and whether the protein and energy intake patterns of USA adults were consistent with the PLH over this time period.

We assessed protein intake in three ways. First, we used absolute protein intake, expressed in grams per day in the original NHANES data. Second, since daily protein requirements vary between individuals, particularly due to body size (Pellett, 1990), we calculated normalized protein intake in grams per kilogram of total body mass per day. This body-proportionate measure of protein intake should be more closely linked to individual protein requirements than absolute protein intake (FAO/WHO/UNU, 2002), and may provide a more

nuanced way of testing the PLH. Third, we calculated dietary protein density as the percentage of total daily energy intake from protein.

For adult USA females and males, we hypothesized that: 1) absolute protein intake remained constant from 2005-2006 to 2015-2016; 2) normalized protein intake, i.e., body-proportionate protein intake, remained consistent from 2005-2006 to 2015-2016; 3) the protein density of the diet was inversely associated with EI throughout the 2005-2006 to 2015-2016 time period.

METHODS

Data

For this analysis, we used data from the National Health and Nutrition Examination Surveys (NHANES) yearly cross-sectional datasets from 2005-2006 through 2015-2016, the most current survey cycle for which data are available. NHANES is a USA-based, continuous, nationally-representative, cross-sectional assessment of the health and nutritional status of the non-institutionalized, household, civilian population (Johnson et al., 2013). Since 1999, NHANES has been conducted continuously, visiting 30 sites within the USA every 2 years and releasing data to the public in 2-year cycles (~10 000 adults and children per cycle). NHANES data are gathered by the Centers for Disease Control and Prevention (CDC) using a complex, stratified, multistage probability cluster sampling design (Johnson et al., 2013), resulting in sample weights assigned to each respondent within survey cycles to generate nationally-representative data profiles.

The previously-collected NHANES datasets used for this analysis include information collected from home interviews, followed by a health examination performed at a mobile examination center. Dietary data were collected via two 24-h dietary recall interviews, the first

taking place in-person at the mobile examination center (CDC, 2009a), and second taking place over the phone 3-10 days later (CDC, 2009b); only data from the in-person interview are included in this analysis. 24-h dietary recalls were conducted by trained interviews using the US Department of Agriculture's (USDA) validated (Moshfegh et al. 2008, Blanton et al., 2006; Rumpler et al., 2008) Automated Multiple-Pass Method (USDA, n.d.).

For this analyses, we extracted variables from the demographics, dietary, and examination data modules in each survey, and all analyses used full sample weights per best practices for weighted survey data (Korn & Graubard, 1999; CDC, 2018; Rosinger & Ice, 2019). Data were extracted for adults aged 18-60 years. The age range was selected to include adult individuals whose protein requirements are not yet substantially impacted by age-related changes in protein requirements (Pellett, 1990; Campbell et al, 1994; Morais et al., 2006), such as those linked to a loss of lean body mass with age (Forbes, 1976).

Analysis was also limited to individuals whose total daily EI was ≥ 1.4 × basal metabolic rate (BMR), estimated via the Schofield (1985) equations. The NHANES datasets contain numerous low reported EI values from respondents, a few hundred kilocalories per day or less. Such values are unlikely to represent habitual 24-hour EI, and are likely the result of under-reporting of energy consumed by NHANES respondents (Murakami & Livingstone, 2015). Therefore, we only included data from individuals unlikely to have under-reported their EI (Livingstone & Black, 2003), and whose total daily EI of ≥ 1.4 × BMR was adequate for at least a "sedentary or light activity lifestyle" classification of habitual physical activity according to the FAO/WHO/UNU (2001:38) definition. NHANES variables extracted were sex, age (yrs), height (cm), weight (kg), BMI (kg/m^2), protein consumption (g/day), and energy intake (kcal/day).

Protein intake was subsequently defined in three ways. 1) Absolute protein intake: total individual daily protein intake, in grams per day (g/day); 2) Normalized protein intake: total individual daily protein intake per unit of total body mass, in grams per kilogram per day (g/kg/day); 3) Dietary protein density: percent contribution of protein to total individual daily energy intake, assuming a caloric density of 4 kilocalories per gram of protein.

Analysis

Females and males were considered separately for all analyses, but figure axes were scaled identically for both sexes to aid comparability. In all analyses, data were weighted according to the appropriate values for that particular variable and NHANES survey year. Mean age was compared between the first and last survey years via independent-samples Welch's *t*-tests (Ruxton, 2006; Derrick et al., 2016), while mean anthropometric characteristics and dietary intake variables were compared between the first and last survey years via ANCOVA models with age as the covariate. Multiple regression models, controlling for age, were used to assess the relationship between dietary protein density (% total energy/day) and total EI within sexes in the first and last survey years, and interaction terms were used to test whether this effect differed between survey years for each sex. Statistical analyses were performed in JMP Pro 14 with significance set at $p < 0.05$.

RESULTS

Weighted means of BMI for USA females and males are shown for the survey years 2005-06 through 2015-16 in Figure 3-2. The *y*-axis scale corresponds to the range defined by the WHO as *pre-obese* (25.0 – 30.0 kg/m^2) (WHO, 2006).

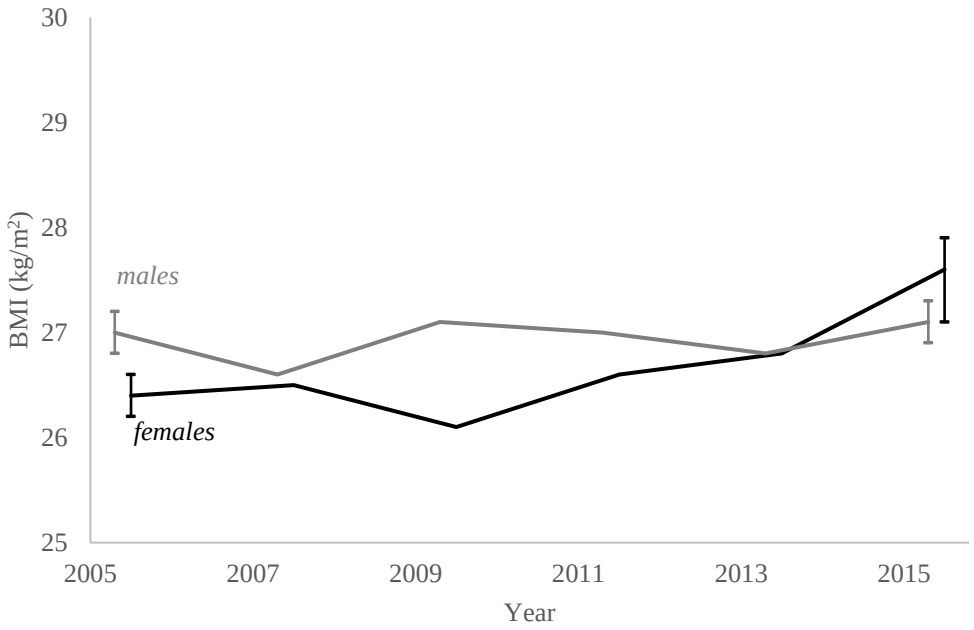

Figure 3-2 Weighted means of BMI (kg/m^2) for USA females (black line) and males (grey line), NHANES survey years 2005-06 through 2015-16. SEM is shown for first and last data points. Values are dithered along the *x*-axis to aid visibility.

Females

For USA females, descriptive statistics for age, anthropometric characteristics, and dietary intake values from NHANES survey years 2005-2006 through 2015-2016 are shown in Table 3-1 by survey year. Sample sizes ranged from ~570 to ~750, and are unequal for some variables within survey years due to missing data.

Table 3-1 Females: Descriptive statistics for age, anthropometric characteristics, and dietary intake values from NHANES survey years 2005-2006 through 2015-2016. Values are weighted mean ± SEM and sample size (N).

	2005-2006	2007-2008	2009-2010	2011-2012	2013-2014	2015-2016
Age (yrs)	37.5 ± 0.4 (753)	38.9 ± 0.5 (658)	38.8 ± 0.4 (706)	39.1 ± 0.5 (664)	37.7 ± 0.4 (714)	40.2 ± 0.5 (584)
Weight (kg)	70.2 ± 0.6 (744)	70.5 ± 0.7 (639)	69.2 ± 0.7 (693)	70.8 ± 0.7 (645)	71.1 ± 0.7 (702)	72.7 ± 0.8 (574)
Height (cm)	163.0 ± 0.2 (746)	162.9 ± 0.3 (639)	162.7 ± 0.3 (693)	163.2 ± 0.3 (646)	162.8 ± 0.3 (702)	162.2 ± 0.3 (573)
BMI (kg/m^2)	26.4 ± 0.2 (744)	26.5 ± 0.2 (639)	26.1 ± 0.2 (693)	26.6 ± 0.2 (645)	26.8 ± 0.2 (701)	27.6 ± 0.3 (573)
Energy intake (kcal/day)	2631 ± 25 (753)	2668 ± 29 (658)	2610 ± 23 (706)	2678 ± 28 (664)	2679 ± 28 (714)	2674 ± 27 (584)
Protein intake						
Absolute (g/day)	96.4 ± 1.2 (753)	93.7 ± 1.4 (658)	92.5 ± 1.2 (706)	92.4 ± 1.2 (664)	96.0 ± 1.3 (714)	95.1 ± 1.3 (584)
Normalized (g/kg/day)	1.44 ± 0.02 (744)	1.39 ± 0.02 (639)	1.39 ± 0.02 (693)	1.36 ± 0.02 (645)	1.43 ± 0.02 (702)	1.37 ± 0.02 (574)
Density (% energy/day)	14.9 ± 0.2 (753)	14.2 ± 0.2 (658)	14.3 ± 0.2 (706)	13.9 ± 0.1 (664)	14.6 ± 0.2 (714)	14.4 ± 0.2 (584)

Comparisons of age, anthropometric characteristics, and dietary intake values between the 2005-2006 and 2015-2016 surveys are shown in Table 3-2. The age of the sample increased between survey years; subsequent analyses control for age. Weight and BMI increased as well, but there was no significant change in height. Total EI increased slightly (< 50 kca/day) but significantly. Neither absolute protein intake (g/day) nor normalized protein intake (g/kg/day) changed between survey years. However, dietary protein density (% energy/day) decreased slightly but significantly (Table 3-2). The main effect of dietary protein density on EI, controlling for age, was significant in 2005-2006 (t ratio for parameter = -4.67, $p < 0.001$) and in 2015-2016 (t ratio for parameter = -4.81, $p < 0.001$), but this effect was not different between the two time periods (survey year × dietary protein density interaction term, $p = 0.940$; Figure 3-3).

Table 3-2 Females: comparison (ANCOVA[a]) of age, anthropometric characteristics, and dietary intake values (mean ± SEM) between NHANES survey years 2005-2006 and 2015-2016.

	2005-2006	2015-2016	*p*-value
Age (yrs)	37.5 ± 0.4	40.2 ± 0.5	< 0.001[b]
Weight (kg)	70.2 ± 0.6	72.7 ± 0.8	0.035
Height (cm)	163.0 ± 0.2	162.2 ± 0.3	0.069
BMI (kg/m^2)	26.4 ± 0.2	27.6 ± 0.3	0.009
Energy intake (kcal/day)	2631 ± 25	2674 ± 27	0.046
Protein intake			
Absolute (g/day)	96.4 ± 1.2	95.1 ± 1.3	0.677
Normalized (g/kg/day)	1.44 ± 0.02	1.37 ± 0.02	0.196
Density (% energy/day)	14.9 ± 0.2	14.4 ± 0.2	0.033

[a] covariate: age

[b] independent-samples Welch's *t*-test

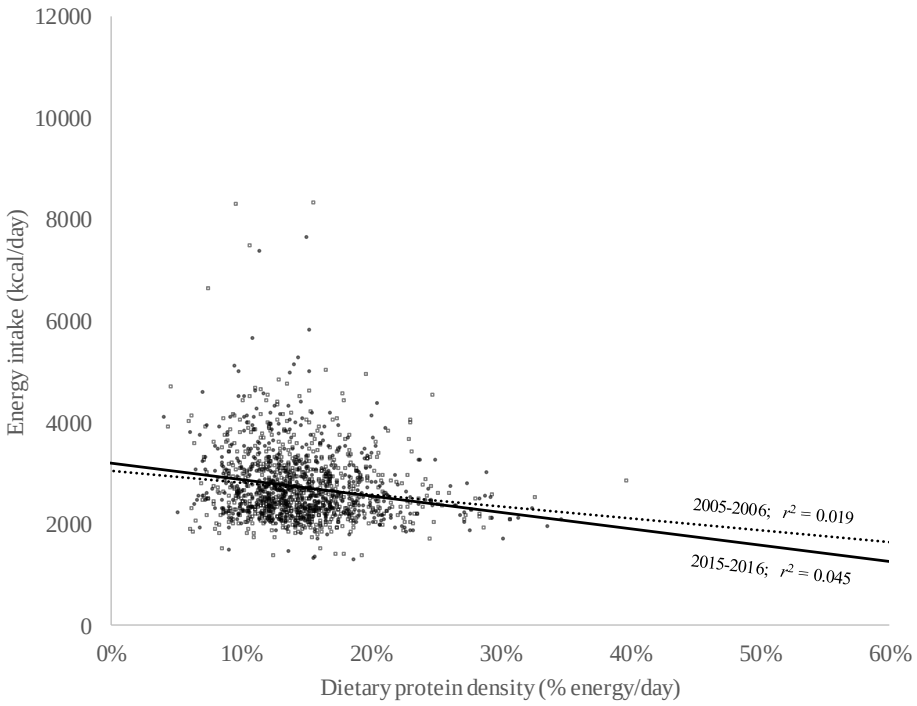

Figure 3-3 Females: relationship between energy intake (kcal/day) and dietary protein density (% energy/day) in NHANES survey years 2005-2006 (open squares, dashed line) and 2015-2016 (filled circles, solid line), with OLS regression lines by survey year.

Figure 3-4 shows a dual plot of mean BMI (kg/m^2) and absolute protein intake (g/day) from 2005-2006 to 2015-2016. Absolute protein intake remained steady over time, with a very shallow decrease from 2005 to 2011, followed by a rebound. BMI, on the other hand, exhibited a a clear increasing trend over time, interrupted only by a slight decrease from 2007 to 2009.

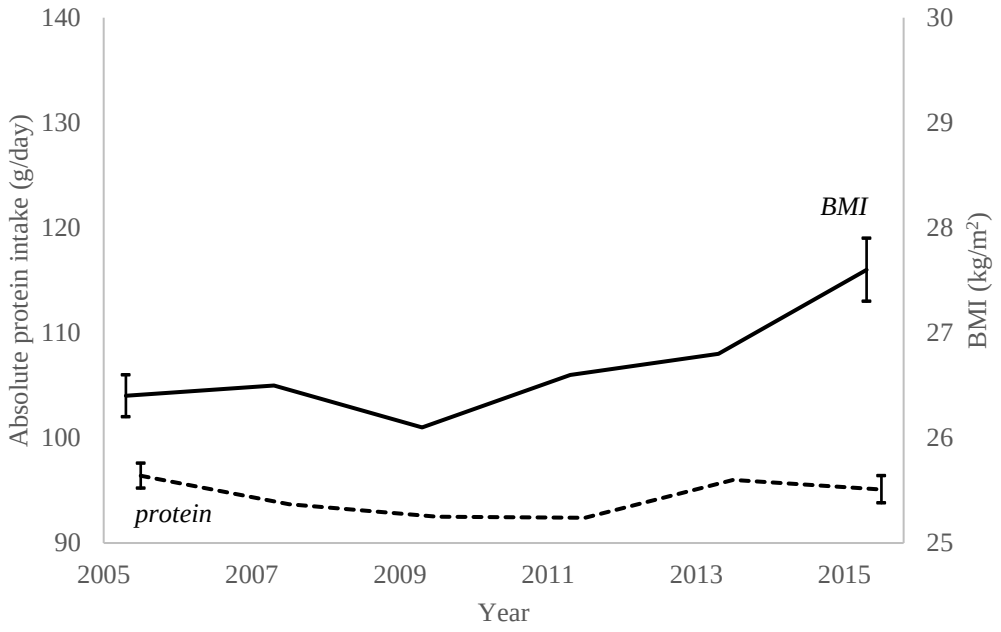

Figure 3-4 Females: dual plot of weighted means of BMI (kg/m^2; solid line) and absolute protein intake (g/day; dashed line), NHANES survey years 2005-06 through 2015-16. SEM is shown for first and last data points. Values are dithered along the *x*-axis to aid visibility.

Figure 3-5 shows a dual plot of mean weight (kg) and normalized protein intake (g/kg/day) over the 10 years of NHANES survey data. Normalized protein intake exhibited a shallow, relatively steady decrease from 2005 to 2011, followed by a peak in 2013 and a return to previous levels by 2015. Weight showed an overall increase from 2005 to 2015, with a slight dip in 2009.

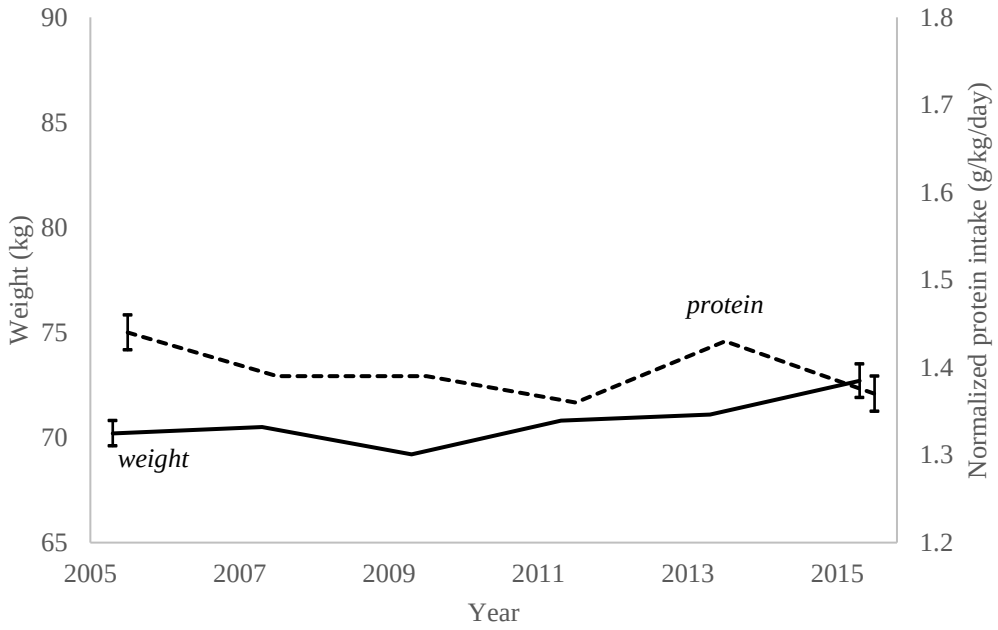

Figure 3-5 Females: dual plot of weighted means of weight (kg; solid line) and normalized protein intake (g/kg/day; dashed line), NHANES survey years 2005-06 through 2015-16. SEM is shown for first and last data points. Values are dithered along the *x*-axis to aid visibility.

Dual plots of EI (kcal/day) and dietary protein density (% energy/day) from NHANES survey years 2005-2006 through 2015-2016 are shown in Figure 3-6. EI remained relatively constant, increasing slightly by 2015 after a dip in 2009. Dietary protein density fluctuated from year to year, with no clear overall trend, and values in 2015 were similar to those in 2005.

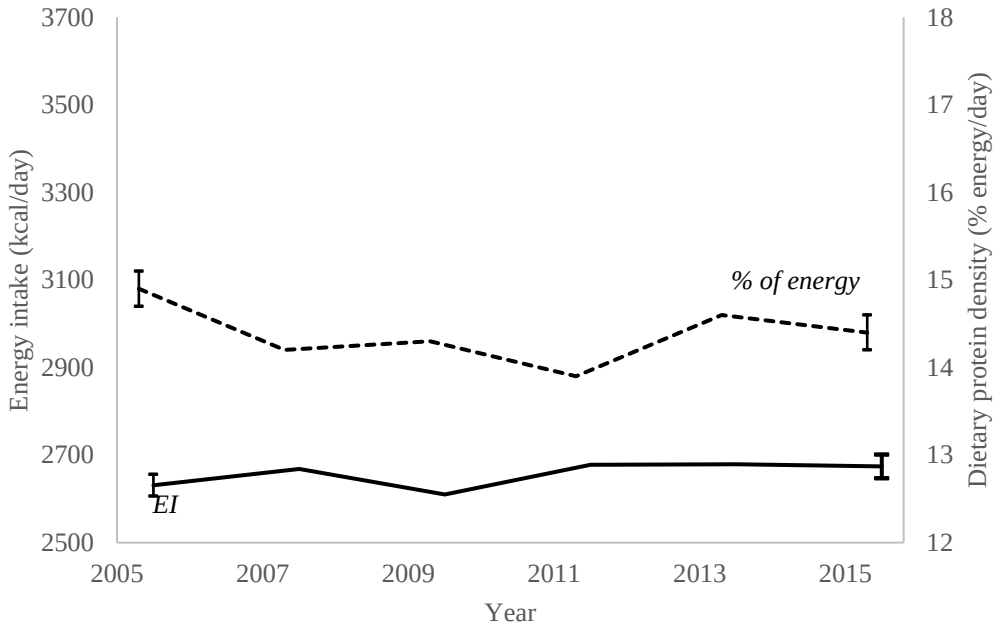

Figure 3-6 Females: dual plot of weighted means of EI (kcal/day; solid line) and dietary protein density (% total energy/day; dashed line), NHANES survey years 2005-06 through 2015-16. SEM is shown for first and last data points. Values are dithered along the *x*-axis to aid visibility.

Males

For USA males, descriptive statistics for age, anthropometric characteristics, and dietary intake values from NHANES survey years 2005-2006 through 2015-2016 are shown in Table 3-3 by survey year. Sample sizes ranged from ~730 to ~890, and are unequal for some variables within survey years due to missing data.

Table 3-3 Males: Descriptive statistics for age, anthropometric characteristics, and dietary intake values from NHANES survey years 2005-2006 through 2015-2016. Values are weighted mean ± SEM and sample size (N).

MALES	2005-2006	2007-2008	2009-2010	2011-2012	2013-2014	2015-2016
Age (yrs)	37.6 ± 0.4 (828)	37.3 ± 0.4 (836)	38.6 ± 0.4 (885)	37.5 ± 0.4 (798)	40.0 ± 0.5 (756)	40.0 ± 0.4 (742)
Weight (kg)	85.2 ± 0.6 (812)	83.5 ± 0.6 (824)	84.7 ± 0.6 (872)	84.1 ± 0.7 (789)	83.6 ± 0.7 (751)	83.7 ± 0.6 (733)
Height (cm)	177.4 ± 0.3 (815)	177.0 ± 0.3 (826)	176.7 ± 0.2 (876)	176.4 ± 0.3 (791)	176.2 ± 0.3 (751)	175.7 ± 0.3 (734)
BMI (kg/m^2)	27.0 ± 0.2 (811)	26.6 ± 0.2 (824)	27.1 ± 0.2 (872)	27.0 ± 0.2 (789)	26.8 ± 0.2 (751)	27.1 ± 0.2 (733)
Energy intake (kcal/day)	3623 ± 35 (828)	3622 ± 37 (836)	3614 ± 34 (885)	3624 ± 33 (798)	3587 ± 39 (756)	3348 ± 34 (742)
Protein intake						
Absolute (g/day)	133.9 ± 1.8 (828)	133.5 ± 1.8 (836)	136.0 ± 1.7 (885)	130.0 ± 1.7 (798)	134.6 ± 2.1 (756)	126.5 ± 1.8 (742)
Normalized (g/kg/day)	1.64 ± 0.02 (812)	1.65 ± 0.03 (824)	1.66 ± 0.02 (872)	1.59 ± 0.02 (789)	1.67 ± 0.03 (751)	1.56 ± 0.02 (733)
Density (% energy/day)	14.9 ± 0.1 (828)	14.8 ± 0.1 (836)	15.2 ± 0.1 (885)	14.4 ± 0.1 (798)	15.2 ± 0.2 (756)	15.3 ± 0.2 (742)

Comparisons of age, anthropometric characteristics, and dietary intake values between the 2005-2006 and 2015-2016 surveys are shown in Table 3-4. The age of the sample increased between survey years; subsequent analyses control for age. Weight and height both decreased, while BMI remained unchanged. Total EI decreased significantly (-275 kcal/day). Absolute protein intake (g/day) decreased between survey years. However, neither normalized protein intake (g/kg/day) nor dietary protein density (% energy/day) changed significantly (Table 3-4). The main effect of dietary protein density on EI, controlling for age, was significant in 2005-2006 (t ratio for parameter = -2.81, p = 0.005) and in 2015-2016 (t ratio for parameter = -3.40, p = 0.001), but this effect was not different between the two time periods (survey year × dietary protein density interaction term, p = 0.907; Figure 3-7).

Table 3-4 Males: comparison (ANCOVA[a]) of age, anthropometric characteristics, and dietary intake values (mean ± SEM) between NHANES survey years 2005-2006 and 2015-2016.

	2005-2006	2015-2016	*p*-value
Age (yrs)	37.6 ± 0.4	40.0 ± 0.4	< 0.001[b]
Weight (kg)	85.2 ± 0.6	83.7 ± 0.6	0.013
Height (cm)	177.4 ± 0.3	175.7 ± 0.3	< 0.001
BMI (kg/m^2)	27.0 ± 0.2	27.1 ± 0.2	0.522
Energy intake (kcal/day)	3623 ± 35	3348 ± 34	< 0.001
Protein intake			
Absolute (g/day)	133.9 ± 1.8	126.5 ± 1.8	0.011
Normalized (g/kg/day)	1.64 ± 0.02	1.56 ± 0.02	0.142
Density (% energy/day)	14.9 ± 0.1	15.3 ± 0.2	0.087

[a] covariate: age

[b] independent-samples Welch's *t*-test

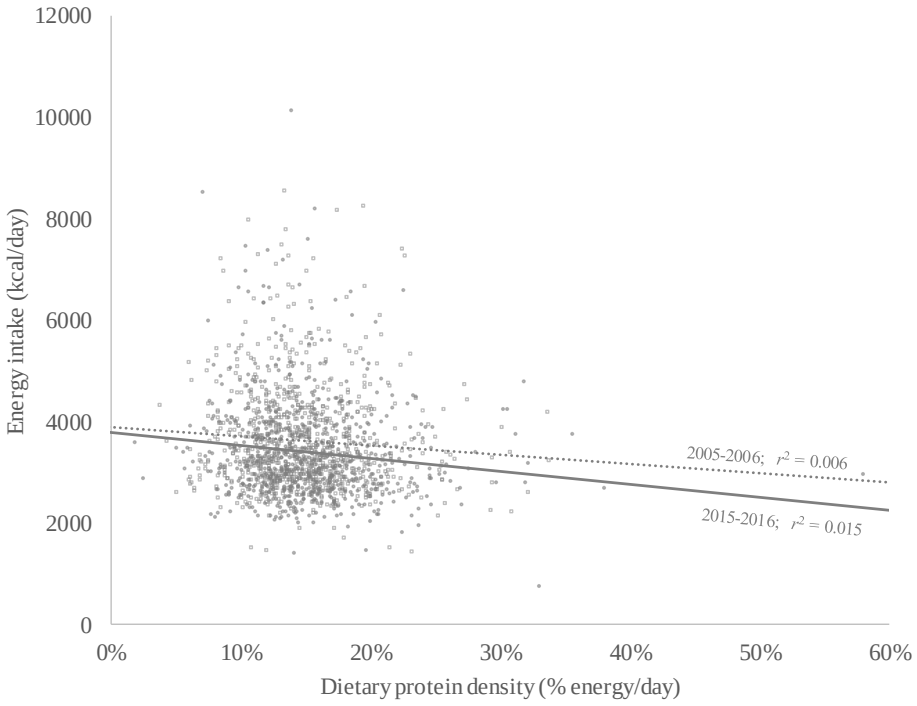

Figure 3-7 Males: relationship between energy intake (kcal/day) and dietary protein density (% energy/day) in NHANES survey years 2005-2006 (open squares, dashed line) and 2015-2016 (filled circles, solid line), with OLS regression lines by survey year.

Figure 3-8 shows a dual plot of mean BMI (kg/m^2) and absolute protein intake (g/day) from 2005-2006 to 2015-2016. Absolute protein intake exhibited fluctuations over time, with a decrease and rebound from 2009 to 2013, followed by a second, more substantial decrease from 2013 to 2015. BMI showed a lesser degree of variation over time, and values in 2015 remained similar to the initial values in 2005.

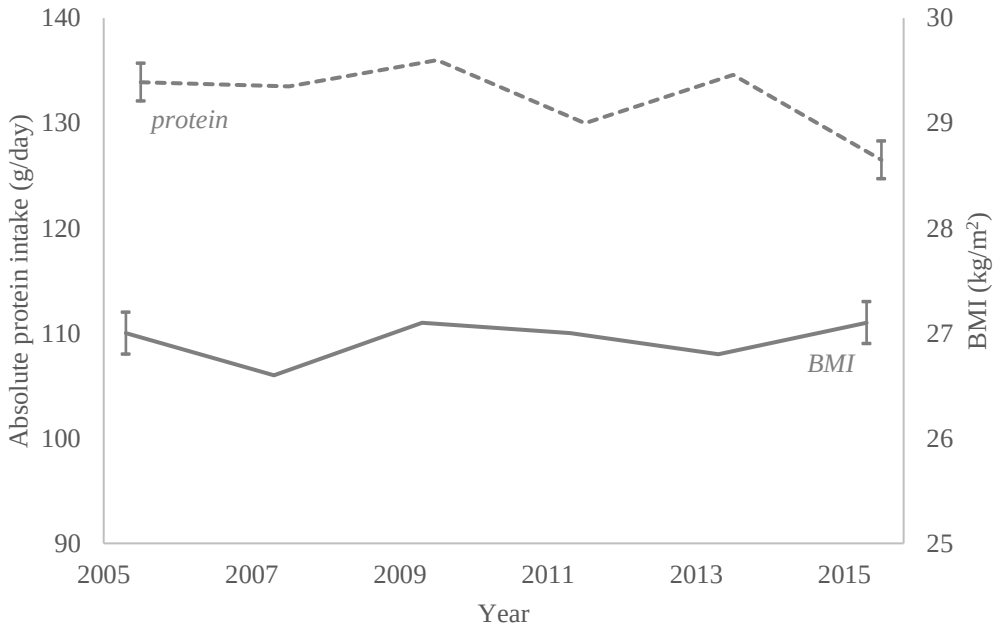

Figure 3-8 Males: dual plot of weighted means of BMI (kg/m^2; solid line) and absolute protein intake (g/day; dashed line), NHANES survey years 2005-06 through 2015-16. SEM is shown for first and last data points. Values are dithered along the *x*-axis to aid visibility.

Dual plots of weight (kg) and normalized protein intake (g/kg/day) from NHANES survey years 2005-2006 through 2015-2016 are shown in Figure 3-9. Weight declined slightly from 2005 to 2007, then rebounded in 2009, but resumed a steady but shallow decrease over the remainder of the study period. Normalized protein intake displayed substantial fluctuations between 2009 and 2015, with no clear overall trend.

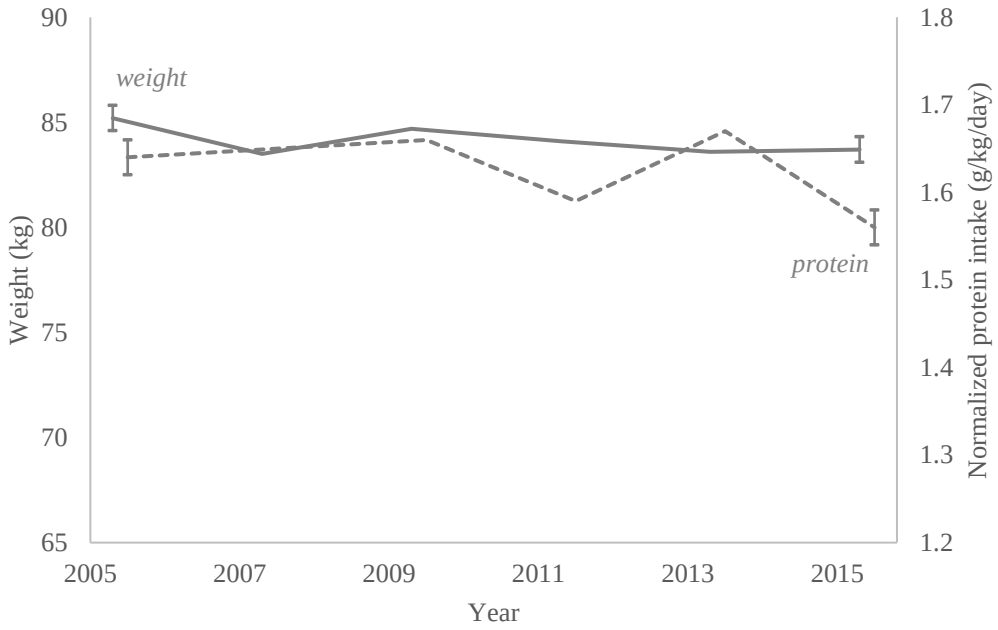

Figure 3-9 Males: dual plot of weighted means of weight (kg; solid line) and normalized protein intake (g/kg/day; dashed line), NHANES survey years 2005-06 through 2015-16. SEM is shown for first and last data points. Values are dithered along the *x*-axis to aid visibility.

Figure 3-10 shows dual plots of energy intake (kcal/day) and dietary protein density (% energy/day) over the 10 years of the NHANES survey period. Energy intake remained constant over the beginning of the survey period, before exhibiting a notable drop from 2013 to 2015. Dietary protein density also showed a notable drop, but earlier, in 2011. Subsequently, dietary protein density returned to levels comparable to 2005 to 2009.

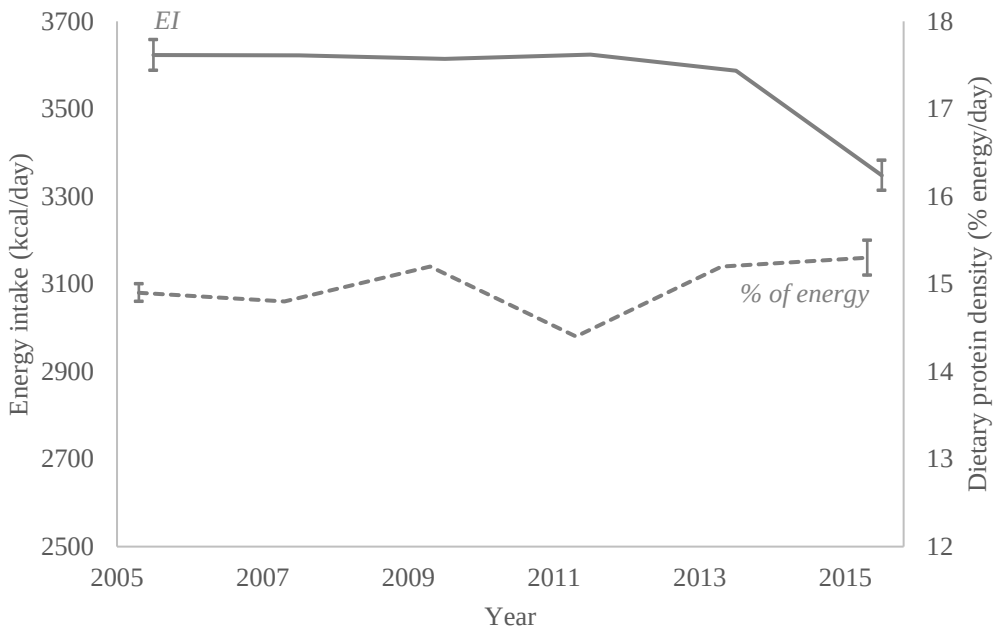

Figure 3-10 Males: dual plot of weighted means of EI (kcal/day; solid line) and dietary protein density (% total energy/day; dashed line), NHANES survey years 2005-06 through 2015-16. SEM is shown for first and last data points. Values are dithered along the *x*-axis to aid visibility.

DISCUSSION

The goal of this study was to test the predictions of the PLH, a theoretical framework that may link shifts in the protein characteristics of the diet to changes in individual food intake, using population-level dietary and anthropometric data. The data were extracted from NHANES, a continuous cross-sectional medical and nutritional survey of the USA population, for the survey years 2005-2005 through 2015-2016. NHANES data have been used for a variety of purposes by human biologists, including questions of diet and growth (Wiley, 2005), allostatic load (Geronimus et al., 2006), and hydration (Rosinger et al., 2016).

In the NHANES data we found, as expected, that females' BMI increased significantly between NHANES survey years 2005-2006 and 2015-2016, from 26.4 to 27.6 kg/m^2. Unexpectedly, however, we found that males' BMI did not change between 2005-2006 (27.0

kg/m^2) and 2015-2016 (27.1 kg/m^2). The weighted means of both female and male BMI remained well within the range defined by the WHO as *pre-obese* (25.0 – 30.0 kg/m^2) (WHO, 2006) during all years of the survey, indicating a high prevalence of overweight in the adult USA population during this time period. Thus, the relatively minor increase in BMI for females, and the lack of an increase in BMI for males, may at least partially be the result of the population as a whole already being substantially overweight by the beginning of the survey period in 2005-2006.

For the dietary analysis, we distinguished between absolute and normalized protein intake, and dietary protein density. Absolute protein intake is total daily protein intake, in g/day. We also converted absolute protein intake to normalized protein intake, calculated as g/kg/day.. Finally, we also analyzed protein intake in terms of its relation to the individual's overall energy intake, i.e., dietary protein density in % of energy/day.

First, according to the assumptions of the PLH, we hypothesized that absolute protein intake (g/day) would remain stable in the USA over time. The data for females supported this hypothesis, with absolute protein intake remaining steady over time, and with mean values in 2015-2016 not differing significantly from those in 2005-2006. On the other hand, the data for males were inconsistent with the hypothesis. Males' absolute protein intake displayed clear fluctuations over time, with mean values in 2015-2016 being significantly lower than those in 2005-2006.

These patterns of absolute protein intake were not matched by concurrent trends in BMI. For females, BMI increased steadily and significantly over time, despite the constancy of absolute protein intake. Conversely, male BMI remained unchanged between 2005-2006 and 2015-2016, despite the decrease in absolute protein intake. In other words, a change or lack of

change in absolute protein intake was not linked with an equivalent (or inverse) change or lack of change in BMI for either sex. However, since individual protein requirements vary between individuals due to body size, among other factors (Pellett, 1990), absolute protein intake may not provide an ideal test of the PLH, because this variable can be confounded by population-level shifts in body size.

Second, we hypothesized that *normalized* protein intake (g/kg/day), a body-proportional measure of protein consumption, would remain unchanged over time. Since this variable is adjusted for body size, any changes in normalized protein intake should occur independently of changes in population weight over the same time period. In contrast, absolute protein intake could be expected to increase or decrease with body weight, thus complicating any analysis of absolute protein intake in a population undergoing secular changes in body size. In accordance with our predictions, we found that normalized protein intake (g/kg/day) remained unchanged for both sexes between NHANES survey years 2005-2006 and 2015-2016, despite significant changes in weight for both females (increased) and males (decreased) over this time period. This is consistent with the idea that individuals consume a quantity of protein at a stable proportion of overall body size (Figure 3-1).

For females, the lack of change in normalized protein intake (g/kg/day), as well as the lack of change over time in absolute protein intake (g/day), were both, in themselves, consistent with our predictions regarding the stability of protein intake over time. However, female weight increased from 2005-2006 to 2015-2016. In light of this observation, we would have expected absolute protein intake (g/day) to increase as well, since the larger female body size should have driven a greater absolute demand for protein. Thus, from the perspective of changes in body size,

the lack of change over time in female absolute protein intake (g/day) was also contrary to our expectations.

For males, the opposite situation emerged regarding normalized protein intake and body size. Male normalized protein intake (g/kg/day) remained unchanged from 2005-2006 to 2015-2016, consistent with our hypothesis of stable protein intake over time. The decrease in male absolute protein intake (g/day) during this time period was, therefore, inconsistent with the hypothesis. On the other hand, the decreased absolute protein intake (g/day) was associated with a concurrent decrease in male weight, consistent with our expectations regarding the link between body size and absolute protein demand.

Third, we hypothesized that the protein density of the diet (% total energy/day) would be inversely associated with total EI; that is, an increase in EI should be driven by a lower percentage of protein in the diet, while a decrease in EI should be driven by a higher percentage of dietary protein. For females, this prediction was met, albeit with only a slight effect size. Female total EI did increase significantly over the 10 years of NHANES survey data, from 2631 kcal/day in 2005-2006 to 2674 kcal/day in 2015-2016; the magnitude of this change, an increase of ~45 kcal/day, is miniscule. Nonetheless, the increase in EI was accompanied by a small but significant decrease in dietary protein density (% total energy/day), from 14.9% in 2005-2006 to 14.4% in 2015-2016.

For males, on the other hand, the data were not consistent with our predictions. Male total EI decreased significantly, from 3623 kcal/day in 2005-2006 to 3348 kcal/day in 2015-2016, and this decrease of ~275 kcal/day was of greater magnitude than the change in EI observed for females. However, males' dietary protein density (% total energy/day) did not change

significantly between NHANES survey years 2005-2006 and 2015-2016, contrary to our predictions.

Overall, our results provide mixed support for the PLH in the USA adult population for the time period from 2005-2016. The stability of female absolute protein intake (g/day) was consistent with the PLH, while the decrease in male absolute protein intake (g/day) was not. However, we argue that data on absolute protein intake (g/day) may not provide an ideal test of the PLH because they may be confounded by changes in body size. Rather, our data were consistent with the concept that dietary behavior is driven to match the quantity of protein consumed to a physiological requirement that is proportional to body size, i.e., that normalized protein intake (g/kg/day) should remain constant over time (and in a variety of nutritional environments). Our data support this aspect of the PLH for both females and males. It is worth noting that both females and males consumed protein at a much higher body-proportional rate, ~1.4-1.6 g/kg/day, than the 0.8 g/kg/day recommended in the Dietary Reference Intakes (Institute of Medicine, 2005) for USA adults. By this measure, the USA diet is very high in protein.

By the same principle of body-proportional protein requirements, normalized protein intake (g/kg/day) should be disassociated from individual body size. This, again, was supported by our data for both females and males, since normalized protein intake (g/kg/day) was unchanged between NHANES survey years 2005-2006 and 2015-2016 despite significant shifts in weight for both sexes over this time period.

We also expected that these observed shifts in weight would be matched by concurrent shifts in *absolute* protein intake (g/day); that is, we expected a decrease in weight to be associated with a decrease in absolute protein intake (g/day). The decrease in males' body weight

was indeed matched with the expected decrease in absolute protein intake (g/day), while females exhibited no change in this aspect of dietary protein consumption, despite a significant increase in weight.

On the other hand, we did not find consistent support for an inverse relationship between total EI and the proportion of protein in the diet. We observed that a decreased dietary protein density (% total energy/day) was associated with a small, but significant, increase in total EI for females. Males exhibited a more substantial decrease in total EI over time. Notably, the available evidence for the anorexigenic (i.e., appetite-suppressing) effects of high-protein diets is considerably stronger than the evidence for the orexigenic (i.e., appetite-stimulating) effects of low-protein diets (e.g., Martens et al., 2013, 2014; see reviews in Davidenko et al., 2013; Morrison & Laeger, 2015). Thus, we expected to find a clear association between males' decreased EI and increased dietary protein density (% total energy/day), but this was not the case.

We aimed to explore the possible links between the protein characteristics of the USA diet and total EI among adults, with potential repercussions for changes in the prevalence of overweight and obesity. While many previous studies have also analyzed protein intake among USA adults, most have focused on health outcomes, rather than on dietary behavior. For example, Pasiakos et al. (2015) also utilized data from NHANES surveys (2001-2010), and found that higher normalized protein intake (g/kg/day) were associated with lower BMI and waist circumference among USA adults, along with healthier blood cholesterol profiles. Although Pasiakos et al. (2015) included EI values as covariates in their regression models, they did not directly report these EI values as outcome measures. Thus, it is unclear if the reported

link between high protein intake and lower BMI is mediated by lower EI, as predicted by the PLH.

More recently, in another analysis of NHANES data (2001-2014), Berryman et al. (2018) reported that the majority of the USA population exceeds minimum protein intake recommendations, in agreement with our findings. However, Berryman et al. (2018) also note that this high protein intake is not excessive, according to the Dietary Reference Intake values. The authors include dietary protein density (% total energy/day) in their study, but do not report total EI values. Again, the goal of Berryman et al. (2018) was to interpret USA adult protein consumption from a public-health perspective, but without a direct examination of how protein consumption may interact with other aspects of dietary behavior, such as total EI.

One of the few recent studies to explicitly link population-level dietary protein characteristics with total EI (again, using NHANES data), is Steele et al.'s (2017) analysis of so-called "ultra-processed foods" in the USA diet. They found that an increased prevalence of ultra-processed foods in the diet was associated with a decrease in dietary protein density (% energy from protein); that is, ultra-processed foods contribute little protein to the diet. Subsequently, Steele et al. (2017) also found that increased consumption of ultra-processed foods, i.e., low-protein foods, was related to an increase in total EI, while absolute protein intake remained "relatively constant" (Steele et al., 2017:114), consistent with the PLH.

However, Steele et al. (2017) only analyzed data from one NHANES survey cycle, 2009-2010, so it is unclear if their findings are representative of a population trend. Additionally, the authors do not report sample characteristics that may influence protein requirements or EI, such as age, sex, or overall body size. This could be particularly problematic because the authors include all NHANES respondents aged ≥ 2 years and ≤ 80 years; thus, the sample contains many

individuals (e.g., infants, elderly) whose protein and energy requirements are likely to differ markedly from the population norm. Further research is needed to link the available population-level data on dietary protein intake to EI to related variables of interest, such as BMI and obesity prevalence.

CONCLUSIONS

First, we found that absolute protein intake (g/day) remained constant from 2005-2006 to 2015-2016 in adult USA females, consistent with the Protein Leverage Hypothesis. However, male absolute protein intake (g/day) decreased significantly over this time period, inconsistent with the Protein Leverage Hypothesis. Second, we showed that normalized protein intake (g/kg/day) remained constant from 2005-2006 to 2015-2016 in adult USA females and males, despite changes in body weight in both sexes, consistent with our predictions. Third, we found the predicted inverse relationship between dietary protein density (% total energy/day) and total EI in females, but not in males, despite a substantial decrease in male EI over the 10-year survey period. Taken together, these data provide mixed support for the Protein Leverage Hypothesis.

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CHAPTER 4. PROTEIN QUANTITY, PROTEIN QUALITY, AND ENERGY INTAKE: A TEST OF THE PROTEIN LEVERAGE HYPOTHESIS

INTRODUCTION

The Protein Leverage Hypothesis (PLH) proposes that protein intake is under tighter physiological regulation than energy intake (Simpson & Raubenheimer, 2005). It predicts that lower-protein diets will result in excess energy intake, as individuals are physiologically driven to over-consume food to meet protein requirements. Conversely, higher-protein diets are expected to result in lower energy intake, as individuals are able to meet their constant protein requirement with less total food consumption (Figure 4-1).

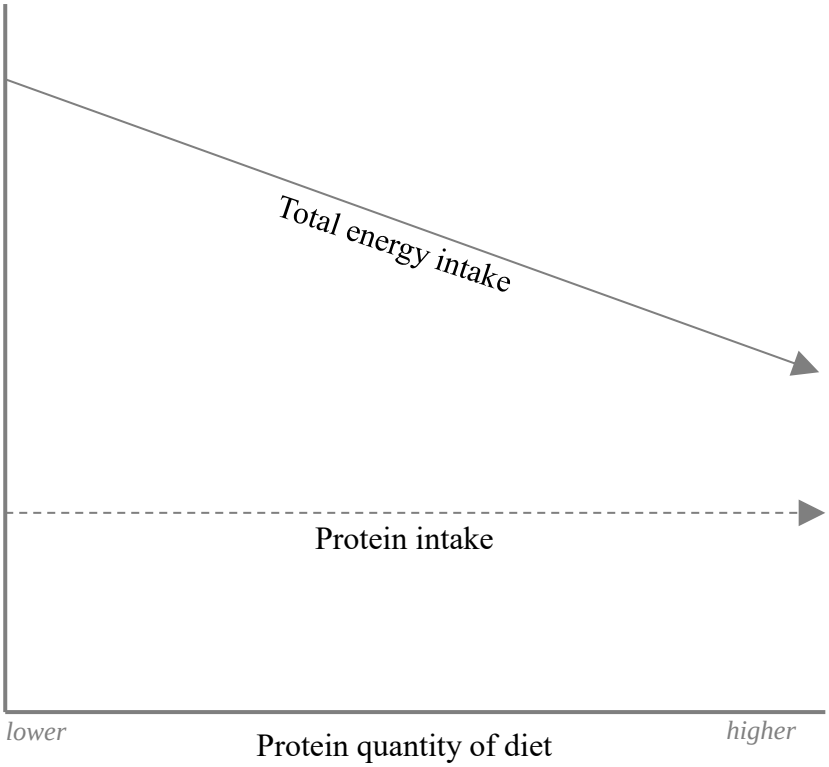

Figure 4-1 Schematic representation of the Protein Leverage Hypothesis

The practical consequences of the PLH for human nutritional epidemiology are argued as follows. Highly processed foods are becoming increasingly prevalent in diets worldwide, due to the Nutrition Transition (e.g., Popkin, 1993, 2006) or comparable processes. These processed

foods are calorie-dense and rich in simple carbohydrates, but deficient in dietary fiber, protein, and micronutrients (Cordain et al., 2005). They may also contain high amounts of added sugars, added sodium, and potentially unhealthy fats (e.g., *trans* fatty acids, saturated fats). Since highly-processed foods tend also to be protein-deficient (Mauron, 1990), individuals are physiologically driven to over-consume these foods in order to meet protein requirements (Simpson & Raubenheimer, 2005). The result of this over-consumption is an excess intake of total calories, and possibly an excess intake of added sugars, added sodium, and unhealthy fats as well. In socioeconomic terms, the situation is compounded by the overall higher monetary cost of protein-rich foods; the lower cost of carbohydrate-dense foods "may bias consumers towards diets high in carbohydrate energy, leading them to consume excessive energy to meet their dietary protein needs" (Brooks et al., 2010:887).

Experimental evidence for the PLH in humans has been equivocal. Some investigators have found support for the PLH (Poppitt et al. 1998; Simpson et al., 2003; Weigle et al., 2005), while others have not (Marmonier et al., 2000; Raben et al., 2003; Griffioen-Roose et al., 2011, 2012; Belza et al., 2013), and most have found mixed results (Stubbs et al., 1996; Gosby et al., 2011; Martens et al., 2013, 2014). The ambiguity of these results may in part be due to methodological limitations. Specifically, the comparisons of *ad libitum* energy intake on low- and high-protein menus of whole food items (e.g., Simpson et al., 2003; Gosby et al., 2011) may be confounded by differences in the taste, texture, and cultural value of foods.

Additionally, most studies to date have ignored differences in protein quality. This is also a potential confounding factor, since proteins of different quality (i.e., plant- *vs*. animal-source protein) may exert different effects on total food consumption (Morrison & Laeger, 2015; Figure 4-2).

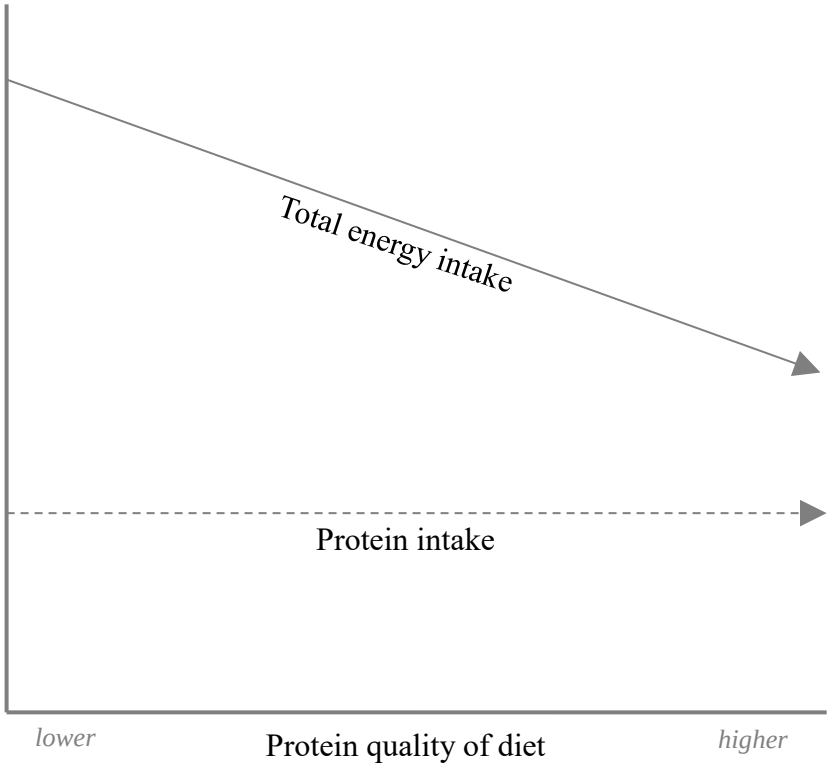

Figure 4-2 Proposed extension of the Protein Leverage Hypothesis

Dietary proteins from various plant or animal sources can differ in numerous nutritional characterstics, such as digestibility or amino acid composition, which can subsequently be used to define the quality of the protein (e.g., FAO/WHO, 1991). In particular, proteins with varied amino acid compositions may have different effects on satiety, possibly due to nutrient-specific responses of orexigenic or anorexigenic hormones (Veldhorst et al., 2008). Also, dietary proteins must provide the body with not only nitrogen, but with essential amino acids (Institute of Medicine, 2005). Thus, higher-quality proteins containing a greater proportion of essential amino acids allow the body to meet its physiological requirements more easily. For these reasons, we propose and test an extension of the PLH, in which higher-quality proteins are associated with decreased EI (Figure 4-2).

Here, we present results from an experimental test of the PLH, using liquid diets to overcome some of the limitations of prior research. Our objectives are to 1) determine if dietary protein quantity (10% *vs*. 25% of energy from protein) influences daily energy intake (EI) and 2) determine if dietary protein quality (plant- *vs*. animal-source) influences daily EI. Specifically, we hypothesize that total daily *ad libitum* EI will be greater on the low-quantity diet (10% protein) and on the low-quality diet (plant-source protein).

METHODS

Participants

We recruited a convenience sample of healthy adults from the Boulder, CO area aged 20 to 45 years. The age range of eligible participants was selected to include individuals who are fully grown adults (≥ 20 yrs), yet whose protein requirements are not yet substantially impacted by increased age (≤ 45 yrs). Since protein requirements may increase with age (Pellett, 1990; Campbell et al, 1994; Morais et al., 2006), likely due to a progressive loss of lean body mass and concurrent increase in protein demand with age (Forbes, 1976), the inclusion of older adults could introduce a confounding factor.

Participation was also limited to individuals with a body mass index (BMI) between 20.0 and 30.0 kg/m^2; this range includes individuals defined by the WHO as *normal weight* (18.5 – 25.0 kg/m^2), and *pre-obese* (25.0 – 30.0 kg/m^2) (WHO, 2006). This BMI range was intended to only include individuals of relatively healthy weight status, since underweight or obese individuals may have metabolic characteristics that would confound the results of this study. For example, underweight individuals may show increased insulin sensitivity (Tayek et al., 1997), and high-protein diets may induce metabolic changes in obese individuals over and above the changes in total energy intake hypothesized in this study (Skov et al., 1999; Farnsworth et al.,

2003). Additionally, highly physically active individuals reporting ≥ 2.5 hours of moderate or vigorous physical activity per week were excluded from this study, since protein requirements are known to be greater in competitive athletes and other individuals with very high physical activity levels (Lemon, 1998; Tarnopolsky, 2004). Finally, participants were non-pregnant and non-lactating, did not report currently being on a high-protein or weight-loss diet, and did not report having diabetes, eating disorders, or other metabolic disorders.

Power analysis & sample size

A power analysis was conducted with GLIMMPSE v 2.2.5 (glimmpse.samplesizeshop.org; Kreidler et al., 2013) to determine the total sample size necessary for this study. A sample size of N = 18 was required to achieve power ≥ 0.80 at α = 0.05 under the following assumptions:

- Statistical family: multivariate approach to repeated measures (Hotelling-Lawley Trace)
- Hypothesis type: main effect of dietary treatment on daily energy intake over repeated measures
- Bonferroni correction of α for four post-hoc comparisons: 0.0125
- Grand mean: 2,400 kcal/day
- Effect size: ± 200 kcal/day
- Variability: ± 300 kcal/day
- Correlation of energy intakes among treatments: r = 0.30

The assumed grand mean of 2,400 kcal/day was based on data from two 3-day dietary intervention studies (Cornier, personal communication), which are likely to better reflect typical daily energy intake of USA adults in an intervention setting than in a free-living setting. The effect size of ± 200 kcal/day represents what we would consider a satisfactory demonstration of

the PLH, based on the range of effect sizes reported by previous tests of the PLH, e.g., 136 kcal/day (Martens et al., 2014), 260 kcal/day (Gosby et al., 2011), 441 kcal/day (Weigle et al., 2005), 507 kcal/day (Martens et al., 2013). The assumed variability of ± 300 kcal/day is derived from two previous studies which measured total daily *ad libitum* energy intake of participants constrained to purely liquid diets for multiple days (Meier et al., 1993; Mustad et al., 1999); this variability is lower than what would be expected on a free-living diet of normal foods. The assumed correlation of energy intakes among treatments of $r = 0.30$ is calculated from previously-collected, multiday dietary data from free-living women Cali, Colombia (Dufour et al., 2015; Dufour, unpublished data). We expected the correlation among treatments to be higher in this liquid diet intervention study than in a free-living context, but additional data were not available. Therefore, we used the conservative value of $r = 0.30$.

Experimental design

This study used a single-blind, full-factorial, randomized, repeated-measures, cross-over design, in which each participant subsisted for 48-hour treatment phases on each of four liquid diet formulas which differed in protein quantity and protein quality. Participants were provided with 9000 kcal of the liquid diet for each 48-hour treatment phase (Figure 4-3), to be consumed *ad libitum* with unrestricted non-caloric beverage intake. Participants were permitted to consume caffeine, artificial sweeteners, tobacco, marijuana, and other substances known to potentially influence appetite and taste perception, as long as participants maintained their habitual intake of these substances on all four liquid diet formulas. Thus, we rely on the repeated-measures design, in which all participants are their own controls, to mitigate the potential confounding effects of the many dietary and non-dietary consumables that could impact our measurements of EI.

There was a 1-month washout between diets. Female participants underwent each treatment phase at the same self-reported stage of the menstrual cycle, to account for cycle-dependent changes in appetite and food intake (Dalvit, 1981; Lissner et al., 1988; Johnson et al., 1994; Buffenstein et al., 1995; Dye & Blundell, 1997), likely due to progesterone antagonism of estradiol's anorexigenic effects during the luteal phase (Czaja 1978; Hirschberg, 2012).

Figure 4-3 9000 kcal (48-hour supply) of liquid diet

Liquid diets were custom-made at the Nutrition Services Laboratory at the Denver Clinical & Translational Research Center (CTRC), University of Colorado Denver, and designed to be as similar as possible in taste, texture, and energy density (~1.5 kcal/g). Liquid diet formulas differed in protein quantity (10% *vs*. 25% energy from protein) and in protein quality (plant- *vs*. animal-source protein). We defined foods with a higher Protein Digestibility Corrected Amino Acid Score (PDCAAS; FAO/WHO, 1991) to be "higher-quality", and foods

with a lower PDCAAS to be "lower-quality." For this study, whey and pea were selected as the primary protein sources due to their differing protein quality: whey protein is considered a higher-quality protein with a PDCAAS of 1.0, while pea protein is a lower-quality protein with a PDCAAS of 0.7. For each diet formula, equal quantities of the same four flavors were provided: vanilla, chocolate, strawberry, and coffee. The diet formulas were designed to be as similar as possible in taste, texture, smell and appearance. Table 4-1 lists the macronutrient sources and composition of the four diet formulas.

Table 4-1 Description of liquid diet formulas

	Macronutrient sources			Macronutrient energy breakdown (% of energy)		
Diet formula	Protein	Carbohydrate	Fat	Protein	Carbohydrate	Fat
Low-quantity Plant protein	Pea protein	Maltodextrin, sucrose	Canola oil	10	60	30
Low-quantity Animal protein	Whey	Maltodextrin, sucrose	Canola oil	10	60	30
High-quantity Plant protein	Pea protein	Maltodextrin, sucrose	Canola oil	25	45	30
High-quantity Animal protein	Whey	Maltodextrin, sucrose	Canola oil	25	45	30

Procedures

Participant recruitment was performed at the Boulder CTRC, University of Colorado Boulder. While a final sample size of N = 18 will be required for full power, preliminary data for 14 participants are presented here. This study is ongoing, and additional data from participants will be incorporated into the analysis until the final sample size is achieved.

Each participant underwent a total of four dietary treatment periods. Each dietary treatment period lasted 48 hours, beginning and ending at the Boulder CTRC. At the beginning of the period, participants were given a supply of the liquid diet, weighed to provide 9000 kcal to be consumed *ad libitum* over 48 hours. Participants were not aware of the energy density of the liquid diet or of the total energy made available to them, and were instructed not to discard any unconsumed liquid diet. At the end of the 48 hour treatment period, participants returned to the Boulder CTRC with any unconsumed liquid diet, which was subsequently weighed back to calculate total daily energy intake (EI). EI values were converted to percentage of each participant's estimated energy requirement (EER) based on FAO/WHO/UNU (2001) recommendations.

Statistical analysis

To analyze the independent effects of treatment phase, protein quantity, and protein quality on EI in our repeated-measures design, we fit a linear mixed-effects model with a restricted maximum likelihood estimator (REML) (Richardson & Welsh, 1995). Individual participant ID was entered as the random effect, while treatment phase, protein quantity, or protein quality was entered as the fixed effect. The Benjamini-Hochberg correction as used for multiple comparisons (Benjamini & Hochberg, 1995). Descriptive statistics reported for EI are least squares means ± SE. Statistical analyses were performed in JMP Pro 14 with significance set at $p < 0.05$.

RESULTS

Data were collected from 14 participants, 9 females and 5 males. Means of participant age, anthropometric characteristics, and EER are shown in Table 4-2.

Table 4-2 Participant characteristics (9 females, 5 males)

Characteristic	Mean ± SD
Age (yrs)	27 ± 6
Height (m)	1.71 ± 0.16
Weight (kg)	67 ± 13
BMI (kg/m^2)	23.3 ± 4.2
Estimated energy requirement (EER) (kcal/day)	2290 ± 355

There was no fixed effect of treatment phase on total daily EI, expressed as either kcal/day ($F_{3,25.2} = 0.48$, $p = 0.699$) or as percentage of EER ($F_{3,25.5} = 0.45$, $p = 0.717$); i.e., there were no repeated-measures differences in EI on treatment phases 1, 2, 3, and 4, independent of which dietary formula participants had been randomly assigned to for that phase. Participants tolerated the liquid diet protocols well, with none experiencing gastrointestinal stress, anxiety, irritability, or excessive feelings of hunger. Daily *ad libitum* EI ranged from 795 to 4073 kcal, and from 30% to 180% of EER.

To portray this variability, Figures 4-4 and 4-5 show boxplots of daily EI (kcal/day) on diets of different protein quantity (10% *vs*. 25%) and on diets of different protein quality (pea *vs*. whey). These boxplots are illustrative but not analytically rigorous, since they do not account for the covariance among EI values from the same individuals.

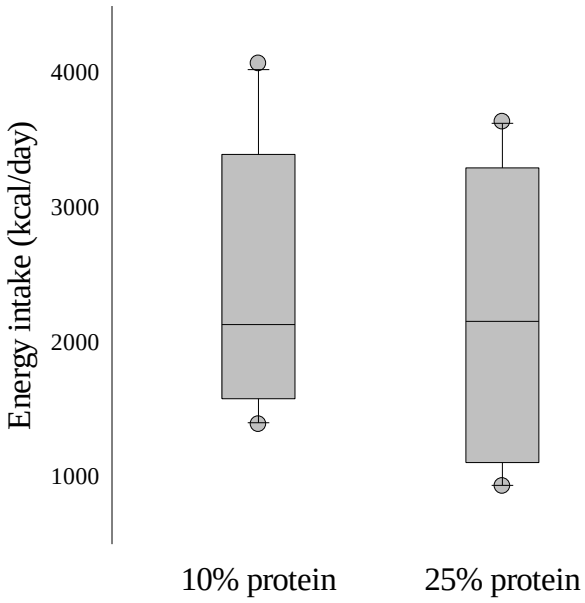

Figure 4-4 Boxplots of daily EI (kcal/day) for diets of different protein quantity. Boxplots show 10th, 25th, 50th, 75th, and 90th percentiles; circles indicate points beyond this range.

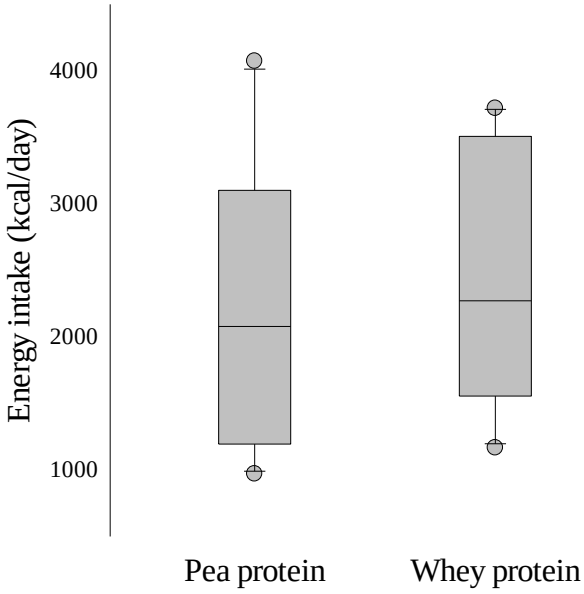

Figure 4-5 Boxplots of daily EI (kcal/day) for diets of different protein quantity. Boxplots show 10th, 25th, 50th, 75th, and 90th percentiles; circles indicate points beyond this range.

Figure 4-6 shows biplots of individual EI (kcal/day) on four combinations of liquid diet formulas. Each plot compares EI on diets with different protein quantities (10% *vs*. 25%) within a given level of protein quality (upper plots), or EI on diets of different protein quality (pea *vs*. whey) within a given level of protein quantity (lower plots). Diagonal lines are lines of unity ($y = x$); points along these lines represent identical individual EI on the two diets under comparison.

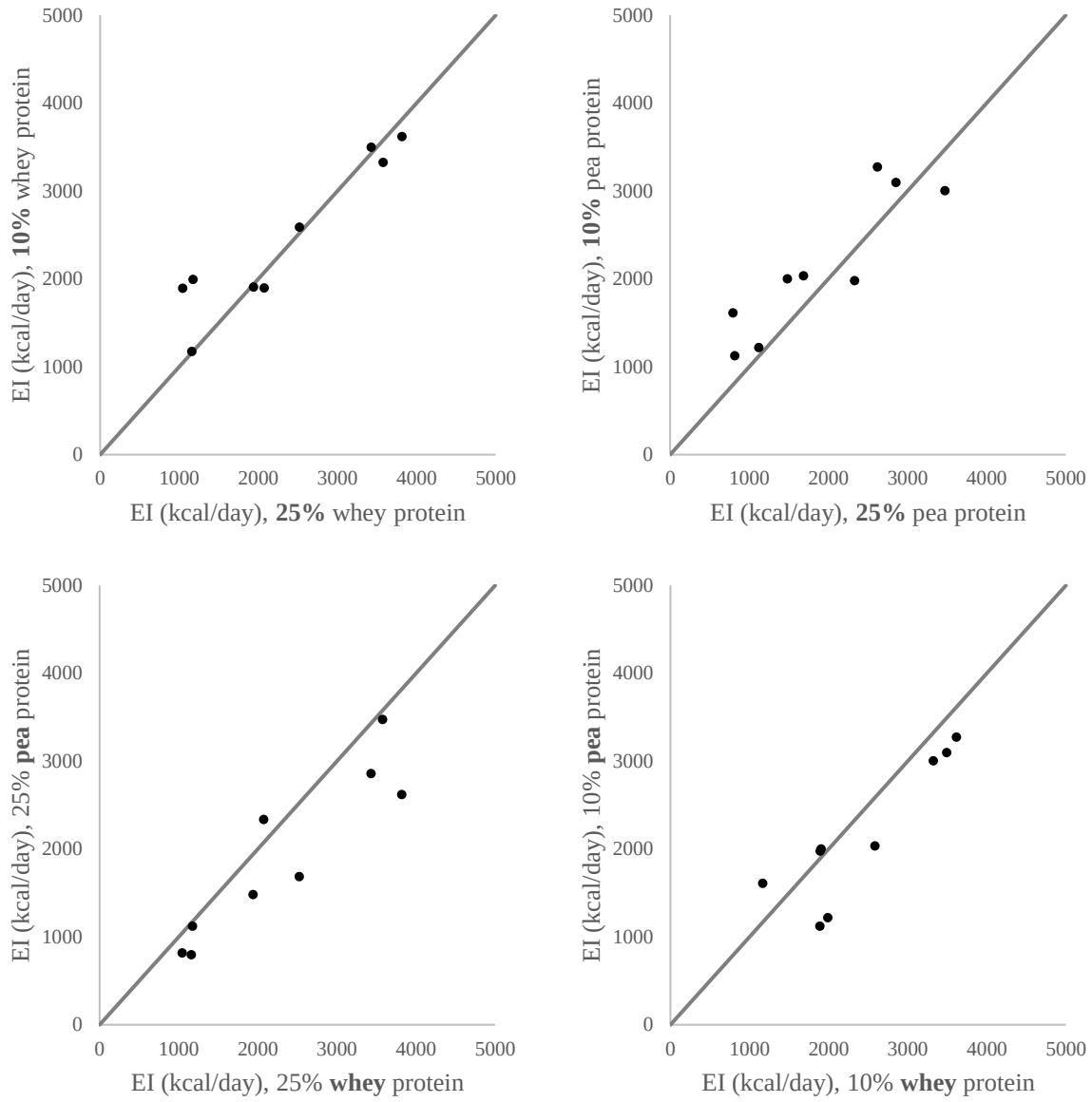

Figure 4-6 Biplots of individual EI (kcal/day) differing in either protein quantity (10% vs. 25%, upper plots) or protein quality (pea *vs*. whey, lower plots), with lines of unity.

In the repeated-measures analysis, there was no fixed effect of protein quantity, i.e., 10% *vs*. 25% protein, on total daily EI (kcal/day; $p = 0.094$). There was, however, an effect of protein quality, i.e., pea *vs*. whey protein, on total daily EI ($p = 0.036$), with significantly greater EI on the whey-protein test meals (Table 4-3).

Table 4-3 Fixed effect of protein quantity and protein quality on total daily EI (kcal/day). Values are least squares means ± SE.

	Protein quantity		*F*	df	*p*-value[a]
	10% (low)	25% (high)			
EI, kcal/day	2419 ± 295	2221 ± 293	3.01	1, 27.2	0.094
	Protein quality				
	Pea (low)	Whey (high)			
EI, kcal/day	2151 ± 298	2447 ± 296	7.86	1, 27.1	0.036

[a] Benjamini-Hochberg correction

Similarly, when total daily EI was expressed as % of EER, there was no fixed effect of protein quantity ($p = 0.089$), but a significant effect of protein quality ($p = 0.028$), with greater EI on the whey-protein test meals (Table 4-4).

Table 4-4 Fixed effect of protein quantity and protein quality on total daily EI (% of EER). Values are least squares means ± SE.

	Protein quantity		*F*	df	*p*-value[a]
	10% (low)	25% (high)			
EI, % of EER	106 ± 12	97 ± 12	3.18	1, 27.4	0.089
	Protein quality				
	Pea (low)	Whey (high)			
EI, % of EER	93 ± 12	107 ± 12	8.48	1, 27.3	0.028

[a] Benjamini-Hochberg correction

DISCUSSION

In this experimental test of the PLH, we asked whether the protein characteristics of *ad libitum* liquid diets influenced participants' total EI. First, we found that dietary protein quantity (10% *vs*. 25% of energy) did not affect daily *ad libitum* EI, inconsistent with the PLH. Second, we found that dietary protein quality (plant *vs*. animal-source) did significantly affect daily *ad libitum* EI, with EI being greater on the high-quality, animal-source protein (whey) than on the low-quality, plant-source protein (pea). The magnitude of the difference, ~300 kcal/day, was relatively small, but still greater than the 200 kcal/day effect size we had defined as biologically meaningful in our power analysis. On the other hand, the direction of the difference was opposite to our expectations: in our proposed extension of the PLH, we had hypothesized that EI would be greater on the *low-quality* (pea) diet, not the high-quality (whey) diet. Therefore, these results are also inconsistent with our predictions.

Our liquid diets were designed to mitigate the confounding effects of varying taste, texture, smell, and cultural value of whole foods with differing protein characteristics. These sensory characteristics of foods can potentially influence individual satiety and energy intake. Satiety and energy intake can also be affected by portion sizes and the energy densities of foods (Kissileff et al., 1984; Kral & Rolls, 2004), or the variety of foods available in the diet (Brondel et al., 2009). Again, our liquid diets were comparable in energy density, and the same four flavors were provided to participants for each diet, so these factors should not have confounded our assessments of EI. Also, participants in this study were not aware of the energy density of the liquid diets or of the total energy made available to them for the treatment period. In contrast, previous tests of the PLH using whole-food diets (e.g., Simpson et al., 2003; Gosby et al., 2011; Griffioen-Roose et al., 2011, 2012; Martens et al., 2013, 2014) may have been confounded by

factors unrelated to protein; that is, the differences in EI reported in these studies may have been driven in part by differences in the sensory characteristics, energy densities, portion sizes, or variety of various high-protein and low-protein whole food items.

In the current study, all participants successfully tolerated the 48-hour *ad libitum* diet protocols, yet EI varied greatly, both between and within individuals. This variability is evident in Figure 4-6, which plots individual pairs of EI values on diets with different protein characteristics. According to the null hypothesis of no effect of protein characteristics on EI, all points in Figure 4-6 would be expected to fall on the line of unity, representing equal EI on the two dietary formulas under comparison. Conversely, under our research hypotheses that lower-quantity (10% protein) and lower-quality (pea protein) diets should drive increased EI, all points would be expected to fall above the line of unity (i.e., the residuals should be positive). In Figure 4-6, some points do fall along or near the line of unity, indicating no difference in EI on the diets, consistent with the null hypothesis. At the same time, many points also deviate substantially from the line of unity, indicating a difference in EI on two diets differing in protein characteristics; however, the residuals are not consistently positive, as predicted by our research hypotheses.

Additionally, there was no repeated-measures effect of treatment phase on EI, suggesting that the temporal sequence of the four dietary treatments did not result in a training effect that confounded the analysis of EI. During the course of the study, qualitative self-reports from participants suggested that EI may have been higher on the 2nd treatment phase, regardless of the liquid diet formula, due to participants becoming more comfortable with the protocol. Conversely, some participants reported feelings of fatigue with the protocol during the 3rd treatment phase, but subsequently felt motivated to complete the study during the 4th treatment

phase. While these subjective factors could potentially have generated a training effect that influenced variability in EI and thereby confounded the analysis of protein characteristics and EI, our analysis found no such effect.

The unusual level of variability in EI observed in this study may have been linked to the use of liquid dietary treatments. For instance, several experimental studies have demonstrated the high satiety value of soups (Kissileff, 1984; Rolls et al., 1990; Himaya & Louis-Sylvestre, 1998; Mattes, 2005; Flood & Rolls, 2007), suggesting that EI should be reduced on a liquid diet. On the other hand, Almiron-Roig et al. (2004) reported no difference in satiety or subsequent EI from solid or liquid food preloads, while numerous studies have found lower satiety and/or greater EI when dietary energy was consumed in liquid form, as meal replacements or beverages (DiMeglio & Mattes, 2000; Rothacker & Watemberg, 2004; Mourao et al., 2007; Tieken et al., 2007; Stull et al., 2008; Leidy et al., 2010a).

Thus, the evidence is equivocal regarding the relative satiating effects of food energy in liquid form. Indeed, these effects may be mediated by cognitive factors, e.g., the different dietary qualities associated with labels such as “soups” or “beverages” (Mattes, 2006a,b). While it is clear that the physical form of the diet (solid *vs*. liquid) can have an effect on food intake, the direction and magnitude of the effect is still uncertain, with “soups” generally being associated with relatively greater satiety than “beverages”. More research is needed to explore the cognitive associations of full-meal replacements such as “protein shakes”, and whether these associations have an impact on satiety and *ad libitum* EI. Nonetheless, our repeated-measures design should ensure that any effects of liquid diets on satiety, even if these differed between individuals, e.g., due to personal experience with protein shakes or liquid meal replacements in this population, did not confound our analysis of protein characteristics and EI.

The lack of an effect of protein quantity on daily *ad libitum* EI was also surprising in light of the links between high-protein diets and weight loss reported extensively in the literature (e.g., see reviews in Westerterp-Plantenga & Lejeune, 2005; Veldhorst et al., 2008; Westerterp-Plantenga et al., 2009; Martens & Westerterp-Plantenga, 2014; Leidy et al., 2015), potentially due to mechanisms such as the satiating effects of protein (Vandewater & Vickers, 1996; Leidy et al., 2010b; Fromentin et al., 2012) or increased metabolic rates on high-protein diets (Bray et al., 2015). For example, Weigle et al. (2005) found that an increase in dietary protein from 15% to 30% of total energy resulted in sustained reductions in appetite, EI, and body weight over a 2-week period. Similarly, Clifton et al. (2008) found that a high-protein diet (34% of energy) contributed significantly to weight loss over 12 weeks compared to a high-carbohydrate diet; however, this study used restricted-calorie diets specifically to induce weight loss. In a longer-term, year-long study, Soenen et al. (2012) demonstrated that the high-protein component of a dietary intervention, rather than a low-carbohydrate component, was responsible for sustained weight loss. The Soenen et al. (2012) study was, however, also a weight-loss intervention with calorie restriction.

What is less clear is how high-protein diets influence satiety and EI in less-clinical contexts. Indeed, population-level observational studies of protein intake tend to focus on health outcomes rather than dietary behavior *per se*. For instance, Pasiakos et al. (2015) analyzed data from the National Health and Nutrition Examination Surveys (NHANES) from 2001-2010, finding that higher-protein diets were associated with lower BMI and waist circumference, and more favorable blood cholesterol profiles, among USA adults. While these authors included EI as covariates in their regression models, they did not present EI as an outcome in itself. More recently, Berryman et al. (2018) also analyzed protein intake trends in the NHANES data from

2001-2014. They report that the majority of the US population exceeds minimum protein intake recommendations, although the intake is not excessive, according to the Dietary Reference Intake values. Although dietary protein densities (as % of EI) are included in this study, total EI values are not reported. Further research can link the available population-level data on protein intake to EI and related variables of interest, such as BMI.

Additionally, our study examined the effect of protein quality (plant *vs*. animal-source) on EI, independent of protein quantity (10% *vs*. 25% of energy). We hypothesized that the lower-quality plant-source protein, i.e., pea protein, would be associated with increased EI, since proteins with varied amino acid compositions are proposed to have different effects on satiety, possibly due to different nutrient-related responses of orexigenic or anorexigenic hormones (Veldhorst et al., 2008). We did find an effect of protein quality on EI, but it was opposite to what we had predicted, with the lower-quality plant-source protein being associated with *decreased* EI.

Few previous studies have explicitly examined the relationship between protein quality and EI. Hall et al. (2003) and Veldhorst et al. (2009) did compare the effects of whey-based and casein-based preloads on subsequent *ad libitum* EI, finding a greater satiating effect (i.e., lower EI) of whey protein. Alfenas et al. (2010) found the opposite effect, reporting lower EI on a casein-based diet than on a whey-based diet. However, whey and casein are two protein sources of equal quality (PDCAAS = 1.0). On the other hand, Martens et al. (2013) compared EI on two protein sources of different quality: soy protein and whey protein with α-lactalbumin. While these authors report that EI was related to the protein quantity of experimental diets (5%, 15%, or 30% of energy), there was no effect of protein type.

Notably, while Hall et al. (2003) and Veldhorst et al. (2009) distinguish between "fast"-digesting protein (whey) and "slow"-digesting protein (casein) according to the definitions of Boirie et al. (1997), none of these studies quantify the differences in quality among their protein sources, or define what is meant by protein quality. Instead, their analyses are framed more generally in terms of protein types or sources. Indeed, even a recent review of protein-dependent regulation of feeding and metabolism states that "both the quantity and quality of dietary protein can markedly influence food intake" (Morrison & Laeger, 2015:256), but the authors do not actually define protein quality. Additional research is needed to extend the limited clinical data available on protein types and EI to free-living, population-level contexts, and to more rigorously connect the effects of different protein types to quantifiable measures of protein quality.

Strengths and limitations

The primary strength of this experimental test of the PLH is the repeated-measures crossover design, in which research participants acted as their own controls. This increases our confidence than any within-participants differences (or lack of differences) in EI among diet formulas were the result of differences in protein quantity and protein quality, rather than differences in age, sex, or individual energy requirements, metabolic characteristics, or physical activity levels.

Next, our experimental treatments took the form of liquid diets ("protein shakes") that differed in protein quantity and protein quality, but were similar in appearance, taste, texture, and smell. Since our study used homogenous liquid diets, it is more likely that we were able to isolate the effects of protein quantity and protein quality on energy intake. An additional strength of this study is that 48-hour EI was assessed via weigh-back of liquid diets with known energy densities. Therefore, we are confident in the accuracy of the reported EI values.

A primary limitation of this study is the incomplete sample. While a final sample size of N = 18 will be required for full power, preliminary data for 14 participants were presented here. It is possible that the additional data from the remaining participants will alter our findings, including the statistical significance or non-significance of protein quantity or quality on EI. However, the likelihood of this is lessened by the high degree of variability in EI observed in these preliminary data.

Another limitation of this study is that we collected no data on participants' energy expenditure, habitual EI, or physical activity levels during the treatment periods. Therefore, we were unable to assess participants' energy balance. Similarly, participants' energy requirements were not measured directly, but were instead estimated from the FAO/WHO/UNU (2001) equations, so EI results expressed as a percentage of EER are estimates as well.

Additionally, the 48-hour duration of the treatment periods, while longer than that of many previous studies, may still not have been long enough for protein leverage effects to manifest. While the duration was longer than that of "pre-load" designs, which typically occur over a period of several hours (Poppitt et al., 1998; Marmonier et al., 2000; Griffioen-Roose et al., 2011), the duration was not as long as previous studies of protein and EI (Gosby et al., 2011, 2012; Martens et al., 2013, 2014). However, many of these previous studies were weight-loss interventions, with restricted-calorie diets, rather than *ad libitum* feeding (Clifton et al., 2008; Soenen et al., 2012, 2013).

A related issue is the experimental duration in the context of liquid diets. Previous studies have successfully had participants subsist on a liquid diet for a week (Meier et al., 1993) or even longer (Brown et al., 1983; Donnelly et al., 1991; Lean et al., 2013). Again, however, these longer-duration studies tend to be weight-loss interventions (Hart & Warriner, 2005) or other

medical interventions related to aerobic fitness and body composition (Bryner et al., 1999), improved blood lipid profiles (Mustad et al., 1999), etc. It is unlikely that participant compliance could have been maintained for a similarly long duration in this study, which did not offer participants any potential health benefits.

Although our liquid dietary formulas were designed to overcome several limitations of previous research, there is also a range of potential limitations associated with the liquid diets. While the liquid dietary formulas used in this study were of similar energy densities, they were not identical, ranging from 1.23 to 1.75 kcal/g. Energy densities differed slightly among the four flavors offered for each dietary formula, and also between the high-protein and low-protein formulas. They did not, however, differ between whey-protein and pea-protein formulas. Differences in energy density are known to have a potential effect on satiety and EI (Kral & Rolls, 2004); therefore, these minor variations among dietary formulas could have confounded the analysis of EI values.

The dietary formulas were also designed to be as similar as possible in taste, texture, smell, and appearance. Participants were offered the same four flavors for each dietary formula (vanilla, chocolate, strawberry, coffee), and the flavoring agents were identical for each formula (e.g., vanilla extract or fresh blended strawberries in each case). However, we did not rigorously test the sensory equivalence of the dietary formulas. Informal single-blind tests were performed among researchers and staff at the Denver CTRC Nutrition Services, but no formal double-blind data were collected.

Also, informal qualitative observations suggest that participants were able to identify the protein quantity or protein quality of certain dietary formulas, or at least, that some participants believed they were able to do so. However, no participant was able to correctly identify all four

dietary formulas after completing the study. An improvement to the study design would be to provide participants with samples of each of the four dietary formulas in a preliminary phase of the protocol. The participant's ability to discriminate among the dietary formulas could then be subsequently incorporated as a covariate into the repeated-measures analysis.

Finally, compared to solid whole foods, liquid diets may have different effects on satiety and EI, but the direction and magnitude of these effects are uncertain. Liquid calories delivered in the form of "soups" to eat tend to have a high satiety value (e.g., Flood & Rolls, 2007), while liquid calories delivered in the form of "beverages" to drink tend to have a low satiety value (e.g., Leidy et al., 2010a). It is unclear whether the "protein shake" diets used in this study would have satiety effects more similar to "soups" or to "beverages"; indeed, this distinction is likely mediated by cognitive factors (Mattes, 2006a,b).

Regardless of whether the liquid form of the "protein shakes" increased or decreased participants' satiety (independently of protein content, energy density, etc.), or whether the form-linked satiety effects were different among individuals, the repeated-measures design should ensure that this aspect of the experimental diets did not confound the analysis of EI. However, the reported effect sizes should be interpreted with caution, since these may have been influenced by the liquid form of the diet.

CONCLUSIONS

In this experimental test of the PLH, we custom-designed homogenous liquid diets to minimize the potential impact of taste, texture, smell, and other sensory qualities of whole foods on satiety and energy intake. Using a repeated-measures, crossover design, we found that dietary protein quantity (10% *vs*. 25% of energy) did not influence daily *ad libitum* EI. We also found that dietary protein quality (plant- *vs*. animal-source) did influence daily *ad libitum* EI, with

greater EI on the high-quality protein, contrary to expectations. Our results are inconsistent with the Protein Leverage Hypothesis.

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CHAPTER 5. EFFECTS OF PROTEIN QUANTITY AND QUALITY ON PLASMA GHRELIN LEVELS

INTRODUCTION

The Protein Leverage Hypothesis (PLH), proposed by Simpson & Raubenheimer (2005), states that the intake of protein is under tighter physiological regulation than the intake of total energy. Specifically, the PLH predicts that a high-protein diet will result in low intake of total energy, since the individual's constant protein requirement can be met with a relatively low quantity of total food consumed. Conversely, a low-protein diet is predicted to lead to increased energy intake, since a greater quantity of the low-protein food must be consumed in order to meet the individual's protein needs.

While a number of experimental studies have shown support for protein leverage (Poppitt et al. 1998; Simpson et al., 2003; Weigle et al., 2005), what is yet unclear is a potential physiological mechanism for *how* individual dietary behavior may be modified to ensure adequate protein intake. Morrison et al. (2012), for example, point out that the observed homeostatic regulation of protein consumption lacks a plausible physiological pathway. In fact, a number of pathways may be involved, including hypothalamic and hepatic signaling and other neuroendocrine systems (e.g., Kalra et al., 1991; Kuo et al., 2007; Magni et al., 2009; Fromentin et al., 2012), along with taste and satiety effects that may confound other mechanisms driving protein intake. Further research is needed to explore the potential physiological mechanisms underlying the protein-linked differences in energy intake observed in some, but not all, previous tests of the PLH. Here, we present results from an experiment testing a potential physiological pathway that may underlie protein leverage: plasma ghrelin response.

Ghrelin is a peptide hormone secreted by cells in the gastrointestinal tract. The hormone functions as a neuropeptide, acting on the hypothalamus to stimulate hunger as well as

gastrointestinal motility and gastric acid secretion. Ghrelin levels are highest immediately preceding voluntary meal initiation (Cummings et al., 2004) and decline rapidly following a meal (Cummings et al., 2001; Shiiya et al., 2002; Jakubowicz et al., 2012). Thus, the postprandial reduction in plasma ghrelin can be used as a biomarker of satiety (de Graaf et al., 2004), that is, the inter-meal reduction in hunger that delays the voluntary onset of the next meal. *Satiety* is distinct from *satiation*, the intra-meal reduction in hunger that leads to cessation of food intake (Green et al., 1997; Gerstein et al., 2004). Additionally, some evidence suggests that ghrelin responds especially powerfully to protein (Tannous dit El Khoury et al., 2006; Foster-Schubert et al., 2008). Furthermore, proteins of different quality (i.e., plant- *vs*. animal-source protein) may exert different effects on total food consumption (Morrison & Laeger, 2015), possibly due to nutrient-specific responses of orexigenic or anorexigenic hormones (Veldhorst et al., 2008). Therefore, we also test whether proteins of different quality exert varied effects on satiety, independently of protein quantity.

In this study, our objectives are to 1) determine if the dietary protein quantity of a test meal (10% *vs*. 25% of energy) influences postprandial plasma ghrelin response and 2) determine if the dietary protein quality (plant- *vs*. animal-source) of a test meal influences postprandial plasma ghrelin response. We hypothesize that high-quantity (25% protein) test meals and high-quality (whey protein) test meals will induce a greater plasma ghrelin response, and hence be associated with a greater satiety value.

METHODS

Participants

We recruited a convenience sample of healthy adults from the Boulder, CO area aged 20 to 45 years, with body mass index (BMI) between 20.0 and 30.0 kg/m^2 and reporting ≤ 2.5 hours

of moderate or vigorous physical activity per week. While the final sample size for this study will be N = 18, we present preliminary data here for 10 participants. This study is ongoing, and additional data from participants will be incorporated into the analysis until the final sample size is achieved. Participants were non-pregnant and non-lactating, did not report currently being on a high-protein or weight-loss diet, and did not report having diabetes, eating disorders, or other metabolic disorders.

Experimental design

The study used a single-blind, full-factorial, randomized, repeated-measures, cross-over design. Each participant underwent a total of four experimental treatments, each of which consisted of a liquid test meal (e.g., "protein shake") and concurrent blood draws to assess plasma ghrelin levels at 0, 30, 60, and 90 minutes postprandial. Liquid test meals differed in protein quantity (10% *vs*. 25% energy from protein) and in protein quality (plant- *vs*. animal-source protein) (Table 5-1). The liquid test meals were custom-made at the Nutrition Services Laboratory at the Denver Clinical & Translational Research Center (CTRC), University of Colorado Denver, and designed to be as similar as possible in taste, texture, and energy density.

Table 5-1 Description of test meals

	Macronutrient sources			Macronutrient energy breakdown (% of energy)		
Meal type	Protein	Carbohydrate	Fat	Protein	Carbohydrate	Fat
Low-quantity Plant protein	Pea protein	Maltodextrin, sucrose	Canola oil	10	60	30
Low-quantity Animal protein	Whey	Maltodextrin, sucrose	Canola oil	10	60	30
High-quantity Plant protein	Pea protein	Maltodextrin, sucrose	Canola oil	25	45	30
High-quantity Animal protein	Whey	Maltodextrin, sucrose	Canola oil	25	45	30

Procedures

Experimental protocols were performed at the Boulder CTRC, University of Colorado Boulder. Participants arrived at the CTRC at the same time in the morning for each of the four experimental treatments, without having consumed breakfast. For the previous 48 hours, participants had subsisted solely on the liquid test meal formula assigned for that experimental treatment phase (see Chapter 4).

Upon arriving at the Boulder CTRC, an IV needle was inserted at the median cubital vein by a Boulder CTRC phlebotomist. Next, participants consumed the set-calorie liquid test meal, equal to 20% of the participant's estimated energy requirement (EER) based on FAO/WHO/UNU (2001) recommendations. Immediately upon completion of the test meal (0 min), a 4.0-mL blood sample was drawn into an EDTA-treated tube (Hosoda et al. 2004). At 30, 60, and 90 min after completion of the test meal, an additional 4.0-mL blood sample was drawn, for a total of 4 blood samples per participant per experimental treatment (16.0 mL total).

Blood samples were subsequently sent to the Core Laboratory of the University of Colorado Hospital CTRC in Aurora, CO for radioimmunoassay (Millipore) of total plasma ghrelin levels (pg/mL). While only acylated ghrelin is bioactive (Kojima et al., 1999), total ghrelin as measured here is considered a valid proxy for acylated ghrelin, since the ratio between the two remains constant under a variety of conditions (Ariyasu et al., 2002; Murakami et al., 2002). We defined plasma ghrelin response as the percentage of the lowest value observed at 30, 60, or 90 min relative to the initial sample (0 min). We also calculated plasma ghrelin area under the curve (AUC) over 90 minutes according to the trapezoidal rule (Wolever & Jenkins, 1986).

All participants underwent the test meal and blood sampling protocol for each of the four test meal formulas, in random order. For each participant, the four protocols all began at the same time of day (between 0730 and 1000), to account for the circadian rhythm of spontaneous ghrelin secretion (Natalucci et al., 2005). There was a 1-month washout period between experimental treatments, and female participants underwent each treatment at the same self-reported stage of the menstrual cycle. This was to account for cycle-dependent changes in appetite (Dalvit, 1981; Lissner et al., 1988; Johnson et al., 1994), which are likely due to progesterone antagonism of estradiol's anorexigenic effects during the luteal phase (Czaja 1978; Hirschberg, 2012).

Statistical analysis

To analyze the independent effects of treatment phase, protein quantity, and protein quality on plasma ghrelin response and plasma ghrelin AUC in our repeated-measures design, we fit a linear mixed-effects model with a restricted maximum likelihood estimator (REML) (Richardson & Welsh, 1995). Individual participant ID was entered as the random effect, while treatment phase, protein quantity, or protein quality was entered as the fixed effect. The χ^2 test

for independence was used to compare the frequencies of participants reaching minimum plasma ghrelin levels at different time points on different test meal formulas, with Cramér's V as the measure of association. Levene's test was used to assess homogeneity of variance of plasma ghrelin values among different test meal formulas at different time points. The Benjamini-Hochberg correction as used for multiple comparisons (Benjamini & Hochberg, 1995). Statistical analyses were performed in JMP Pro 14 with significance set at $p < 0.05$.

RESULTS

For 10 participants, means (± SD) of age, anthropometric characteristics, BMI, EER, and test meal energy content are shown in Table 5-2.

Table 5-2 Participant characteristics (7 females, 3 males).

Characteristic	Mean ± SD
Age (yrs)	28 ± 6
Height (m)	1.71 ± 0.16
Weight (kg)	67 ± 13
BMI (kg/m^2)	23.3 ± 4.2
Estimated energy requirement (EER) (kcal/day)	2290 ± 355
Test meal energy content (kcal)	458 ± 71

Mean plasma ghrelin levels over the 90-min experimental period for each of the four test meals are shown in Table 5-3, in both absolute (pg/mL) and relative (% of initial value (0 min)) terms. When controlled for individual participant ID, initial (0 min) absolute ghrelin levels

(pg/mL) did not significantly differ by either test meal formula ($F_{3,19.7} = 2.12$, $p = 0.130$) or experimental sequence ($F_{3,18.9} = 0.95$, $p = 0.435$).

Table 5-3 Absolute and relative plasma ghrelin levels over 90 min for the four test meals. Values are mean ± SD.

		Absolute plasma ghrelin levels (pg/mL)			
Test meal type	N	0 min	30 min	60 min	90 min
10% pea	4	1464 ± 345	1127 ± 345	1062 ± 213	1094 ± 343
10% whey	9	1121 ± 393	925 ± 349	896 ± 309	971 ± 376
25% pea	7	1306 ± 357	1031 ± 208	1024 ± 242	940 ± 165
25% whey	6	1019 ± 292	805 ± 240	759 ± 181	789 ± 300
		Relative plasma ghrelin levels (% of initial value)			
Test meal type	N	0 min	30 min	60 min	90 min
10% pea	4	100	77 ± 5	73 ± 5	74 ± 5
10% whey	9	100	83 ± 16	77 ± 9	83 ± 13
25% pea	7	100	81 ± 12	81 ± 21	76 ± 21
25% whey	6	100	80 ± 8	77 ± 12	78 ± 18

Summary statistics for the overall changes in plasma ghrelin levels over 90 min are shown in Table 5-4. Data included plasma ghrelin response (% of minimum to maximum value) and plasma ghrelin AUC ($\times 10^3$ pg/mL · 90 min).

Table 5-4 Plasma ghrelin response and plasma ghrelin AUC for the four test meals. Values are mean ± SD.

Test meal type	Plasma ghrelin response (% min:max)	Plasma ghrelin AUC ($\times 10^3$ pg/mL · 90 min)
10% pea	69 ± 4	104 ± 26
10% whey	76 ± 13	80 ± 36
25% pea	72 ± 19	95 ± 19
25% whey	70 ± 11	74 ± 20

Individual absolute plasma ghrelin levels (pg/mL) over 90 postprandial minutes are shown in Figure 5-1. Initial ghrelin values varied between ~580-1890 pg/mL, with no clear trend over time among the four test meals.

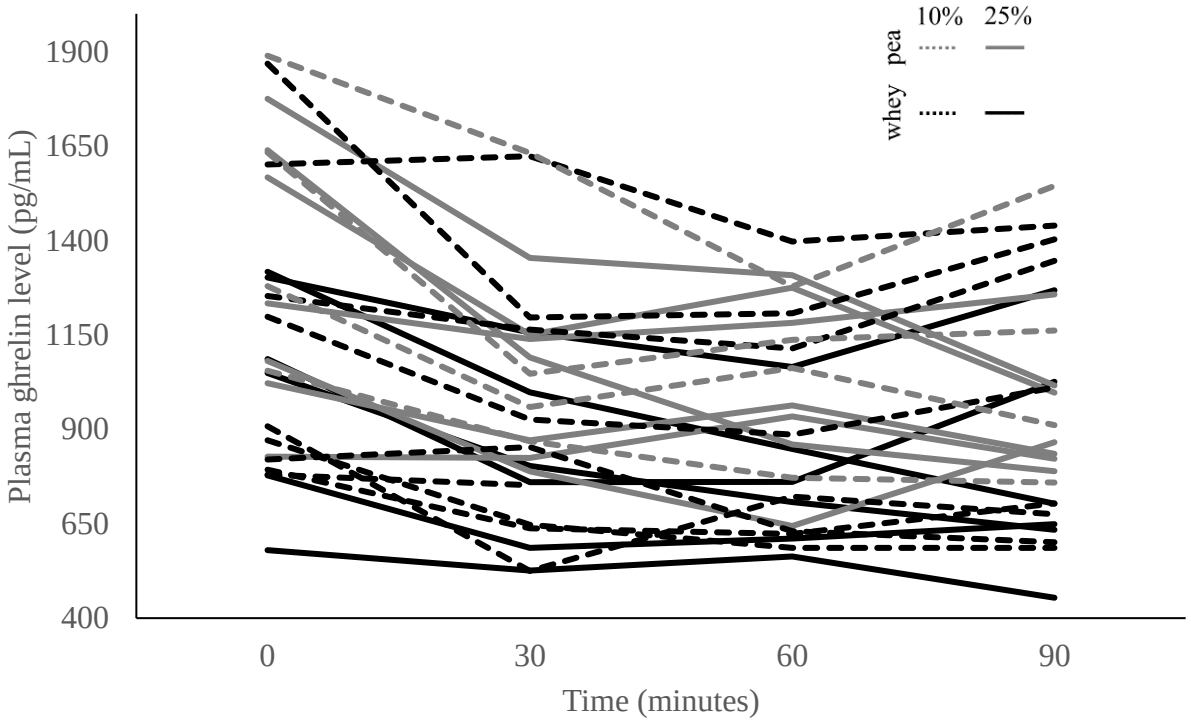

Figure 5-1 Individual postprandial plasma ghrelin values (pg/mL) over 90 minutes. Solid black: 25% whey protein; solid grey: 25% pea protein; dashed black: 10% whey protein; dashed grey: 10% pea protein.

In 58% of cases, participants reached their minimum plasma ghrelin level within 60 min, indicating a ghrelin nadir. The remaining 42% of cases reached their minimum at 90 min (the last blood sample taken), which may indicate a ghrelin nadir (Table 5-5a). When these categories were collapsed into time frames of ≤ 60 min or ≥ 90 min, and into either pea-protein *vs*. whey-protein or 10% protein *vs*. 25% protein meals, there appeared to be a trend earlier minimum plasma ghrelin levels on the whey-protein meals, but the trend was not statistically significant. On the other hand, there was a statistically significant trend toward earlier minimum plasma ghrelin levels on the 10% protein test meals (Table 5-5c).

Table 5-5 a) Numbers of participants reaching their minimum plasma ghrelin level at 30, 60, or 90 min on the four test meals. b) Number of participants reaching their plasma ghrelin nadir at ≤ 60 or ≥ 90 min on pea-protein *vs*. whey-protein test meals. c) Number of participants reaching their plasma ghrelin nadir at ≤ 60 or ≥ 90 min on 10% protein *vs*. 25% protein test meals.

a

Test meal type	30 min	60 min	90 min
10% pea	1	1	2
10% whey	3	5	1
25% pea	1	1	5
25% whey	2	1	3

b

Test meal type	≤ 60 min	≥ 90 min	
Pea protein	4	7	$\chi^2_1 = 3.55$
Whey protein	11	4	$p = 0.059$ V = 0.370

c

Test meal type	≤ 60 min	≥ 90 min	
10% protein	10	3	$\chi^2_1 = 3.94$
25% protein	5	8	$p = 0.047$ V = 0.389

Individual relative plasma ghrelin levels (% change from initial value) over 90 postprandial minutes are shown in Figure 5-2. Three individuals experienced an increase in plasma ghrelin values following the initiation of the protocol at 0 minutes. There was no clear trend over time in changes to plasma ghrelin levels among the four test meals.

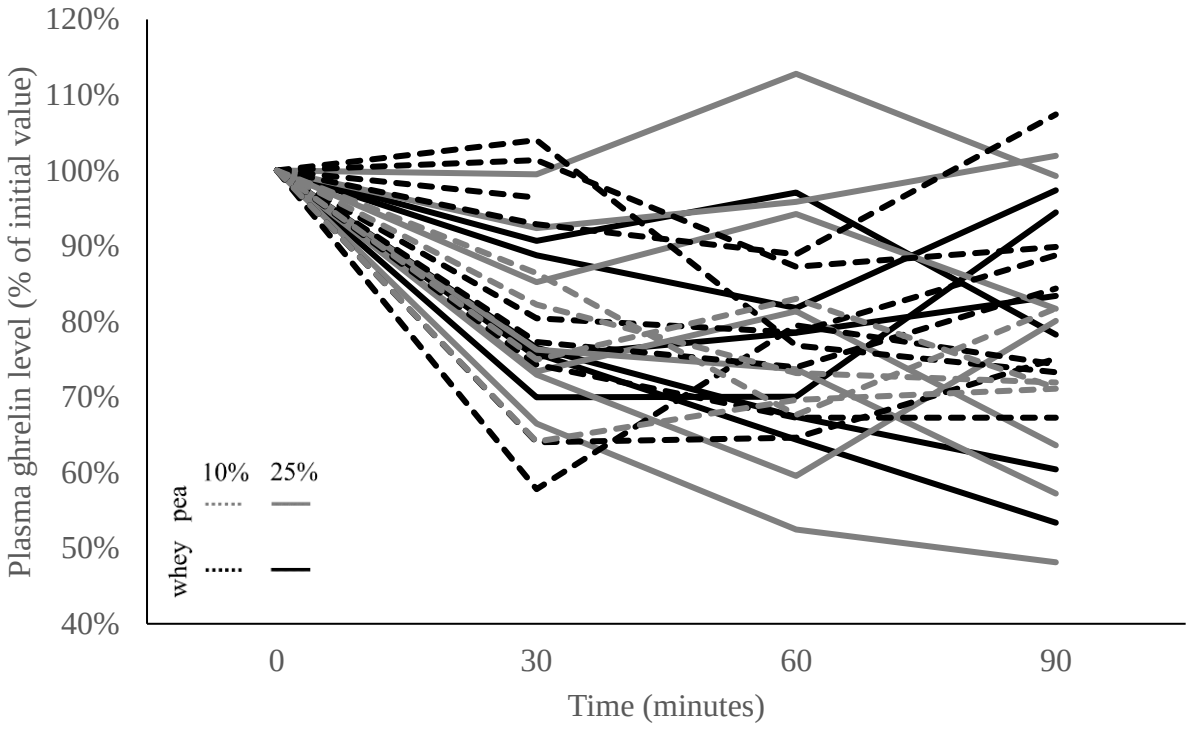

Figure 5-2 Individual postprandial plasma ghrelin values (% change from initial value) over 90 minutes. Solid black: 25% whey protein; solid grey: 25% pea protein; dashed black: 10% whey protein; dashed grey: 10% pea protein.

To better portray differences among test meals, Figure 5-3 shows means of relative plasma ghrelin levels (% change from initial value) over 90 postprandial minutes for each of the four test meals, with bars indicating SD. 60- and 90-minute relative plasma ghrelin levels were significantly more variable for the 25% pea-protein *vs*. the 10% pea-protein test meals (Levene's test, $F_{1,20} = 10.50$, $p_{B\text{-}H} = 0.010$).

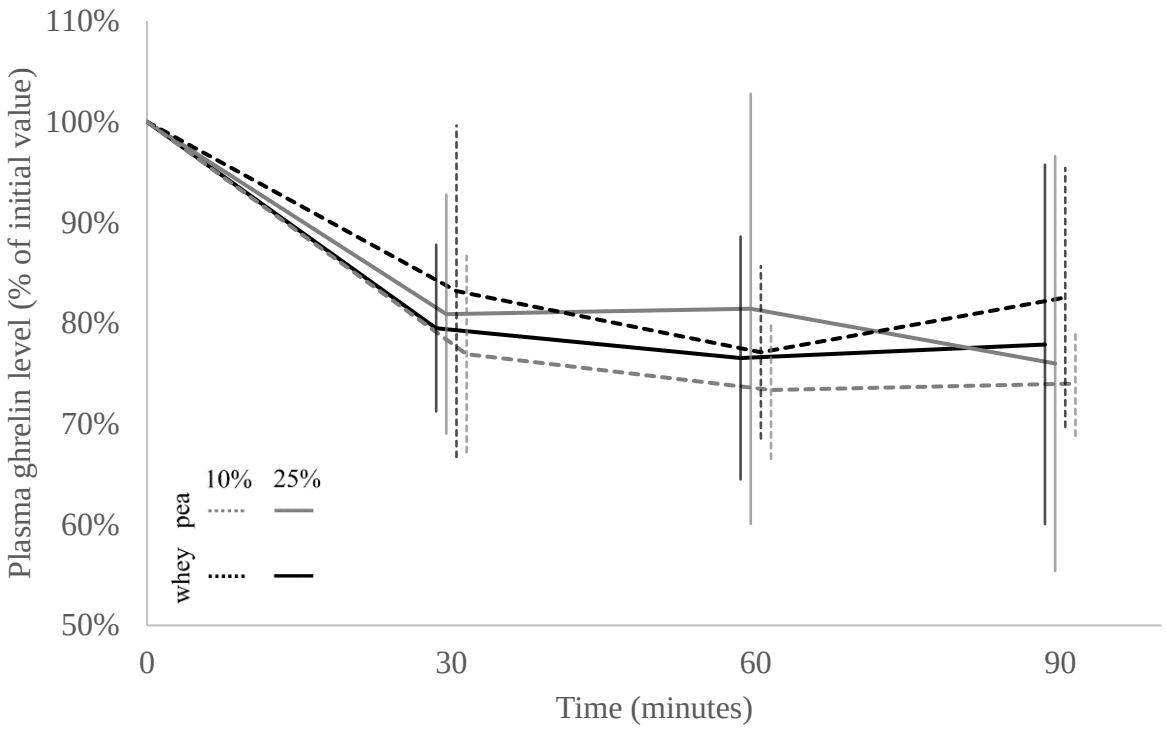

Figure 5-3 Mean ± SD postprandial plasma ghrelin values (% change from initial value) over 90 minutes. Values are dithered along the *x*-axis to aid visibility. Solid black: 25% whey protein; solid grey: 25% pea protein; dashed black: 10% whey protein; dashed grey: 10% pea protein.

In the repeated-measures analysis, there was no fixed effect of experimental phase on plasma ghrelin response ($p = 0.776$) or on plasma ghrelin AUC ($p = 0.549$); that is, there were no differences in plasma ghrelin values in experimental phases 1, 2, 3, and 4, independent of which test meal formula participants had been randomly assigned to for that phase (Table 5-6). There was also no fixed effect of protein quantity, i.e., 10% *vs*. 25% protein, on plasma ghrelin response ($p_{B\text{-}H} = 0.661$) or on plasma ghrelin AUC ($p_{B\text{-}H} = 0.855$). Finally, there was no fixed effect of protein quality, i.e., pea protein *vs*. whey protein, on plasma ghrelin response ($p_{B\text{-}H} = 0.716$) or on plasma ghrelin AUC ($p_{B\text{-}H} = 0.081$) (Table 5-6).

Table 5-6 Fixed effect of experimental phase, protein quantity (10% *vs*. 25%), or protein quality (pea *vs*. whey) on plasma ghrelin response and plasma ghrelin AUC.

	Plasma ghrelin response			Plasma ghrelin AUC		
Effect	*F*	df	*p*-value	*F*	df	*p*-value
Experimental phase	0.37	3, 20.4	0.776	0.72	3, 17.8	0.549
Protein quantity (10% *vs*. 25%)	0.48	1, 22.0	0.661[a]	0.03	1, 18.7	0.855[b]
Protein quality (pea *vs*. whey)	0.14	1, 22.1	0.716[a]	3.95	1, 19.3	0.081[b]

[a,b] Benjamini-Hochberg correction

DISCUSSION

The objective of this study was to determine whether test meals differing in either protein quantity (10% *vs*. 25% of energy) or protein quality (pea *vs*. whey protein) exerted differential effects on satiety. Our liquid test meals were designed to mitigate the confounding effects of varying taste, texture, and other sensory qualities of whole foods with differing protein characteristics on satiety. As a biomarker of satiety, we measured plasma levels of ghrelin, an appetite-stimulating peptide hormone. Based on the PLH, we hypothesized that high-quantity (25% protein) test meals would have a greater satiety value, i.e., induce a greater plasma ghrelin response. By extension, we also hypothesized that high-quality (whey protein) test meals would also have a greater satiety value, i.e., induce a greater plasma ghrelin response.

In the experiment, we found that test meals differing in dietary protein quantity (10% *vs*. 25% of energy) did not consistently influence postprandial plasma ghrelin response or AUC. This suggests that postprandial ghrelin response may not be the mechanism linking dietary protein intake with total energy intake. Further, we found that test meals differing in dietary protein quality (plant *vs*. animal-source) also did not consistently influence postprandial plasma

ghrelin response, although there was a slight (but non-significant) trend toward greater plasma ghrelin AUC on the pea protein test meal.

Absolute plasma ghrelin values at the initial sample (0 min) varied substantially, ranging from ~580-1890 pg/mL. This was unsurprising in light of the known variability in 24-h spontaneous ghrelin secretion patterns (Purnell et al., 2003; Natalucci et al., 2005; Spiegel et al., 2011), and in comparison to comparable dietary intervention studies (Erdmann et al., 2003; Gottero et al., 2003; Foster-Schubert et al., 2008). There was some evidence that 60- and 90-minute variability in plasma ghrelin levels was greater on the 25% pea-protein test meal than on the 10% pea-protein test meal (there were no differences in variability between protein types, or between protein quantities within whey-protein test meals). However, this result does not account for the inter-individual correlation between plasma ghrelin values.

Unexpectedly, three participants exhibited an increase in plasma ghrelin values following the administration of the test meal and initiation of the blood draw protocol at 0 minutes, suggesting that these individuals had not yet reached peak morning ghrelin (Shiiya et al., 2002; Purnell et al., 2003; Natalucci et al., 2005). Nonetheless, the majority of participants displayed the expected and rapid postprandial drop in plasma ghrelin levels (Cummings et al., 2001; Shiiya et al., 2002; Jakubowicz et al., 2012).

In 15 out of 26 samples (58%), participants reached their minimum plasma ghrelin level within 60 min, indicating the postprandial ghrelin nadir. However, for the remaining 11 samples (42%) in which the minimum was reached at 90 min (the last blood sample taken), it cannot be determined whether the nadir was reached, since it is possible that ghrelin levels were still in decline at this time point. Previous studies of plasma ghrelin responses to morning test meals have tended to observe ghrelin nadirs within ~60 min of food consumption (Shiiya et al., 2002;

Erdmann et al., 2003; Gottero et al., 2003; Reynolds et al., 2009), although some studies have reported substantially longer times to nadir, on the order of ~180 min (Tannous dit El Khoury et al., 2006; Foster-Schubert et al., 2008). Therefore, we cannot state with confidence that the participants who did not reach their minimum ghrelin level until 90 min were necessarily near to their true postprandial ghrelin nadir.

There appeared to be a trend for participants on the two pea-protein test meals to reach their minimum plasma ghrelin level at ≥ 90 min, as opposed to ≤ 60 min for participants on the two whey-protein meals, which would suggest a lesser satiety value for the lower-quality, pea-protein test meals, but this trend was not statistically significant. On the other hand, there was a significant trend toward earlier attainment of minimum plasma ghrelin level, indicative of a greater satiety value, on the low-quantity, 10% protein test meals. This was contrary to our expectations of a *lesser* satiety value for the low-quantity protein meals. However, this analysis of time to minimum plasma ghrelin level does not account for the correlation among measurements within individuals, so it must be regarded as suggestive only.

In terms of the magnitude of postprandial changes in plasma ghrelin levels, participants in this study reached a minimum level of ~70-75% of the initial level (0 min), although there was a high degree of variability. The magnitude of this relative change is comparable to that reported in previous studies (Shiiya et al., 2002; Erdmann et al., 2003; Gottero et al., 2003). On the other hand, many previous studies report much lower absolute postprandial ghrelin levels, on the order of ~250-560 pg/mL, variability notwithstanding (Erdmann et al., 2003; Gottero et al., 2003; Foster-Schubert et al., 2008). In contrast, minimum plasma ghrelin levels observed in this study ranged from ~525-1400 pg/mL. The fact that absolute plasma ghrelin levels remained comparatively high in this study, despite a postprandial drop, could be the result of our test meals

being too low in energy (see Callahan et al., 2004); however, our mean test meal content, ~460 kcal, was in fact greater than the 400 used by Gottero et al. (2003), who reported ghrelin nadirs of 265 ± 45 pg/mL after 60 min.

Another possibility is that our participants had not yet reached peak morning ghrelin levels at the time that the test meals were administered (0730 to 1000). Therefore, the postprandial decrease in ghrelin could have been attenuated by the ongoing circadian increase (Shiiya et al., 2002; Natalucci et al., 2005; Spiegel et al., 2011). Unfortunately, we lack fasting ghrelin data for our participants that would allow us to evaluate this possibility.

Finally, we found no fixed effect of the sequence of experimental treatments, independent of the protein characteristics of the test meal, on initial plasma ghrelin levels (0 min), plasma ghrelin response, or plasma ghrelin AUC. Initial plasma ghrelin levels (0 min) also did not vary by test meal formula. Thus, it appears that there was neither a temporal training effect nor a stochastic confounder that would have influenced our analysis of protein characteristics and satiety.

Ghrelin and satiety

The main outcome measure of this experiment was the plasma level of ghrelin, a peptide hormone secreted by cells in the gastrointestinal tract. Notably, ghrelin is the only known orexigenic (i.e., appetite-stimulating) hormone (Cummings, 2006; Higgins et al., 2007; Howick et al., 2017; Gissey et al., 2019; Hougland, 2019), acting as a neuropeptide on the hypothalamus to stimulate hunger, gastrointestinal motility, and gastric acid secretion. Ghrelin was only recently discovered as the endogenous ligand for the growth hormone secretagogue receptor 1α (Kojima et al., 1999; Müller et al., 2015), but it stimulated a great deal of research interest and

was rapidly and thoroughly described (Asakawa et al., 2001; Kojima et al., 2004; Kojima & Kangawa, 2005; Ueno et al., 2005).

Beyond structural-functional descriptions, researchers also quickly noted the biobehavioral role of ghrelin in the regulation of appetite, food intake, and energy balance, beginning only a year after the initial discovery (Tschöp et al., 2000; Wren et al., 2001; Cummings et al., 2001, 2004; Shiiya et al., 2002; Schmid et al., 2005; Bowen et al., 2006a,b). Numerous review articles have subsequently highlighted the importance of ghrelin as a target for nutritional-epidemiological and public-health research focused on diet and body weight (Cummings, 2006; Castañeda et al., 2010; De Vriese et al., 2010; Howick et al., 2017). In the short term, experiments have demonstrated that plasma ghrelin levels are at a peak preceding voluntary meal initiation (Cummings et al., 2004) and rapidly decline to a nadir immediately following a meal (Cummings et al., 2001; Shiiya et al., 2002; Jakubowicz et al., 2012). Therefore, the postprandial reduction in plasma ghrelin levels serves as a physiological indicator of satiety (de Graaf et al., 2004); furthermore, there is some evidence that ghrelin exhibits a particularly sustained postprandial response to protein (Tannous dit El Khoury et al., 2006; Foster-Schubert et al., 2008; Prudom et al., 2010; but see Erdmann et al., 2003).

The question of what constitutes satiety, as well as how to quantify this phenomenon, has been a topic of debate in the nutrition literature (e.g., Green et al., 1997; Merrill et al., 2002; Cardello et al., 2005). Nonetheless, the consensus is that *satiety* represents the inter-meal reduction in hunger that delays voluntary meal onset, and may also reduce energy intake (EI) during the next meal, whereas *satiation* refers to the intra-meal reduction in hunger that leads to the termination of a meal, and possibly a reduction in EI during that meal (Gerstein et al., 2004).

Thus, a measure like the postprandial reduction in plasma ghrelin levels can be considered an indicator of satiety, rather than satiation.

Most previous work in this area has used subjective measures of appetite or fullness to quantify satiety (Green et al., 1997; Drapeau et al., 2007), although efforts have also been made to quantify satiety through hormonal biomarkers such as cholecystokinin (CCK), glucagon-like peptide 1 (GLP-1), leptin, and ghrelin, among others (de Graaf et al., 2004). Some studies have examined the differential effects of protein and other macronutrients on ghrelin response (Blom et al., 2006; Bowen et al., 2006a,b; Lejeune et al., 2006; Gosby et al., 2016), but most previous research on the effects of protein on satiety specifically have utilized subjective measures to quantify satiety (Rolls et al., 1988; Vandewater & Vickers, 1996). Additional work is needed to extend the use of biomarkers to explicitly quantify the satiety effects of protein and other macronutrients.

Besides macronutrient composition, there are many other aspects of food that can influence satiety. For instance, both energy density and portion size can exert effects on satiety (Kissileff et al., 1984; Kral & Rolls, 2004). In this experiment, however, the four test meals were of comparable energy density, and the energy content of the test meals was standardized at 20% of EER. Therefore, our satiety results should not have been confounded by such differences among test meals.

Additionally, the liquid form of our test meals could also have influenced our assessments of satiety, although the potential direction and magnitude of this influence is unclear. On the one hand, several experimental studies have demonstrated the high satiety value of liquid calories consumed as “soups” (Himaya & Louis-Sylvestre, 1998; Mattes, 2005; Flood & Rolls, 2007). On the other hand, numerous studies have also shown that liquid energy

delivered in the form of “meal replacements” or “beverages” has a relatively low satiety value (DiMeglio & Mattes, 2000; Mourao et al., 2007; Leidy et al., 2010a). Thus, the evidence is equivocal regarding the relative satiating effects of liquid foods. The repeated-measures design of our experiment should ensure that any effects of the liquid form of the test meals did not systematically influence our assessments of satiety.

Regardless of any demonstration of the satiety effects of protein, what is still missing is a plausible physiological mechanism by which such effects could manifest. Although this question has been examined before, within the past decade researchers such as Morrison et al. (2012) called for continuing the search for mechanisms of homeostatic regulation of protein intake, indicating that a consensus had not yet been reached. Nevertheless, recent findings suggest several potential avenues for increased investigation.

First, animal models have highlighted the importance of lower gut hormones and hypothalamic signaling in the regulation of protein intake. For example, Pezeshki et al. (2015) found that whey protein increased the tissue expression and plasma concentration of GLP-1, an anorexigenic (i.e., appetite-suppressing) lower-gut hormone in obesity-prone rats. Subsequently, Pezeshki et al. (2016) also found a decreased plasma concentration of most essential amino acids in obesity-prone rats on a protein-restricted diet. On the other hand, Hu et al. (2018) recently reported that only dietary fat, not protein or carbohydrate, regulates EI in mice. The authors found that hypothalamic hunger pathways were unresponsive to dietary protein content, and that mice regulate food consumption primarily to meet an energy target rather than a protein target.

In humans, investigation of the satiety effects of protein (Vandewater & Vickers, 1996; Leidy et al., 2010b; Fromentin et al., 2012) have also emphasized the importance of gut hormones and hypothalamic signaling. Veldhorst et al. (2008) and Belza et al. (2013) found that

protein-induced satiety was linked with relatively high GLP-1 release, while Brennan et al. (2012) associated the suppressed EI on high-protein diets with sustained suppression of ghrelin and stimulation of CCK, another anorexigenic lower-gut hormone (see also Blom et al., 2006; Bowen et al., 2006a,b; Lejeune et al., 2006; Weterterp-Plantenga, 2008). Veldhorst et al. (2009) also reported that a high-protein breakfast suppressed ghrelin release, stimulated GLP-1, and was associated with higher plasma concentrations of certain amino acids. However, Veldhorst et al. (2009) found no association between these hormonal responses and satiety rating or EI. Likewise, Raben et al. (2003) showed that isocaloric meals high in protein, fat, carbohydrate, or alcohol did not lead to differences in satiety, EI, or hormonal response, although the high-alcohol meal did greatly suppress the anorexigenic hormone leptin.

In addition to gut hormone responses, researchers have also examined metabolic effects of dietary protein that may be linked to its greater satiety value (Bray et al., 2015), particularly increased diet-induced thermogenesis (DIT) due to protein intake (Westerterp-Plantenga & Lejeune, 2005; Weterterp-Plantenga, 2008). Both Scott & Devore (2005) and Lejeune et al. (2006), for instance, detected higher DIT associated with higher-protein diets. However, the link between increased DIT and increased satiety, and hence lower EI, is still unclear. One challenge of research in this area is that thermogenic measurements must generally be taken in a laboratory setting, limiting the possibility of investigating DIT in free-living populations.

The current evidence regarding mechanisms of protein regulation in humans was summarized by Morrison & Laeger (2015) in a recent review. They report that certain amino acids, particularly leucine, can suppress food intake by acting locally within the brainstem and hypothalamus. They also conclude that high-protein diets induce satiety via stimulation of gut hormones and vagal signaling, although the precise hormonal pathways involved are still

uncertain. Morrison & Laeger (2015) also discuss more recent evidence that the metabolic hormone fibroblast growth factor 21 (FGF-21) mediates adaptive changes in food intake in situations of restricted total protein or individual amino acid intake (e.g., Kharitonenkov et al., 2005; Laeger et al., 2014; Owen et al., 2014; Gosby et al. 2016); the authors highlight FGF-21 as a potentially fruitful target for additional research in dietary protein regulation, particularly since FGF-21 may be the first hormone known to be activated specifically by protein or amino acid deprivation (Laeger et al., 2014).

Despite the substantial recent progress in elucidating the mechanisms of protein regulation, Morrison & Laeger (2015) also emphasize a number of outstanding questions that remain. First, is human dietary protein intake regulated to a set target point? Models such as the PLH imply (but do not necessarily explicate) that such a set point exists; however, protein could still exert more powerful effects on satiety and EI than the other macronutrients without a set point. Second, what guides macronutrient selection in human dietary selection, especially the selection between low-protein and high-protein diets (Morrison & Laeger, 2015)?

The answer to this second question is of crucial importance in translating a possible physiological mechanism governing protein intake to the dietary behavior of individual organisms. For example, consider a nutritional scenario in which an organism enters a protein deficit, and is physiologically driven to redress this deficit, i.e., to increase protein intake. Could the organism seek out higher-protein foods in the environment, thereby shifting the usual macronutrient composition of its diet? Or could the organism increase its protein intake by simply consuming more of its usual diet, thereby also increasing total EI as a consequence? Both outcomes could be construed as the organism "prioritizing protein intake," yet each has different biological implications. The first case would require the ability of the organism to gauge the

protein characteristics of foods through external sensory cues, or through hepatic signals that rapidly translate to discriminatory feeding behavior. The second case links total EI inextricably to the protein composition of the diet, potentially leading to problematic consequences such as insufficient EI on a high-protein diet. Both empirical and theoretical work is needed to clarify if and how the satiety effects of protein (or other macronutrients) translate into food preferences or food choices at the organismal level.

Strengths and limitations

The primary strength of this study is the repeated-measures crossover design, in which research participants acted as their own controls. This increases our confidence than any within-participants differences (or lack of differences) in plasma ghrelin response between the various dietary formulas were the result of differences in protein quantity and protein quality, rather than differences in age, sex, metabolic traits, or other individual characteristics that may drive variation in ghrelin dynamics.

Another strength of our study is the use of liquid test meals ("protein shakes") that differed in protein quantity and protein quality, but were similar in appearance, taste, texture, and smell. Since these sensory qualities of foods could potentially influence individual satiety, we are confident that our homogenous liquid dietary treatments isolated the effects of protein quantity and protein quality on energy intake with minimal confounding from other satiety-linked food characteristics.

A primary limitation of this study is the incomplete sample. While the final sample size for this experiment will be N = 18, preliminary data for 10 participants were presented here. It is possible that the additional data from the remaining participants will alter our findings, including the statistical significance or non-significance of protein quantity or quality on plasma ghrelin

values. However, the likelihood of this is lessened by the high degree of variability in plasma ghrelin values observed in these preliminary data.

Another limitation of this study is the relatively short duration (90 min) of the blood sampling protocol used for the collection of ghrelin data. While some participants reached a ghrelin nadir during this time period, not all did. Thus, the difference between maximum and minimum plasma ghrelin levels may have been greater than indicated for participants that did not reach a nadir; i.e., the maximum-minimum values may be conservative for these individuals. Also, some participants' plasma ghrelin levels increased following administration of the liquid test meal, indicating that they may not have reached peak morning ghrelin (Shiiya et al., 2002; Purnell et al., 2003; Natalucci et al., 2005). An improvement to the experimental design would involve a separate, preliminary analysis of each participant's fasting morning plasma ghrelin levels to determine the individual timing of morning peak ghrelin. This would ensure that the liquid test meal protocols could be timed to succeed peak ghrelin whenever possible. Additionally, the duration of the postprandial blood draw protocol could be extended to 120 minutes or more, to increase the likelihood of capturing the postprandial ghrelin nadir for all participants.

Additionally, while participants all consumed a 20% of EER test meal at the beginning of the acute feeding study, a preceding 48-hour *ad libitum* feeding study (see Chapter 4) prevented a standard pre-test 12-hour fast from being imposed. Furthermore, participants' energy requirements, used to estimate EER and thus the energy content of the test meals, were not measured directly but were instead estimated from the FAO/WHO/UNU (2001) equations.

Finally, this study used measures of total ghrelin, not of acylated ghrelin specifically. This is potentially problematic, since most biological actions of ghrelin, specifically those related

to appetite and satiety, require acylated ghrelin (Kojima et al., 1999; van der Lely et al., 2004; Cummings, 2006). On the other hand, total ghrelin as measured here can be considered a valid proxy for acylated ghrelin, since the ratio between acylated and total ghrelin remains stable under a variety of conditions (Ariyasu et al., 2002; Murakami et al., 2002; Lucidi et al., 2004; Marzullo et al., 2004; Druce et al., 2006; Weickert et al., 2008). Thus, while total ghrelin is not a direct measure of a bioactive hormone, it can provide a legitimate relative indication of one (namely, acylated ghrelin). A related issue is that ghrelin levels are correlated with insulin (Cummings et al., 2004) and HDL cholesterol levels (Purnell et al., 2003), and ghrelin's action could be modulated by various other metabolic hormones such as GLP-1 (Lejeune et al., 2006), CCK (Bowen et al., 2006b), insulin-like-growth factor 1 (Müller et al., 2015), or leptin (Cummings et al., 2004; but see Schmid et al., 2005). Some or all of these could be measured concurrently with ghrelin to provide additional physiological contextualization of any observed ghrelin-related effects.

CONCLUSIONS

We found that dietary protein quantity (10% *vs*. 25% of energy) did not consistently influence postprandial plasma ghrelin response. We also found that dietary protein quality (plant- *vs*. animal-source) did not influence postprandial plasma ghrelin response. Thus, our results do not indicate that diets of higher protein quantity exert a greater satiety effect, as assessed by plasma ghrelin response, inconsistent with the Protein Leverage Hypothesis. Our results also do not indicate that diets of higher protein quality exert a greater satiety effect. More research is needed to explore the potential physiological mechanisms underlying macronutrient-specific satiety.

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CHAPTER 6. DISCUSSION & CONCLUSIONS

PROJECT GOALS

The goal of this project was to test the Protein Leverage Hypothesis (PLH), a theoretical framework that may link population-level changes in dietary composition with individual energy intake, and hence in weight status (e.g., obesity) and associated health outcomes (Simpson & Raubenheimer, 2005). According to the PLH, the food intake of humans and other animals is primarily constrained by the need to meet a protein intake target. Therefore, if the diet has a low proportion of protein, the individual will be physiologically driven to consume a larger total quantity of food in order for the protein requirement to be met. Conversely, if the diet is rich in protein, then less food must be consumed overall to meet the protein target. Thus, low-protein diets are predicted to drive increased (possibly excessive) energy intake (EI). High-protein diets, on the other hand, are predicted to result in decreased (possibly deficient) EI (Simpson et al., 2003).

The reason that the PLH can be of interest to nutritional anthropologists is that it suggests a physiological mechanism by which population-level shifts in dietary characteristics, such as an increased preponderance of low-protein foods, may translate into individual shifts in dietary behavior, such as increased EI and subsequent weight gain. More specifically, the PLH suggests a possible mechanism behind the so-called "obesity epidemic," a phenomenon afflicting the USA and many other populations worldwide (James et al., 2001; Stein & Colditz, 2004; Caballero, 2007), and which anthropologists have investigated with a view toward uncovering the evolutionary and environmental drivers of the epidemic (Brown & Konner, 1987; Thompson & Gordon-Larsen, 2011).

Assuming that this phenomenon is driven primarily by increased EI, the PLH predicts that individuals are being physiologically driven to over-consume a low-protein diet – such as one characterized by high-fat, high-carbohydrate "empty calories" (Cordain et al., 2005; Brooks et al., 2010) – in order to meet their protein targets. While the socioeconomic and historical factors leading to the emergence of such diets are a topic of ongoing research (e.g., Popkin, 1993, 2006), if it can be shown that these diets have become preponderant in certain populations, the PLH provides a potential physiological mechanism.

Specifically, a PLH-driven mechanism would suggest that individuals in high-obesity populations like the USA are over-consuming energy because the contemporary diet of ultra-processed diet is low in protein (Maruon, 1990; Steele et al., 2017); excess energy intake would therefore be an unintended side effect of a behavior adaptation to protein restriction. This would contradict a prevailing view in nutritional anthropology, namely, that individuals are physiologically driven to maximize energy intake. In particular, the human craving for high-calorie foods, alongside the ability to efficiently store surplus dietary energy, are framed as formerly-adaptive traits that have become maladaptive in contemporary obesogenic environments (Egger & Swinburn, 1997), which have rapidly become saturated with cheap, high-calorie, readily available foods (Lieberman, 2003, 2006; Drewnowski & Specter, 2004).

The PLH, if true, would have further implications within biological anthropology. For example, the role of meat-eating in human evolution has long been a topic of both research and debate within paeloanthropology (and historically, within archaeology as well). The fact that meat and possibly other animal foods became a part of hominin diets at some point in evolutionary history, even an important part of the diet, is well-accepted (Stanford & Bunn, 2001). There is less agreement on exactly how hominins at different points in evolutionary

history accessed animal foods (e.g., through direct hunting, secondary scavenging, etc.), and what the biological benefits and repurcussions of such a dietary shift may have been.

Much of the research has focused on the energetics of encephalization; that is, how hominins were able to meet the energy costs of an ever-increasing, metabolically expensive brain (Fish & Lockwood, 2003; Isler & van Schaik, 2006, 2009). Prominent models suggest a metabolic trade-off as the mechanism, with the energy trade-off occuring between brain size and gut size (Aiello & Wheeler, 1995), between muscle mass and fat mass (Leonard et al., 2007), between brain size and energy allocated to locomotion, growth, and reproduction (Navarrete et al., 2011). In addition to such calorie-for-calorie trade-offs to subsidize the high metabolic costs of the brain, others have argued that an increase in dietary quality would also have been necessary to support the costs of brain evolution (Snodgrass et al., 2009; Isler & van Schaik, 2014).

An assumption shared by some of these models is that meat is an energy-dense food that could play a key role in hominin energetics. This is not necessarily true. If it is low in fat content, then meat may not be a particularly energy-dense food compared to other potential foods available in the diet (although meat is, by definition, a high-quality protein source (Milton, 1999)). More fundamentally, the PLH (if true) would force a radical reconsideration of models linking increased consumption of meat (or other animal-source foods) with hominin encephalization. This is because the PLH predicts that an increase in dietary protein would lead to a *reduction* in hominin energy intake, contrary to the very notion of meat as superior metabolic fuel for the brain. Thus, according to the PLH, an increase in hominin protein intake would generate an additional energetic deficit on top of the costs of encephaliztion, regardless of the nutritional benefits of meat.

Finally, the PLH has implications for primate dietary ecology, particularly the debate over protein as a limiting factor in primate diets. Oftedal (1991) suggested that primates (including humans) have comparatively low protein requirements due to their slow life histories. Thus, protein should not be the primary limiting factor in primate diets. However, Chapman et al. (2004, 2015), building on work by Milton (1979), subsequently argued that the ratio of protein to fiber within the nutritional environment is a strong predictor of primate population density. This would suggest that protein availability is, in fact, a limiting factor that constrains primate feeding behavior.

While the importance of the protein-to-fiber model in limiting primate population density and feeding behavior has subsequently been questioned (Gogarten et al., 2012; Johnson et al., 2017), other work has continued to explore and refine this concept, for example, by highlighting the importance of available nitrogen vs. total nitrogen in primate foods (Wallis et al., 2012), or of habitat protein concentration in the selection of high-protein leaves by folivores (Ganzhorn et al., 2017). The PLH would tend to support the protein-to-fiber perspective, since it forefronts the importance of dietary protein intake at the possible expense of other nutritional requirements, including total energy intake.

Observational evidence regarding the PLH specifically has been reported for some species of nonhuman primates. Felton et al. (2009) did find evidence of protein prioritization in spider monkeys. These primates maintained a constant daily protein intake, while their total energy intake fluctuated in response to the varying nutritional composition of available food items. That is, as changes in available food items resulted in an increased protein density of the diet, energy intake fell (and vice versa), consistent with the PLH.

On the other hand, Rothman et al. (2011) found that mountain gorillas in Uganda, while apparently preferring protein-rich foods, did not always prioritize protein intake. During certain times of the year, they replaced protein-rich leaves with carbohydrate-rich foods. Total non-protein energy intake remained invariant throughout the year for these primates, while they actually over-consumed protein when leaves were the primary component of the diet. Thus, the data reported by Rothman et al. (2011) are consistent with mountain gorillas prioritizing non-protein energy intake, rather than total protein intake as proposed by the PLH. Interestingly, during the periods of high leaf consumption, i.e., high protein intake, the protein density of the mountain gorillas' diet (~31% of energy) was in the upper range of high-protein weight-loss diets for humans. Meanwhile, during the periods of high fruit consumption, i.e., high carbohydrate intake, the protein density was comparable to the ~15% of energy observed in many human populations.

Taken together, the observational data reported by Felton et al. (2009) and Rothman et al. (2011) indicate that more research is required to understand the role of dietary protein density in the feeding behavior of wild nonhuman primates. In particular, additional data are needed on changes in food availability (e.g., due to seasonality), and how these affect the protein-linked feeding behaviors of primate species with different body sizes, dietary niches, and social structures.

SUMMARY OF MAIN RESULTS

There were three main components to this research study. First, we analyzed population-level data on dietary intake and anthropometry for USA adults from the time period 2005-20006 through 2015-2016 to uncover any trends supportive of the PLH (Chapter 3). We used data from the National Health and Nutrition Examination Surveys (NHANES), a set of continuous, cross-

sectional, representative medical and dietary surveys of the USA population. This analysis provided a population-level backdrop for our subsequent experimental studies. We found mixed support for the PLH: protein intake did remain steady over time, as expected, but the evidence for an inverse link between dietary protein content and individual EI was equivocal.

Second, we conducted an *ad libitum* feeding experiment (Chapter 4) to further test the proposed link between the protein characteristics of the diet and individual EI. In this experiment, participants subsisted for 48 hours on 4 different dietary formulas differing in protein content and protein quality. In this experiment, our results were inconsistent with the PLH. We found that protein content (10% *vs*. 25%) of total energy had no effect on daily *ad libitum* EI. We did find that protein quality (plant- *vs*. animal-source protein) had on effect on daily *ad libitum* EI, but the effect was contrary to our predictions: EI was greater on the high-quality protein diets.

Third, we conducted an acute hormone and satiety study (Chapter 5) to investigate how dietary protein characteristics affected not EI, but a related phenomenon: satiety. Using the same 4 dietary formulas as the ad libitum feeding experiment, we tested the effects of protein content and protein quality on the postprandial response of ghrelin, an important hunger-stimulating hormone. Again, our experimental results were inconsistent with the PLH. We found that neither protein content (10% *vs*. 25%) nor protein quality (plant- *vs*. animal-source protein) exerted differential effects on the postprandial ghrelin response, a biomarker of satiety.

Our two experiments were designed to improve on previous work in several ways. First, the experiments both used a full-factorial, randomized, repeated-measures, cross-over design to independently test the effects of protein content and protein quality on individual EI (Chapter 4) and on a biomarker of satiety, plasma ghrelin levels (Chapter 5). In this design, participants are

their own controls, mitigating the confounding effects of inter-individual differences in metabolism, energy flux, and dietary habits. Additionally, the *ad libitum* feeding experiment employed isocaloric, homogenized liquid diets (i.e., "protein shakes"). Previous studies of dietary protein and EI using whole foods may have been confounded by differences in sensory qualities (e.g., taste, texture, smell) of such foods, thereby influencing measures of EI, satiety, and other dietary outcomes. Our liquid diets mitigated such confounders, increasing our confidence in the observed EI values. Finally, both experiments explicitly and independently assessed the effects of protein content and protein quality on dietary outcomes; few previous studies have incorporated protein quality (or even defined it) in this context.

A primary limitation of the *ad libitum* EI study is that we did not measure energy expenditure during the treatment periods; therefore, we were unable to assess participants' energy balance. Additionally, participants' energy requirements were estimated from the FAO/WHO/UNU (2001) equations, so EI results expressed as a percentage of estimated energy requirements (EER) were themselves estimates. Also, the 48-hour duration of the *ad libitum* treatment periods, while longer than that of many previous studies related to dietary protein and EI, may still not have been long enough for protein leverage effects to manifest.

A primary limitation of the acute hormone and satiety study is the relatively short duration (90 minutes) of the blood sampling protocol used for the collection of ghrelin data. While some participants reached a ghrelin nadir during this time period, not all did. Thus, the difference between maximum and minimum ghrelin levels may have been greater than indicated for some participants. Additionally, while participants all consumed a 20% of EER test meal at the beginning of the acute feeding study, the preceding 48-hour *ad libitum* EI study prevented a standard pre-test 12-hour fast from being imposed. Again, participants' energy requirements,

used to calculate the energy content of the liquid test meals, were estimated from the FAO/WHO/UNU (2001) equations, not measured directly.

Overall, the three components of this research study failed to provide consistent evidence in support of the PLH. The results are difficult to reconcile with the well-documented anorexigenic (i.e., appetite-suppressing) qualities of high-protein diets reported in the literature, or with the less well-supported but still notable orexigenic (i.e., appetite-stimulating) qualities of low-protein diets (Davidenko et al., 2013; Morrison & Laeger, 2015). This suggests that other mechanisms may be at play in the complex interrelationship between the environment, the nutritional characteristics of the diet, and individual feeding behavior.

ADDITIONAL IMPLICATIONS FOR NUTRITIONAL ANTHROPOLOGY

These investigation of the PLH raise a number of additional questions of relevance to nutritional anthropology. To begin with, the PLH frames the question of food choice in a particular way that is not the only logical possibility. Assume that an animal is in a protein deficit, detects that deficit through some internal physiological mechanism, and is thereby driven to rectify the deficit through a modification of its feeding behavior. Would the animal *select a higher-protein diet*? Or would it simply *consume more of the original diet*? Both possibilities would be consistent with the concept of "prioritizing protein intake" to reestablish a nutritional balance, yet each possibility also has very different behavioral implications.

Figure 6-1 provides a schematic representation of these two behavioral responses to protein deficit. The original (baseline) diet is represented to consist of 25% protein and 75% other macronutrients, with the size of each component corresponding to absolute quantities of macronutrient (e.g., grams or calories). The right-hand side of the figure portrays the behavior assumed by the PLH: individual protein requirements drive behavioral change in *total food*

intake, not *food selection*. In this case, the protein deficit is redressed by increasing the overall consumption of the diet, without a change in the nutritional characteristics of the diet. Thus, in Figure 6-1, the right-hand schema portrays an increase in total energy intake, represented by an increase in the height of the component columns, while the proportions of macronutrients remain constant (i.e., protein is maintained at 25% of the diet).

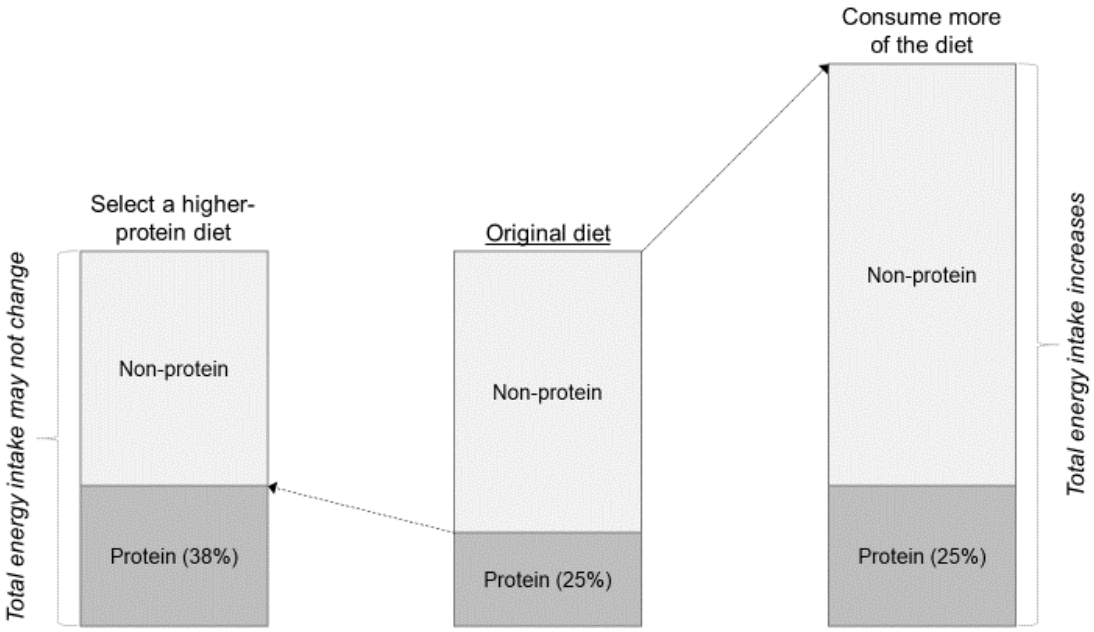

Figure 6-1 Schematic representation of two possible dietary-behavioral responses to protein deficit. Left: select a higher-protein diet; right: consume more of the original diet. Both strategies result in increased protein intake, but with different implications for total energy intake.

From a physiological standpoint, this is a conceptually simple model of behavioral change, because it only requires an internal protein-sensing mechanism, which then engages with the animal's general orexigenic responses. In other words, the animal is able to increase its protein intake (and thereby rectify its protein deficiency) by up-regulating its original food intake behavior, without the need to ingest different kinds or proportions of food items from the environment, but with a necessary increase in total energy consumption.

The left-hand side of Figure 6-1 represents a very different model of food choice. In this scenario, the animal responds to its protein deficit by targeting dietary protein directly: it selects higher-protein foods from the environment, and hence consumes an overall higher-protein diet. In terms of absolute protein intake, the outcome is the same as in the previous model. More protein is consumed, and the deficit is corrected. However, the animal is now consuming a qualitatively different diet than the original diet, by definition: the percentage of protein has increased. In this model, it is also possible that the consumption of greater quantities of protein results in an increased total energy intake, but *not necessarily*. Depending on the relative macronutrient compositions of the higher-protein foods selected, in comparison to the lower-protein foods in the original diet, total energy intake may increase, or decrease, or remain unchanged. This food-selection model is somewhat more complex than the diet-quantity model, because it requires not only an internal protein-sensing mechanism, but also the ability of the organism to discriminate the protein characteristics of the diet in some way and change its behavior accordingly.

Figure 6-2 portrays the same two behavioral models as Figure 6-1, except in response to an excess of protein rather than a protein deficit. Again, the right-hand side of the figure represents the diet-quantity model, in which the animal down-regulates its native food-intake behavior to consume less of the original diet, resulting in decreased protein intake along with a necessary decrease in total energy intake. The food-selection model represented on the left-hand side of Figure 6-2, on the other hand, involves the animal de-selecting protein in its diet. The result is a decrease in absolute protein intake, with a necessary shift in the macronutrient composition of the diet, but without a necessary change in total energy intake.

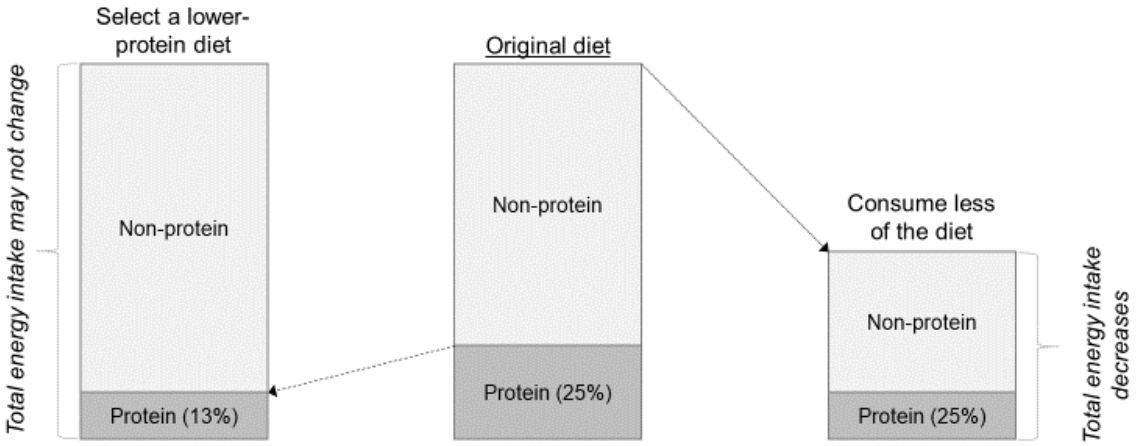

Figure 6-2 Schematic representation of two possible dietary-behavioral responses to excess dietary protein. Left: select a lower-protein diet; right: consume less of the original diet. Both strategies result in decreased protein intake, but with different implications for total energy intake.

The conceptually-simpler diet-quantity model is the one assumed by the PLH, as well as by our *ad libitum* feeding experiment. Participants in the study were only able to respond to protein-related physiological cues (e.g., an excess or deficit of protein) by consuming more or less of an experimental diet with set macronutrient proportions. They had no option of selecting higher- or lower-protein foods within the diet. Therefore, it is possible that our experiment could have detected protein-driven changes in food intake, if only participants had the ability to select foods of different protein contents.

On the other hand, an experiment of this type would still raise a further question: how are animals able to discriminate among foods with different protein contents? The fact that animals are capable of such discrimination is not in dispute. For instance, nonhuman primates routinely select foods with distinctive protein characteristics, e.g., ripe fruits or young leaves (Oftedal, 1991; Felton et al., 2009; Ganzhorn et al., 2017). In fact, the evolution of trichromatic vision in cattarhine primates (and *Alouatta*) is likely to be linked to food-choice behaviors, including

protein discrimination (Dominy & Lucas, 2001; Lucas et al., 2003; Surridge et al., 2003). There is also evidence for human primates selecting high-protein foods after undergoing protein restriction (Deutsch et al., 1989; White et al., 2000; Griffioen-Roose et al., 2012), with sensory abilities (in this case, taste) again appearing to play a key role in food-discrimination ability (Griffioen-Roose et al., 2014)

While these observations suggest the importance of external sensory cues in the ability to discriminate dietary protein, there are many other possible mechanisms involved, and much recent work has been aimed at uncovering them. In a recent review, Morrison & Laeger (2015) highlight three primary mechanisms that are likely to drive the response to dietary protein: 1) direct actions of specific amino acids within the brain; 2) neural or hormonal signals derived form the gastrointestinal tract (GIT); 3) additional endocrine signals, particularly fibroblast growth factor 21 (FGF21), which has received much recent attention (Kharitonenkov et al., 2005; Laeger et al., 2014a; Owen et al., 2014; Gosby et al. 2016; see Chapter 5).

Evidence regarding the direct effects of amino acids comes primarily, but not exclusively, from rodent models. For example, earlier work has also shown that rats can distinguish between diets varying only slightly in amino acid content and composition (Hrupka et al., 1997; Torii & Niijima, 2001). This could be due to specific amino acids suppressing food intake via afferents of the vagus nerve (Tomé et al., 2009; Jordi et al., 2013). Schwartz (2013) presents evidence that the anorexigenic effects of high-protein diets may be driven by the amino acid leucine acting locally within the hypothalamus; indeed leucine may act as a unique metabolic signaling molecule in the brain (Laeger et al., 2014; Morrison & Laeger, 2015).

However, one difficulty with these individual amino acid-based mechanisms is that circulating concentrations of blood amino acids are strongly buffered by metabolic adaptations of

the liver and skeletal muscle during protein deficit; therefore, low-protein diets generally result in only small-scale and short-term changes to circulating amino acid levels (Kalhan et al., 2011; Laeger et al., 2014b).

Subsequently, Anthony & Gietzen, (2013) have shown that rats quickly detect and subsequently avoid diets that are deficient in a single amino acid, even reducing total food intake in response. This avoidant behavior may be the result of depletion of the limiting amino acid, triggering the activation of general control nondepressible 2 (GCN2), a serine/threonine kinase, within the anterior cortex (Hao et al., 2005; Maurin et al., 2005). GCN2 acts as an amino acid sensor that plays a key role in modulating amino acid metabolism, linking amino acid availability to protein synthesis (Zhang et al., 2002; Kilberg et al., 2012). Thus, its activation may represent a unique mechanism for the detection of amino acid deficits in the brain.

In addition to the potential protein-sensing mechanisms involving individual amino acids, other work has also highlighted the importance of the GIT in detecting and signaling protein intake. Mithieux (2013) argues that nutrient sensing by the extrinsic GIT nervous system is fundamental to the high satiety effects of protein, and in its stimulation of food intake control mechanisms by the central nervous system, possibly as the result of sensing of intestinal gluconeogenesis in the portal vein (Mithieux et al, 2005). In a review of brain mechanisms controlling protein and energy intake, Davidenko et al. (2013) expand on this notion, pointing out that protein sensing may begin in the oral cavity, before reaching the GIT. They do, however, emphasize that the mechanism of protein-induced satiety most probably lies in signaling to the brain through the vagus nerve afferents. This process likely involves the gastric hormones cholecystokinin (CKK) and peptide YY, with additional pathways in the form of post-absorptive signaling (e.g., levels of circulating amino acids) and again, the direct influence of amino acids

in the brain. Fromentin et al. (2012) also review evidence that protein-induced satiety is most likely driven by sensing within the GIT or the portal vein, subsequently transmitted to the brain via endocrine signals (e.g., GIT hormones) and/or neural signals (e.g., vagus nerve afferents).

The most well-supported data regarding the physiological mechanisms of protein sensing are illustrated by Morrison & Laeger (2015:258) in a summary figure, reproduced here as Figure 6-3. Despite the wealth of research portrayed in this figure, from a nutritional-anthropological standpoint, one potential limitation is indicated by the shaded circle in the upper right added by the current author (RL Bender).

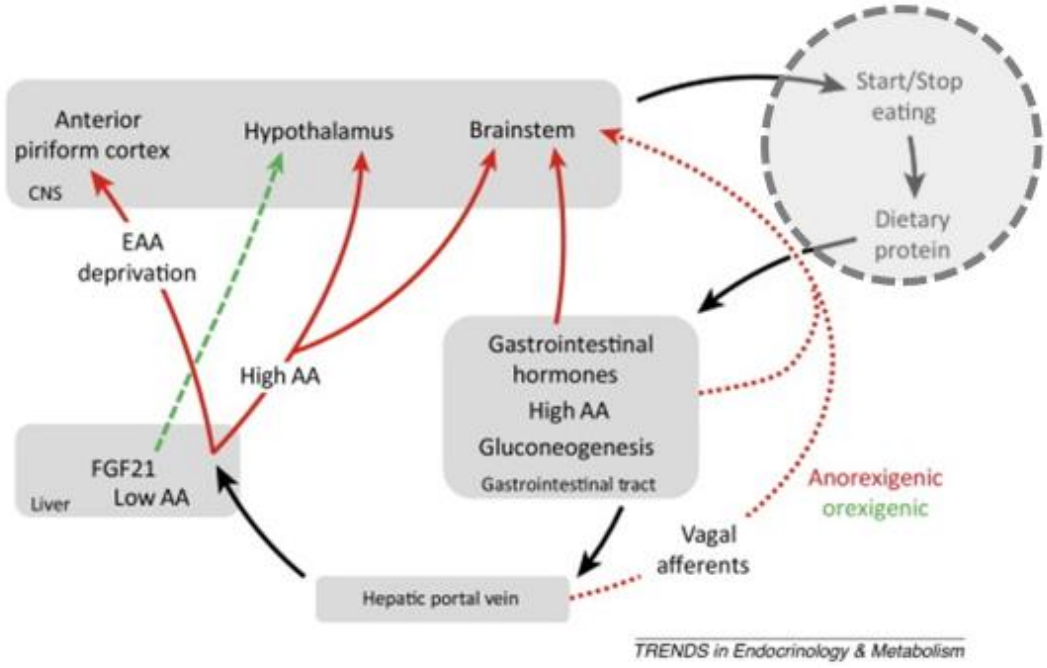

Figure 6-3 Mechanisms through which changes in dietary protein intake are detected and communicated to the brain; reproduced from Morrison & Laeger (2015:258). Shaded circle in the upper right added by the current author (RL Bender).

As argued previously, a basic issue in dietary protein regulation is the question of how the signals from a protein-sensing mechanism would be translated into changes in an animal's

dietary behavior in a particular nutritional environment, given that a protein-sensing mechanism exists in the first place. Few studies of protein-linked satiety have addressed this question (but see Berthoud et al., 2012; Davidenko et al., 2013). Thus, the two components highlighted by the shaded circle in Figure 6-3 leave important questions unanswered: start/stop eating *what*, exactly? What are the sources of dietary protein?

Figure 6-4 attempts to explore these questions, at the expense of some loss of physiological detail regarding protein sensing and signaling. Here, the nutritional environment is represented as the fundamental context in which protein regulation must operate. The types and quantities of foods available to an animal, not to mention the protein characteristics of those foods, are influenced by a vast range of factors, ranging from ecological (seasonality, competition) and biochemical (protein availability, nutrient density) to bioenergetic (procurement cost, energy density) and social (cultural influences, economic constraints). Against this backdrop, a sequence is conceived in which an initial bout of food intake, highly constrained in its dietary characteristics by the environment, enters the protein sensing domain. Here, a variety of physiological mechanisms, potentially including hormonal or neural signals from soft tissues, direct amino acid effects, and perceptive signals (e.g., visual, olfactory), provide a protein signal to the brain; these mechanisms are likely to interact in complex ways, as ably demonstrated by the literature reviewed.

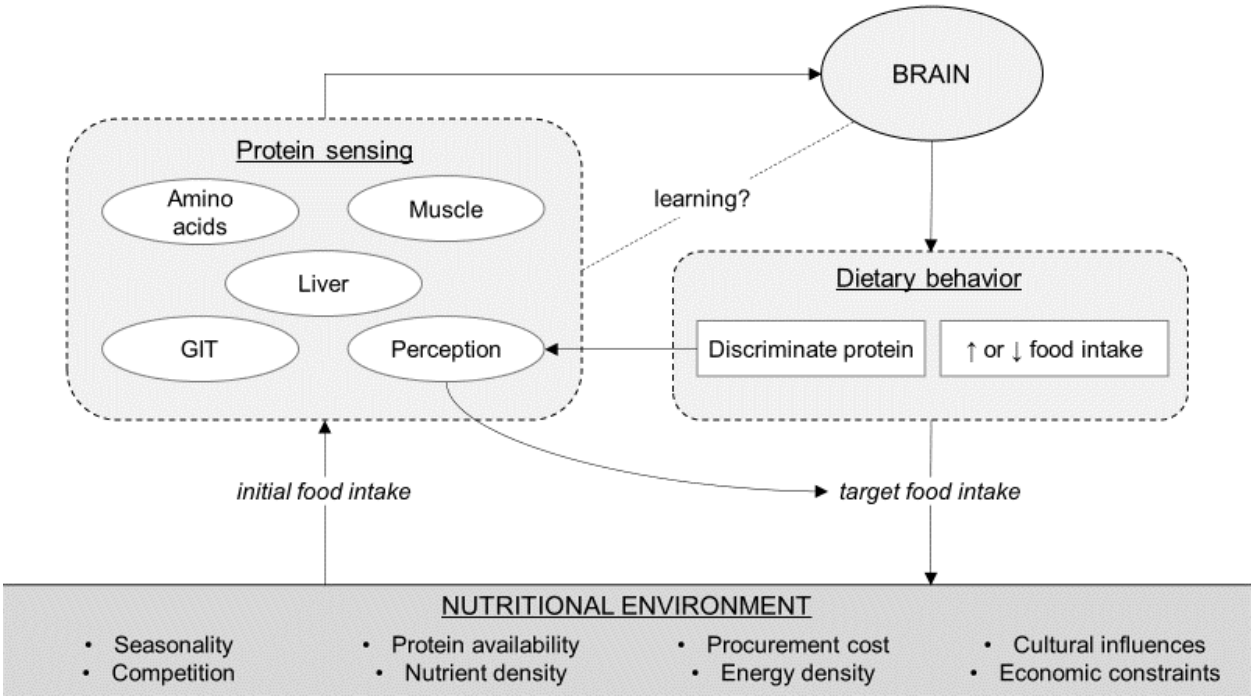

Figure 6-4 Schematic representation of the proposed interactions among the nutritional environment, protein-sensing mechanisms, and changes in dietary behavior.

Subsequently, in the case of protein deficit or excess, the brain triggers a shift in dietary behavior to rectify the imbalance. Again, as argued previously, there are at least two possible pathways for such a behavioral shift to manifest. One pathway is to simply increase or decrease total food intake. In that case, following the behavioral shift, target food intake remains qualitatively identical to initial food intake, only the quantity has changed. Nonetheless, target food intake is still fundamentally mediated by the nutritional environment; specifically, an increase in food intake driven by a protein deficit may simply not be achievable in a particular environment.

The other pathway for dietary-behavioral shift portrayed in Figure 6-4 involves protein discrimination. That is, the animal seeks to modify not only the quantities, but also the qualities of the foods it consumes, again within the constraints of the nutritional environment. The

contention illustrated in Figure 6-4 is that the ability to discriminate protein, not only to maintain an initial diet, but also to shift to a qualitatively different pattern of food intake, requires some form of protein perception ability. The animal must be able to distinguish different foods by external sensory cues, and to link those cues to protein signals. This process may be mediated by learning, e.g., trial and error or observing conspecifics.

The purpose here is not to advocate one particular physiological model of protein sensing over another. Instead, it is simply to emphasize that animals operate within complex physical (and often, social) nutritional environments, and this should be taken into account when considering protein regulation. It is often not taken into account. A pertinent example of this disconnect is the relationship between national-level dietary and anthropometric data in the USA, as examined in Chapter 3. As shown by multiple studies (e.g., Berryman et al., 2018), the USA adult diet is certainly not deficient in protein. On the contrary, our results showed mean adult body-proportional intake, ~1.4-1.6 g/kg/day over the period from 2005-2006 to 2015-2016 for both women and men, was about two times the 0.8 g/kg/day recommended in the Dietary Reference Intakes (Institute of Medicine, 2005) for USA adults. Assuming that this dietary recommendation is valid, the USA would thus be characterized as having a very high-protein diet. Therefore, not only the PLH, but also the extensive independent literature on high-protein diets for weight loss (Veldhorst et al., 2008; Westerterp-Plantenga et al., 2009; Leidy et al., 2015), predict that population-level EI in the USA should be low.

Why, then, does the USA have such high rates of obesity (Flegal et al., 2002, 2012)? There is a seeming disconnect between the high-protein nature of the national diet, and the expected anthropometric consequences of the diet. More generally, how can the crucial dietary necessary of protein be reconciled with its well-documented high satiety value? While the

appetite-suppressing effects of high-protein diets might be considered beneficial in the context of a weight-loss intervention, these same effects would seem positively harmful in a free-living ecological setting. In short, what is so detrimental about excess protein intake that an organism would sacrifice something as crucial as total EI in order to avoid it?

One potential culprit is an important physiological constraint on protein metabolism: deamination. In order for amino acids to be used as metabolic fuel, the amino group (i.e., the nitrogen-bearing group) must first be removed in order for the organic group to be catabolized in aerobic metabolism (TCA cycle) or anaerobic metabolism. (Figure 6-5).

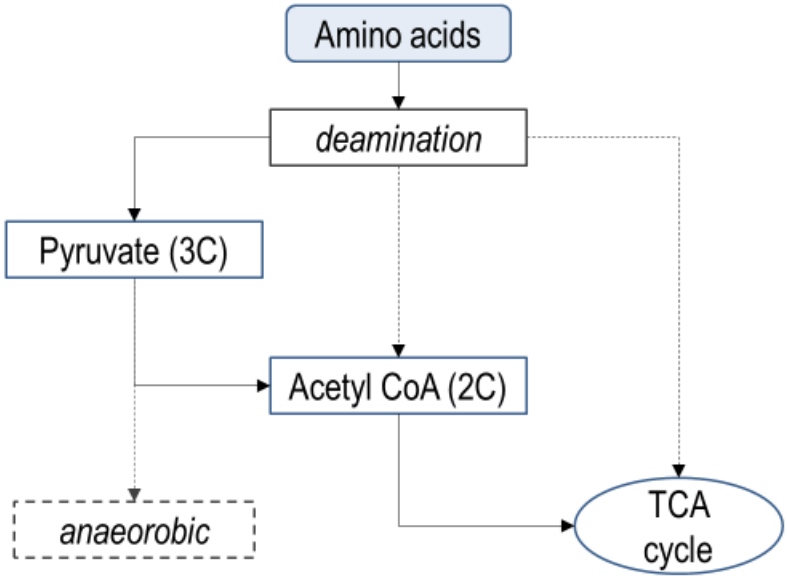

Figure 6-5 Basic pathways of amino acid catabolism for usable energy.

This deamination process occurs primarily in the liver, with some action of the kidneys. The cleaved amine groups must subsequently be converted to ammonia (NH_3); since ammonia is highly toxic, it must be further metabolized to urea $(NH_2)_2CO$, again primarily in the liver and (to a lesser extent) the kidneys. Finally, urea is excreted in the form of urine, an important component of renal physiology. Thus, one consequence of a high-protein diet is an increase in

urine-specific gravity (Martin et al., 2006), due to the need to excrete excess nitrogen (i.e., excess urea). Greater water intake is required on a high-protein diet to avoid dehydration (Cuenca-Sánchez et al., 2015); conversely, dehydration is a mechanism of rapid initial weight loss on high-protein diets (Yang & Van Itallie, 1976).

Could increased fluid flux due to deamination, the risk of dehydration, and concurrent stress on the liver and kidneys (Denke, 2001; Friedman, 2004) be drivers of protein-avoidance mechanisms, and help to account for the somewhat puzzling fact that animals will apparently sacrifice EI in order to evade a protein excess? Indeed, the poorly-documented yet intriguing historical phenomenon of "rabbit starvation" (Lieb, 1929; Speth & Spielmann, 1983) suggests that chronic high protein intake, in the absence of sufficient dietary fat to spare bodily lean tissues from catabolism, can lead to a range of pathologies beginning with nausea and diarrhea and ending with death. Cordain et al. (2000) argue that the symptoms of "rabbit starvation" most likely stem from the finite capacity of the liver to up-regulate the enzymes necessary for urea synthesis on a very high-protein diet. Using data from Rudman et al. (1973), Cordain et al. (2000) calculated that dietary protein densities of > 35% of total energy would be likely to result in hyperammonemia and hyperaminoacidemia, and subsequently in the "rabbit starvation" syndrome; Bilsborough & Mann (2006) arrive at the same conclusion.

Suggestive as these results may be, the fact remains that a dietary protein density of 35% or more represents a rather extreme scenario for humans. Pathologies such as hyperammonemia and hyperaminoacidemia are not particularly useful in explaining dietary regulation at the much finer scales of protein intake assumed by the PLH and the weight-loss literature. In fact, there appears to be little evidence of any harmful effects of consuming protein at a rate typical for the USA (Martin et al., 2005; Friedman et al., 2012). Thus, additional investigation is needed to

elucidate the deleterious effects of high protein consumption, not at extraordinary levels, but at realistic levels relevant to the daily experiences of humans in typical populations.

CONCLUSIONS

In this project, we set out to test the Protein Leverage Hypothesis (PLH), a theoretical framework that could help researchers link population-level dietary changes with individual energy intake. Using data from national-level health and nutrition surveys, as well as two dietary experiments, we found clear evidence for only one component of the PLH: the consistency of absolute protein intake over time. On the other hand, our data did not support another fundamental component of the PLH: an inverse relationship between the protein content of the diet and total energy intake. Overall, the data collected in this research study failed to provide consistent evidence for the PLH. Future research is needed to explore other physiological, evolutionary, ecological, and sociocultural mechanisms that help us to address the question: why do we eat what we eat?

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APPENDIX A. UNIVERSITY OF COLORADO DENVER, SCIENTIFIC ADVISORY & REVIEW COMMITTEE (SARC) PROTOCOL

University of Colorado Boulder
Scientific Advisory and Review Committee (SARC) Protocol

Date Submitted: 2nd re-submission September 29, 2017 (1st re-submission August 7, 2017; original submission March 6, 2017)
Protocol Full Title: Do protein content and protein quality influence human food intake? Testing the Protein Leverage Hypothesis

Principal Investigator: Richard L Bender

General Utilization Information
Is this study: (Check all that apply)
☒ Outpatient Boulder Patient Visit ☐ Inpatient UCH ☐ Inpatient Children's Hospital ☐ No
☐ Outpatient UCH ☐ Outpatient Children's Hospital

How many participants do you anticipate screening? 45
How many participants do you expect to enroll/complete the study? 21 enroll/18 complete

I. Hypotheses and Specific Aims

The main objective of this study is to collect data for a dissertation project that studies the effects of dietary protein quantity and dietary protein quality on total energy intake in a free-living human population from the Boulder, CO area. In this experiment, each participant will undergo four dietary treatments. Each dietary treatment consists of a 48 hr period in which the participant subsists exclusively on one of four differently-formulated liquid diets (e.g., "protein shakes") varying in protein quantity and protein quality. The primary outcome measure is total individual energy intake under each of the four liquid diets; secondary outcome measures are hormonal response to each of the four liquid diets and subjective measures of hunger and satiety under each of the four liquid diets.

Objective 1
Quantitatively evaluate the effects of protein quantity and protein quality on total energy intake.

Q1: Do participants consume more/less energy on any of the liquid diets?

Objective 2
Quantitatively evaluate the effects of protein quantity and protein quality on plasma ghrelin response, a biomarker of satiety (de Graaf et al., 2004).

Q2: Do participants have a greater/lesser plasma ghrelin response on any of the liquid diets?

Objective 3
Quantitatively explore the effects of protein quantity and protein quality on subjective feelings of hunger and satiety.

Q3: Do participants report greater/lesser feelings of hunger and satiety on any of the liquid diets?

II. Background and Significance

Background
A fundamental goal of nutritional anthropology is to explore the physiological and sociocultural factors that drive differences in eating behavior both within and between populations. The goal of this project is to experimentally test the Protein Leverage Hypothesis (PLH), a potential explanatory framework that may link population-level shifts in dietary composition to changes in individual energy intake.

The PLH, proposed by Simpson and Raubenheimer (2005), suggests that protein intake is under tighter physiological regulation than carbohydrate or fat intake. Therefore, dietary behavior should optimize protein intake to meet individual protein requirements, even at the cost of over- or under-consuming the other macronutrients (and hence, over- or under-consuming total energy). Thus, if the protein density of a diet decreases, then individuals are predicted to over-consume the diet in order to meet their protein requirements, and consequently, total energy intake would increase. Conversely, a shift to a higher-protein diet should lead to a decrease in energy intake, since individuals can meet their protein requirements with less total food consumption.

There have been several experimental tests of the PLH, but the available evidence is equivocal. Some investigators have found support for protein leverage (Poppitt et al., 1998; Simpson et al., 2003; Weigle et al., 2005; Gosby et al., 2011; Martens et al., 2013, 2014), but others have not (Stubbs et al., 1996; Marmonier et al., 2000; Raben et al., 2003; Griffioen-Roose et al., 2011). Most studies to date have assessed total food consumption, either following or concurrent with a protein treatment, by *ad libitum* consumption of a mixed diet. This is problematic because individuals likely differ in taste and texture preference, and this may confound the effect of the protein treatment on total energy intake. Additionally, most studies to date have assessed dietary protein only in terms of quantity, not quality. This is potentially a confounding factor, since proteins of different quality (e.g., plant-derived vs. animal-derived proteins) may exert stronger or weaker leveraging effects on total energy intake.

Significance
Our experimental test of the PLH will improve on previous studies in three ways. First, our use of homogenous liquid diets removes the confounding effect of taste or texture differences on individual eating behavior. Second, our experiment will isolate the independent effects of both protein quantity and protein quality on total energy intake. Third, the within-participants crossover design of our study allows all participants to be their own controls, removes any confounding effect of treatment order, and increases the statistical power of the analysis. In sum, our study will experimentally test the PLH, an explanatory framework with the potential to link population-level shifts in dietary composition to individual eating behaviors, in a precise and internally-controlled way.
Preliminary Studies We were unable to conduct preliminary research for this dietary intervention study for budgetary reasons. However, our co-investigators, Dr. Marc-Andre Cornier and Dr. Tanya Halliday (Anschutz Medical Center), have experience in successfully conducting dietary intervention studies (e.g., Cornier et al., 2005, 2006, 2007, 2010).

III. Research Design and Methods (Be sure to include a statistical analyses plan and sample size justification as part of this section)

DESIGN
This is a repeated-measures, randomized, crossover design. There are four experimental dietary treatments (diets A, B, C, and D), and all participants will undergo all four treatments in random order (phases 1, 2, 3, and 4).

Randomization
Concatenated Latin square, e.g.,

Table 1: Representation of Latin square design

Participant ID#	Phase 1	Phase 2	Phase 3	Phase 4
001	Diet A	Diet B	Diet C	Diet D
002	Diet B	Diet A	Diet D	Diet C
003	Diet C	Diet D	Diet A	Diet B
004	Diet D	Diet C	Diet B	Diet A
…	…	…	…	…
…	…	…	…	…
…etc.	…	…	…	…

Controls

Since this is a repeated-measures crossover design, all participants act as their own controls.

Power analysis & sample size

A power analysis was conducted with GLIMMPSE v 2.2.5 (glimmpse.samplesizeshop.org; Kreidler et al., 2013) to determine the total sample size necessary for this study. A sample size of N = 18 is required to achieve power ≥ 0.80 at $\alpha = 0.05$ under the following assumptions:

- Statistical family: multivariate approach to repeated measures (Hotelling-Lawley Trace)
- Hypothesis type: main effect of dietary treatment on daily energy intake over repeated measures
- Bonferroni correction of α for four post-hoc comparisons (see *Data analysis plan* below): 0.0125
- Grand mean: 2,400 kcal/day
- Effect size: ± 200 kcal/day
- Variability: ± 300 kcal/day
- Correlation of energy intakes among treatments: $r = 0.30$

The assumed grand mean of 2,400 kcal/day is based on data from two 3-day dietary intervention studies (Cornier, personal communication), which are likely to better reflect typical daily energy intake of USA adults in an intervention setting than in a free-living setting. The effect size of ± 200 kcal/day represents what we would consider a satisfactory demonstration of the PLH, based on the range of effect sizes reported by previous tests of the PLH, e.g., 136 kcal/day (Martens et al., 2014), 260 kcal/day (Gosby et al., 2011), 441 kcal/day (Weigle et al., 2005), 507 kcal/day (Martens et al., 2013). The assumed variability of ± 300 kcal/day is derived from two previous studies which measured total daily *ad libitum* energy intake of participants constrained to purely liquid diets for multiple days (Meier et al., 1993; Mustad et al., 1999); this variability is lower than what would be expected on a free-living diet of normal foods. The assumed correlation of energy intakes among treatments of $r = 0.30$ is calculated from previously-collected, multiday dietary data from free-living women Cali, Colombia (Dufour et al., 2015; Dufour, unpublished data). We expect the correlation among treatments to be higher in this liquid diet intervention study than in a free-living context, but additional data are not available. Therefore, we use the conservative value of $r = 0.30$.

Assuming a dropout rate of 15% due to compliance and/or tolerability issues, the total sample size to be recruited for the study is $18 + 2.7 \approx 21$ participants. Dropouts will be replaced until the required sample size of 18 will be met.

Data analysis plan

There are three outcome measures for this study: 1) total energy intake on each of the dietary treatments; 2) plasma ghrelin response on each of the dietary treatments; 3) self-reported hunger and satiety on each of the dietary treatments. The data will be analyzed as follows.

1) Energy intake

For each dietary treatment, total *ad libitum* food intake (in grams) will be measured over a 48-hour period. Food intake values will subsequently be converted to total energy intake values, based on the energy density of the liquid diets. These data will be analyzed via repeated-measures ANOVA to detect any significant between-treatment differences in total energy intake. Total energy intake on each dietary treatment is not compared to usual energy intake, but rather to energy intake on one of the other dietary treatments according to the following four pre-planned post-hoc comparisons:

A) Varying protein quantity within a given level of protein quality:
    1. HpHq (high-protein/high-quality) vs. LpHq (low-protein/high-quality)
    2. HpLq (high-protein/low-quality) vs. LpLq (low-protein/low-quality)
B) Varying protein quality within a given level of protein quantity:
    1. HpHq (high-protein/high-quality) vs. HpLq (high-protein/low-quality)
    2. LpHq (low-protein/high-quality) vs. LpLq (low-protein/low-quality)

2) Plasma total ghrelin response
For each dietary treatment, plasma ghrelin area-under-the-curve (AUC) will be measured in an acute feeding study following each 48-hour *ad libitum* dietary treatment period, with 4 blood samples taken at 30-minute intervals over a 90-minute period. These data will be analyzed via repeated-measures ANOVA to detect any significant between-treatment differences in plasma ghrelin response according to the following four pre-planned post-hoc comparisons:

A) Varying protein quantity within a given level of protein quality:
    1. HpHq (high-protein/high-quality) vs. LpHq (low-protein/high-quality)
    2. HpLq (high-protein/low-quality) vs. LpLq (low-protein/low-quality)
B) Varying protein quality within a given level of protein quantity:
    1. HpHq (high-protein/high-quality) vs. HpLq (high-protein/low-quality)
    2. LpHq (low-protein/high-quality) vs. LpLq (low-protein/low-quality)

3) Self-reported hunger and satiety
For each dietary treatment, multiple aspects of self-reported hunger and satiety will be assessed with visual analogue scale (VAS) surveys, as detailed in the *Dietary treatments & data collection* and *Experimental protocols & timeline* sections below. VAS data will be collected during each 48-hour *ad libitum* dietary treatment, as well as during the acute feeding study following each 48-hour treatment period (concurrent with the plasma ghrelin protocol described above). For the 48-hour *ad libitum* component, the mean difference between preprandial and postprandial self-reported hunger (*How hungry do you feel?*) and self-reported satiety (*How satisfied do you feel?*) will be assessed. For the acute feeding study, AUC of self-reported hunger (*How hungry do you feel?*) and self-reported satiety (*How satisfied do you feel?*) at 30-minute intervals over 90 minutes will be assessed. All VAS data will be analyzed via repeated-measures ANOVA to detect any significant between-treatment differences in self-reported hunger and satiety according to the following four pre-planned post-hoc comparisons:

A) Varying protein quantity within a given level of protein quality:
    1. HpHq (high-protein/high-quality) vs. LpHq (low-protein/high-quality)
    2. HpLq (high-protein/low-quality) vs. LpLq (low-protein/low-quality)
B) Varying protein quality within a given level of protein quantity:
    1. HpHq (high-protein/high-quality) vs. HpLq (high-protein/low-quality)
    2. LpHq (low-protein/high-quality) vs. LpLq (low-protein/low-quality)

Duration
All participants will undergo 4 experimental dietary treatments, each of which will last 48 hrs. The 48-hr treatment duration mirrors that of Simpson et al. (2003), the foundational demonstration of protein leverage that we are referencing in this work. Studies of longer duration, on the order of 12-14 days, have generally produced evidence in support of the PLH (Weigle et al., 2005; Martens et al., 2013). On the other hand, shorter-duration studies (< 24 hrs) have produced mixed results: some have found evidence in support of the PLH (Poppitt et al., 1998), and some against (Marmonier et al., 2000; Griffioen-Roose et al., 2011). The mixed results of the short-duration studies may indicate that a < 24-hr period is insufficient to allow for physiological responses to changes in energy intake or diet composition to emerge (de Castro, 1998). Hence, we have chosen 48 hrs as a treatment period that mirrors the duration of the main previous test of the PLH (i.e., Simpson et al., 2003), and that should allow sufficient time for protein-leveraging effects to emerge (e.g., Weigle et al., 2005) without imposing excess burden on participants. There will be 4-week washout periods between treatments. Thus, the total duration of the study will be ~16 weeks. All experimental procedures will take place at or through the Clinical & Translational Research Center (CTRC) on the University of Colorado Boulder (CU Boulder) campus.

METHODS

This description of procedures is divided into two segments: A) Dietary treatments & data collection, B) Experimental protocols & timeline.

A) Dietary treatments & data collection

Dietary treatments

The four dietary treatments will be custom-produced in the Nutrition Services lab at the Denver CTRC, under the supervision of Janine Higgins, PhD, Nutrition Research Director. For this study, the Denver CTRC is providing nutritional consultation to the PI, as well as the facilities and materials to create the dietary treatments, but the Denver CTRC is not directly involved in the research protocol itself. All participant recruitment, enrollment, data collection, etc. will take place at or through the Boulder CTRC only. The four treatments will differ in protein quantity and/or quality, but they will be identical in energy density. In terms of energy derived from each macronutrient, the four treatments will be:

- HpHq (high-protein/high-quality): 25% energy from whey protein, 45% energy from carbohydrate, 30% energy from fat
- LpHq (low-protein/high-quality): 10% energy from whey protein, 60% energy from carbohydrate, 30% energy from fat
- HpLq (high-protein/low-quality): 25% energy from pea protein, 45% energy from carbohydrate, 30% energy from fat
- LpLq (low-protein/low-quality): 10% energy from pea protein, 60% energy from carbohydrate, 30% energy from fat

We define foods with a higher Protein Digestibility Corrected Amino Acid Score (PDCAAS) to be "high-quality", and foods with a lower PDCAAS to be "low-quality." For this study, whey and pea were selected as the primary protein sources due to their differing protein quality: whey protein is considered a higher-quality protein with a PDCAAS of 1.0, while pea protein is a lower-quality protein with a PDCAAS of 0.7. For each dietary treatment, participants will be provided with 4 different flavors: vanilla, chocolate, strawberry, and coffee. All participants will be given the same flavors for all dietary treatments to avoid any confounding effects of between-participant differences in flavor preferences.

Table 2 lists the specific ingredients necessary to produce a 2,000-kcal portion of each dietary treatment. These recipes are for vanilla-flavored diets; the recipes for other flavors are similar except that different flavoring ingredients are used (e.g., chocolate, coffee, or strawberries instead of vanilla). For all dietary treatments, the primary fat source (canola oil) and the primary carbohydrate source (polycose powder) are identical.

Table 2: Quantities (g) of ingredients required to produce a 2,000-kcal portion of each dietary treatment (vanilla flavor)

	HpHq	LpHq	HpLq	LpLq
Coconut milk (vanilla)	550	530	-	-
Whey protein isolate powder	74	30	-	-
Pea milk (Ripple™ vanilla)	-	-	500	500
Pea protein (vanilla)	-	-	68	16
Water	-	-	60	44
Polycose powder	56	80	18	66
Oil (canola)	22	24	20	22
Sugar (granulated white)	34	50	48	50
Vanilla flavor (imitation, alcohol-free)	10	10	10	10

Table 3 shows the macronutrient and micronutrient contents of the four dietary treatments. Values are for a 2,000-kcal portion of the vanilla flavor; participants will have access to at least 9,000 kcal of each dietary treatment for each 48-hour treatment period (4,500 kcal/day). Micronutrient contents vary slightly according to flavor; for example, the strawberry-flavored dietary treatments contain whole fresh strawberries, and therefore contain additional fiber and Vitamin C.

Table 3: Macronutrient and micronutrient composition of a 2,000-kcal portion of each dietary treatment (vanilla flavor)

Nutrient	HpHq	LpHq	HpLq	LpLq
Protein (g)	126.5	51.0	129.8	56.3
Fat (g)	65.0	67.9	70.4	66.5
Carb (g)	226.8	298.5	233.5	306.3
Fiber (g)	0.0	0.0	3.8	0.9
Calcium (mg)	611	503	2413	2033
Iron (mg)	0	0	23	14
Magnesium (mg)	185	177	1	1
Potassium (mg)	976	523	2,281	1,708
Sodium (mg)	512	284	1,400	721
Vitamin C (mg)	0	0	0	0
Vitamin A (IU)	2,305	2,208	2,160	2,135
Vitamin D (IU)	553	530	518	512

Table 4 shows the essential amino acid (EAA) content of each of the four dietary treatments (vanilla flavor), expressed as milligrams of amino acid per gram of total protein, in comparison to the adult EAA requirements provided by the WHO/FAO/UNU (2007). These values indicate that all four dietary treatments meet the minimum WHO/FAO/UNU (2007) requirements, except that the two low-quality treatments (HpLq and LpLq) are not sufficient in methionine + cysteine density.

Table 4: Relative EAA requirements compared to relative EAA composition of each dietary treatment (mg amino acid per g total protein; vanilla flavor)

	Requirement[a]	HpHq	LpHq	HpLq	LpLq
Histidine	15	15	15	25	25
Isoleucine	30	50	50	48	48
Leucine	59	99	99	84	84
Lysine	45	77	77	74	74
Methionine + Cysteine	22	27	27	19	19
Phenylalanine + Tyrosine	38	54	54	92	92
Threonine	23	71	71	41	41
Tryptophan	6	15	15	10	10
Valine	39	47	47	50	50

[a]*From WHO/FAO/UNU (2007:150)*

Table 5 lists the absolute EAA content, in milligrams, of each of the four dietary treatments (vanilla flavor). Values are for a 2,000-kcal portion; participants will have access to at least 9,000 kcal of each dietary treatment for each 48-hour treatment period (4,500 kcal/day). The values can be compared to the adult EAA requirements provided by the WHO/FAO/UNU (2007), based on an assumed body mass of 83.1 kg. This is the mean body mass for adult USA males calculated from the NHANES 2009-2010 data (CDC, 2010). For the HpHq and HpLq dietary treatments, a 2,000-kcal portion is sufficient to meet or exceed all EAA requirements. For the LpHq dietary treatment, however, a 2,201-kcal portion is needed to meet all requirements, while for the LpLq dietary treatment, a 2,393-kcal portion is needed to meet all requirements. A daily energy intake of 2,393 kcal is ~66% of the mean of 3,624 kcal calculated for an 83.1-kg adult male from the NHANES 2009-2010 data (CDC, 2010), and participants will be provided with 4,500 kcal/day of each dietary treatment. Therefore, participants in this study should be able to meet all EAA requirements, even on the lowest-protein and lowest-quality dietary treatment.

Table 5: Absolute EAA requirements (mg amino acid per day; vanilla flavor) compared to absolute EAA composition of each dietary treatment (mg amino acid per 2,000 kcal)

	Requirement[a]	HpHq	LpHq	HpLq	LpLq
Histidine	831	1,873	755	3,259	1,414
Isoleucine	1,662	6,289	2,535	6,180	2,681
Leucine	3,241	12,577	5,070	10,918	4,737
Lysine	2,493	9,743	3,927	9,555	4,146
Methionine + Cysteine	1,247	3,416	1,377	2,402	1,042
Phenylalanine + Tyrosine	2,078	6,808	2,744	11,957	5,188
Threonine	1,247	8,971	3,616	5,258	2,281
Tryptophan	332	1,949	785	1,337	580
Valine	2,161	5,884	2,372	6,530	2,833

[a]From WHO/FAO/UNU (2007:150); based on mean adult male body mass of 83.1 kg (CDC, 2010)

As detailed in the *Experimental protocols & timeline* below, participants subsist exclusively on one of the 4 liquid diets during each treatment phase, with no other foods allowed. They are, however, permitted to consume unlimited quantities of water and other non-caloric beverages, as shown in Table 6.

Table 6: List of allowed and disallowed beverages

Allowed	Disallowed
Water	Fruit juices
Black coffee (with or without no-calorie sweeteners)	Coffee drinks with sugar and/or dairy (milk, cream)
Black, herbal or green tea (with or without no-calorie sweeteners)	Tea drinks with sugar, honey, and/or dairy (milk, cream)
Zero-calorie soft drinks (example: Diet Coke)	Non-diet soft drinks (example: regular Coke)
Zero-calorie sports drinks (example: Powerade Zero)	Non-diet sports drinks (example: regular Powerade)
	Energy drinks
	Alcoholic beverages (beer, wine, liquor, mixed drinks)
	Smoothies, milkshakes, other protein shakes not provided by the research team

Self-reported measures of representativeness of previous day's food intake, physical activity, and sleep duration

At the beginning of each treatment phase, additional data will be collected from each participant via the *Initial Survey* instrument. Participants will be asked four sets of questions to assess: 1) when they had their last meal or snack; 2) when they went to bed and woke up, and whether this conforms to their usual pattern; 3) how much moderate/vigorous physical activity they conducted the previous day, and whether the overall physical activity conforms to the participant's usual pattern; 4) and whether their previous day's diet and food intake conformed to their usual pattern. Any significant interaction effects of these variables with the dietary treatments will be included in the repeated-measures ANOVA models.

Acute feeding study

At the end of each 48-hour *ad libitum* dietary treatment phase, all participants will return to the Boulder CTRC to undergo a 90-minute acute feeding study. This will involve the consumption of a set-calorie test meal of the same liquid diet formula that was consumed during the preceding *ad libitum* phase, as well as the collection of blood samples and VAS data (both described in additional detail below). The acute feeding study begins with participants completing the 1st prompt of the *Clinic Survey*, a VAS survey of 4 questions repeated in five prompts. Next, an IV is inserted by a Boulder CTRC phlebotomist. Then, participants consume a test "breakfast" meal of their liquid diet formula for that treatment phase, equal to 20% of their daily energy requirement, as estimated by the FAO/WHO/UNU (2001) recommendations. Immediately upon completion of the test meal (0 min), participants complete the 2nd prompt of the *Clinic Survey* and the 1st 4.0-mL blood sample is drawn. At 30, 60, and 90 min after completion of the test meal, participants again complete a prompt of the *Clinic Survey*, for a total of 5 VAS prompts (4 questions each) and 4 blood samples per participant for the acute feeding study.

Blood draws

Blood samples will be drawn at the end of each 48-hour treatment phase. All blood draws will be performed at the Boulder CTRC by trained in-house personnel. Each participant will undergo 1 venipuncture (IV insertion) and 4 blood draws over a 90-minute period in each of the 4 dietary treatment phases, for a total of 4 venipunctures and 16 blood draws per participant overall. Each 4.0 mL blood sample will be drawn into an EDTA- treated tube for subsequent analysis of plasma ghrelin levels. Ghrelin levels are highest immediately preceding voluntary meal initiation (Cummings et al., 2004) and decline rapidly following a meal (Cummings et al., 2001; Jakubowicz et al., 2012). Thus, the postprandial reduction in plasma ghrelin will be used as a biomarker of satiety (de Graaf et al., 2004), providing a physiological context for the main outcome measure. Specifically, the 90-minute AUC of plasma ghrelin levels (from 4 blood samples) will be compared among the 4 dietary treatments using repeated-measures ANOVA. Sample tubes will be labeled with confidential participant ID numbers, not participants' names or other identifiers, and will be frozen and stored at the Boulder CTRC until they are sent to the Core Laboratory of the University of Colorado Hospital CTRC in Aurora, CO for analysis.

Self-reported measures of hunger and satiety

Participants will self-report their feelings of hunger and satiety using the *Shake Surveys* during each 48-hour *ad libitum* dietary treatment period, and the *Clinic Survey* during each acute feeding study. These surveys all use VAS to assess self-rated hunger and satiety. VAS use a 100mm horizontal line, with words/phrases anchored at each end of the line, to describe the extremes of response to a particular question. For example, the question "How hungry do you feel?" is anchored by the phrase "I am not hungry at all" at the left end of the line, and by the phrase "I have never been more hungry" at the right end of the line. Participants make a pen or pencil mark across the line at the point that corresponds to their feelings for each question. These responses are subsequently quantified by measuring the distance of the mark down the 100 mm line; scores for each question therefore range continuously from 0 to 100. The VAS method has been shown to be valid and reliable in studies of appetite sensations (Parker et al., 2004; Flint et al., 2000), particularly in within-participant, repeated-measures designs (Stubbs et al., 2000).

For this study, the VAS surveys each include 4 questions to gauge hunger and satiety: 1) *How hungry do you feel?* 2) *How satisfied do you feel?*, 3) *How full do you feel?*, 4) *How much do you think you can eat?* These questions are listed twice on each copy of the *Shake Surveys*, with instructions for the questions to be answered both immediately before and immediately after each *ad libitum* meal during the 48-hour treatment period. Each participant is provided with 15 paper copies of the *Shake Survey*, with more available upon request from the research team. The same 4 VAS questions are listed 5 times on the *Clinic Survey*, with prompts to complete a question set immediately before the acute test meal, then at 0, 30, 60, and 90 minutes following completion of the test meal. For the 48-hour *ad libitum* component, the mean difference between preprandial and postprandial self-reported hunger and self-reported satiety will be assessed. For the acute feeding study, AUC of self-reported hunger and self-reported satiety at 30-minute intervals over 90 minutes will be assessed.

Self-reported tolerance and compliance

At the end of each treatment period, participants will fill out a *Final Survey*, an instrument designed to assess tolerance of the liquid dietary treatment and compliance with the study protocols. Specifically, participants are asked whether they consumed any solid foods or disallowed beverages (i.e., caloric beverages) during the treatment period. The *Final Survey* also includes seven additional VAS prompts to gauge participants' overall feelings of hunger, food cravings, and comfort throughout the treatment period, and two open-ended questions to assess negative side-effects

of the dietary treatments (e.g., stomach pain, stress, anxiety) and other comments about the protocol. Taken together, these self-reported results will be assessed by the research team to determine whether the participant was noncompliant or could not adequately tolerate the treatment. In either case, the participant would be removed from the study. Dropouts will be replaced until the required sample size is met.

Additionally, the *Final Survey* includes five prompts to gauge the sensory qualities of the food: 1) *Visual appeal of food*, 2) *Smell of food*, 3) *Taste of food*, 4) *Aftertaste of food*, 5) *Texture of food*. These variables are not part of the main analyses, but will be compared among the dietary treatments to ensure that they do not differ in sensory qualities, which would confound the main analyses.

Summary of data collection instruments

1. *Initial Survey*
    - 1 question to assess the timing and general composition of the last caloric meal consumed before the treatment period begins
    - 2 questions to assess bedtime the previous evening and wake-up time the morning of the treatment period (used to evaluate sleep duration prior to the treatment period)
    - 2 questions to assess hours of moderate/vigorous exercise the day before the treatment period, and representativeness of this physical activity level (used to evaluate representativeness of physical activity level preceding each treatment period)
    - 2 questions to assess representativeness of dietary composition and consumption the day before the treatment period (used to evaluate representativeness of total daily food intake preceding each treatment period)
    - Completed once per treatment period, before the treatment period begins
2. *Shake Surveys*
    - 2 sets of 4 VAS questions to gauge pre- and postprandial hunger and satiety
    - Completed at every snack or meal during the 48-hr *ad libitum* period
3. *Final Survey*
    - 2 prompts to report any additional calorie consumption during the 48-hr *ad libitum* period (used to assess participant compliance with the study protocol)
    - 5 VAS questions to gauge sensory qualities of dietary treatments
    - 7 VAS questions to gauge overall feelings of hunger, satiety, and comfort throughout the 48-hr *ad libitum* period (used to assess participant tolerance of dietary treatments)
    - 2 open-ended questions to report any negative physical, mental, or emotional symptoms of the dietary treatments and any other participant concerns (used to assess participant tolerance of dietary treatments)
    - Completed once per treatment period, at the end of the treatment period
4. *Clinic Survey*
    - 5 sets of 4 VAS questions to gauge pre- and postprandial hunger and satiety
    - Completed once per treatment period, during the acute feeding study following the 48-hr *ad libitum* period

B) Experimental protocols & timeline

Pre-screening

Before visiting the Boulder CTRC, all potential participants will be pre-screened by the PI via telephone, using the *Pre-Screening Script*. The pre-screening is intended to ensure that the inclusion and exclusion criteria are met before any potential participant takes the time to visit the Boulder CTRC.

Enrollment & 1st treatment phase

All participant recruitment, enrollment, and data collection will take place at or through the Boulder CTRC. During enrollment, potential participants will first be familiarized with all procedures, risks, time commitments, and monetary compensation associated with the study. Second, informed consent will be obtained from those potential participants who choose to join the study. Third, the take-home *Guidelines for Participants* document will be distributed and reviewed. This document describes 1) the dietary protocols that participants are to follow during the free-living portion of the treatment period, 2) additional beverages that are allowed or disallowed during the

treatment period, 3) potential discomforts and risks associated with the liquid diets, and 4) contact information for the research team, emergency medical personnel, and the CU Boulder IRB. Fourth, all participants will undergo a medical history intake and physical examination to ensure that exclusion criteria are met and that they can safely participate. Fifth, participant data will be recorded on the *Participant Intake Form.* Finally, enrolled participants will immediately begin the 1st of four dietary treatment phases.

Enrollment

1. Potential participants arrive at the Boulder CTRC in the morning (according to the availability of a Boulder CTRC physician; see below) following a 12-hour overnight fast and are familiarized with the procedures, dietary restrictions, potential risks, time commitments, and monetary compensation associated with the study.
2. Informed consent is obtained from those participants wishing to join the study. Participants are assigned a confidential ID number for data identification.
3. The *Guidelines for Participants* are reviewed and each participant receives a paper copy. This document is also made available to each participant in three ways as a PDF file: 1) as an email attachment, 2) uploaded to a private Facebook page accessible only by participants, 3) uploaded as a Google Doc to a folder accessible only by participants. This will allow participants to have ready access to the study guidelines at all times, and removes the need for participants to carry a paper copy of the document if they do not wish to.
4. In accordance with Boulder CTRC regulations, participants undergo a medical history intake and physical examination by a Boulder CTRC physician. This should take approximately 15 minutes.
5. Participant data (age, sex, weight, height) are recorded on the *Participant Intake Form.*

1st dietary treatment phase

1. Data on representativeness of previous day's food intake, physical activity, and sleep duration are collected from each participant via the *Initial Survey.*
2. Participants are issued a 48-hr supply (9,000 kcal total) of the liquid diet they have been assigned for that phase; participants may request more of the diet at any time by contacting the PI.
3. Participants receive the *Shake Surveys* (15 copies) and *Final Survey* and depart the Boulder CTRC with their 48-hr liquid diet supply.
4. *Ad libitum* treatment period: for the next 48 hrs, participants subsist exclusively on the liquid diet they have been assigned for that phase. Participants consume as much or as little of the liquid diet as they wish, at any time.
    a. Whenever participants wish to consume a meal or snack, the following steps are followed:
        i. Complete page 1 of a *Shake Survey*.
        ii. Consume an *ad libitum* quantity of the liquid diet.
        iii. Complete page 2 of the *Shake Survey*.
    b. As detailed in the *Guidelines for Participants*, participants may not consume any other food items during the treatment period, including liquid foods such as soups or broths. They also may not consume any caloric beverages. Participants may, however, consume unlimited quantities of non-caloric beverages.
5. At the end of the 48-hr period:
    a. Participants complete the *Final Survey* 48 hours after the treatment period began, i.e., at the same time of the morning that they received their liquid diet supply, and return to the Boulder CTRC with all unconsumed portions of the liquid diet (along with all original containers) for weigh-back.
    b. Participants complete the acute feeding component of the dietary treatment phase; procedures begin at the same time of day as the *ad libitum* period 48 hours prior.
        i. Participants complete prompt 1 of a *Clinic Survey* (4 VAS survey questions repeated in 5 prompts)
        ii. An IV is inserted.
        iii. Participants consume a "breakfast" meal of their liquid diet for that phase (i.e., the same diet they have been consuming for the previous 48 hours), equal to 20% of their daily energy requirement, as estimated according to the FAO/WHO/UNU (2001) recommendations.
        iv. Immediately upon completion of the "breakfast" meal (0 min), a blood sample is drawn into a 4.0ml EDTA-treated tube and the participant completes prompt 2 of the *Clinic Survey*.

v. At 30 min, 60 min, and 90 min after the meal, blood samples are again drawn and participants complete prompts 3, 4, and 5 of the *Clinic Survey*, for a total of 4 blood samples and 5 sets of VAS data.

vi. The IV is removed, and the 1st dietary treatment phase is complete.

2nd, 3rd, & 4th treatment phases

There are a total of 4 treatment phases to the study, enabling each participant to undergo each of the 4 dietary treatments in random order. For example, during treatment phase 1, one participant may be on the HpHq diet while another participant is on the LpHq diet. As detailed above, the 1st treatment phase will begin immediately following the enrollment process at the Boulder CTRC. The subsequent treatment phases (2nd, 3rd, and 4th) will follow exactly the same procedures as the 1st treatment phase, except that participants will begin the treatment phase immediately upon arrival at the Boulder CTRC (i.e., they do not repeat the enrollment procedures or the medical history intake and physical examination). For each treatment phase, participants will be instructed to fast for 12 hours (overnight) before beginning each new treatment. Also, the 2nd, 3rd, and 4th treatment phases will begin at the same time of day as the 1st treatment phase for each participant; e.g., if a participant began the 1st treatment phase at 9:30am following enrollment, medical history intake, etc., then that participant will also begin each subsequent treatment phase at 9:30am. Likewise, the acute feeding component at the end of each treatment phase also begins at the same time of day that the treatment phase itself begin (9:30am, in this example).

Upon completion of each phase, participants will have a 4-week washout period before beginning the next phase with a different dietary treatment. The washout period will allow participants to return to a physiological baseline between treatment phases. It will also ensure that female participants can begin each treatment phase at the same point of their menstrual cycles, specifically the follicular phase (as determined by participant self-report of menses), since ad libitum food intake is known to vary over the menstrual cycle in adult females (Lissner et al., 1988; Buffenstein et al., 1995; Dye & Blundell, 1997). A female undergraduate research assistant will be employed to assist in scheduling the female participants' treatment periods, such that the treatment periods all begin at the (self-reported) follicular phase of the menstrual cycle for each individual. Males will also be held to the same washout schedule, to eliminate any confounding effect of different washout periods. Data from the *Final Survey* will be examined to ensure that each participant will be able to comfortably complete the next phase without undue burden. This process will continue until all participants have completed all 4 phases.

Overall timeline

The total time commitment for participants is 8 weeks, or approximately 112 days. Of these 112 days of enrollment in the study, there are 8 days of active participation, i.e., 8 days on the dietary treatments with 8 visits to the Boulder CTRC. The remaining days represent the washout periods (inactive participation) between the 4 dietary treatments. The specific breakdown of the total time commitment is as follows: 1) Enrollment at the Boulder CTRC and beginning of 1st dietary treatment phase (1.5 hrs), followed by a 48-hr *ad libitum* dietary treatment period, an acute feeding component at the Boulder CTRC (2.0 hrs), and a subsequent 4-week washout period; 2) 3 additional dietary treatment periods, each involving an initial visit to the Boulder CTRC (0.5 hrs), followed by a 48-hr *ad libitum* dietary treatment period, an acute feeding component at the Boulder CTRC (2.0 hrs), and a subsequent 4-week washout period. The individual visits to the Boulder CTRC are described in detail in Table 7.

Table 7: Summary of participant visits to the Boulder CTRC

Visit #	***Procedures/Tools***	***Location***	***How much time the visit will take***
Visit 1 (Enrollment & Phase 1)	• Project overview and consent process • Medical history and physical examination • Completion of *Initial Survey* Distribution of Phase 1 diet, *Shake Surveys*, & *Final Survey*	Boulder CTRC	1.5 hrs
Visit 2 (Phase 1)	• Return of any unconsumed portions of Phase 1 diet, *Shake Surveys*, & *Final Survey* • Acute feeding component: 1) consumption of 20% of daily energy requirement of Phase 1 diet, 2) 4 blood draws and completion of *Clinic Survey* over 90 minutes	Boulder CTRC	2.0 hrs
Visit 3 (Phase 2)	• Completion of *Initial Survey* • Distribution of Phase 2 diet	Boulder CTRC	0.5 hr
Visit 4 (Phase 2)	• Return of any unconsumed portions of Phase 2 diet, *Shake Surveys*, & *Final Survey* • Acute feeding component: 1) consumption of 20% of daily energy requirement of Phase 2 diet, 2) 4 blood draws and completion of *Clinic Survey* over 90 minutes	Boulder CTRC	2.0 hrs
Visit 5 (Phase 3)	• Completion of *Initial Survey* • Distribution of Phase 3 diet	Boulder CTRC	0.5 hr
Visit 6 (Phase 3)	• Return of any unconsumed portions of Phase 3 diet, *Shake Surveys*, & *Final Survey* • Acute feeding component: 1) consumption of 20% of daily energy requirement of Phase 3 diet, 2) 4 blood draws and completion of *Clinic Survey* over 90 minutes	Boulder CTRC	2.0 hrs
Visit 7 (Phase 4)	• Completion of *Initial Survey* • Distribution of Phase 4 diet	Boulder CTRC	0.5 hr
Visit 8 (Phase 4)	• Return of any unconsumed portions of Phase 4 diet, *Shake Surveys*, & *Final Survey* • Acute feeding component: 1) consumption of 20% of daily energy requirement of Phase 4 diet, 2) 4 blood draws and completion of *Clinic Survey* over 90 minutes	Boulder CTRC	2.0 hrs

V. Data Management and Security (include plans for assuring data accuracy & protocol compliance

There are 4 categories of data to be collected in this study: 1) enrollment data and personal characteristics, 2) plasma ghrelin data from blood draws, 3) total quantity of food consumed (i.e., total energy intake) during each dietary

treatment phase, 4) self-reported hunger, satiety, and experiential data collected with the *Initial Survey*, *Shake Survey*, *Final Survey*, and *Clinic Survey* instruments. Management of each of these 4 categories of data is described in detail below.

1. Enrollment data and personal characteristics

Before the dietary treatment phases of the study begin, all participants will undergo the consent process as well as a medical history intake and physical examination at the Boulder CTRC. The medical history intake and physical examination will be conducted by a Boulder CTRC physician. The goal of these procedures is not to collect data for direct analysis in this study, but rather to ensure that exclusion criteria are met and that the participant would be able to safely participate in the study. Therefore, the medical findings will not be shared with the PI or other members of the research team.

Once participants have consented to participate in the study, they will be assigned a confidential participant ID number known only to the PI. The hardcopy key of participant names and confidential participant ID numbers will be stored in a locked file cabinet within a locked office in the Hale Sciences building (room Hale 126). Only the PI and Faculty Advisor/CI will have access to this file cabinet. This key is the only document, either hardcopy or electronic, in which participant names and confidential participant ID numbers will appear together. Following the conclusion of the study and coding of all collected data (described below), the key will be shredded.

The only other data recorded during the enrollment process (after consent has been obtained) are participant age, sex, weight, height, and self-described physical activity characteristics. These data will be recorded by the PI on the hardcopy Participant Intake Form. The PI will identify each of these data sheets using only the confidential participant ID numbers; participants' names or other personal identifying information will not appear on any Participant Intake Form. These anonymous data sheets will be stored in a locked file cabinet within a locked office in the Hale Sciences building (room Hale 126). At the conclusion of the study, the participant data will be coded and transferred electronically to the PI's password-protected Redcap account. At this point, the original anonymous hardcopy participant data sheets will be shredded. Only the PI will have access to the electronic files, which he will access through his office computer (password-protected with 15-min automatic logoff).

2. Plasma ghrelin data from blood draws

During each of the 4 dietary treatment phases, each participant will undergo a venipuncture (IV placement) and 4 blood draws at the Boulder CTRC. Since these blood draws will occur in the presence of the PI, he will inform the phlebotomist of the relevant confidential participant ID with which to label each blood tube; the blood tubes will not be labeled with participant' names or other personal identifiers. For analysis of plasma ghrelin levels, blood tubes will be sent to the Core Laboratory of the University of Colorado Hospital CTRC in Aurora, CO via prearranged courier.

The results of the ghrelin analyses will be sent in hardcopy from the University of Colorado Hospital CTRC to the Boulder CTRC, where they will subsequently be obtained by the PI. Note that the ghrelin datasheets will include confidential participant ID numbers, but not participants' names. The PI will store the anonymous data sheets in a locked file cabinet within a locked office in the Hale Sciences building (room Hale 126). Only the PI and Faculty Advisor/CI will have access to this file cabinet. At the conclusion of the study, the ghrelin data will be coded and transferred to an electronic spreadsheet saved to a private folder in the PI's password-protected Redcap account (cloud server). At this point, the original anonymous hardcopy ghrelin data sheets will be shredded. Only the PI will have access to the electronic files, which he will access through his office computer (password-protected with 15-min automatic logoff).

3. Food consumed in each treatment phase

At the end of each of the 4 dietary treatment phases, participants will return any unused portions of the liquid diet and all original food containers to the Boulder CTRC for weigh-back and calculation of total food consumed (and hence total energy intake). These data will be recorded electronically in the PI's password-protected Redcap account and will be identified only by the confidential participant ID numbers, not the participants' names. Only the PI will have access to these electronic files, which he will access through his office computer (password-protected with 15-min automatic logoff).

4. Self-reported survey data

Each participant will self-report data on 4 hardcopy instruments (*Initial Survey*, *Shake Surveys*, *Final Survey*, and *Clinic Surveys*) during each of the 4 dietary treatment phases. Since participants will record these data themselves, the instruments will be labeled with the confidential participant ID numbers by the PI before they are distributed to the participants. The anonymous data sheets will be stored in a locked file cabinet within a locked office in the Hale Sciences building (room Hale 126). Only the PI and Faculty Advisor/CI will have access to this file cabinet. At the conclusion of the study, the survey data will be coded and transferred electronically to the PI's password-protected Redcap account. At this point, the original anonymous hardcopy survey data sheets will be shredded. Only the PI will have access to the electronic files, which he will access through his office computer (password-protected with 15-min automatic logoff).

VI. Human Subjects

A. Subject Description

The total number of participants we plan to enroll for this study is 21 (Table 8). Of these, we expect 18 to complete the study.

Table 8: Participants to be enrolled

Participant Population(s)	***Number to be enrolled in each group***
Adults from the Boulder, CO area	21

As detailed below, the participants for this study will be adults (aged 20-45) from the Boulder, CO area. Inclusion criteria will be assessed using the *Pre-Screening Script* during the pre-screening process, before potential participants are asked to come to the Boulder CTRC.

Inclusion criteria

1. Age 20-45 yrs
2. Non-pregnant and non-lactating if female
3. Body mass index (BMI) between 20.0 and 30.0 kg/m^2
4. From the Boulder, CO area

The age range of eligible participants was selected to include individuals who are fully grown adults (≥ 20 yrs), yet whose protein requirements are not yet substantially impacted by increased age (≤ 45 yrs). Since protein requirements change with age (Pellett, 1990; Campbell et al, 1994; Morais et al., 2006), likely due to a loss of lean body mass with age (Forbes, 1976), the inclusion of older adults could introduce a confounding factor into this protein-intake study.

Participation is limited to individuals with a BMI between 20.0 and 30.0 kg/m^2; this range includes individuals defined by the WHO as *normal weight* (18.5 – 25.0 kg/m^2), and *pre-obese* (25.0 – 30.0 kg/m^2) (WHO, 2006). This BMI range is intended to be narrow enough to only include individuals of relatively healthy weight status, since underweight or obese individuals may have metabolic characteristics that would confound the results of this study. For example, underweight individuals may show increased insulin sensitivity (Tayek et al., 1997), and high-protein diets may induce metabolic changes in obese individuals over and above the changes in total energy intake hypothesized in this study (Skov et al., 1999; Farnsworth et al., 2003).

Exclusion criteria

Determined by medical history intake & physical examination:

1. Has a family history of diabetes, other metabolic disorder, or eating disorder

Determined by self-report during pre-screening:

2. Currently following an intentionally high-protein diet
3. Currently following a weight-loss diet
4. Highly physically active (i.e., report engaging in > 150 min of moderate to vigorous exercise per week)
5. Has irregular menstrual cycle if female
6. Does not consume animal foods (e.g., vegan)
7. Allergic to whey or pea products and derivatives

8. Allergic to nuts

This study will exclude individuals with a family history of diabetes mellitus (either Type I or Type II), other metabolic disorders (e.g., Prader-Willi syndrome), or eating disorders (e.g., anorexia nervosa, bulimia nervosa). All of these conditions can influence an individual's eating behavior, physiological response to food, and/or psychological response to food, and this in turn could confound both the physiological and self-reported outcome measures of this dietary intervention study. This exclusion criterion will be assessed during a medical history intake and physical examination, performed by a Boulder CTRC physician before participants begin any treatments in accordance with standard Boulder CTRC procedure.

The remaining exclusion criteria will be assessed during the pre-screening process using the *Pre-Screening Script*. Highly physically active individuals are excluded from this study, since protein requirements are known to be greater in competitive athletes and other individuals with very high physical activity levels (Lemon, 1998; Tarnopolsky, 2004). Thus, including highly active participants could confound measures of daily protein intake in this study. A qualitative assessment of physical activity will be made over the telephone using the *Pre-Screening Script*. Participants will be excluded if they report engaging in > 150 min of moderate to vigorous exercise per week. Participants will also self-report their previous day's physical activity level and previous night's sleep duration during the administration of the *Initial Surveys*, as detailed above.

Additionally, female participants will begin each dietary treatment phase during the follicular phase (as determined by participant self-report of menses) of their menstrual cycles, since ad libitum food intake is known to vary over the menstrual cycle in adult females (Lissner et al., 1988; Buffenstein et al., 1995; Dye & Blundell, 1997), particularly under the influence of increased progesterone in the luteal phase. Therefore, female participants will be excluded during pre-screening if they report irregular menstrual cycling.

Finally, two of the liquid dietary treatments will contain whey protein (an animal-derived protein from cow's milk), while the other two will contain pea protein (a plant-derived protein). Also, although the liquid dietary treatments will not contain nuts as an ingredient, they will be prepared in a facility that handles nuts (the Nutrition Services lab at the Denver CTRC). Thus, the study should not include anyone who is allergic to these products or their derivatives, or anyone who does not wish to consume animal products.

B. Provisions for Data and Safety Monitoring of Participants

Self-reported data for each participant (i.e., the data collected with the *Initial Survey*, *Shake Survey*, *Final Survey*, and *Clinic Survey* instruments) will be analyzed by the PI immediately upon completion of each of the four dietary treatment phases of the study. The main purpose of these interim data analyses is to ensure that participants have not experienced undue physical, mental, or emotional distress during the treatment phase. Page 2 of the *Final Survey* is specifically intended to collect data for this purpose: seven VAS prompts to assess participants' overall feelings of hunger, satiety, and comfort during the dietary treatment phase, and two open-ended questions allowing participants to describe any other negative physical, mental, or emotional experiences during the dietary treatment phase. Any participants who self-report such negative experiences will be contacted privately by the PI and asked if they wish to continue into the next dietary treatment phase of the study. Participants will be reminded that their safety is the top priority and that voluntary withdrawal from the study will not be held against them.

C. Risk to Participants

Risks of dietary treatment

During each of the four dietary treatments, participants will subsist exclusively on a single liquid diet (plus approved beverages) for a 48-hr period. Participants may find this diet to be monotonous, displeasing, and/or unsatisfying. They may experience hunger, irritation, food cravings, and/or gastrointestinal discomfort while on the liquid diet. Also, participants will need continual access to the liquid diet throughout each 48-hr period, which may require that participants carry containers of the diet with them to work, school, etc. This, along with the prohibition against consuming other foods or beverages during each 48-hr period, may be disruptive to the participants' daily activities and social interactions.

Risks of blood collection
The collection of blood samples will require venipuncture, which participants may find uncomfortable or painful. Additional risks of venipuncture include: excessive bleeding, fainting or feeling light-headed, hematoma (blood accumulating under the skin), infection (a slight risk any time the skin is broken), and multiple punctures to locate veins. Although the blood samples collected for this study will only be analyzed for total ghrelin levels, blood contains other information that participants may wish to keep private (e.g., cholesterol levels).

Risks of data storage
Some data will initially be collected on hardcopy data sheets. It is possible that these sheets could be misplaced or stolen. Some data will be originally stored in the form of electronic spreadsheets; all hardcopy data will eventually be converted to electronic format. It is possible that electronic data files could be accessed by unauthorized personnel either from the PI's office computer or from the cloud server.

D. Plan to Minimize Risk to Participants
Management of dietary treatment risks
Self-reported survey data will be frequently monitored throughout the study. Specifically, each participant's data from both the VAS and open-ended sections of the *Final Survey* will be analyzed immediately upon completion of each dietary treatment phase to ensure that participants have not experienced undue physical, mental, or emotional distress during the treatment phase. Also, the *Guidelines for Participants* document is made available to all participants in four ways: 1) as a paper copy, 2) as an email attachment, 3) uploaded to a private Facebook page accessible only by participants, 4) uploaded as a Google Doc to a folder accessible only by participants. This will allow participants to have ready access to the study guidelines at all times, and removes the need for participants to carry a paper copy of the document if they do not wish to. This should help to reduce the disruptiveness of the dietary treatment protocol to the participants' daily activities and social interactions.

Management of blood collection risks
All blood collections will be performed by a phlebotomist at the Boulder CTRC, in the presence of trained and experienced personnel who can respond to any emergencies. To prevent unauthorized access or analysis, blood samples will be frozen and stored at the Boulder CTRC. The samples will only leave this location when they are sent to the University of Colorado Hospital CTRC for analysis, via the weekly courier system already established by the Boulder CTRC. Additionally, blood sample tubes will only be labeled with confidential participant ID numbers, not participants' names or other personal identifiers.

Management of data storage risks:
As soon as any anonymous hardcopy data sheets are collected by the PI, they will be stored in a locked file cabinet within a locked office in the Hale Sciences building (room Hale 126). Only the PI and Faculty Advisor/CI will have access to this file cabinet. At the conclusion of the study all data will be transferred electronically to the PI's password-protected Redcap account, and all original hardcopy data sheets (including the participant ID key) will be shredded. Only the PI will have access to the electronic files, which he will access through his office computer (password-protected with 15-min automatic logoff to prevent unauthorized access).

E. Potential benefits of the study
At the conclusion of the study, participants will learn how much of each dietary treatment they consumed. This may be of personal interest to some participants, but otherwise there are no direct benefits to participating in this study. The results obtained from this study may help us to better understand the physiological drivers of food consumption. This, in turn, may help us to explain how shifts in the dietary protein supply may influence the total caloric intake of a population.

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APPENDIX B. UNIVERSITY OF COLORADO BOULDER, INSTITUTIONAL REVIEW BOARD (IRB) PROTOCOL

TITLE**: Do protein content and protein quality influence human food intake? Testing the Protein Leverage Hypothesis**

PROTOCOL VERSION DATE**:** November 30, 2017

VERSION**:** 2

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Name: Darna Dufour
Role in project: Faculty Advisor

I. OBJECTIVES

The main objective of this study is to collect data for a dissertation project that studies the effects of dietary protein quantity and dietary protein quality on total energy intake in a free-living human population from the Boulder, CO area. In this experiment, each participant will undergo four dietary treatments. Each dietary treatment consists of a 48 hr period in which the participant subsists exclusively on one of four differently-formulated liquid diets (e.g., "protein shakes") varying in protein quantity and protein quality. The primary outcome measure is total individual energy intake under each of the four liquid diets; secondary outcome measures are hormonal response to each of the four liquid diets and subjective measures of hunger and satiety under each of the four liquid diets.

Objective 1
Quantitatively evaluate the effects of protein quantity and protein quality on total energy intake.

Q1: Do participants consume more/less energy on any of the liquid diets?

Objective 2
Quantitatively evaluate the effects of protein quantity and protein quality on plasma ghrelin response, a biomarker of satiety (de Graaf et al., 2004).

Q2: Do participants have a greater/lesser plasma ghrelin response on any of the liquid diets?

Objective 3
Quantitatively explore the effects of protein quantity and protein quality on subjective feelings of hunger and satiety.

Q3: Do participants report greater/lesser feelings of hunger and satiety on any of the liquid diets?

II. BACKGROUND AND SIGNIFICANCE

Background
A fundamental goal of nutritional anthropology is to explore the physiological and sociocultural factors that drive differences in eating behavior both within and between populations. The goal of this project is to experimentally test the Protein Leverage Hypothesis (PLH), a potential explanatory framework that may link population-level shifts in dietary composition to changes in individual energy intake.

The PLH, proposed by Simpson and Raubenheimer (2005), suggests that protein intake is under tighter physiological regulation than carbohydrate or fat intake. Therefore, dietary behavior should optimize protein intake to meet individual protein requirements, even at the cost of over- or under-consuming the other macronutrients (and hence, over- or under-consuming total energy). Thus, if the protein density of a diet decreases, then individuals are predicted to over-consume the diet in order to meet their protein requirements, and consequently, total energy intake would increase. Conversely, a shift to a higher-protein diet should lead to a decrease in energy intake, since individuals can meet their protein requirements with less total food consumption.

There have been several experimental tests of the PLH, but the available evidence is equivocal. Some investigators have found support for protein leverage (Poppitt et al., 1998; Simpson et al., 2003; Weigle et al., 2005; Gosby et al., 2011; Martens et al., 2013, 2014), but others have not (Stubbs et al., 1996; Marmonier et al., 2000; Raben et al., 2003; Griffioen-Roose et al., 2011). Most studies to date have assessed total food consumption, either following or concurrent with a protein treatment, by ad libitum consumption of a mixed diet. This is problematic because individuals likely differ in taste and texture

preference, and this may confound the effect of the protein treatment on total energy intake. Additionally, most studies to date have assessed dietary protein only in terms of quantity, not quality. This is potentially a confounding factor, since proteins of different quality (e.g., plant-derived vs. animal-derived proteins) may exert stronger or weaker leveraging effects on total energy intake.

Significance

Our experimental test of the PLH will improve on previous studies in three ways. First, our use of homogenous liquid diets removes the confounding effect of taste or texture differences on individual eating behavior. Second, our experiment will isolate the independent effects of both protein quantity and protein quality on total energy intake. Third, the within-participants crossover design of our study allows all participants to be their own controls, removes any confounding effect of treatment order, and increases the statistical power of the analysis. In sum, our study will experimentally test the PLH, an explanatory framework with the potential to link population-level shifts in dietary composition to individual eating behaviors, in a precise and internally-controlled way.

III. PRELIMINARY STUDIES

We were unable to conduct preliminary research for this dietary intervention study for budgetary reasons. However, our collaborators, Dr. Marc-Andre Cornier and Dr. Tanya Halliday (Anschutz Medical Campus), have experience in successfully conducting dietary intervention studies (e.g., Cornier et al., 2005, 2006, 2007, 2010).

IV. RESEARCH STUDY DESIGN

DESIGN

This is a repeated-measures, randomized, crossover design. There are four experimental dietary treatments (diets A, B, C, and D), and all participants will undergo all four treatments in random order (phases 1, 2, 3, and 4).

Randomization

Concatenated Latin square, e.g.,

Table 1: Representation of Latin square design

Participant ID#	Phase 1	Phase 2	Phase 3	Phase 4
001	Diet A	Diet B	Diet C	Diet D
002	Diet B	Diet A	Diet D	Diet C
003	Diet C	Diet D	Diet A	Diet B
004	Diet D	Diet C	Diet B	Diet A
…	…	…	…	…
…	…	…	…	…
…etc.	…	…	…	…

Controls

Since this is a repeated-measures crossover design, all participants act as their own controls.

Power analysis & sample size

A power analysis was conducted with GLIMMPSE v 2.2.5 (glimmpse.samplesizeshop.org; Kreidler et al., 2013) to determine the total sample size necessary for this study. A sample size of N = 18 is required to achieve power ≥ 0.80 at $\alpha = 0.05$ under the following assumptions:

- Statistical family: multivariate approach to repeated measures (Hotelling-Lawley Trace)
- Hypothesis type: main effect of dietary treatment on daily energy intake over repeated measures
- Bonferroni correction of α for four post-hoc comparisons (see *Data analysis plan* below): 0.0125
- Grand mean: 2,400 kcal/day
- Effect size: ± 200 kcal/day
- Variability: ± 300 kcal/day
- Correlation of energy intakes among treatments: $r = 0.30$

The assumptions of our power analysis were derived with the assistance of our collaborators, Dr. Marc-Andre Cornier and Dr. Tanya Halliday (Anschutz Medical Campus), both of whom have experience in successfully conducting dietary intervention studies. The assumed grand mean of 2,400 kcal/day is based on data from two 3-day dietary intervention studies (Cornier, personal communication), which are likely to better reflect typical daily energy intake of USA adults in an intervention setting than in a free-living setting. The effect size of ± 200 kcal/day represents what we would consider a satisfactory demonstration of the PLH, based on the range of effect sizes reported by previous tests of the PLH, e.g., 136 kcal/day (Martens et al., 2014), 260 kcal/day (Gosby et al., 2011), 441 kcal/day (Weigle et al., 2005), 507 kcal/day (Martens et al., 2013). The assumed variability of ± 300 kcal/day is derived from two previous studies which measured total daily *ad libitum* energy intake of participants constrained to purely liquid diets for multiple days (Meier et al., 1993; Mustad et al., 1999); this variability is lower than what would be expected on a free-living diet of normal foods. The assumed correlation of energy intakes among treatments of $r = 0.30$ is calculated from previously-collected, multiday dietary data from free-living women Cali, Colombia (Dufour et al., 2015; Dufour, unpublished data). We expect the correlation among treatments to be higher in this liquid diet intervention study than in a free-living context, but additional data are not available. Therefore, we use the conservative value of $r = 0.30$.

Assuming a dropout rate of 15% due to compliance and/or tolerability issues, the total sample size to be recruited for the study is $18 + 2.7 \approx 21$ participants. Dropouts will be replaced until the required sample size of 18 will be met.

Data analysis plan

There are three outcome measures for this study: 1) total energy intake on each of the dietary treatments; 2) plasma ghrelin response on each of the dietary treatments; 3) self-reported hunger and satiety on each of the dietary treatments. Our collaborators, Dr. Marc-Andre Cornier and Dr. Tanya Halliday (Anschutz Medical Campus) will participate in the data analysis, but not in the data collection, and therefore will only work with anonymous data (section XIII: DATA MANAGEMENT). The data will be analyzed as follows:

1) Energy intake

For each dietary treatment, total *ad libitum* food intake (in grams) will be measured over a 48-hour period. Food intake values will subsequently be converted to total energy intake values, based on the energy density of the liquid diets. These data will be analyzed via repeated-measures ANOVA to detect any significant between-treatment differences in total energy intake. Total energy intake on each dietary

treatment is not compared to usual energy intake, but rather to energy intake on one of the other dietary treatments according to the following four pre-planned post-hoc comparisons:

A) Varying protein quantity within a given level of protein quality:
    1. HpHq (high-protein/high-quality) vs. LpHq (low-protein/high-quality)
    2. HpLq (high-protein/low-quality) vs. LpLq (low-protein/low-quality)
B) Varying protein quality within a given level of protein quantity:
    1. HpHq (high-protein/high-quality) vs. HpLq (high-protein/low-quality)
    2. LpHq (low-protein/high-quality) vs. LpLq (low-protein/low-quality)

2) Plasma total ghrelin response

For each dietary treatment, plasma ghrelin area-under-the-curve (AUC) will be measured in an acute feeding study following each 48-hour *ad libitum* dietary treatment period, with 4 blood samples taken at 30-minute intervals over a 90-minute period. These data will be analyzed via repeated-measures ANOVA to detect any significant between-treatment differences in plasma ghrelin response according to the following four pre-planned post-hoc comparisons:

A) Varying protein quantity within a given level of protein quality:
    1. HpHq (high-protein/high-quality) vs. LpHq (low-protein/high-quality)
    2. HpLq (high-protein/low-quality) vs. LpLq (low-protein/low-quality)
B) Varying protein quality within a given level of protein quantity:
    1. HpHq (high-protein/high-quality) vs. HpLq (high-protein/low-quality)
    2. LpHq (low-protein/high-quality) vs. LpLq (low-protein/low-quality)

3) Self-reported hunger and satiety

For each dietary treatment, multiple aspects of self-reported hunger and satiety will be assessed with visual analogue scale (VAS) surveys, as detailed in the *Dietary treatments & data collection* and *Experimental protocols & timeline* sections below. VAS data will be collected during each 48-hour *ad libitum* dietary treatment, as well as during the acute feeding study following each 48-hour treatment period (concurrent with the plasma ghrelin protocol described above). For the 48-hour *ad libitum* component, the mean difference between preprandial and postprandial self-reported hunger (*How hungry do you feel?*) and self-reported satiety (*How satisfied do you feel?*) will be assessed. For the acute feeding study, AUC of self-reported hunger (*How hungry do you feel?*) and self-reported satiety (*How satisfied do you feel?*) at 30-minute intervals over 90 minutes will be assessed. All VAS data will be analyzed via repeated-measures ANOVA to detect any significant between-treatment differences in self-reported hunger and satiety according to the following four pre-planned post-hoc comparisons:

A) Varying protein quantity within a given level of protein quality:
    1. HpHq (high-protein/high-quality) vs. LpHq (low-protein/high-quality)
    2. HpLq (high-protein/low-quality) vs. LpLq (low-protein/low-quality)
B) Varying protein quality within a given level of protein quantity:
    1. HpHq (high-protein/high-quality) vs. HpLq (high-protein/low-quality)
    2. LpHq (low-protein/high-quality) vs. LpLq (low-protein/low-quality)

Duration

All participants will undergo 4 experimental dietary treatments, each of which will last 48 hrs. The 48-hr treatment duration mirrors that of Simpson et al. (2003), the foundational demonstration of protein leverage that we are referencing in this work. Studies of longer duration, on the order of 12-14 days, have generally produced evidence in support of the PLH (Weigle et al., 2005; Martens et al., 2013). On the other hand, shorter-duration studies (< 24 hrs) have produced mixed results: some have found evidence in support of the PLH (Poppitt et al., 1998), and some against (Marmonier et al., 2000; Griffioen-Roose et al., 2011). The mixed results of the short-duration studies may indicate that a < 24-hr period is

insufficient to allow for physiological responses to changes in energy intake or diet composition to emerge (de Castro, 1998). Hence, we have chosen 48 hrs as a treatment period that mirrors the duration of the main previous test of the PLH (i.e., Simpson et al., 2003), and that should allow sufficient time for protein-leveraging effects to emerge (e.g., Weigle et al., 2005) without imposing excess burden on participants. There will be 4-week washout periods between treatments. Thus, the total duration of the study will be ~16 weeks. All experimental procedures will take place at or through the Clinical & Translational Research Center (CTRC) on the University of Colorado Boulder (CU Boulder) campus. Our collaborators, Dr. Marc-Andre Cornier and Dr. Tanya Halliday (Anschutz Medical Campus) have advised us on our study design and procedures, and will participate in the analysis of anonymous data, but they will not be interacting, intervening, or collecting data from any participant.

V. ABOUT THE SUBJECTS

The total number of participants we plan to enroll for this study is 21 (Table 2). Of these, we expect 18 to complete the study.

Table 2: Participants to be enrolled

Participant Population(s)	*Number to be enrolled in each group*
Adults from the Boulder, CO area	21

As detailed below, the participants for this study will be adults (aged 20-45) from the Boulder, CO area. Inclusion criteria will be assessed using the *Pre-Screening Script* during the pre-screening process, before potential participants are asked to come to the Boulder CTRC.

Inclusion criteria

5. Age 20-45 yrs
6. Non-pregnant and non-lactating if female
7. Body mass index (BMI) between 20.0 and 30.0 kg/m^2
8. From the Boulder, CO area

The age range of eligible participants was selected to include individuals who are fully grown adults (≥ 20 yrs), yet whose protein requirements are not yet substantially impacted by increased age (≤ 45 yrs). Since protein requirements change with age (Pellett, 1990; Campbell et al, 1994; Morais et al., 2006), likely due to a loss of lean body mass with age (Forbes, 1976), the inclusion of older adults could introduce a confounding factor into this protein-intake study.

Participation is limited to individuals with a BMI between 20.0 and 30.0 kg/m^2; this range includes individuals defined by the WHO as *normal weight* (18.5 – 25.0 kg/m^2), and *pre-obese* (25.0 – 30.0 kg/m^2) (WHO, 2006). This BMI range is intended to be narrow enough to only include individuals of relatively healthy weight status, since underweight or obese individuals may have metabolic characteristics that would confound the results of this study. For example, underweight individuals may show increased insulin sensitivity (Tayek et al., 1997), and high-protein diets may induce metabolic changes in obese individuals over and above the changes in total energy intake hypothesized in this study (Skov et al., 1999; Farnsworth et al., 2003).

Exclusion criteria

Determined by medical history intake & physical examination:

9. Has a family history of diabetes, other metabolic disorder, or eating disorder

Determined by self-report during pre-screening:

10. Currently following an intentionally high-protein diet
11. Currently following a weight-loss diet

12. Highly physically active (i.e., report engaging in > 150 min of moderate to vigorous exercise per week)
13. Has irregular menstrual cycle if female
14. Does not consume animal foods (e.g., vegan)
15. Allergic to whey or pea products and derivatives
16. Allergic to nuts

This study will exclude individuals with a family history of diabetes mellitus (either Type I or Type II), other metabolic disorders (e.g., Prader-Willi syndrome), or eating disorders (e.g., anorexia nervosa, bulimia nervosa). All of these conditions can influence an individual's eating behavior, physiological response to food, and/or psychological response to food, and this in turn could confound both the physiological and self-reported outcome measures of this dietary intervention study. This exclusion criterion will be assessed during a medical history intake and physical examination, performed by a Boulder CTRC physician before participants begin any treatments in accordance with standard Boulder CTRC procedure.

The remaining exclusion criteria will be assessed during the pre-screening process using the *Pre-Screening Script*. Highly physically active individuals are excluded from this study, since protein requirements are known to be greater in competitive athletes and other individuals with very high physical activity levels (Lemon, 1998; Tarnopolsky, 2004). Thus, including highly active participants could confound measures of daily protein intake in this study. A qualitative assessment of physical activity will be made over the telephone using the *Pre-Screening Script*. Participants will be excluded if they report engaging in > 150 min of moderate to vigorous exercise per week. Participants will also self-report their previous day's physical activity level and previous night's sleep duration during the administration of the *Initial Surveys*, as detailed above.

Additionally, female participants will begin each dietary treatment phase during the follicular phase (as determined by participant self-report of menses) of their menstrual cycles, since ad libitum food intake is known to vary over the menstrual cycle in adult females (Lissner et al., 1988; Buffenstein et al., 1995; Dye & Blundell, 1997), particularly under the influence of increased progesterone in the luteal phase. Therefore, female participants will be excluded during pre-screening if they report irregular menstrual cycling.

Finally, two of the liquid dietary treatments will contain whey protein (an animal-derived protein from cow's milk), while the other two will contain pea protein (a plant-derived protein). Also, although the liquid dietary treatments will not contain nuts as an ingredient, they will be prepared in a facility that handles nuts (the Nutrition Services lab at the Denver CTRC). Thus, the study should not include anyone who is allergic to these products or their derivatives, or anyone who does not wish to consume animal products.

VI. VULNERABLE POPULATIONS

N/A (No Vulnerable Populations will be recruited.)

VII. RECRUITMENT METHODS

A convenience sample of participants will be drawn from the Boulder, CO area. Recruitment will be facilitated by 1) paper flyers posted in approved areas throughout the CU Boulder campus and in nearby businesses and 2) word of mouth. The flyers will briefly list the main procedures of the study, as well as the inclusion/exclusion criteria, and will include a unique email address for the project. Potential participants will be invited to express their interest in the study via email. Once interest has been

expressed, the PI will individually reply to each potential participant by email to schedule a pre-screening telephone conversation at a day and time that is convenient for the potential participant.

During recruitment, the PI will emphasize the following to avoid undue influence and/or coercion: 1) there are potential risks associated with the study (section XV: RISKS TO PARTICIPANTS), 2) participants may voluntarily withdraw from the study at any time and it will not be held against them (section XIV: WITHDRAWAL OF PARTICIPANTS), 3) participants will receive monetary compensation for any portion of the study that they do complete; full completion of the study is not necessary in order for compensation to be received (section VIII: COMPENSATION).

Table 3: Recruitment materials

List recruitment methods/materials and attach a copy of each in eRA
1. *Recruitment flyer*

VIII. COMPENSATION

Participants will be provided with monetary compensation for their participation in the study. Payments will be made in cash at the end of each completed dietary treatment phase. Participants who fully complete the study (all 4 dietary treatment phases) will receive a total of $300. In the event of early withdrawal, this amount will be prorated across the 4 treatment phases according to the following schedule:

Table 4: Monetary compensation schedule

Phase 1:	$60
Phase 2:	$70
Phase 3:	$80
Phase 4:	$90
Total:	$300

IX. CONSENT PROCESS

Consent will be obtained at the Boulder CTRC before the first dietary treatment begins. During the consent process, the PI will review the consent form in detail with all potential participants, ensuring that they fully understand the time commitment and all potential risks associated with the study. Additionally, the PI will emphasize that participants may choose to withdraw from the study at any time without repercussion.

X. PROCESS TO DOCUMENT CONSENT IN WRITING

Written consent, in the form of a signed consent document, will be obtained from all participants who agree to join the study. A complete copy of the consent document will be offered to all participants who sign the document.

XI. PROCEDURES

This description of procedures is divided into two segments: A) Dietary treatments & data collection, B) Experimental protocols & timeline. The protocols for the dietary treatments, self-reported measures of

hunger & satiety, and acute feeding study (i.e., blood draws and ghrelin analyses) were developed with the assistance of our collaborators, Dr. Marc-Andre Cornier and Dr. Tanya Halliday (Anschutz Medical Campus).

A) Dietary treatments & data collection

Dietary treatments

The four dietary treatments will be custom-produced in the Nutrition Services lab at the Denver CTRC, under the supervision of Janine Higgins, PhD, Nutrition Research Director. For this study, the Denver CTRC is providing nutritional consultation to the PI, as well as the facilities and materials to create the dietary treatments, but the Denver CTRC is not directly involved in the research protocol itself. All participant recruitment, enrollment, data collection, etc. will take place at or through the Boulder CTRC only. The four treatments will differ in protein quantity and/or quality, but they will be identical in energy density. In terms of energy derived from each macronutrient, the four treatments will be:

- HpHq (high-protein/high-quality): 25% energy from whey protein, 45% energy from carbohydrate, 30% energy from fat
- LpHq (low-protein/high-quality): 10% energy from whey protein, 60% energy from carbohydrate, 30% energy from fat
- HpLq (high-protein/low-quality): 25% energy from pea protein, 45% energy from carbohydrate, 30% energy from fat
- LpLq (low-protein/low-quality): 10% energy from pea protein, 60% energy from carbohydrate, 30% energy from fat

We define foods with a higher Protein Digestibility Corrected Amino Acid Score (PDCAAS) to be "high-quality", and foods with a lower PDCAAS to be "low-quality." For this study, whey and pea were selected as the primary protein sources due to their differing protein quality: whey protein is considered a higher-quality protein with a PDCAAS of 1.0, while pea protein is a lower-quality protein with a PDCAAS of 0.7. For each dietary treatment, participants will be provided with 4 different flavors: vanilla, chocolate, strawberry, and coffee. All participants will be given the same flavors for all dietary treatments to avoid any confounding effects of between-participant differences in flavor preferences.

Table 5 lists the specific ingredients necessary to produce a 2,000-kcal portion of each dietary treatment. These recipes are for vanilla-flavored diets; the recipes for other flavors are similar except that different flavoring ingredients are used (e.g., chocolate, coffee, or strawberries instead of vanilla). For all dietary treatments, the primary fat source (canola oil) and the primary carbohydrate source (polycose powder) are identical.

Table 5: Quantities (g) of ingredients required to produce a 2,000-kcal portion of each dietary treatment (vanilla flavor)

	HpHq	LpHq	HpLq	LpLq
Coconut milk (vanilla)	550	530	-	-
Whey protein isolate powder	74	30	-	-
Pea milk (Ripple™ vanilla)	-	-	500	500
Pea protein (vanilla)	-	-	68	16
Water	-	-	60	44
Polycose powder	56	80	18	66
Oil (canola)	22	24	20	22
Sugar (granulated white)	34	50	48	50

Vanilla flavor (imitation, alcohol-free)	10	10	10	10

Table 6 shows the macronutrient and micronutrient contents of the four dietary treatments. Values are for a 2,000-kcal portion of the vanilla flavor; participants will have access to at least 9,000 kcal of each dietary treatment for each 48-hour treatment period (4,500 kcal/day). Micronutrient contents vary slightly according to flavor; for example, the strawberry-flavored dietary treatments contain whole fresh strawberries, and therefore contain additional fiber and Vitamin C.

Table 6: Macronutrient and micronutrient composition of a 2,000-kcal portion of each dietary treatment (vanilla flavor)

Nutrient	HpHq	LpHq	HpLq	LpLq
Protein (g)	126.5	51.0	129.8	56.3
Fat (g)	65.0	67.9	70.4	66.5
Carb (g)	226.8	298.5	233.5	306.3
Fiber (g)	0.0	0.0	3.8	0.9
Calcium (mg)	611	503	2413	2033
Iron (mg)	0	0	23	14
Magnesium (mg)	185	177	1	1
Potassium (mg)	976	523	2,281	1,708
Sodium (mg)	512	284	1,400	721
Vitamin C (mg)	0	0	0	0
Vitamin A (IU)	2,305	2,208	2,160	2,135
Vitamin D (IU)	553	530	518	512

Table 7 shows the essential amino acid (EAA) content of each of the four dietary treatments (vanilla flavor), expressed as milligrams of amino acid per gram of total protein, in comparison to the adult EAA requirements provided by the WHO/FAO/UNU (2007). These values indicate that all four dietary treatments meet the minimum WHO/FAO/UNU (2007) requirements, except that the two low-quality treatments (HpLq and LpLq) are not sufficient in methionine + cysteine density.

Table 7: Relative EAA requirements compared to relative EAA composition of each dietary treatment (mg amino acid per g total protein; vanilla flavor)

	Requirement[a]	HpHq	LpHq	HpLq	LpLq
Histidine	15	15	15	25	25
Isoleucine	30	50	50	48	48
Leucine	59	99	99	84	84
Lysine	45	77	77	74	74
Methionine + Cysteine	22	27	27	19	19
Phenylalanine + Tyrosine	38	54	54	92	92
Threonine	23	71	71	41	41
Tryptophan	6	15	15	10	10
Valine	39	47	47	50	50

[a]*From WHO/FAO/UNU (2007:150)*

Table 8 lists the absolute EAA content, in milligrams, of each of the four dietary treatments (vanilla flavor). Values are for a 2,000-kcal portion; participants will have access to at least 9,000 kcal of each dietary treatment for each 48-hour treatment period (4,500 kcal/day). The values can be compared to the adult EAA requirements provided by the WHO/FAO/UNU (2007), based on an assumed body mass of 83.1 kg. This is the mean body mass for adult USA males calculated from the NHANES 2009-2010 data (CDC, 2010). For the HpHq and HpLq dietary treatments, a 2,000-kcal portion is sufficient to meet or exceed all EAA requirements. For the LpHq dietary treatment, however, a 2,201-kcal portion is needed to meet all requirements, while for the LpLq dietary treatment, a 2,393-kcal portion is needed to meet all requirements. A daily energy intake of 2,393 kcal is ~66% of the mean of 3,624 kcal calculated for an 83.1-kg adult male from the NHANES 2009-2010 data (CDC, 2010), and participants will be provided with 4,500 kcal/day of each dietary treatment. Therefore, participants in this study should be able to meet all EAA requirements, even on the lowest-protein and lowest-quality dietary treatment.

Table 8: Absolute EAA requirements (mg amino acid per day; vanilla flavor) compared to absolute EAA composition of each dietary treatment (mg amino acid per 2,000 kcal)

	Requirement[a]	HpHq	LpHq	HpLq	LpLq
Histidine	831	1,873	755	3,259	1,414
Isoleucine	1,662	6,289	2,535	6,180	2,681
Leucine	3,241	12,577	5,070	10,918	4,737
Lysine	2,493	9,743	3,927	9,555	4,146
Methionine + Cysteine	1,247	3,416	1,377	2,402	1,042
Phenylalanine + Tyrosine	2,078	6,808	2,744	11,957	5,188
Threonine	1,247	8,971	3,616	5,258	2,281
Tryptophan	332	1,949	785	1,337	580
Valine	2,161	5,884	2,372	6,530	2,833

[a]*From WHO/FAO/UNU (2007:150); based on mean adult male body mass of 83.1 kg (CDC, 2010)*

As detailed in the *Experimental protocols & timeline* below, participants subsist exclusively on one of the 4 liquid diets during each treatment phase, with no other foods allowed. They are, however, permitted to consume unlimited quantities of water and other non-caloric beverages, as shown in Table 9.

Table 9: List of allowed and disallowed beverages

Allowed	Disallowed
Water	Fruit juices
Black coffee (with or without no-calorie sweeteners)	Coffee drinks with sugar and/or dairy (milk, cream)
Black, herbal or green tea (with or without no-calorie sweeteners)	Tea drinks with sugar, honey, and/or dairy (milk, cream)
Zero-calorie soft drinks (example: Diet Coke)	Non-diet soft drinks (example: regular Coke)
Zero-calorie sports drinks (example: Powerade Zero)	Non-diet sports drinks (example: regular Powerade)
	Energy drinks
	Alcoholic beverages (beer, wine, liquor, mixed drinks)
	Smoothies, milkshakes, other protein shakes not provided by the research team

Self-reported measures of representativeness of previous day's food intake, physical activity, and sleep duration

At the beginning of each treatment phase, additional data will be collected from each participant via the *Initial Survey* instrument. Participants will be asked four sets of questions to assess: 1) when they had their last meal or snack; 2) when they went to bed and woke up, and whether this conforms to their usual pattern; 3) how much moderate/vigorous physical activity they conducted the previous day, and whether the overall physical activity conforms to the participant's usual pattern; 4) and whether their previous day's diet and food intake conformed to their usual pattern. Any significant interaction effects of these variables with the dietary treatments will be included in the repeated-measures ANOVA models.

Acute feeding study

At the end of each 48-hour *ad libitum* dietary treatment phase, all participants will return to the Boulder CTRC to undergo a 90-minute acute feeding study. This will involve the consumption of a set-calorie test meal of the same liquid diet formula that was consumed during the preceding *ad libitum* phase, as well as the collection of blood samples and VAS data (both described in additional detail below). The acute feeding study begins with participants completing the 1st prompt of the *Clinic Survey*, a VAS survey of 4 questions repeated in five prompts. Next, an IV is inserted by a Boulder CTRC phlebotomist. Then, participants consume a test "breakfast" meal of their liquid diet formula for that treatment phase, equal to 20% of their daily energy requirement, as estimated by the FAO/WHO/UNU (2001) recommendations. Immediately upon completion of the test meal (0 min), participants complete the 2nd prompt of the *Clinic Survey* and the 1st 4.0-mL blood sample is drawn. At 30, 60, and 90 min after completion of the test meal, participants again complete a prompt of the *Clinic Survey*, for a total of 5 VAS prompts (4 questions each) and 4 blood samples per participant for the acute feeding study.

Blood draws

Blood samples will be drawn at the end of each 48-hour treatment phase. All blood draws will be performed at the Boulder CTRC by trained in-house personnel. Each participant will undergo 1 venipuncture (IV insertion) and 4 blood draws over a 90-minute period in each of the 4 dietary treatment phases, for a total of 4 venipunctures and 16 blood draws per participant overall. Each 4.0 mL blood sample will be drawn into an EDTA- treated tube for subsequent analysis of plasma ghrelin levels. Ghrelin levels are highest immediately preceding voluntary meal initiation (Cummings et al., 2004) and decline rapidly following a meal (Cummings et al., 2001; Jakubowicz et al., 2012). Thus, the postprandial reduction in plasma ghrelin will be used as a biomarker of satiety (de Graaf et al., 2004), providing a physiological context for the main outcome measure. Specifically, the 90-minute AUC of plasma ghrelin levels (from 4 blood samples) will be compared among the 4 dietary treatments using repeated-measures ANOVA. Sample tubes will be labeled with confidential participant ID numbers, not participants' names or other identifiers, and will be frozen and stored at the Boulder CTRC until they are sent to the Core Laboratory of the University of Colorado Hospital CTRC in Aurora, CO for analysis.

Self-reported measures of hunger and satiety

Participants will self-report their feelings of hunger and satiety using the *Shake Surveys* during each 48-hour *ad libitum* dietary treatment period, and the *Clinic Survey* during each acute feeding study. These surveys all use VAS to assess self-rated hunger and satiety. VAS use a 100mm horizontal line, with words/phrases anchored at each end of the line, to describe the extremes of response to a particular question. For example, the question "How hungry do you feel?" is anchored by the phrase "I am not hungry at all" at the left end of the line, and by the phrase "I have never been more hungry" at the right end of the line. Participants make a pen or pencil mark across the line at the point that corresponds to their feelings for each question. These responses are subsequently quantified by measuring the distance of the mark down the 100 mm line; scores for each question therefore range continuously from 0 to 100. The VAS method has been shown to be valid and reliable in studies of appetite sensations (Parker et al., 2004; Flint et al., 2000), particularly in within-participant, repeated-measures designs (Stubbs et al., 2000).

For this study, the VAS surveys each include 4 questions to gauge hunger and satiety: 1) *How hungry do you feel?* 2) *How satisfied do you feel?*, 3) *How full do you feel?*, 4) *How much do you think you can eat?* These questions are listed twice on each copy of the *Shake Surveys*, with instructions for the questions to be answered both immediately before and immediately after each *ad libitum* meal during the 48-hour treatment period. Each participant is provided with 15 paper copies of the *Shake Survey*, with more available upon request from the research team. The same 4 VAS questions are listed 5 times on the *Clinic Survey*, with prompts to complete a question set immediately before the acute test meal, then at 0, 30, 60, and 90 minutes following completion of the test meal. For the 48-hour *ad libitum* component, the mean difference between preprandial and postprandial self-reported hunger and self-reported satiety will be assessed. For the acute feeding study, AUC of self-reported hunger and self-reported satiety at 30-minute intervals over 90 minutes will be assessed.

Self-reported tolerance and compliance

At the end of each treatment period, participants will fill out a *Final Survey*, an instrument designed to assess tolerance of the liquid dietary treatment and compliance with the study protocols. Specifically, participants are asked whether they consumed any solid foods or disallowed beverages (i.e., caloric beverages) during the treatment period. The *Final Survey* also includes seven additional VAS prompts to gauge participants' overall feelings of hunger, food cravings, and comfort throughout the treatment period, and two open-ended questions to assess negative side-effects of the dietary treatments (e.g., stomach pain, stress, anxiety) and other comments about the protocol. Taken together, these self-reported results will be assessed by the research team to determine whether the participant was noncompliant or

could not adequately tolerate the treatment. In either case, the participant would be removed from the study. Dropouts will be replaced until the required sample size is met.

Additionally, the *Final Survey* includes five prompts to gauge the sensory qualities of the food: 1) *Visual appeal of food*, 2) *Smell of food*, 3) *Taste of food*, 4) *Aftertaste of food*, 5) *Texture of food*. These variables are not part of the main analyses, but will be compared among the dietary treatments to ensure that they do not differ in sensory qualities, which would confound the main analyses.

Summary of data collection instruments

5. *Initial Survey*

- 1 question to assess the timing and general composition of the last caloric meal consumed before the treatment period begins
- 2 questions to assess bedtime the previous evening and wake-up time the morning of the treatment period (used to evaluate sleep duration prior to the treatment period)
- 2 questions to assess hours of moderate/vigorous exercise the day before the treatment period, and representativeness of this physical activity level (used to evaluate representativeness of physical activity level preceding each treatment period)
- 2 questions to assess representativeness of dietary composition and consumption the day before the treatment period (used to evaluate representativeness of total daily food intake preceding each treatment period)
- Completed once per treatment period, before the treatment period begins

6. *Shake Surveys*

- 2 sets of 4 VAS questions to gauge pre- and postprandial hunger and satiety
- Completed at every snack or meal during the 48-hr *ad libitum* period

7. *Final Survey*

- 2 prompts to report any additional calorie consumption during the 48-hr *ad libitum* period (used to assess participant compliance with the study protocol)
- 5 VAS questions to gauge sensory qualities of dietary treatments
- 7 VAS questions to gauge overall feelings of hunger, satiety, and comfort throughout the 48-hr *ad libitum* period (used to assess participant tolerance of dietary treatments)
- 2 open-ended questions to report any negative physical, mental, or emotional symptoms of the dietary treatments and any other participant concerns (used to assess participant tolerance of dietary treatments)
- Completed once per treatment period, at the end of the treatment period

8. *Clinic Survey*

- 5 sets of 4 VAS questions to gauge pre- and postprandial hunger and satiety
- Completed once per treatment period, during the acute feeding study following the 48-hr *ad libitum* period

B) Experimental protocols & timeline

Pre-screening

Before visiting the Boulder CTRC, all potential participants will be pre-screened by the PI via telephone, using the *Pre-Screening Script*. The pre-screening is intended to ensure that the inclusion and exclusion criteria are met before any potential participant takes the time to visit the Boulder CTRC.

Enrollment & 1st treatment phase

All participant recruitment, enrollment, and data collection will take place at or through the Boulder CTRC. During enrollment, potential participants will first be familiarized with all procedures, risks, time commitments, and monetary compensation associated with the study. Second, informed consent will be obtained from those potential participants who choose to join the study. Third, the take-home *Guidelines for Participants* document will be distributed and reviewed. This document describes 1) the dietary protocols that participants are to follow during the free-living portion of the treatment period, 2) additional beverages that are allowed or disallowed during the treatment period, 3) potential discomforts and risks associated with the liquid diets, and 4) contact information for the research team, emergency medical personnel, and the CU Boulder IRB. Fourth, all participants will undergo a medical history intake and physical examination to ensure that exclusion criteria are met and that they can safely participate. Fifth, participant data will be recorded on the *Participant Intake Form.* Finally, enrolled participants will immediately begin the 1st of four dietary treatment phases.

Enrollment

6. Potential participants arrive at the Boulder CTRC in the morning (according to the availability of a Boulder CTRC physician; see below) following a 12-hour overnight fast and are familiarized with the procedures, dietary restrictions, potential risks, time commitments, and monetary compensation associated with the study.
7. Informed consent is obtained from those participants wishing to join the study. Participants are assigned a confidential ID number for data identification.
8. The *Guidelines for Participants* are reviewed and each participant receives a paper copy. This document is also made available to each participant in three ways as a PDF file: 1) as an email attachment, 2) uploaded to a private Facebook page accessible only by participants, 3) uploaded as a Google Doc to a folder accessible only by participants. This will allow participants to have ready access to the study guidelines at all times, and removes the need for participants to carry a paper copy of the document if they do not wish to.
9. In accordance with Boulder CTRC regulations, participants undergo a medical history intake and physical examination by a Boulder CTRC physician. This should take approximately 15 minutes.
10. Participant data (age, sex, weight, height) are recorded on the *Participant Intake Form.*

1st dietary treatment phase

6. Data on representativeness of previous day's food intake, physical activity, and sleep duration are collected from each participant via the *Initial Survey.*
7. Participants are issued a 48-hr supply (9,000 kcal total) of the liquid diet they have been assigned for that phase; participants may request more of the diet at any time by contacting the PI.
8. Participants receive the *Shake Surveys* (15 copies) and *Final Survey* and depart the Boulder CTRC with their 48-hr liquid diet supply.
9. *Ad libitum* treatment period: for the next 48 hrs, participants subsist exclusively on the liquid diet they have been assigned for that phase. Participants consume as much or as little of the liquid diet as they wish, at any time.
    a. Whenever participants wish to consume a meal or snack, the following steps are followed:
        i. Complete page 1 of a *Shake Survey*.
        ii. Consume an *ad libitum* quantity of the liquid diet.
        iii. Complete page 2 of the *Shake Survey*.
    b. As detailed in the *Guidelines for Participants*, participants may not consume any other food items during the treatment period, including liquid foods such as soups or broths.

They also may not consume any caloric beverages. Participants may, however, consume unlimited quantities of non-caloric beverages.

10. At the end of the 48-hr period:
    a. Participants complete the *Final Survey* 48 hours after the treatment period began, i.e., at the same time of the morning that they received their liquid diet supply, and return to the Boulder CTRC with all unconsumed portions of the liquid diet (along with all original containers) for weigh-back.
    b. Participants complete the acute feeding component of the dietary treatment phase; procedures begin at the same time of day as the *ad libitum* period 48 hours prior.
        i. Participants complete prompt 1 of a *Clinic Survey* (4 VAS survey questions repeated in 5 prompts)
        ii. An IV is inserted.
        iii. Participants consume a "breakfast" meal of their liquid diet for that phase (i.e., the same diet they have been consuming for the previous 48 hours), equal to 20% of their daily energy requirement, as estimated according to the FAO/WHO/UNU (2001) recommendations.
        iv. Immediately upon completion of the "breakfast" meal (0 min), a blood sample is drawn into a 4.0ml EDTA-treated tube and the participant completes prompt 2 of the *Clinic Survey*.
        v. At 30 min, 60 min, and 90 min after the meal, blood samples are again drawn and participants complete prompts 3, 4, and 5 of the *Clinic Survey*, for a total of 4 blood samples and 5 sets of VAS data.
        vi. The IV is removed, and the 1st dietary treatment phase is complete.

2nd, 3rd, & 4th treatment phases

There are a total of 4 treatment phases to the study, enabling each participant to undergo each of the 4 dietary treatments in random order. For example, during treatment phase 1, one participant may be on the HpHq diet while another participant is on the LpHq diet. As detailed above, the 1st treatment phase will begin immediately following the enrollment process at the Boulder CTRC. The subsequent treatment phases (2nd, 3rd, and 4th) will follow exactly the same procedures as the 1st treatment phase, except that participants will begin the treatment phase immediately upon arrival at the Boulder CTRC (i.e., they do not repeat the enrollment procedures or the medical history intake and physical examination). For each treatment phase, participants will be instructed to fast for 12 hours (overnight) before beginning each new treatment. Also, the 2nd, 3rd, and 4th treatment phases will begin at the same time of day as the 1st treatment phase for each participant; e.g., if a participant began the 1st treatment phase at 9:30am following enrollment, medical history intake, etc., then that participant will also begin each subsequent treatment phase at 9:30am. Likewise, the acute feeding component at the end of each treatment phase also begins at the same time of day that the treatment phase itself begin (9:30am, in this example).

Upon completion of each phase, participants will have a 4-week washout period before beginning the next phase with a different dietary treatment. The washout period will allow participants to return to a physiological baseline between treatment phases. It will also ensure that female participants can begin each treatment phase at the same point of their menstrual cycles, specifically the follicular phase (as determined by participant self-report of menses), since ad libitum food intake is known to vary over the menstrual cycle in adult females (Lissner et al., 1988; Buffenstein et al., 1995; Dye & Blundell, 1997). A female undergraduate research assistant will be employed to assist in scheduling the female participants' treatment periods, such that the treatment periods all begin at the (self-reported) follicular phase of the menstrual cycle for each individual. Males will also be held to the same washout schedule, to eliminate any confounding effect of different washout periods. Data from the *Final Survey* will be examined to ensure that each participant will be able to comfortably complete the next phase without undue burden. This process will continue until all participants have completed all 4 phases.

Overall timeline

The total time commitment for participants is 8 weeks, or approximately 112 days. Of these 112 days of enrollment in the study, there are 8 days of active participation, i.e., 8 days on the dietary treatments with 8 visits to the Boulder CTRC. The remaining days represent the washout periods (inactive participation) between the 4 dietary treatments. The specific breakdown of the total time commitment is as follows: 1) Enrollment at the Boulder CTRC and beginning of 1st dietary treatment phase (1.5 hrs), followed by a 48-hr *ad libitum* dietary treatment period, an acute feeding component at the Boulder CTRC (2.0 hrs), and a subsequent 4-week washout period; 2) 3 additional dietary treatment periods, each involving an initial visit to the Boulder CTRC (0.5 hrs), followed by a 48-hr *ad libitum* dietary treatment period, an acute feeding component at the Boulder CTRC (2.0 hrs), and a subsequent 4-week washout period. The individual visits to the Boulder CTRC are described in detail in Table 10.

Table 10: Summary of participant visits to the Boulder CTRC

Visit #	***Procedures/Tools***	***Location***	***How much time the visit will take***
Visit 1 (Enrollment & Phase 1)	• Project overview and consent process • Medical history and physical examination • Completion of *Initial Survey* Distribution of Phase 1 diet, *Shake Surveys*, & *Final Survey*	Boulder CTRC	1.5 hrs
Visit 2 (Phase 1)	• Return of any unconsumed portions of Phase 1 diet, *Shake Surveys*, & *Final Survey* • Acute feeding component: 1) consumption of 20% of daily energy requirement of Phase 1 diet, 2) 4 blood draws and completion of *Clinic Survey* over 90 minutes	Boulder CTRC	2.0 hrs
Visit 3 (Phase 2)	• Completion of *Initial Survey* • Distribution of Phase 2 diet	Boulder CTRC	0.5 hr
Visit 4 (Phase 2)	• Return of any unconsumed portions of Phase 2 diet, *Shake Surveys*, & *Final Survey* • Acute feeding component: 1) consumption of 20% of daily energy requirement of Phase 2 diet, 2) 4 blood draws and completion of *Clinic Survey* over 90 minutes	Boulder CTRC	2.0 hrs
Visit 5 (Phase 3)	• Completion of *Initial Survey* • Distribution of Phase 3 diet	Boulder CTRC	0.5 hr

Visit 6 (Phase 3)	• Return of any unconsumed portions of Phase 3 diet, *Shake Surveys*, & *Final Survey* • Acute feeding component: 1) consumption of 20% of daily energy requirement of Phase 3 diet, 2) 4 blood draws and completion of *Clinic Survey* over 90 minutes	Boulder CTRC	2.0 hrs
Visit 7 (Phase 4)	• Completion of *Initial Survey* • Distribution of Phase 4 diet	Boulder CTRC	0.5 hr
Visit 8 (Phase 4)	• Return of any unconsumed portions of Phase 4 diet, *Shake Surveys*, & *Final Survey* • Acute feeding component: 1) consumption of 20% of daily energy requirement of Phase 4 diet, 2) 4 blood draws and completion of *Clinic Survey* over 90 minutes	Boulder CTRC	2.0 hrs

XII. SPECIMEN MANAGEMENT

The only specimens to be analyzed for this study are blood samples collected at the Boulder CTRC during each dietary treatment phase (section XI: PROCEDURES): 4 samples per participant in each of the 4 dietary treatment phases, for a total of 16 samples per participant overall. Analysis of blood samples for plasma ghrelin levels will take place at the Core Laboratory of the University of Colorado Hospital CTRC in Aurora, CO. Frozen blood samples are sent from the Boulder CTRC to the University of Colorado Hospital CTRC once per week via prearranged courier. Blood tubes will be labeled with confidential participant ID numbers, not participant names or other identifying information (section XIII: DATA MANAGEMENT).

XIII. DATA MANAGEMENT

There are 5 categories of data to be collected in this study: 1) enrollment data and personal characteristics, 2) self-reported measures of representativeness of previous day's food intake, physical activity, and sleep duration, 3) plasma ghrelin data from blood draws, 4) total quantity of food consumed (i.e., total energy intake) during each dietary treatment phase, 5) self-reported hunger, satiety, and experiential data collected with the Initial Survey, Shake Survey, Clinic Survey, and Final Survey instruments. Management of each of these 5 categories of data is described in detail below.

1. Enrollment data and personal characteristics

Before the dietary treatment phases of the study begin, all participants will undergo the consent process as well as a medical history intake and physical examination (see section XI: PROCEDURES) at the Boulder CTRC. The medical history intake and physical examination will be conducted by a Boulder CTRC physician. The goal of these procedures is not to collect data for direct analysis in this study, but rather to ensure that exclusion criteria are met and that the participant would be able to safely participate

in the study. Therefore, the medical findings will not be shared with the PI or other members of the research team.

Once participants have consented to participate in the study, they will be assigned a confidential participant ID number known only to the PI. The hardcopy key of participant names and confidential participant ID numbers will be stored in a locked file cabinet within a locked office in the Hale Sciences building (room Hale 126). Only the PI and Faculty Advisor/CI will have access to this file cabinet. This key is the only document, either hardcopy or electronic, in which participant names and confidential participant ID numbers will appear together. Following the conclusion of the study and coding of all collected data (described below), the key will be shredded.

The only other data recorded during the enrollment process (after consent has been obtained) are participant age, sex, weight, and height. These data will be recorded by the PI on the hardcopy Participant Intake Form. The PI will identify each of these data sheets using only the confidential participant ID numbers; participants' names or other personal identifying information will not appear on any Participant Intake Form. These anonymous data sheets will be stored in a locked file cabinet within a locked office in the Hale Sciences building (room Hale 126). At the conclusion of the study, the participant data will be coded and transferred electronically to the PI's password-protected Redcap account. At this point, the original anonymous hardcopy participant data sheets will be shredded. Only the PI will have access to the electronic files, which he will access through his office computer (password-protected with 15-min automatic logoff).

3. Plasma active ghrelin data from blood draws

During each of the 4 dietary treatment phases, each participant will undergo a venipuncture and 4 blood draws at the Boulder CTRC (see section XI: PROCEDURES). Since these blood draws will occur in the presence of the PI, he will inform the phlebotomist of the relevant confidential participant ID with which to label each blood tube; the blood tubes will not be labeled with participant' names or other personal identifiers. For analysis of plasma ghrelin levels, blood tubes will be sent to the Core Laboratory of the University of Colorado Hospital CTRC in Aurora, CO via prearranged courier (see section XII: SPECIMEN MANAGEMENT).

The results of the ghrelin analyses will be sent in hardcopy from the University of Colorado Hospital CTRC to the Boulder CTRC, where they will subsequently be obtained by the PI. Note that the ghrelin datasheets will include confidential participant ID numbers, but not participants' names. The PI will store the anonymous data sheets in a locked file cabinet within a locked office in the Hale Sciences building (room Hale 126). Only the PI and Faculty Advisor/CI will have access to this file cabinet. At the conclusion of the study, the ghrelin data will be coded and transferred to an electronic spreadsheet saved to a private folder in the PI's password-protected Redcap account (cloud server). At this point, the original anonymous hardcopy ghrelin data sheets will be shredded. Only the PI will have access to the electronic files, which he will access through his office computer (password-protected with 15-min automatic logoff).

4. Food consumed in each treatment phase

At the end of each of the 4 dietary treatment phases, the PI will collect any unused portions of the liquid diet and all original food containers from each participant at the Boulder CTRC (see section XI: PROCEDURES). The PI will then weigh back the unconsumed food and calculate total food consumed (and hence total energy intake) during each dietary treatment phase. These data will be recorded electronically in the PI's password-protected Redcap account and will be identified only by the confidential participant ID numbers, not the participants' names. Only the PI will have access to these

electronic files, which he will access through his office computer (password-protected with 15-min automatic logoff).

5. Self-reported hunger, satiety, and experiential data

Each participant will self-report hunger, satiety, and experiential data on 3 hardcopy instruments (Shake Surveys, Clinic Survey, and Final Survey) during each of the 4 dietary treatment phases. The data sheets will list only the participants' confidential ID numbers, not their names or other identifying information. Upon receipt by the PI, the anonymous data sheets will be stored in a locked file cabinet within a locked office in the Hale Sciences building (room Hale 126). Only the PI and Faculty Advisor/CI will have access to this file cabinet. At the conclusion of the study, the survey data will be coded and transferred electronically to the PI's password-protected Redcap account. At this point, the original de-identified hardcopy survey data sheets will be shredded. Only the PI will have access to the electronic files, which he will access through his office computer (password-protected with 15-min automatic logoff).

XIV. WITHDRAWAL OF PARTICIPANTS

Participants will have the option to withdraw at any time during the study. Additionally, participants will be withdrawn from the study if they do not comply with study procedures, specifically: consuming other foods or disallowed beverages during a dietary treatment phase; discarding or misplacing unconsumed portions of the dietary treatment. Participants will also be withdrawn if they report intolerable psychological or physical side effects of the blood draw procedures or of a dietary treatment (e.g., emotional or gastrointestinal distress). These factors will be assessed via the *Final Survey* instrument (as described in section XI: PROCEDURES). Withdrawal will not be held against participants, and withdrawn participants will still receive monetary compensation commensurate with their degree of completion of the study (as detailed in section VIII: COMPENSATION).

Withdrawn participants will be replaced as needed to meet the proposed sample size of 18. Due to the repeated-measures design of this study, any data gathered from withdrawn participants will not be usable in testing the food intake and satiety hypotheses.

XV. RISKS TO PARTICIPANTS

Risks of dietary treatment

During each of the four dietary treatments, participants will subsist exclusively on a single liquid diet (plus approved beverages) for a 48-hr period. Participants may find this diet to be monotonous, displeasing, and/or unsatisfying. They may experience hunger, irritation, food cravings, and/or gastrointestinal discomfort while on the liquid diet. Also, participants will need continual access to the liquid diet throughout each 48-hr period, which may require that participants carry containers of the diet with them to work, school, etc. This, along with the prohibition against consuming other foods or beverages during each 48-hr period, may be disruptive to the participants' daily activities and social interactions.

Risks of blood collection

The collection of blood samples will require venipuncture, which participants may find uncomfortable or painful. Additional risks of venipuncture include: excessive bleeding, fainting or feeling light-headed, hematoma (blood accumulating under the skin), infection (a slight risk any time the skin is broken), and multiple punctures to locate veins. Although the blood samples collected for this study will only be analyzed for total ghrelin levels, blood contains other information that participants may wish to keep private (e.g., cholesterol levels).

Risks of data storage

Some data will initially be collected on hardcopy data sheets. It is possible that these sheets could be misplaced or stolen. Some data will be originally stored in the form of electronic spreadsheets; all hardcopy data will eventually be converted to electronic format. It is possible that electronic data files could be accessed by unauthorized personnel either from the PI's office computer or from the cloud server.

XVI. MANAGEMENT OF RISKS

Management of dietary treatment risks

Self-reported survey data will be frequently monitored throughout the study. Specifically, each participant's data from both the VAS and open-ended sections of the *Final Survey* will be analyzed immediately upon completion of each dietary treatment phase to ensure that participants have not experienced undue physical, mental, or emotional distress during the treatment phase. Also, the *Guidelines for Participants* document is made available to all participants in four ways: 1) as a paper copy, 2) as an email attachment, 3) uploaded to a private Facebook page accessible only by participants, 4) uploaded as a Google Doc to a folder accessible only by participants. This will allow participants to have ready access to the study guidelines at all times, and removes the need for participants to carry a paper copy of the document if they do not wish to. This should help to reduce the disruptiveness of the dietary treatment protocol to the participants' daily activities and social interactions.

Management of blood collection risks

All blood collections will be performed by a phlebotomist at the Boulder CTRC, in the presence of trained and experienced personnel who can respond to any emergencies. To prevent unauthorized access or analysis, blood samples will be frozen and stored at the Boulder CTRC. The samples will only leave this location when they are sent to the University of Colorado Hospital CTRC for analysis, via the weekly courier system already established by the Boulder CTRC. Additionally, blood sample tubes will only be labeled with confidential participant ID numbers, not participants' names or other personal identifiers.

Management of data storage risks:

As soon as any anonymous hardcopy data sheets are collected by the PI, they will be stored in a locked file cabinet within a locked office in the Hale Sciences building (room Hale 126). Only the PI and Faculty Advisor/CI will have access to this file cabinet. At the conclusion of the study all data will be transferred electronically to the PI's password-protected Redcap account, and all original hardcopy data sheets (including the participant ID key) will be shredded. Only the PI will have access to the electronic files, which he will access through his office computer (password-protected with 15-min automatic logoff to prevent unauthorized access).

XVII. POTENTIAL BENEFITS

At the conclusion of the study, participants will learn how much of each dietary treatment they consumed. This may be of personal interest to some participants, but otherwise there are no direct benefits to participating in this study. The results obtained from this study may help us to better understand the physiological drivers of food consumption. This, in turn, may help us to explain how shifts in the dietary protein supply may influence the total caloric intake of a population.

XVIII. PROVISIONS TO MONITOR THE DATA FOR THE SAFETY OF PARTICIPANTS

Self-reported data for each participant (i.e., the data collected with the *Initial Survey*, *Shake Survey*, *Final Survey*, and *Clinic Survey* instruments) will be analyzed by the PI immediately upon completion of each of the four dietary treatment phases of the study. The main purpose of these interim data analyses is to ensure that participants have not experienced undue physical, mental, or emotional distress during the treatment phase. Page 2 of the *Final Survey* is specifically intended to collect data for this purpose: seven

VAS prompts to assess participants' overall feelings of hunger, satiety, and comfort during the dietary treatment phase, and two open-ended questions allowing participants to describe any other negative physical, mental, or emotional experiences during the dietary treatment phase. Any participants who self-report such negative experiences will be contacted privately by the PI and asked if they wish to continue into the next dietary treatment phase of the study. Participants will be reminded that their safety is the top priority and that voluntary withdrawal from the study will not be held against them.

XIX. PROVISIONS TO PROTECT THE PRIVACY INTERESTS OF PARTICIPANTS

Consenting participants will be assigned a confidential study identification number (unrelated to any personal identifying information), which will be used on all study documentation (e.g., *Shake Surveys*) in lieu of the participants' names or other direct identifiers. Also, participants may not want to advertise the fact that they are participating in a dietary study. Since participants may need to carry portions of the liquid diets with them throughout their daily activities, the diets will be provided in generic bottles and containers free of any markings from the Boulder CTRC, Denver CCTSI, or CU Department of Anthropology.

XX. MEDICAL CARE AND COMPENSATION FOR INJURY

N/A (This research involves only minimal risk.)

XXI. COST TO PARTICIPANTS

The only anticipated cost to participants is transportation to and from the Boulder CTRC on 8 occasions (see section XI: PROCEDURES). This may incur the costs of parking, fuel, or public transportation.

XXII. DRUG ADMINISTRATION

N/A (No drugs will be administered.)

XXIII. INVESTIGATIONAL DEVICES

N/A (No Investigational Devices will be used.)

XXIV. MULTI-SITE STUDIES

N/A (This study will be conducted at the Boulder CTRC only.)

XXV. SHARING OF RESULTS WITH PARTICIPANTS

At the conclusion of the study, each participant will be provided with the following individualized results: order of dietary treatments received; total consumption (in both grams and kilocalories) of each dietary treatment.

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APPENDIX C. UNIVERSITY OF COLORADO BOULDER, INSTITUTIONAL REVIEW BOARD (IRB) CONSENT FORM

Title of research study: *Do protein content and protein quality influence human food intake? Testing the Protein Leverage Hypothesis*

IRB Protocol Number**:** 17-0543

Investigator**:** Richard Bender

Purpose of the Study

The purpose of the study is to find out how different types and quantities of protein in a diet influence how much people eat throughout the day. There is some previous research showing that people find high-protein foods to be more satisfying, so they eat less food overall on a high-protein diet than on a low-protein diet.

Some of these previous studies assigned people to two groups, one with a "menu" of high-protein foods and one with a "menu" of low-protein foods, then measured which group consumed more food overall. One potential problem with this approach is that high- and low-protein foods tend to have different tastes and textures, and this could skew the results of the study. For example, a high-protein diet containing lots of meats and cheeses might be more (or less) appealing to someone than a low-protein diet containing lots of grains and vegetables, just because of difference in taste or texture.

The purpose of our research study is to test this idea in a more precise way. In our study, people will not be assigned to different groups with high- or low-protein "menus." Instead, our research team has designed 4 different kinds of specially-designed protein shakes with different types and quantities of protein, but identical taste and texture. Participants in our study will consume the 1st type of protein shake exclusively for a 48-hour period, then the 2nd type of protein shake for another 48-hour period, and so on until each participant has tried each of the 4 different protein shakes. By measuring how much of each different protein shake people consume, our research team will be able to see if protein content really influences how much people eat overall. We hope that the results of this research will help us to better understand how dietary shifts around the world are influencing how much people eat. This, in turn, could help us to better understand the links between diet and health.

We invite you to take part in this research study because you have expressed interest in participating, and because the research team has determined that you would be an appropriate participant in this study.

Over the course of this research study, you will undergo 4 different dietary treatments, each of which will last 48 hours. There will be a 4-week recovery period between each of the dietary treatments. So, we expect that you will be in this research study for a total of about 16 weeks.

We expect about 21 people will be in this research study.

Explanation of Procedures
If you agree to participate in this research study, you will be asked to do the following:

1) Travel to the Clinical and Translational Research Center located on the west side of the third floor of the Wardenburg Health Center on the main campus of the University of Colorado Boulder (Boulder CTRC) on 8 separate occasions – once at the beginning and once at the end of each of the 4 different 48-hour treatment periods.

2) Subsist on a liquid protein-shake diet during each of the 4 different 48-hour treatment periods. During these periods, you will be asked to only consume the specially-designed protein shakes provided to you by the research team, but no other foods. You may find this special protein shake diet to be monotonous, unpleasant, or unsatisfying. You may have feelings of hunger, irritation, or food cravings. Also, since this will be a change from the usual foods that you eat, you could experience bloating, cramps, or other physical discomforts. Since you will need continual access to your protein shakes throughout each 48-hour period, you may need to carry containers with you to school, work, etc. This could be disruptive to your normal daily activities.

3) During visits 2, 4, 6, and 8 – that is, at the end of each of the 4 different 48-hour treatment periods – you will have an IV needle inserted into your vein. When the needle goes into a vein, it hurts for a short time and there may be swelling around where the needle goes into the skin. There is a small chance you may feel lightheaded or faint. A risk of blood clot forming in the vein is about 1 in 100. The risk of infection or significant blood loss is less than 1 in 1,000. Once the IV needle is inserted, 4 small blood samples will be taken. For each blood sample, approximately 1 teaspoon (4 ml) of blood will be drawn into a tube every 30 minutes over the course of one and a half hours at the Boulder CTRC. This will happen during each 48-hour treatment period, for a total of 4 IV needle insertions and 16 blood samples taken. The blood samples will be drawn after a standardized protein-shake “breakfast” that you consume at the Boulder CTRC. They will be analyzed for ghrelin, a hormone that is related to hunger. This will help us to understand how your appetite responds to the different protein shakes.

4) Answer a set of survey questions before, during, and after each of the 4 different 48-hour treatment periods. The surveys are meant to gauge how hungry you feel throughout the treatment period, how satisfying you find the protein shakes, and whether you experience any discomfort or side effects.

A more detailed timeline for this research study is on the following page:

- Visit 1: Enrollment at the Boulder CTRC & beginning of 1st treatment period (about 1.5 hours)

  Enrollment
  - Arrive at the Boulder CTRC in the morning, following a 12-hour overnight fast. In other words, you will be asked to refrain from consuming any food or calorie-containing beverages for 12 hours before you are scheduled to arrive at the Boulder CTRC.
  - Meet with the research team to learn more about the study and to be familiarized with all procedures, commitments, and potential risks. Receive a Guidelines for Participants document. Sign the consent form (this document) if you do agree to participate in the study.
  - You will also have medical screening tests to help us decide if you meet the requirements to continue further in the study. These tests will be performed at the Boulder CTRC. The medical screening tests include:
    - A physician and other medical professionals will conduct a full physical examination to confirm that you are in a good state of health. You will be asked about your health history.
    - Medical care staff will measure/collect all of the following information: your vital signs (blood pressure, heart rate, body temperature), your height, and your weight.
  - Provide a few more pieces of preliminary information (age, sex, height, weight).

  1st treatment period
  - Complete the Initial Survey. This is a short interview in which the Principal Investigator (Richard Bender) will ask you about your diet, physical activity, and sleep patterns on the previous day.
  - Receive a 48-hour supply of a specially-designed protein shake (1st of 4 different kinds) and take-home Shake Survey and Final Survey documents.
  - Depart the Boulder CTRC with your protein shake supply. For next 48 hours, you are instructed to consume only the protein shakes and approved beverages (listed in the Guidelines for Participants), but no other foods. Whenever you have a meal or snack, you are asked to complete a Shake Survey (1 page).

- Visit 2: end of 1st treatment period (about 2 hours)
  - At the end of the 48-hour treatment period, you are asked to complete the Final Survey (2 pages) and to return to the Boulder CTRC in the morning with any leftover protein shakes and all original containers.
  - Have an IV needle inserted in your vein by a qualified Boulder CTRC staff member.
  - Consume a pre-measured protein-shake “breakfast.”
  - Over the next hour and half, you will have 1 teaspoon (4 ml) of your blood drawn through the IV needle into a tube every 30 minutes by a qualified

Boulder CTRC staff member (4 blood draws total). During the same time period, you are asked to complete the Clinic Survey (2 pages).
    - Have the IV needle removed and depart the Boulder CTRC.

- [4-week break]

- Visit 3: Beginning of 2nd treatment period (about half an hour)
    - Arrive at the Boulder CTRC in the morning. Procedures are identical to 1st visit, except that you won't need to go through the enrollment procedures or medical screening tests again. You will again complete an Initial Survey and receive the Shake Surveys, Final Survey, and receive a 48-hour supply of protein shakes. This time, you'll be consuming a different kind of protein shake (2nd of 4 different kinds) for the 48-hour treatment period.

- Visit 4: end of 2nd treatment period (about 2 hours)
    - Arrive at the Boulder CTRC in the morning. Procedures are identical to the 2nd visit. You will again complete a Final Survey and return any leftover protein shakes and all original containers. Then, you will again have an IV needle inserted, have your blood drawn 4 times over an hour-and-a-half period, and complete a Clinic Survey during the same time period.

- [4-week break]

- Visit 5: beginning of 3rd treatment period (about half an hour)
    - Arrive at the Boulder CTRC in the morning. Procedures are identical to 1st visit, except that you won't need to go through the enrollment procedures or medical screening tests again. You will again complete an Initial Survey and receive the Shake Surveys, Final Survey, and receive a 48-hour supply of protein shakes. This time, you'll be consuming a different kind of protein shake (3rd of 4 different kinds) for the 48-hour treatment period.

- Visit 6: end of 3rd treatment period (about 2 hours)
    - Arrive at the Boulder CTRC in the morning. Procedures are identical to the 2nd visit. You will again complete a Final Survey and return any leftover protein shakes and all original containers. Then, you will again have an IV needle inserted, have your blood drawn 4 times over an hour-and-a-half period, and complete a Clinic Survey during the same time period.

- [4-week break]

- Visit 7: beginning of 4th treatment period (about half an hour)
    - Arrive at the Boulder CTRC in the morning. Procedures are identical to 1st visit, except that you won't need to go through the enrollment procedures or medical screening tests again. You will again complete an Initial Survey and receive the Shake Surveys, Final Survey, and receive a 48-hour

supply of protein shakes. This time, you'll be consuming a different kind of protein shake (4th of 4 different kinds) for the 48-hour treatment period.

- Visit 8: end of 4th treatment period (about 2 hours)
  - Arrive at the Boulder CTRC in the morning. Procedures are identical to the 2nd visit. You will again complete a Final Survey and return any leftover protein shakes and all original containers. Then, you will again have an IV needle inserted, have your blood drawn 4 times over an hour-and-a-half period, and complete a Clinic Survey during the same time period.

You will receive all 4 of the different protein shakes over the course of the study, but the order of protein shakes you receive will be chose by chance, like flipping a coin. You will not be told the order of protein shakes you are getting; however, the Principal Investigator will know.

Voluntary Participation and Withdrawal
Whether or not you take part in this research is your choice. You can leave the research at any time and it will not be held against you.

The person in charge of the research study or the sponsor can remove you from the research study without your approval. Possible reasons for removal include non-compliance with study procedures, or the research team believing that any negative side effects from the study are too great.

If you decide to leave the research, contact the investigator so that the investigator can remove you from the pool of participants. Any data collected from you will not be used in the research.

If you are a CU Boulder student or employee, taking part in this research is not part of your class work or duties. You can refuse to enroll, or withdraw after enrolling at any time, with no effect on your class standing, grades, or job at CU Boulder. You will not be offered or receive any special consideration if you take part in this research.

Potential Benefits
We cannot promise any benefits to you or others from your taking part in this research. However, possible benefits include knowing how much food you consumed on different kinds of high- and low-protein diets.

Confidentiality
Information obtained about you for this study will be kept confidential to the extent allowed by law. Research information that identifies you may be shared with the University of Colorado Boulder Institutional Review Board (IRB) and others who are responsible for ensuring compliance with laws and regulations related to research, including people on behalf of the Office for Human Research Protections. The information from this research may be published for scientific purposes; however, your identity will not be given out.

Cost of Participation
Taking part in this research study may lead to added costs to you. Over the course of the study, you will need to travel to and from the Boulder CTRC (on the University of Colorado Boulder campus) a total of 8 times. This may incur the costs of parking, fuel, or public transportation.

Payment for Participation
If you agree to take part in this research study, we will pay you a total of $300 for your time and effort. Payments will be made in cash with a receipt at the end of each 48-hour treatment period: $60 at the end of the 1st treatment period, $70 at the end of the 2nd treatment period, $80 at the end of the 3rd treatment period, and $90 at the end of the 4th treatment period ($300 total). If you decide to withdraw from the study early, we will pay you for the proportion of the study that you did complete, as follows:

Treatment period	Payment
1st treatment period	$60
2nd treatment period	$70
3rd treatment period	$80
4th treatment period	$90
Total	$300

It is important to know that payment for participation is taxable income.

Questions
If you have questions, concerns, or complaints, or think the research has hurt you, talk to the research team:

Principal Investigator: Richard Bender (719-332-7825; richard.bender@colorado.edu)

Co-Investigator/Faculty Advisor: Darna Dufour (303-492-6061; darna.dufour@colorado.edu)

This research has been reviewed and approved by an Institutional Review Board (IRB). You may talk to them at (303) 735-3702 or irbadmin@colorado.edu if:

- Your questions, concerns, or complaints are not being answered by the research team.
- You cannot reach the research team.
- You want to talk to someone besides the research team.
- You have questions about your rights as a research subject.
- You want to get information or provide input about this research

Signatures

Your signature documents your permission to take part in this research.

Signature of subject	Date
Printed name of subject	
Signature of person obtaining consent	Date
Printed name of person obtaining consent	

APPENDIX D. EXPERIMENTAL INSTRUMENTS

University of Colorado **Boulder**

DIETARY PROTEIN STUDY

Are you an adult from the Boulder area who is…

…between 20**-45 years old?**

…sedentary or moderately active?

…not diabetic?

…not currently on a high**-protein diet or weight-loss diet?**

…not a vegan or allergic to n**uts, whey, or peas?**

If so, you may be a candidate for our dietary protein study!

The study will involve:

- **8 visits to the CU Boulder CTRC (located at Wardenburg Health Center)**
- **4 separate 48-hour protein shake diets**
- **4 in-house blood draw procedures**
- **A 4-week break between each diet**
- **Payment of up to $300**

For more information, please contact:

Richard Bender (CUproteinstudy@gmail.com)
Department of Anthropology
University of Colorado Boulder

PRE-SCREENING SCRIPT

Potential participant _____ Date _____
*(record **first name only**)*

Read to potential participant:

Hi, thanks so much for your interest in our study, and for taking the time to talk with me. My name's Richard Bender. I'm a PhD candidate in the Anthropology department, and I'm the Principal Investigator of this study. I'd like to talk with you for a few minutes and ask you some questions about yourself, just to see if you would be eligible for the study. It should only take about 10 minutes and I won't be recording any of your personal information. Is this okay with you?

[ ] NO → That's okay. Thanks again for your time, and have a good day.
[ ] YES
↓
Great. Is this a good time for you to talk? I can call back at another time if you'd prefer. We can also meet in person if you'd prefer that to talking over the phone.

[ ] NO *(not a good time/prefer to meet in person)* → [reschedule]
[ ] YES *(okay to talk now)*
↓
Great. Now I'd like to ask you a few questions about yourself. Again, I'm not going to keep this information – this is just to see if you'd be a good fit for our study. First, how old are you?

[ ] *under 20 or over 45* → Okay. Unfortunately, that puts you outside of our preferred age range. Thank you for taking the
time to talk with me. Have a good day.
[ ] *between 20 and 45*
↓
What is your weight and your height?

_____ kg = _____ OR _____ lbs = _____ x 703 = _____
_____ m ⇒ _____ m^2 ____ ft ____ in ⇒ _____ in ⇒ _____ in^2

[ ] *BMI under 20.0 or over 30.0* → Okay. Unfortunately, that puts you outside of our preferred range for this study. Thank
you for taking the time to talk with me. Have a good day.
[ ] *BMI between 20.0 and 30.0*
↓
FEMALES: Are you currently pregnant or lactating, or do you plan to become pregnant during the next 4 months?

[ ] YES → Okay. Unfortunately, you might not be the best fit for this particular study. Thank you for taking the time to talk
with me. Have a good day.
[ ] NO
↓
FEMALES: Would you describe yourself as having an irregular menstrual cycle?

[ ] YES → Okay. Unfortunately, you might not be the best fit for this particular study. Thank you for taking the time to talk
with me. Have a good day.
[ ] NO
↓
Okay. Let's talk briefly about your physical activity level. First, about how many hours of moderate or vigorous exercise do you usually do each week? This includes things like running, biking, or rock climbing.

[ ] > *2.5 hrs* → Okay. Unfortunately, that puts you above our preferred activity level. Thank you for taking the time to talk with me. Have a good day.
[ ] ≤ *2.5 hrs*
↓
Would you consider yourself to be a competitive athlete?

[ ] YES → Okay. Unfortunately, that puts you above our preferred activity level. Thank you for taking the time to talk with
me. Have a good day.
[ ] NO
↓
Are you currently on a high-protein diet or on a weight-loss diet?

[ ] YES → Okay. Unfortunately, you might not be the best fit for this particular study. Thank you for taking the time to talk
with me. Have a good day.
[ ] NO
↓
We're almost finished. I just have a few more questions about your health and diet, to make sure that it would be safe for you to be a part of this study. Do you currently have an eating disorder, diabetes, or metabolic disorder?

[ ] YES → Okay. Unfortunately, it might not be safe for you to participate in this study. Thank you for taking the time to talk
with me. Have a good day.
[ ] NO
↓
Great. During this study, you'll be asked to consume four different protein shakes that were specially designed by the research team. Some of these protein shakes might contain animal products. Would you be comfortable consuming something that contains animal products?

[ ] NO → Okay. Unfortunately, it might be uncomfortable for you to participate in this study. Thank you for taking the
time to talk with me. Have a good day.
[ ] YES
↓
The protein shakes will contain either whey (from milk) or peas. Are you allergic to either of these things?

[ ] YES → Okay. Unfortunately, it might not be safe for you to participate in this study. Thank you for taking the time to talk
with me. Have a good day.
[ ] NO
↓
Finally, the protein shakes will be made in a facility that handles nuts. Are you allergic to any nuts?

[ ] YES → Okay. Unfortunately, it might not be safe for you to participate in this study. Thank you for taking the time to talk
with me. Have a good day.
[ ] NO
↓
Excellent. It looks like you could be a great addition to our study! If you'd like to participate, we can set up a time for you to come meet the research team and get enrolled in the study.

Discuss enrollment visit with potential participant and set up day/time.

PARTICIPANT INTAKE FORM

Participant ID _____ Date _____

*Complete **before** recording any data*

Consent obtained?
[ ] NO
[ ] YES, date _____

Pre-screening completed?
[ ] NO
[ ] YES, date _____

Study familiarization completed?
[ ] NO
[ ] YES, date _____

Guidelines for Participants issued?
[ ] NO
[ ] YES, date _____

Medical history intake completed?
[ ] NO
[ ] YES, date _____

Physical examination completed?
[ ] NO
[ ] YES, date _____

Consent obtained **and** all other inclusion/exclusion criteria met?
[ ] NO
[ ] YES, date _____

*Complete only **after** consent obtained and all other inclusion/exclusion criteria met*

Age (years) _____ Sex _____

Weight _____ kg / _____ lbs Height _____ inches / _____ cm

SHAKE TRACKING FORM

Participant ID _____

Phase _____

Date _____

Bottle ID	Weight in	Weight out	Difference	ED	EI
1V					
2V					
3C					
4C					
5S					
6S					
7F					
8F					

Total 48hrs: _____

Total 24hrs: _____

Participant daily ER _____ 20% _____

Flavor _____ ED_____ BF weight _____

Cup	BF weight in	Total in	Weight out	BF weight out	EI kcal	EI %

A/B	ED	C/D	ED
Vanilla	1.33	Vanilla	1.38
Chocolate	1.65	Chocolate	1.62
Strawberry	1.30	Strawberry	1.23
Coffee	1.74	Coffee	1.72

CU Boulder Protein Study

GUIDELINES FOR PARTICIPANTS

Thank you for participating in this study! This research would not be possible without you and we appreciate your time and effort. In order for this study to be successful, it's important for all participants to adhere to the following guidelines. *If you have questions at any point, please don't hesitate to get in touch with us (see "contacts" below).*

Protein Shakes

- For the next 48 hours, you'll be consuming only the CU Protein Shakes that you received at the CTRC. You may have as much or as little of these CU Protein Shakes as you wish, any time you feel like it, but **no other foods** (including liquid foods like soups or broths).
- You should have plenty of the CU Protein Shakes to last you through the 48-hour period, but if you think you're running low, just let Richard know and he'll provide you with more.
- After 48 hours, you will return any unused CU Protein Shakes, **and** the containers they came in, to the Boulder CTRC.

**** Important*: Please do not discard any unused CU Protein Shakes or containers!**

**** Important*: Please do not rinse or wash out any containers!**

Beverages

- For the next 48 hours, you can drink as much water as you wish. You may also drink other beverages as long as they have **no calories**. Here are some examples:
- Zero-calorie beverages that are **allowed**:
  - Water
  - Black coffee, with or without zero-calorie sweeteners
  - Black, herbal or green tea, with or without zero-calorie sweeteners
  - Zero-calorie soft drinks (example: Diet Coke)
  - Zero-calorie sports drinks (example: Powerade Zero)
- Calorie-containing beverages that are **not allowed**:
  - Fruit juices
  - Smoothies, milkshakes, other protein shakes not received from the CTRC
  - Non-diet soft drinks (example: regular Coke)
  - Non-diet sports drinks (example: regular Powerade)
  - Energy drinks
  - Alcoholic beverages (beer, wine, liquor, mixed drinks)
  - Coffee drinks with sugar and/or dairy (milk, cream)
  - Tea drinks with sugar, honey, and/or dairy (milk, cream)

Health and Safety

- The CU Protein Shakes you received from the CTRC have been designed to give you all the vitamins, minerals, and nutrients you need for a healthy diet. However, if you feel like you're not getting adequate energy or nutrition, please let us know right away.
- Since this is not your usual diet, you might not consume as much food as you normally do. So, you might experience hunger or food cravings. You might also experience digestive issues like gas or bloating.
- Since this study will impact your daily routine, you might also have feelings of stress, anxiety, or irritation.
- If you have a medical emergency, call 911. If you have other medical complaints, contact the CTRC at (303) 735-2304. After hours, call (303) 206-6339 (physician pager).
- **If any of these symptoms or experiences become intolerable, please don't hesitate to contact us. If you do not feel comfortable participating in this study, you may withdraw and it will not be held against you.**

Your health and safety are our top priority!

Contacts

- Principal Investigator: Richard L Bender (719-332-7825; richard.bender@colorado.edu or CUproteinstudy@gmail.com)
- Co-Investigator/Faculty Advisor: Darna L Dufour (303-492-6061; darna.dufour@colorado.edu)

This research has been reviewed and approved by an Institutional Review Board ("IRB"). You may talk to them at (303) 735-3702 or irbadmin@colorado.edu if:

- *Your questions, concerns, or complaints are not being answered by the research team.*
- *You cannot reach the research team.*
- *You want to talk to someone besides the research team.*
- *You have questions about your rights as a research subject.*
- *You want to get information or provide input about this research.*

INITIAL SURVEY (complete at the Boulder CTRC)

Date _____/_____/201__ Time _____:_____am

1. When was the last time you had a meal or snack?
(This includes caloric beverages like soda, fruit juice, and alcohol)

Time _____:_____am/pm

What did you have?

_____

2. About what time did you go to bed last night?

Time _____:_____am/pm

Was this *earlier than usual*, *later than usual*, or *about the same*? (circle one)

3. About what time did you wake up this morning?

Time _____:_____am/pm

Was this *earlier than usual*, *later than usual*, or *about the same*? (circle one)

4. About how many hours of moderate or vigorous exercise did you do yesterday? This includes things like running, biking, or rock climbing.

_____ hours

5. Think about your overall level of physical activity yesterday:

Was it *less than usual*, *more than usual*, or *about the same*? (circle one)

6. Think of the meals you ate yesterday. Did you eat about the same kinds of foods that you normally eat? Or was your diet unusual yesterday?

How was your diet unusual? (or N/A)

_____

7. Think about how much food you ate overall yesterday:

Was it *less than usual*, *more than usual*, or *about the same*? (circle one)

SHAKE SURVEY (complete on your own each time you have some of your CU Protein Shake)

Date _____/_____/201__ Time _____:_____am/pm

*Please answer the following questions **just before** you have your CU Protein Shake:*

How hungry do you feel?

I am not hungry at all _____ I have never been more hungry

How satisfied do you feel?

I am completely empty _____ I cannot have any more

How full do you feel?

Not at all full _____ Totally full

How much do you think you can eat?

Nothing at all _____ A lot

*Please answer the following questions **just after** you have your CU Protein Shake:*

How hungry do you feel?

I am not hungry at all _____ I have never been more hungry

How satisfied do you feel?

I am completely empty _____ I cannot have any more

How full do you feel?

Not at all full _____ Totally full

How much do you think you can eat?

Nothing at all _____ A lot

CLINIC SURVEY (complete at the Boulder CTRC)

Date _____/_____/201__ Time _____:_____am/pm

*1. Please answer the following questions **at the beginning** of your clinic visit:*

How hungry do you feel?

I am not hungry at all _____ I have never been more hungry

How satisfied do you feel?

I am completely empty _____ I cannot have any more

How full do you feel?

Not at all full _____ Totally full

How much do you think you can eat?

Nothing at all _____ A lot

** You will now have an IV placed by trained Boulder CTRC personnel. The IV will stay in place for 90 minutes, and a small blood sample will be drawn every 30 minutes**

Now, please consume all of the CU Protein Shake provided by the research team.

*2. Please answer the following questions **just after** you have your CU Protein Shake:*

How hungry do you feel?

I am not hungry at all _____ I have never been more hungry

How satisfied do you feel?

I am completely empty _____ I cannot have any more

How full do you feel?

Not at all full _____ Totally full

How much do you think you can eat?

Nothing at all _____ A lot

*3. Please answer these questions **30 minutes after** you have your CU Protein Shake:*

How hungry do you feel?

I am not hungry at all _____ I have never been more hungry

How satisfied do you feel?

I am completely empty _____ I cannot have any more

How full do you feel?

Not at all full _____ Totally full

How much do you think you can eat?

Nothing at all _____ A lot

*4. Please answer these questions **60 minutes after** you have your CU Protein Shake:*

How hungry do you feel?

I am not hungry at all _____ I have never been more hungry

How satisfied do you feel?

I am completely empty _____ I cannot have any more

How full do you feel?

Not at all full _____ Totally full

How much do you think you can eat?

Nothing at all _____ A lot

*5. Please answer these questions **90 minutes after** you have your CU Protein Shake:*

How hungry do you feel?

I am not hungry at all _____ I have never been more hungry

How satisfied do you feel?

I am completely empty _____ I cannot have any more

How full do you feel?

Not at all full _____ Totally full

How much do you think you can eat?

Nothing at all _____ A lot

FINAL SURVEY (complete on your own)

Date _____/_____/201__

*At the end of your 48-hour diet, please answer the following questions about your experience **overall**:*

Did you have any other food besides your CU Protein Shakes during the last 48 hours?

☐ No

☐ Yes (please describe):

_____

Did you have any calorie-containing beverages (see *Guidelines for Participants*) during the last 48 hours?

☐ No

☐ Yes (please describe):

_____

Visual appeal of food overall

Good _____ Bad

Smell of food overall

Good _____ Bad

Taste of food overall

Good _____ Bad

Aftertaste of food overall

Much _____ None

Texture of food overall

Good _____ Bad

How hungry did you feel overall?

I was not hungry at all _____ I had never been more hungry

How satisfied did you feel overall?

I was completely empty _____ I could not have any more

How full did you feel overall?

Not at all full _____ Totally full

How much did you crave other foods?

No cravings at all _____ Constant cravings

How pleasant was the experience overall?

Not pleasant at all _____ Very pleasant

How disruptive was the experience overall?

Very disruptive _____ Not disruptive at all

How comfortable was the experience overall?

Not comfortable at all _____ Very comfortable

Please describe any negative physical, mental, or emotional symptoms you experienced (stomach pain, stress, anxiety, etc.):

_____

_____

Please list any other thoughts or comments about the experience you'd like to share:

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Thank you for completing this phase of the experiment!