© 2013 Kristy Du

BREAKFAST DIETARY PROTEIN SOURCES AFFECT SATIETY AND COGNITIVE PERFORMANCE

BY

KRISTY DU

THESIS

Submitted in partial fulfillment of the requirements
for the degree of Master of Science in Nutritional Sciences
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2013

Urbana, Illinois

Master's Committee:

Professor Rodney Johnson, Chair
Professor J. Lee Beverly, Director of Research
Associate Professor Justin Rhodes

Abstract

There is considerable truth to the saying that "Breakfast is the most important meal of the day," with regular breakfast consumers generally having better macro- and micronutrient intakes, healthier body weights, and quality lifestyle. The growing bodies and developing brains of children and adolescents make them more reliant on regular intake of nutrients and more susceptible to impairments of cognitive performance if the first meal of the day is skipped. While dietary protein has been shown to increase satiety, it has not been well studied as a component of breakfast. The objective of this study was to provide insight on the influence of protein level and composition at breakfast (BF) on subsequent meal size and learning and memory performance. Male Sprague-Dawley rats were entrained to a meal-feeding schedule which included an overnight fast before being provided one of three isocaloric diets as BF equivalent to 20% of average daily intake. Two of the diets were moderately high protein diets (35% energy from protein), with protein provided from either egg white or wheat gluten. The basal diet provided 20% energy from protein, and protein was provided equally by egg white and wheat gluten. Rats provided the egg white diet consumed less during the subsequent period while those that were fed the wheat gluten diet consumed more when compared to those fed the basal diet, suggesting satiation from high protein diets is influenced by protein source. In another cohort of rats, Barnes maze was used to test spatial learning and memory following the same BF protocol. Rats that were not provided breakfast moved slower and displayed compromised working and long-term memory. Rats fed higher dietary protein BF were more active and learned more quickly. Rats fed the egg white diet had better working and long-term memory, whereas rats fed the wheat gluten diet moved faster, but performed test with less accuracy. Together, the present study demonstrated different protein sources at breakfast having varying effects on satiety and

cognitive performance, suggesting the importance of the protein component of breakfast on subsequent satiety and cognitive performance.

Acknowledgements

Many people are to be recognized in my development and growth into the scientist and researcher I am today.

My advisor Dr. J. Lee Beverly has been so supportive, and his evident dedication to science and teaching fueled my passion for learning, discovery, and research. His invaluable insight and guidance were strongly influential into my training of becoming of an inquisitive and passionate scientist. I would also like to thank my thesis committee members, Drs. Rodney Johnson and Justin Rhodes for their dedicated time and input that have enhanced my scientific knowledge and improved the quality of my research skills.

There are many past and present members of the Beverly lab I would like to thank, as they made my research experience both manageable and fun. Andrew Cooper has been very important for his resourcefulness, technical knowledge and skills in the lab. I must also thank Chiara Barbieri for her tremendous patience in teaching me so many of the intricate lab techniques and example setting diligence and commitment. Lastly, Jonathan Mun has been there for me a great deal throughout the entire duration of my studies, and along the way has challenged me and taught me the importance of paying attention to details both in the lab and in my writing. He provided valuable professional and scientific input that I hope to continue to have and build upon.

I must also thank my family and friends at home in California for all their support and patience with my absence. The decision to pursue graduate studies would never have happened without all the encouragement of my significant other Sean Munoz, who saw my passion for Nutritional Sciences early on as we took Biochemistry together as undergraduates. The drive and passion he displays while pursuing medical school has been incredibly inspiring and continues to motivate me to strive for my own goals. I must also thank my family and friends at home in

California for all their support and patience with my absence. My dad instilled in me the idea of "you are what you eat" throughout my upbringing, unknowing that it would have such deep-seated impact in me. My mom has felt every emotion I experienced during the last two years with every excited, tired, or nervous phone call I'd make.

Table of Contents

Chapter 1: Literature Review 1
1.1 Effects of dietary protein on behavior 1
1.2 Feeding behavior 5
1.3 Learning and memory 14
1.4 Literature cited 20
Chapter 2: Satiety from higher protein diets is dependent on the protein source 27
2.1 Introduction 27
2.2 Materials and Methods 29
2.3 Results 31
2.4 Discussion 32
2.5 Literature Cited 37
2.6 Tables and figures 39
Chapter 3: The source of dietary protein for breakfast influences subsequent learning and memory performance 43
3.1 Introduction 43
3.2 Materials and Methods 45
3.3 Results 47
3.4 Discussion 49
3.5 Literature Cited 53
3.6 Tables and figures 55
Conclusion 64
Appendix 68

Chapter 1: Literature Review

1.1 Effects of dietary protein on behavior

The need for protein was determined when early nutritional scientists noticed animals fed just carbohydrates, fats, and a mix of micronutrients were losing weight and wasting muscle tissues (Carpenter 2003). They also knew that proteins contained nitrogen bound in "amino-bodies". The World Health Organization (WHO) currently defines protein requirement as "the lowest level of dietary protein intake that will balance the losses of nitrogen from the body, and thus maintain the body protein mass, in persons at energy balance with modest levels of physical activity…" (2007). Mammals, including humans, require dietary protein for its constituent amino acids that consequently flow into pathways that maintain vital structures and functions of the body. There are twenty amino acids that are required for life, but nine of these are actually considered indispensible due to humans' inability to synthesize these certain amino acids. These nine essential amino acids can be used to synthesize the other amino acids. The most important function of dietary amino acids is to maintain adequate protein synthesis to balance protein degradation and protein loss from the body through mechanisms such as skin cells shedding, hair growth and secretions in the gut. In addition, amino acids are used for the synthesis of other non-protein compounds (ie. hormones, neurotransmitters, purines, and pyrimidines), energy substrates, and as signaling molecules for the regulation of protein synthesis.

Adults require protein in their diet for maintenance of their health. There is constant turnover of protein, with new protein being synthesized at the same time protein is degraded and lost. So although there is no change in the total amount of protein in the body, adults require protein intake to keep up with protein loss or risk tissue protein wasting. And because the body does not contain a dedicated reserve of amino acids, protein intake needs to occur on a regular

basis. The current estimated average requirement for human adults is 0.66 g/kg/d. It is thought that this level of intake provides adequate nitrogen intake to balance that of output of nitrogenous compounds from the body (mainly as urea excreted through urine, but smaller amounts lost through feces, skin and lungs). A constant energy supply is especially important for the brain (Bourre 2006). Of any organ systems in the body, the brain has the greatest metabolic activity with a very minimal capacity for energy storage. A fully developed brain uses an average of 20% of dietary energy during resting state.

The growth period in an animal's lifespan includes development in length, mass, and function. Young animals from birth through adolescence require ample need for all nutrients, including protein. Proteins supplied through the diet are important for establishing architectural components of the brain and provide the fuel needed for efficient functioning. Lack of nutrients during this major developmental period can have detrimental, long-term changes with associated decreased brain functioning. A newborn baby has a brain weight that is roughly 10% of its body weight, whereas an adult brain is about 2% of body weight (Dekaban and Sadowsky 1978). As the child moves through early development, the brain rapidly grows and develops at a rate well above the rate of the rest of the body. The brain continues to develop well through adolescent years, adapting to more mature ways of thinking (Benton 2008). Throughout the brain, gray matter continues to grow and subsequently thin back through apoptosis connections that were not being stimulated. This plasticity of the brain, termed "synaptic pruning", correlates to increased glucose utilization rates and continues until 16-18 years of age (Chugani 1998). Nutrition can also have short term influences on brain function in children. A given amount of brain tissue of a child 4 years of age uses glucose at a rate twice that of an adult and because of this requires a higher relative continual supply of glucose.

Brain development can be described in two distinct stages (Benton 2010). The critical period is shorter, more precise in its inception and end, and marked by immense sensitivity for environmental stimulus. In the absence of some stimuli, functions later in life may be impaired or even absent. The sensitive time period for development spans a longer time frame, although acquisition can still occur at a later stage, but at a reduced level of efficiency.

Early protein-energy malnutrition (PEM) has been linked to issues with behavior, motor control, language development, and academic performance (Benton 2010). Children ages 9-15 years who were malnourished during their first year of life, showed increased aggressive behavior, hyperactivity, disorderly conduct, and attention problems (Benton 2008). Some of these observations are consistent with damage seen in the cortex and hippocampus; lower neuronal counts, reduced protein synthesis, and reduced neurotransmitter synthesis (Wiggens et al. 1984, Jones and Dyson 1981, Georgieff 2007). An early study showed young rats on a protein deprived diet having lower brain weight and underdeveloped morphology of presynaptic terminals. In addition, children have larger brain weight to total body weight ratio than do adults, with their brains energy utilization being twice the rate of an adult (Benton 2010). A review on the literature of over a hundred studies of human brain growth in regards to weight and circumference showed peaks at ages 7, 12, and 15 years (Epstein 1986). Many studies have looked at the causal link of early nutrition to later academic performance and have seen poorly nourished children having impaired cognitive development, yet find too many environmental and socio-economic factors involved to draw firm conclusions (Morley and Lucas 1997). Undernourished children tend to come from poor populations where parents were likely to not have resources to care for and stimulate children. The high metabolic demands for both development and function suggests that energy provided through the diet is stretched across

multiple pathways, and that during periods of limiting protein and energy certain protein synthesis may be too “extravagant”.

Protein intake needs to be adjusted at other stages of life as well (WHO 2007). Protein requirement for pregnant and lactating women increases to 71 g/d to meet demands for tissue protein deposition and milk production. And while the health of the mother is important, pregnancy and lactation are immensely sensitive period for the brain and body structures and functions of the growing fetus and newborn respectively. Major injuries, traumas, and surgeries are associated with tissue loss, blood loss, and tissue catabolism. In addition, recovering patients tend to have decreased mobility and therefore decreased stimulation of muscle for tissue rebuilding. The lost protein needs to be replaced during recovery, so positive nitrogen balance is required. Protein intake continues to be important during aging to prevent osteoporosis and sarcopenia through its involvement with calcium absorption (Layman 2009). Calcium deposition into bones of elderly is decreased when dietary protein is limiting.

Discussions of protein requirement need to include consideration of protein quality, as each protein source varies in digestibility and essential amino acid composition. The most optimal dietary protein would provide the correct ratio of all 20 amino acids needed to meet our body’s needs (Elango et al. 2009). Plant protein sources (such as rice, wheat, and legumes) tend to have lower levels of lysine and methionine compared to animal protein foods. For this reason, animal protein sources are generally thought of as being “higher quality”. One method for evaluating protein quality is the protein digestibility-corrected amino acid score (PDCAAS), which rates protein sources by the concentration of the limiting amino acid and corrected for true fecal protein digestibility (TD) determined by a rat model. In this case TD is the difference of dietary nitrogen intake the protein food and the nitrogen excreted in the feces.

1.2 Feeding behavior

Understanding the regulation of energy homeostasis, the balance of energy intake to energy expenditure, has generated vast attention due to its implication for weight loss at a time of rising prevalence of obesity worldwide. Under a normal circumstances, feelings of hunger and satiety act as the main regulators of food intake. Environmental and physiological factors control the initiation and termination of hunger and satiety, and these are necessary to understand how to manage overeating. This section of the chapter will focus on the environmental factors that influence satiety and proposed mechanisms involved in inducing satiety.

Satiety is the sensation of being full that reduces desires for continued energy intake. Satiety focuses on fullness due to the prior meal and time period until the next episode of energy consumption. This is different from satiation which describes what is developed during a meal which leads someone to stop eating and controls food intake of only the present meal (Abou-Samra et al. 2011, Benelam 2009). Foods or drinks stimulate sensory and cognitive responses even prior to consumption, continue stimulating signals involved in regulation of energy intake as it is digested and absorbed in the gastrointestinal tract (GIT), and these signals ultimately integrate in the brain to indicate ingestion of energy and stimulate satiety. However, even with such physiological mechanisms regulating food intake, eating remains a voluntary act. Sensory properties of foods can cause initiation of a feeding episode even when satiety signals are present.

A number of external factors are involved in eating, which can also act on other behavioral pathways of the brain to override satiety signals and increase food intake. This review will focus on only a few of these factors. Of these, palatability has arguably been one of the more studied external factor involved in food intake, although lacking an accepted definition due to the

‘palatability’ of a food varying with each individual’s evaluation. Yeomans concludes a review on palatability and the control of appetite by defining palatability as the hedonic evaluation of the taste and smell of food under standardized conditions (Yeomans 1998). Foods higher in energy density tended to be most palatable, and studies have shown palatability reducing satiety, leading to increased food consumption (Bobroff and Kissileff 1986, Yeomans 1998, Benelam 2009). Access to a variety of food, as studied in rats using the cafeteria diet, has been shown to strongly stimulate food intake (Treit et al. 1983). Advertisements of foods can also act as both availability and variety of foods thus stimulating ‘wanting’ (Berthoud 2007). Portion sizes are rightfully a concern as they have increased over the last three decades and has been shown to be positively correlated to increased energy intake (Rolls et al. 2006). Restricted sleep duration has been linked to weight gain, and it is suggested that the alterations in hormone level resulting from shortened sleep duration reduces satiety (Benelam 2009). Modern day lifestyle has also become increasingly stressful, which is being suggested to increase energy intake through its affects on reward processing and increased appetite of “comfort foods” to self-medicate hormonal responses of stress (Berthoud 2007). Eating while watching television or in social situations is said to relax and distract people, making them less sensitive to satiety signals and increasing energy intake (Benelam 2009).

Circadium rhythm and time of day may have a role in energy intake and even macronutrient specific intake. Rats can be trained to anticipate feeding at a specific time of the day and will continue to express those aniticipatory activities when the meal is missed (Mistlberger 2011). The general feeding pattern in humans seems to be that as the day progresses, meal size increases with intermeal intervals decreasing, suggesting eating earlier induces a greater amount of satiety and foods become less satiating later in the day (de Castro

2007). Breakfast meals tend to be when the highest proportion of carbohydrate is ingested and dinner being higher in proportion of fat (Westerterp-Plantenga 1999). However, increased protein consumption at breakfast led to greater meal-related (3 h) fullness and overall (15 h) fullness than increased protein provided at lunch or dinner (Leidy et al. 2009). Overall, this emphasizes the importance of eating at breakfast time and that the macronutrient composition of breakfast needs to be considered to maximize the satiety effects. Food intake is beyond consumption to satisfy energy requirements.

Many studies have compared the effects of various ratios of dietary carbohydrates, proteins, and fats in our diet and their implications on our health. Studies on the effects of various foods on satiety typically provide subjects with a preload of a determined macronutrient content followed by satiety measurements through either a self-reported rating system or subsequent energy intake is recorded. Of these three, protein has been shown to have the greater effect to increase satiety and decrease the consumption at subsequent meals. Fats were suggested to have the least effect on satiety (Rolls et al. 1988, Bensaid et al. 2002, Benelam 2009, Westerterp-Plantenga et al. 2009). Stubbs et al. found their subjects to be hungrier after a high fat breakfast than either a high carbohydrate or high protein breakfast; however, the high protein breakfast suppressed hunger more over the rest of the day (Stubbs et al. 1996). Studying the satiety effects of fats can be complicated by its addition causing foods to be more palatable, as well as it being more energy dense at 9 kcal/g of fat compared to protein and carbohydrate at 4 kcal/g each.

Even between carbohydrate types, the rate of digestion and absorption can affect glucose release and availability. Glycemic index (GI) is a concept originally conceived to classify different types of foods containing carbohydrate relative to their postprandial effects on blood

glucose. GI of a food is measured as the area under the curve of blood glucose versus time following the consumption of a fixed amount of available carbohydrate. This measurement is then compared to that of pure glucose or white bread. Food with a higher GI would then induce a greater increase in blood glucose levels. The same amount of carbohydrate in a lower GI food is digested and absorbed more slowly, remains in the gastrointestinal tract over a more prolonged period, and therefore stimulates nutrient receptors to signal satiety for a longer length of time (Bornet et al. 2007). Although carbohydrate consumption of any source induce some amount of fullness, the difference in satiety between carbohydrates with high GI and low GI can produce a difference in the length of satiety as much as one hour versus 6 hours respectively (Anderson and Woodend 2003). Of the 19 studies reviewed by Bornet et al. that tested low glycemic carbohydrates on satiety, 12 studies demonstrated increased satiety and decreased energy intake following low glycemic carbohydrate preload (Bornet et al. 2007).

The satiating effect of protein is dose dependent, and is most evident at sufficiently high levels. A breakfast of high protein (25% energy from casein) was reported to be more satiating than one of normal protein level (10% energy from casein) 3-4 hours after the breakfast meal (Veldhorst et al. 2009). Griffioen-Roose et al. didn't see an effect between preloads of 7% vs. 25% energy from protein, but that may be due to the subsequent ad libitum meal being only 30 minutes after preload, too short of a period to see a difference in satiety of preloads (Griffioen-Roose et al. 2011).

Few studies have compared protein sources, but the results indicate that different types of protein may induce different levels of satiety. The earliest one of these studies is likely that conducted by Uhe et al in which beef, chicken, and fish protein were compared for its satiating effects, as well as amino acid profiles. They found satiety to be greater after consumption of the

fish protein and speculated, based on plasma amino acid results, that it could be due to increased tryptophan levels inducing serotoninergic activity or slower absorption due to the longer time it took for amino acid to be released in the blood (Uhe et al. 1992). Even between two milk protein sources, it was suggested that whey induces greater satiety than casein (Hall et al 2003, Veldhorst et al 2009). Interestingly, Bowen and colleagues did not find a difference in satiety in the three hours following whey and casein protein preload (Bowen et al. 2006). A study that included casein, whey, pea protein, and egg albumin found food intake to decrease most significantly following consumption of casein and pea protein as compared to whey (Abou-Samra et al. 2011).

The underlying mechanisms that allow dietary protein to increase satiety remain unclear. Several of the proposed mechanisms by which dietary protein induces satiety include: 1) increase in energy expenditure through thermogenesis, 2) increase amino acids in circulation, 3) glucose regulation by way of gluconeogenesis, and 4) increase of satiety signaling hormones (Veldhorst et al 2008).

1) The increase in thermic response to increased protein ingestion may be another possible mechanism for protein-induced satiety. Increased resting state energy expenditure was related to increased oxygen consumption and increased body temperature, as well as increased feelings of satiety. A review concluded protein was always found to induce the greatest thermic effect whether in lower doses or higher doses when compared to carbohydrate and fat (Halton and Hu 2004). Both satiety and 24 h diet-induced themogenesis were found to be synchronously higher in subjects fed a diet that was high protein and high carbohydrate compared to subjects fed high fat meals (Westerterp-Plantenga et al. 1999). 24 h energy expenditure was also found to be greater in subjects fed pork-meat protein than in subject fed soy protein or carbohydrates, and

energy expenditure again correlated with increased satiety (Mikkelsen et al. 2000). Amino acid composition of protein sources will also differ in the energy cost during catabolism (van Milgen 2002). Amino acids can vary largely in their metabolic cost due to difference in carbon chains, how they are involved in metabolic pathways (ie citric acid cycle and urea cycle), and the cofactors each yields.

2) Circulating levels of metabolites, some of which being amino acids, during the postprandial period is thought to have a role in perceived satiety. In 1956, Mellinkoff proposed the aminostatic hypothesis: postulating that changes in blood amino acid concentration can change perceptions of hunger, appetite, and satiety. That is, increasing levels of amino acid is related to an increase in feelings of satiety and decrease in both appetite and hunger. Mellinkoff suggested there exists a satiety center in the brain, and that plasma amino acids in excess of protein synthesis needs serve as satiety signals to control food intake. He tied the level of serum tryptophan as being influenced by the diet and that tryptophan uptake into the central nervous system was dependent on the ratio of tryptophan to total large neutral amino acids in serum (Mellinkoff et al. 1956).

If the aminostatic hypothesis holds true, all factors that influence the rate of dietary protein digestion and absorption can change the rate of appearance of amino acids in circulation, thus causing differences in satiety between protein sources. Similarly to the digestion and metabolism of carbohydrates, different types of proteins are metabolized at different rates and result in distinct postprandial profiles. As an example, casein and whey are two protein types both coming from milk and have been shown to have different postprandial affects (Hall et al 2003). As compared to casein, whey protein is digested, absorbed, and metabolized more quickly, thus producing a larger peak in plasma amino acids within a shorter amount of time

(Boirie et al 1997 and Brun et al 2012). Whey protein is a more soluble and easily digestible protein. During gut digestion, gastric acid causes casein to precipitate and coagulate in the stomach, slowing down absorption and gastric emptying rates. This results in a post-prandial increase in plasma amino acids to be similar to the increase in glucose of a low GI carbohydrate food, smaller peak that is longer in duration. Hall et al. compared the satiating effects of whey and casein protein to plasma amino acids. Their results support the aminostatic mechanism of appetite regulation by showing that the ingestion of whey protein preload creates a greater increase in total plasma amino acid, while inducing greater subjective reporting of fullness and a lower desire to eat profile from casein preload in 3 hours following consumption (Hall et al. 2003).

3) Protein involved in hepatic gluconeogenesis can contribute to satiety through its involvement in glucose homeostasis. Because amino acids can be metabolized in the liver via gluconeogenesis to produce glucose, dietary proteins can have a role in supplying circulating glucose (Westerterp-Plantenga et al 2009, Layman and Baum 2004). Consequently, this process can be involved in protein induced satiety through its involvement in both the modulation of glucose homeostasis and communiating glucose availability to the brain. Hepatic phosphoenolpyruvate carboxylase (PEPCK), a rate limiting enzyme of the gluconeogenesis pathway that converts oxaloacetate to phosphoenolpyruvate, is increased in rats fed a high protein diet whether or not carbohydrates are present in the diet (Azzout-Marniche et al 2006). Glucose 6-phosphate (G6P), an intermediate of gluconeogenesis, in the fed state is directed towards glycogen synthesis, while in the fasted state is made into glucose before being released to peripheral tissues. Veldhorst et al. demonstrated increased gluconeogenesis measured by endogenous glucose production in subjects provided a high protein diet (Veldhorst et al 2012).

4) Lastly, it is thought that during protein digestion, peptide products stimulate the gastrointestinal tract to increase anorexigenic satiety hormones meanwhile decreasing orexigenic hunger homone, ghrelin, to decrease appetite. Some of the more commonly studied anorexigenic hormones include cholecystokinine (CCK), glucagon-like peptide 1 (GLP-1), and peptide YY (PYY). GLP-1 has been shown to be stimulated by gastric contents and volume, and is involved in insulin secretion, acid secretion, and the rate of gastric emptying (Mansour et al. 2013). Intestinal CCK is released upon stimulation by the presence of dietary protein and fat in the duodenum, and it has been demonstrated many times that the presence of CCK in the GIT decreases food intake in a dose-dependent manner (Lieverse et al. 1995, Cummings and Overduin 2007, Wren and Bloom 2007). Casein and milk protein, as well as leucine and isoleucine, but not whey protein, have been shown to stimulate GLP-1 secretion in human intestinal cell culture (Chen and Reimer 2009). This is consistent to results found in human subjects, in which milk protein induced a greater increase of both CCK and GLP-1 than whey protein and pea protein hydrolysate (Diepvens et al. 2008). Casein protein on its own was not included in this study, but it should be noted that milk protein consists of about 80% casein and 20% whey. The results of the Diepvens et al. study is inconsistent with another study comparing the satiating effects of whey and casein proteins in which both CCK and GLP-1 were increased more in whey preload matching increased subjective fullness and decreased desire to eat (Hall et al 2003). Bowen et al. also found increased CKK and decreased ghrelin following protein preloads, consistent to decreased energy intake. This study also compared whey and casein protein, but did not see a significant difference between protein sources on these two satiety hormones (Bowen et al. 2006). High protein diets stimulate increased PYY secretion and deletion of PYY causes mice to be resistant to satiety therefore inducing hyperphagia driven

obesity (Batterham et al. 2006). Evidence is plentiful showing protein ingestion promotes secretion of satiety signaling hormones and decreases ghrelin, a known hunger hormone. The increase and decrease in anorexigenic and orexigenic hormone respectively seems to play a role in decreasing food intake.

Afferent vagus nerve fibers in the GIT express receptors of the gut hormones that may be enhanced by the presence of long-term adiposity signals, leptin and insulin (Cummings and Overduin 2007). The vagus nerve extends from the gut to the brain, and removal of the vagus nerve blocks satiety signaling in rats (Benelam 2009). These signals converge in the hypothalamus to regulate energy balance. Early studies involving lesions and stimulation of the hypothalamus demonstrated the importance of this area of the brain in energy intake and expenditure balance, leading researchers to refer to the hypothalamus as the brain's "satiety center" (Morgane and Jacobs 1969).

Both anorexigenic and orexigenic pathways originate in the arcuate nucleus, which lies proximal to the third ventricle. Anorexigenic neuropeptides of neurons in the arcuate nucleus includes melanocortins; α-melanocyte-stimulating hormone (α-MSH), corticotrophin-releasing hormone (CRH), and cocaine- and amphetamine-regulated transcript (CART). Melanocortins are the product of cleaving pro-opiomelanocortin (POMC). These peptides bind to melanocortin receptors (five known members; MC1R through MC5R) to exert their anorexigenic effects of decreasing energy intake and increasing energy expenditure. A separate yet adjacent area of the arcuate nucleus contains neurons expressing orexigenic neuropeptides, neuropeptide Y (NPY) and agouti-related peptide (AgRP). Increasing NPY causes hyperphagia and obesity (Schwartz et al 2000). Neurons of the arcuate nucleus co-express the mentioned neuropeptides along with leptin, insulin, and ghrelin. Manipulation of the efficiency of these hormones in the arcuate

nucleus would thus alter expression of the neuropeptides in its respective manner. There currently only exist a handful of studies examining the effects of macronutrient diets on the expression of these neuropeptides, and these studies have only chronic manipulation of diets, not single meal effects. Axons of the arcuate nucleus neurons innervate to the peraventricular nucleus (PVN) and lateral hypothalamic area (LHA). Nucleus tractus solitaries (NTS) of the brain stem has also been suggested to be involved in satiety as it also receive input signals from the vagus nerves (Morton et al. 2006, Wren and Bloom 2007, Benelam 2009).

1.3 Learning and memory

Breakfast is often stressed as being the most important meal of the day, with much support for breakfast consumers being linked to having better weight control and an overall more quality nutrient intake profile (Ruxton and Kirk 1997). It was suggested that that decline in breakfast, or even nutritionally adequate breakfast consumption, and the rise in obesity over the past several decades indicating the importance of the first meal of the day on appetite control, diet quality, and risk of chronic diseases (Timlin and Pereira 2007). The consumption of a quality breakfast is linked to better food choices during the rest of the day, with breakfast consumers tending to have lower intakes of fat and higher intakes of fiber and certain micronutrients. More importantly for this thesis, studies show support for breakfast consumption having a positive influence on cognitive performance in children and adolescents, as well as adults and elderly (Hoyland et al., 2009 and Kaplan et al 2001).

Skipping breakfast has been widely seen to impair children’s ability to learn and focus in school. Carbohydrates can be digested and metabolized to provide a quick source of glucose to fuel extensive functions. Increased errors were made during a visual discrimination test when

skipping breakfast resulted in reduced blood glucose levels (Pollitt et al. 1981). A majority of the studies included in the review Hoyland and colleagues conducted that compared consequences of breakfast consumption and breakfast omission found skipping breakfast to result in increased error rates for task that required memory or attention (Hoyland et al. 2009). The opposite effect was observed in breakfast consumers who were better able to memorize and made less errors on tasks that required attention even during the late morning period. These tendencies were even more evident when tests were more complex, demanding, or error rates were accounted for. It should be noted that these impacts of breakfast, appears to be most dramatic in children and adolescents of malnourished or lower socio-economic backgrounds.

Positive cognitive effects were most clear after a breakfast meal compared to meals consumed at other times of the day (Dye et al. 2000). Midday meals have nearly consistently shown to decrease performance (Gibson and Green 2002). Studies on mood and performance after lunch has described subjects feeling more lethargic, clumsy, bored, mentally slow, and was more susceptible to being distracted. However, the effects of the lunch meal on cognitive function may be tied to the natural circadian rhythm causing "postlunch dip," making it difficult to distinguish how much of the declined performance is actually due to the lunch meal. Breakfast being the meal with the strongest effect on cognitive function may suggest a role of the metabolic state following an overnight fast to maintain the supply of fuel and other nutrients to the brain and central nervous system, and followed by the consumption of the first meal of the day replenishing energy stores and "jump-starting" the system's metabolism.

The size of the breakfast meal also needs to be considered, as adolescents provided a larger breakfast (averaging 2653 kJ) had poorer vigilance compared to peers provided a 1628 kJ breakfast (Michaud et al. 1991). On the other hand, too small a breakfast resulted in worse mood

and performance (Wyan et al. 1997). Timlin and Pereira (2007) defined a reasonable size for breakfast to be between 20% and 35% of total daily energy requirements.

Many studies of breakfast's effect on cognitive function have focused primarily on the amount and types of carbohydrates subjects consume at breakfast (Hoyland et al. 2009, Benton et al. 2003, Micha et al. 2011). This is reasonable as the brain's most preferred source of energy is glucose. Glycemic index (GI) may be linked to cognitive performance, although studies have been inconclusive (Gilsenan et al 2009). Given the importance of a steady supply of glucose to optimal brain functioning, foods digested and absorbed more slowly, and therefore providing more steady amounts of glucose to be added into circulation, should ideally result in greater cognitive functioning. In a study conducted with both adults and rats being fed the same diets of either "slowly available glucose", lower GI, or "rapidly available glucose", it was shown that both subject groups benefited in performance when given the lower GI breakfast. The improvement was apparent most significantly in the late morning (around the 3 hour mark post breakfast ingestion) (Benton et al 2002). Elementary school children showed better spatial memory and auditory attention on the day they were fed oatmeal (lower GI) as compared to being fed ready-to-eat breakfast cereals (Mahoney et al 2005). These observations are reinforced by findings that grazing, resulting in energy intake being spread out through the first half of the day and possibly sustaining a constant level of blood glucose, improves cognitive performance (Hewlett et al 2009). Even an afternoon energy-containing snack had beneficial effects on memory, reasoning, and reaction tasks (Kanarek and Swinney 1990).

Another way of looking at post-prandial glycemic response is through glycemic load (GL), which classifies the quality of food similarly to GI, but takes into account both the glycemic index, by evaluation of type of carbohydrate, and amount present in a particular food

per each serving. Foods with higher GL are thought to be most optimal for cognition by maintaining the increase blood glucose level for a more extended period of time. It is suggested that a combined use of both GI and GL be ideal for predicting performance. However, studies that have made use of both measures on performance, looked at the effects at 90 minutes post-ingestion, which may be too early to observe differences (Micha et al 2010, 2011).

Few studies have focused on non-carbohydrate components of breakfast and its subsequent effect on cognitive performance. In a study carried out by Fischer et al. (2001), subjects were given pure, isoenergetic carbohydrates, protein, or fat test meals. It was determined that fat ingestion was best for overall cognitive performance, carbohydrate ingestion showed better short-term memory, and protein ingestion showed better attention and efficiency at a task. They discussed the possibility of protein in breakfast suppressing the release of serotonin, and increasing neurotransmitters involved in the state of alertness. Fat ingestion at breakfast having positive effects is also reflected in elderly, where fat improved attention and protein reduced the rate of forgetting (Kaplan et al. 2001). However, studies in rats have reported increased levels of either polyunsaturated fatty acids or saturated fats to disrupt performance (Dye et al. 2000). This review on macronutrients and mental performance also discussed glucose appearing to enhance short-term, working memory and attention tasks that required higher cognitive demands. Fisher et al. also demonstrates differences in macronutrient consumption resulted in different post-prandial glucose and peptide hormones in which cognitive performance may depend on. In comparison to protein-rich meals, high-carbohydrate breakfasts and lunches induced greater feelings of sleepiness and calmness 2 hours later (Spring et al. 1983). The different macronutrients and its levels seem to have a different role depending on the task in question.

The hippocampus is a brain area important for learning and memory, and it has widely been demonstrated that lesions of the hippocampus causes impairment of these cognitive processes. Glucose levels in the hippocampus decreases during spatial learning tasks at different rates depending on the complexity of the task (Rex et al 2009, McNay et al 2000). Exogenous supply of glucose by injection prevented the decline in glucose and enhanced task performance in related experiments carried out by McNay et al. Decline in glucose corresponded to the difficulty of the hippocampus dependent maze, with a larger decrease in glucose when rats were placed in the more difficult maze. This is a reflection of studies in humans showing greater susceptibility to poorer cognitive performance when blood glucose levels fell (Benton and Nabb 2003).

Dietary protein levels can affect many aspects of behavior, including appetite, mood, and cognition. However, in order to do so individual amino acids must be transported across the blood-brain barrier, the endothelial cells of cerebral capillary. This is a highly regulated, facilitated, Na^+-independent transport process that most efficiently transports essential large neutral amino acids (LNAA). This transport process involves system L1 (SLC7A5, LAT1) transporters, which takes up valine, isoleucine, leucine, tryptophan, phenylalanine, tyrosine, and methionine through both the luminal (blood side) and abluminal (brain side) of the endothelial cell (Hawkins et al 2006).

The concentration of LNAA in the brain's CSF is roughly 10% of that which appears in the plasma, and transport through system L1 occurs in a fashion in which individual amino acids compete for uptake (Hawkins et al. 2006). These concentrations have been shown to affect brain functions and behavioral processes controlled by the brain by altering levels of aromatic amino acids, which are required by neurons for monoamine neurotransmitter synthesis. Tryptophan is

required for serotonin synthesis. Whereas, phenylalanine can be hydrolyzed to form tyrosine, which in turn is required for catecholamine neurotransmitter (dopamine, norepinephrine, and epinephrine) synthesis by the rate-limiting enzyme, tyrosine hydroxylase.

Because all LNAAs compete for uptake by system L1 transporter, changes in plasma concentrations of any one LNAA can alter uptake of catecholamine precursor amino acids, the rate of catecholamine synthesis and release, and therefore brain functions which depend on catecholamines. For example, increases in overall circulating LNAAs can cause decreased uptake of tyrosine, decreasing its availabilility in the brain to synthesize catecholamines. Food ingestion can indirectly alter tyrosine levels in the brain, and a catecholamine synthesis rate is highly responsive to tyrosine levels in the brain (Fernstrom and Fernstrom 1987). This increase in brain tyrosine concentration and hydroxylation rate is not only seen as a response by a single meal, but also in rats fed a protein diets for two weeks (Fernstrom and Fernstrom 1995). By contrast, many studies have also taken advantage of system L1 competitive uptake into the brain by introducing all LNAAs except tyrosine and phenylalanine into the system. In these tyrosine depletion studies, brain catecholamine synthesis decreased (Bongiovanni et al 2012, Fernstrom and Fernstrom 2007). On the other end, Men et al. (1999) has reported release of norepinephrine in the hippocampus as rats were engaged in a spontaneous alternation task. In addition, prior studies from our lab showed hippocampal administration of norepinephrine modulating performance in the Morris water maze.

1.4 Literature cited

1. Abou-Samra R, Keersmaekers L, Brienza D, Mukherjee R, Macé K. Effect of different protein sources on satiation and short-term satiety when consumed as a starter. *Nutrition Journal*. 2011;10(1).

2. Anderson GH, Woodend D. Effect of glycemic carbohydrates on short-term satiety and food intake. *Nutrition Review*. 2003;61(5 II):S17-S26.

3. Anne C B, Ketil S, Johannesdottir GB, Vibeke F, Bentsen BS, Medhus AW. Nissen fundoplication in children with cerebral palsy: Influence on rate of gastric emptying and postprandial symptoms in relation to protein source in caloric liquid meals. *Clinical Nutrition*. 2012.

4. Azzout-Marniche D, Gaudichon C, Blouet C, et al. Liver glyconeogenesis: A pathway to cope with postprandial amino acid excess in high-protein fed rats? *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology*. April 2007;292(4):R1400-R1407.

5. Batterham RL, Heffron H, Kapoor S, et al. Critical role for peptide YY in protein-mediated satiation and body-weight regulation. *Cell Metabolism*. 2006;4(3):223-233.

6. Benelam B. Satiation, satiety and their effects on eating behaviour. *Nutrition Buletin*. 2009;34(2):126-173.

7. Bensaid A, Tomé D, Gietzen D, et al. Protein is more potent than carbohydrate for reducing appetite in rats. *Physiology and Behavior*. 2002;75(4):577.

8. Benton D, Nabb S. Carbohydrate, memory, and mood. *Nutrition Review* 2003;61(s5):S61-S67.

9. Benton D. The influence of dietary status on the cognitive performance of children. *Molecular Nutrition and Food Research*. 2010;54(4):457-470.

10. Benton D. The influence of children's diet on their cognition and behavior. *European Journal of Nutrition.* 2008;47(SUPPL.3):25-37.

11. Benton D, Ruffin M-, Lassel T, et al. The delivery rate of dietary carbohydrates affects cognitive performance in both rats and humans. *Psychopharmacology (Berl)*. 2003;166(1):86-90.

12. Benton D. Carbohydrate ingestion, blood glucose and mood. *Neuroscience & Biobehavioral Reviews*. 2002;26(3):293-308.

13. Berthoud H. Interactions between the “cognitive” and “metabolic” brain in the control of food intake. *Physiology and Behavior* 2007;91(5):486-498.

14. Bobroff EM, Kissileff HR. Effects of changes in palatability on food intake and the cumulative food intake curve in man. *Appetite*. 1986;7(1):85-96.

15. Boirie Y, Dangin M, Gachon P, Vasson M-, Maubois J-, Beaufrère B. Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proceedings of the National Academy of Sciences of the United States of America.* 1997;94(26):14930-14935.

16. Bongiovanni R, Kyser AN, Jaskiw GE. Tyrosine depletion lowers in vivo DOPA synthesis in ventral hippocampus. *European Journal of Pharmacology*. 2012;696(1-3):70-76.

17. Bornet FR, Jardy-Gennetier A, Jacquet N, Stowell J. Glycaemic response to foods: Impact on satiety and long-term weight regulation. *Appetite*. 2007;49(3):535-553.

18. Bourre JM. Effects of nutrients (in food) on the structure and function of the nervous system: Update on dietary requirements for brain. part 2: Macronutrients. *Journal of Nutrition, Health and Aging*. 2006;10(5):386-399.

19. Bowen J, Noakes M, Trenerry C, Clifton PM. Energy intake, ghrelin, and cholecystokinin after different carbohydrate and protein preloads in overweight men. *Journal of Clinical Endocrinology and Metabolism*. 2006;91(4):1477-1483.

20. Carpenter KJ. A short history of nutritional science: Part 2 (1885-1912). *Journal of Nutrition*. 2003;133(4):975-984.

21. Chen Q, Reimer RA. Dairy protein and leucine alter GLP-1 release and mRNA of genes involved in intestinal lipid metabolism in vitro. *Nutrition*. 2009;25(3):340-349.

22. Chugani HT. A critical period of brain development: Studies of cerebral glucose utilization with PET. *Preventive Medicine*. 1998;27(2):184-188.

23. Cummings DE, Overduin J. Gastrointestinal regulation of food intake. *Journal Clinical Investigation*. 2007;117(1):13-23.

24. de Castro JM. The time of day and the proportions of macronutrients eaten are related to total daily food intake. *British Journal of Nutrition*. 2007;98(5):1077-1083.

25. Dekaban AS, Sadowsky D. Changes in brain weights during the span of human life: Relation of brain weights to body heights and body weights. *Annals of Neurology*. 1978;4(4):345-356.

26. Diepvens K, Häberer D, Westerterp-Plantenga M. Different proteins and biopeptides differently affect satiety and anorexigenic/orexigenic hormones in healthy humans. *International Journal of Obesity*. 2008;32(3):510-518.

27. Dye L, Lluch A, Blundell JE. Macronutrients and mental performance. *Nutrition*. 2000;16(10):1021-1034.

28. Elango R, Ball RO, Pencharz PB. Amino acid requirements in humans: With a special emphasis on the metabolic availability of amino acids. *Amino Acids*. 2009;37(1):19-27.

29. Epstein HT. Stages in human brain development. *Developmental Brain Research*. 1986;30(1):114-119.

30. Fernstrom MH, Fernstrom JD. Effect of chronic protein ingestion on rat central nervous system tyrosine levels and in vivo tyrosine hydroxylation rate. *Brain Research*. 1995;672(1):97-103.

31. Fernstrom MH, Fernstrom JD. Protein consumption increases tyrosine concentration and in vivo tyrosine hydroxylation rate in the light-adapted rat retina. *Brain Research*. 1987;401(2):392-396.

32. Fernstrom JD, Fernstrom MH. Tyrosine, phenylalanine, and catecholamine synthesis and function in the brain. *Journal Nutrition*. 2007;137(6):1539S-1547S.

33. Fischer K, Colombani PC, Langhans W, Wenk C. Cognitive performance and its relationship with postprandial metabolic changes after ingestion of different macronutrients in the morning. *British Journal of Nutrition*. 2001;85(3):393-405.

34. Georgieff MK. Nutrition and the developing brain: Nutrient priorities and measurement. *Am Journal of Clinical Nutrition*. 2007;85(2):614S-620S.

35. Gibson EL, Green MW. Nutritional influences on cognitive function: Mechanisms of susceptibility. *Nutrition Research Reviews*. 2002;15(1):169.

36. Gilsenan MB, de Bruin EA, Dye L. The influence of carbohydrate on cognitive performance: A critical evaluation from the perspective of glycaemic load. *British Journal of Nutrition*. 2009;101(7):941-949.

37. Griffioen-Roose S, Mars M, Finlayson G, Blundell JE, De Graaf C. The effect of within-meal protein content and taste on subsequent food choice and satiety. *British Journal of Nutrition*. 2011;106(5):779-788.

38. Hall WL, Millward DJ, Long SJ, Morgan LM. Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite. *British Journal of Nutrition*. 2003;89(2):239-248.

39. Halton TL, Hu FB. The effects of high protein diets on thermogenesis, satiety and weight loss: A critical review. *Journal of the American College of Nutrition*. 2004;23(5):373-385.

40. Hawkins RA, O'Kane RL, Simpson IA, Viña JR. Structure of the blood-brain barrier and its role in the transport of amino acids. *Journal of Nutrition*. 2006;136(1):218S-226S.

41. Hewlett P, Smith A, Lucas E. Grazing, cognitive performance and mood. *Appetite*. 2009;52(1):245-248.

42. Hoyland A, Dye L, Lawton CL. A systematic review of the effect of breakfast on the cognitive performance of children and adolescents. *Nutrition Research Reviews*. 2009;22(2):220-243.

43. Jones DG, Dyson SE. The influence of protein restriction, rehabilitation and changing nutritional status on synaptic development: A quantitative study in rat brain. *Brain Research*. 1981;208(1):97-111.

44. Kanarek RB, Swinney D. Effects of food snacks on cognitive performance in male college students. *Appetite*. 1990;14(1):15-27.

45. Kaplan RJ, Greenwood CE, Winocur G, Wolever TMS. Dietary protein, carbohydrate, and fat enhance memory performance in the healthy elderly. *American Journal of Clinical Nutrition*. 2001;74(5):687-693.

46. Layman DK. Dietary guidelines should reflect new understandings about adult protein needs. *Nutrition and Metabolism*. 2009;6.

47. Layman DK, Baum JI. Dietary protein impact on glycemic control during weight loss. *Journal of Nutrition*. 2004;134(4):968S-973S.

48. Leidy HJ, Bossingham MJ, Mattes RD, Campbell WW. Increased dietary protein consumed at breakfast leads to an initial and sustained feeling of fullness during energy restriction compared to other meal times. *British Journal of Nutrition*. 2009;101(6):798-803.

49. Lieverse RJ, Jansen JBMJ, Masclee AAM, Lamers CBHW. Satiety effects of a physiological dose of cholecystokinin in humans. *Gut*. 1995;36(2):176-179.

50. Mahoney CR, Taylor HA, Kanarek RB, Samuel P. Effect of breakfast composition on cognitive processes in elementary school children. *Physiology and Behavior*. 2005;85(5):635-645.

51. Mansour A, Hosseini S, Larijani B, Pajouhi M, Mohajeri-Tehrani MR. Nutrients related to GLP1 secretory responses. *Nutrition*. 2013.

52. McNay EC, Fries TM, Gold PE. Decreases in rat extracellular hippocampal glucose concentration associated with cognitive demand during a spatial task. *Proceedings of the National Academy of Science of the United States of America*. 2000;97(6):2881-2885.

53. Mellinkoff SM, Frankland M, Boyled D, Greipel M. Relationship between serum amino acid concentration and fluctuations in appetite. *Journal of Applied Physiology*. 1956;8(5):535-538.

54. Men D, McCarty R, Gold PE. Enhanced release of norepinephrine in rat hippocampus during spontaneous alternation tests. *Neurobiology of Learning and Memory*. 1999;71(3):289-300.

55. Micha R, Rogers P, Nelson M. The glycaemic potency of breakfast and cognitive function in school children. *European Journal of Clinical Nutrition*. 2010;64(9):948-957.

56. Micha R, Rogers PJ, Nelson M. Glycaemic index and glycaemic load of breakfast predict cognitive function and mood in school children: A randomised controlled trial. *British Journal Nutrition*. 2011;106(10):1552-1561.

57. Mikkelsen PB, Toubro S, Astrup A. Effect of fat-reduced diets on 24-h energy expenditure: Comparisons between animal protein, vegetable protein, and carbohydrate. *American Journal of Clinical Nutrition*. 2000;72(5):1135-1141.

58. Mistlberger RE. Neurobiology of food anticipatory circadian rhythms. *Physiology and Behavior*. 2011;104(4):535-545.

59. Morgane PJ, Jacobs HL. Hunger and satiety. *World Review of Nutrition and Dietetics*. 1969;10:100-213.

60. Morley R, Lucas A. Nutrition and cognitive development. *British Medical Bulletin*. 1997;53(1):123-134.

61. Morton GJ, Cummings DE, Baskin DG, Barsh GS, Schwartz MW. Central nervous system control of food intake and body weight. *Nature*. 2006;443(7109):289-295.

62. Pollitt E, Leibel RL, Greenfield D. Brief fasting, stress, and cognition in children. *American Journal of Clinical Nutrition*. 1981;34(8):1526-1533.

63. Rex A, Bert B, Fink H, Voigt J-. Stimulus-dependent changes of extracellular glucose in the rat hippocampus determined by in vivo microdialysis. *Physiology and Behavior*. 2009;98(4):467-473.

64. Rolls BJ, Roe LS, Meengs JS. Reductions in portion size and energy density of foods are additive and lead to sustained decreases in energy intake. *American Journal of Clinical Nutrition*. 2006;83(1):11-17.

65. Ruxton C, Kirk T. Breakfast: A review of associations with measures of dietary intake, physiology and biochemistry. *British Journal of Nutrition*. 1997;78(2):199-214.

66. Schwartz MW, Woods SC, Porte Jr. D, Seeley RJ, Baskin DG. Central nervous system control of food intake. *Nature*. 2000;404(6778):661-671.

67. Spring B, Maller O, Wurtman J, Digman L, Cozolino L. Effects of protein and carbohydrate meals on mood and performance: Interactions with sex and age. *Journal of Psychiatric Research*. 1982–1983;17(2):155-167.

68. Stubbs RJ, Van Wyk MCW, Johnstone AM, Harbron CG. Breakfasts high in protein, fat or carbohydrate: Effect on within-day appetite and energy balance. *European Journal of Clinical Nutrition*. 1996;50(7):409-417.

69. Timlin MT, Pereira MA. Breakfast frequency and quality in the etiology of adult obesity and chronic diseases. *Nutrition Review*. 2007;65(6):268-281.

70. Treit D, Spetch ML, Deutsch JA. Variety in the flavor of food enhances eating in the rat: A controlled demonstration. *Physiology and Behavior*. 1983;30(2):207-211.

71. Uhe AM, Collier GR, O'Dea K. A comparison of the effects of beef, chicken and fish protein on satiety and amino acid profiles in lean male subjects. *Journal of Nutrition*. 1992;122(3):467-472.

72. Van Milgen J. Modeling biochemical aspects of energy metabolism in mammals. *Journal of Nutrition*. 2002;132(10):3195-3202.

73. Veldhorst M, Smeets A, Soenen S, et al. Protein-induced satiety: Effects and mechanisms of different proteins. *Physiology and Behavior*. 2008;94(2):300-307.

74. Veldhorst MAB, Nieuwenhuizen AG, Hochstenbach-Waelen A, et al. Dose-dependent satiating effect of whey relative to casein or soy. *Physiology and Behavior*. 2009;96(4-5):675-682.

75. Veldhorst MAB, Nieuwenhuizen AG, Hochstenbach-Waelen A, et al. Comparison of the effects of a high- and normal-casein breakfast on satiety, 'satiety' hormones, plasma amino acids and subsequent energy intake. *British Journal of Nutrition*. 2009;101(2):295-303.

76. Veldhorst MAB, Westerterp KR, Westerterp-Plantenga MS. Gluconeogenesis and protein-induced satiety. *British Journal of Nutrition*. 2012;107(4):595-600.

77. Westerterp-Plantenga MS. Effects of extreme environments on food intake in human subjects. *Proceeding of the Nutrition Society*. 1999;58(4):791-798.

78. Westerterp-Plantenga MS, Nieuwenhuizen A, Tomé D, Soenen S, Westerterp KR, eds. Dietary protein, weight loss, and weight maintenance. ; 2009 *Annual Review of Nutrition*; No. 29.

79. Westerterp-Plantenga MS, Rolland V, Wilson SAJ, Westerterp KR. Satiety related to 24 h diet-induced thermogenesis during high protein/carbohydrate vs high fat diets measured in a respiration chamber. *European Journal of Clinical Nutrition*. 1999;53(6):495-502.

80. Wiggins RC, Fuller G, Enna SJ. Undernutrition and the development of brain neurotransmitter systems. *Life Sciences*. 1984;35(21):2085-2094.

81. Wren A, Bloom S. Gut hormones and appetite control. *Gastroenterology*. 2007;132(6):2116-2130.

82. Yeomans MR. Taste, palatability and the control of appetite. *Proceedings of the Nutrition Society*. 1998;57(4):609-615.

Chapter 2: Satiety from higher protein diets is dependent on the protein source

2.1 Introduction

Protein has been observed to suppress short-term food intake beyond what would be expected by an isoenergetic amount from carbohydrates and fats (Rolls et al. 1987, Bensaid et al. 2002, Anderson and Moore 2004). Diets high in protein are believed to decrease hunger and increase satiety, leading to decreased food intake. In a review of 14 studies that investigated the effects of high protein diets on satiety and subsequent energy intake, Halton and Hu (2004) reported that 11 found preloads of increased protein to increase subjective ratings of satiety and 8 found significantly decreased subsequent energy intake compared to lower protein preloads.

Much less attention has been given to whether protein sources may have varying effects on short-term satiety. The satiety effect from different protein sources has been most clearly demonstrated between whey and casein, with whey tending to be more satiating (Hall et al. 2003, Veldhorst et al. 2009). Postprandial investigation of absorption between the two protein sources show whey proteins are more rapidly digested, inducing a sharper rise in plasma amino acids compared to casein (Boirie et al 1997). Casein was also moted to coagulate in the stomach by precipitation with gastric acid, slowing down the rate of digestion and absorption (Westerterp-Plantenga et al. 2006).

Interestingly, Abou-Samra and colleagues reported casein and pea protein cause a greater decrease in food intake than whey protein (Abou-Samra et al. 2011). In their study, egg white protein was also included as a treatment, but subsequent food intake did not significantly differ from other tested protein sources. However, a liquid preload of egg white protein was reported to result in greater food intake than either a whey or soy protein preload with the whey preloads suppressing food intake greatest (Anderson et al. 2004). Postprandial profiles of anorexigenic

and orexigenic hormones has also been reported to differ between protein sources (Diepvens et al. 2008), suggesting peptide hormones to have a role in difference in satiety. The satiating effects of wheat gluten protein versus other protein sources have been used in two other studies where no significant difference was detected in appetite and satiety (Bensaid et al. 2002, Lang et al. 1998). In another study where overweight subjects were provided either an egg breakfast (18.4 g protein) or bagel-based breakfast (13.6 g protein), greater satiety was reported following the egg breakfast (Vander Wal et al. 2005). In addition, when provided the egg breakfast, energy intake at lunch, the rest of the day, and the following 36 hours were reduced in comparison to bagel-based breakfast.

Protein source may play a role in size of subsequent meal and satiety; however, study design factors, such as differences in amount of protein offered at preload and time to subsequent meal, make it difficult to compare across studies. The objective of the present study was to evaluate the influence of protein level and source on the size of subsequent meals. The hypothesis was that meal size would be reduced by higher protein diets and the reduction in meal size may differ between protein sources. Our study was designed determine whether a breakfast preload containing moderately high protein from egg white protein or wheat gluten protein influenced the size of the subsequent meal. Research on satiating effects of preloads tend to measure satiety by a visual analog scale of subjective feelings related to hunger and satiety, or by size and timing of subsequent meal. Because this study uses rats as an animal model, we intended to use the latter two factors, size and time of next meal, as indicators of satiety.

2.2 Materials and Methods

Animals. Male Sprague-Dawley rats (125 g; Charles River Laboratories) were singly housed in a temperature (26 ± 2 ˚C) controlled room and with free access to water. Upon arrival to our research facility, animals were trained to a reversed 12 h light:dark cycle (lights off at 1200) and to a meal-feeding schedule. Acclimation to the powdered diet and feeding schedule was designed to be a gradual process over 7-10 days. This started with pellet chow being replaced with powdered chow, food provided ad libitum for 10 hours of the dark cycle and progressed into being fed testing diet at restricted meal times. Food intake data was collected during this period to access efficiency of acclimation, and provided figures to based breakfast meal size off of. Food was removed one hour prior to the onset of the light cycle for an overnight fast. At one hour into the dark cycle, animal are given 30 minutes to consume a breakfast-like meal measured to 20% average daily intake of either one of three isocaloric diets. All animal studies were approved by the University of Illinois' Institutional Animal Care and Use Committee (IACUC) and are in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council).

Diet preparation. Diets were prepared to be comparable to AIN-93G (Reeves 1997) with methods adapted from Watson (1996). Separate mineral mixes were used to account for micronutrient differences in the egg white powder and wheat gluten powder. All diet components were purchased from Dyets, Inc. (Bethlehem, PA), with the exception of egg white powder and maltodextrin coming from Harlan (Indianapolis, IN). The basal diet was 60:20:20 (carbohydrate:protein:fat, as a % calories) with protein being provided equally by egg white protein (EW) and wheat gluten protein (WG). The two remaining diets were 45:35:20 with protein provided by either EW or WG.

Satiety study. Animals (n=12) were placed in The Comprehensive Laboratory Animal Monitoring Systems (CLAMS) (Oxymax; Columbus Instruments International, Columbus, OH, USA) that continuously quantified time and amount of food intake. Rats were allowed three days to acclimate to the cages before usable data was collected. Food intake during breakfast was closely monitored so that feeders manually were opened at one hour after the start of the dark phase and were closed as soon as the allotted 20% of average daily intake was consumed. The first phase of this study lasting 8 days during which post-prandial respiratory exchange ratio (RER) profile was collected will be discussed in Appendix 1. Following the last day of this of metabolic activity collection, the second phase focused on the size of a second meal of basal diet after being fed the different breakfast diets. This phase of the study lasted 6 days, with four animals receiving each of the breakfast diets on the first 3 days and diets alternated for the last 3 days. Feeders were removed following the breakfast period and replaced 15 minutes later with the Basal diet to consume ad libitum until one hour prior to the light phase. Meals were defined as being the total of all bouts in which consumption was greater than 0.03 grams and with the time between each bout being no longer than 10 minutes.

Preference study. A follow up study was conducted to assess whether the difference in food intake after breakfast was due to satiety, or whether food preference may have been involved. Rats were singly housed in plexiglass cages (30 x 30 x 38 cm) with the bottoms of the cages fitted with steel wired bottoms resting approximately one inch above the cage bottom in order to measure powdered diet spillage. Animals (n=24) were trained to the reversed light cycle, meal-feeding schedule, and transitioned from pellet AIN93 chow to powdered chow, but were not previously exposed to any of the three testing diets. Animals were randomly assigned to one of the three breakfast pretreatment groups (basal, EW, or WG). The 90 minute preference test

started 15 minutes after the breakfast period. The length of the preference testing period was determined based of the feeding behavior data collected from the previous study with the 90 minutes being a cutoff point in which animals tended to have completed the subsequent meal , yet had not started its next bouts of consumption. Using a cross over design, rats in each breakfast group received each combination of diets (EW/Basal, EW/WG, or Basal/WG) over three consecutive days. Diets in glass jars held by steel rings were attached by suction cups to opposite corners of the cage in random order and consumption was measured by the difference in mass of the jars before and after testing. Rats were provided powdered chow to consume ad libitum later in the day for 5.5 hours before food was removed one hour prior to light onset.

Statistical analysis. Main effect of breakfast treatment diet and subsequent food intake were analyzed by one-way analysis of variance (ANOVA) and Tukey's honest significance test. Values are presented as means ± SEM. Analysis were performed using SAS 9.2 (SAS Institute Inc., Cary, NC).

2.3 Results

In the first part of this study in which rats were housed in the metabolic cages, we took advantage of the scales below the feeders and its direct recording of time and amount of food consumed. Using CLAMS provided insight as to the feeding behavior following consumption of each testing diet for breakfast without disturbances by investigators. When rats were provided the EW diet for breakfast, they consumed the meal slower ($P<0.0001$), taking the entire 30 minutes given (29.4 ± 0.4 minutes), than basal breakfast rats (15.3 ± 0.5 minutes) and WG breakfast rats (20.4 ± 1.1 minutes). Rats given the EW breakfast also consumed less ($P<0.0001$) of the calculated 20% daily total allotted for breakfast (67.9 ± 4.4 % breakfast consumed). The

size of the following meal of the basal diet was greater ($p<0.01$) when animals were provided the WG breakfast (6.8 ± 1.0 g) than when fed either the basal breakfast (4.8 ± 0.6 g) or the EW breakfast (2.9 ± 0.4 g) (Fig. 1). In addition, rats provided the basal and WG breakfast spent significantly more time consuming the following meal than rats provided the EW breakfast (45 ± 6.7, 50 ± 7.0, and 25 ± 3.2 min respectively, $P<0.01$). The effect of breakfast detected in the second meal size was not apparent in the third meal. Neither were significant differences observed in total daily consumption between groups. Because of these observations, a preference versus satiety study was conducted.

In the subsequent study, rats finished the breakfast ration on most occasions; rats provided Basal diet finished their breakfast meal 92% of the time, EW 75% of the time, and WG 79% of the time. When given two options in the period following breakfast, animals fed the WG diet for breakfast consumed more ($P<0.01$) than when fed the basal diet (6.3 ±0.6 g vs. 4.7 ± 0.3 g). Rats fed EW consumed less ($P<0.05$; 3.2 ± 0.3 g) (Fig. 2). When provided a choice that included the EW diet (EW/Basal or EW/WG), rats consumed less of the EW diet ($P<0.01$) (Fig. 3). Basal and WG diets were equally preferred, as seen by the nearly equal consumption when both diets were provided during the preference testing period. There were no differences between groups in regards to breakfast meal size, spillage, or total daily consumption.

2.4 Discussion

The degree of satiation developed by higher protein diets is dependent on the protein source. Food intake following a breakfast of 35% energy from egg white protein was less than when animals were fed isocaloric breakfast of 35% energy from wheat gluten protein or a basal diet with 20% energy made up equally of egg white protein and wheat gluten protein.

Interestingly, rats provided the wheat gluten diet for breakfast consumed more in the following period than rats fed the Basal diet. These results supports satiation from high protein diets is influenced by the protein source, with the egg white protein being more satiating than wheat gluten protein. We were initially interested in both the size of, and time to, the subsequent meal to breakfast as indicators of satiety. However, when feeders were open or made available soon after the breakfast period, rats immediately began eating. This may have been due to disturbances made by investigators or perhaps the allotted 20% of average daily intake was not sufficient after the 14 h overnight fast. In any case, there were significant difference in the size of subsequent meal (CLAMS study) and the amount consumed in following period (preference study).

Many of the prior studies comparing protein sources effect on satiety have focused on two milk protein, casein and whey. The limited number of other studies including non-milk protein sources had mixed results. Egg white protein is considered one of the highest quality protein sources, receiving the full PDCAAS value of 100, with wheat gluten protein being a plant protein source being one of the lower quality protein (Schaafsma 2000). Other protein sources receive scores that generally fall between these two, with animal protein sources falling on the upper end. Even so, several studies have found egg white protein to be less satiating (Anderson et al. 2004, Abou-Samra et al. 2011). However, Semon et al. found that rats switched from a low protein diet to a diet where 40% energy came from egg white protein had the most depressed food intake 90 to 180 min. later, as compared to lactoalbumin, soy, and casein proteins (Semon et al. 1987). And on the other hand, Bensaid et al. conducted a study comparing oral loads of 50% wheat gluten protein and 50% total milk protein and discovered no difference in

effect on food intake (Bensaid et al. 2002). This suggests that hunger, satiety, and subsequent food intake has little to do with protein quality.

Lang et al. is the only other study we are aware of comparing satiating effects of egg white protein with wheat gluten protein; although they also compared casein, gelatin, soy, and pea protein (Lang et al. 1998). In their study, no differences were seen in hunger, satiety, or 24 h energy and macronutrient intake following a lunch of manipulated protein content. This can likely be explained by the large testing lunch (mean of 5193 kJ) provided, which consisted of roughly 22% protein, and only 64% of the protein content manipulated with the testing proteins. A buffet dinner was offered 8 hours later to measure energy intake. The extensive time may have caused subjects to become equally hungry. Lastly, the variety of foods present at dinner may be a confounding factor by stimulating sensory-specific satiety, the tendency for subjects to have increased desires to eat food that has not been tasted (Rolls 1984).

When given a choice, rats in each breakfast treatment group consumed less of the EW diet, preferring the alternate diet regardless of the choice provided. No difference was observed between the amount of basal and WG chosen, but less of the EW diet was consumed. And although the EW diet seemed to be the least preferred of the three diets, prior exposure to the EW diet for breakfast led rats to consume more of EW during the following meal than when provided the other two testing diets for breakfast. Rats in all three breakfast groups were slightly more likely to consume the same type of diet provided at breakfast when given the choice during preference testing, although not at significant value. This suggest that the egg white diet may be less preferred, but not aversive and there may be a small role of neophobia. Semon et al. expressed concerns with initial acceptability of 40% egg white protein and 40% soy protein diets by rats, but showed intake rate to return to normal and similar to rats fed other 40% protein diets

after 30 minutes (Semon et al. 1987). They also conducted a diet choice test ad libitum over 7 days comparing soy, casein, lactalalbumin and egg white protein, with the 40% casein protein diet seemingly being most preferred, but was confounded by rats being trained to on a 15% casein protein diet. Our experiment was designed for rats to be provided standard rodent chow (15% energy from casein protein) during training and non-testing periods to avoid familiarization towards any one testing diet. Regardless of the choice provided during the immediate subsequent period, total intake was smallest in the EW breakfast group. To summarize the preference study results, although the egg white diet was not as preferred as the other two treatment diets, it induced the greatest satiety.

There are a couple of possible mechanism that may be involved in the difference in satiety of the two protein sources used in the present study. Amino acid composition may also be involved, as it has been suggested that increased leucine can induce satiety (Layman 2003). Dietary protein provides the body with its sole source of leucine, which can act as a signaling molecule to stimulate mammalian Target of Rapamycin (mTOR) to signal protein synthesis in certain cells (Woods et al. 2008). Cota and colleagues (2006) reported central administration of leucine to increase hypothalamic mTOR signaling and decrease in food intake. Between the two protein sources used in the present study, the egg white protein has 8.4 g leucine per 100 g protein, whereas the wheat gluten protein has 6.9 g leucine per 100 g protein. Taking into consideration the amount of diet we provided to rats at breakfast, the amount of leucine ingested by rats in each group is 0.10 g, 0.086 g, and 0.054 g for the egg white diet, wheat gluten diet, and Basal diet respectively. There is slightly less leucine in the wheat gluten diet compared to the egg white diet that seems to explain increase food intake in the wheat gluten group, however the amount of leucine from the wheat gluten diet is still greater than that of the Basal diet. Rats

receiving the wheat gluten breakfast had a higher relative dose of leucine than the Basal group, yet was consuming more. This rules out leucine being the predominant mechanism inducing satiety in this study.

Bioavailability may differ between protein sources due to differences in rates of digestion and absorption (Benelam 2009). There is the possibility of wheat gluten protein having lower bioavailability that would decrease circulating leucine, which cannot be dismissed without further analysis of post prandial plasma amino acids. Consequently, these factors could alter the rate of post-prandial hormone signaling and the rate of gastric emptying, both of which have roles in communicating energy homeostasis via the gut-brain axis. Gastric volume alone can cause termination of eating independent of post-gastric, intestinal signaling (Ritter 2004). Additional studies are needed to identify the specific mechanism underlying the satiety effects of protein, as well as the contributing factors between protein sources.

2.5 Literature Cited

1. Abou-Samra R, Keersmaekers L, Brienza D, Mukherjee R, Macé K. Effect of different protein sources on satiation and short-term satiety when consumed as a starter. *Nutrition Journal*. 2011;10(1).

2. Anderson GH, Moore SE. Dietary proteins in the regulation of food intake and body weight in humans. *Journal of Nutrition*. 2004;134(4):974S-979S.

3. Anderson GH, Tecimer SN, Shah D, Zafar TA. Protein source, quantity, and time of consumption determine the effect of proteins on short-term food intake in young men. *Journal of Nutrition*. 2004;134(11):3011-3015.

4. Benelam B. Satiation, satiety and their effects on eating behaviour. *Nutrition Bulletin*. 2009;34(2):126-173.

5. Bensaid A, Tomé D, Gietzen D, et al. Protein is more potent than carbohydrate for reducing appetite in rats. *Physiology and Behavior*. 2002;75(4):577.

6. Boirie Y, Dangin M, Gachon P, Vasson M-, Maubois J-, Beaufrère B. Slow and fast dietary proteins differently modulate postprandial protein accretion. *Proceedings of the National Academy of Sciences of the United States of America*. 1997;94(26):14930-14935.

7. Cota D, Proulx K, Smith KAB, et al. Hypothalamic mTOR signaling regulates food intake. *Science*. 2006;312(5775):927-930.

8. Diepvens K, Häberer D, Westerterp-Plantenga M. Different proteins and biopeptides differently affect satiety and anorexigenic/orexigenic hormones in healthy humans. *International Journal of Obesity*. 2008;32(3):510-518.

9. Hall WL, Millward DJ, Long SJ, Morgan LM. Casein and whey exert different effects on plasma amino acid profiles, gastrointestinal hormone secretion and appetite. *British Journal of Nutrition*. 2003;89(2):239-248.

10. Halton TL, Hu FB. The effects of high protein diets on thermogenesis, satiety and weight loss: A critical review. *Journal of the American College of Nutrition*. 2004;23(5):373-385.

11. Lang V, Bellisle F, Oppert J-, et al. Satiating effect of proteins in healthy subjects: A comparison of egg albumin, casein, gelatin, soy protein, pea protein, and wheat gluten. *American Journal of Clinical Nutrition*. 1998;67(6):1197-1204.

12. Layman DK. The role of leucine in weight loss diets and glucose homeostasis. *Journal of Nutrition*. 2003;133(1):261S-267S.

13. Reeves PG. Components of the AIN-93 diets as improvements in the AIN-76A diet. *Journal of Nutrition*. 1997;127(5 SUPPL.):838S-841S.

14. Ritter RC. Gastrointestinal mechanisms of satiation for food. *Physiology and Behavior*. 2004;81(2):249-273.

15. Rolls BJ. Sensory-specific satiety. *Nutrition Review*. 1986;44(3):93-101.

16. Rolls BJ, Hetherington M, Burley VJ. The specificity of satiety: The influence of foods of different macronutrient content on the development of satiety. *Physiology and Behavior*. 1988;43(2):145-153.

17. Schaafsma G. The protein digestibility-corrected amino acid score. *Journal of Nutrition*. 2000;130(7):1865S-1867S.

18. Semon BA, Leung PMB, Rogers QR, Gietzen DW. Effect of type of protein on food intake of rats fed high protein diets. *Physiology and Behavior*. 1987;41(5):451-458.

19. Vander Wal JS, Marth JM, Khosla P, Jen K-C, Dhurandhar NV. Short-term effect of eggs on satiety in overweight and obese subjects. *Journal of the American College of Nutrition*. 2005;24(6):510-515.

20. Veldhorst M, Smeets A, Soenen S, et al. Protein-induced satiety: Effects and mechanisms of different proteins. *Physiology and Behavior*. 2008;94(2):300-307.

21. Watson RR. *Trace elements in laboratory rodents.* Boca Raton, FL: CRC Press; 1996.

22. Westerterp-Plantenga M, Luscombe-Marsh N, Lejeune MPGM, et al. Dietary protein, metabolism, and body-weight regulation: Dose–response effects. *Int J Obes*. 2006;30:S16-S23.

23. Woods SC, Seeley RJ, Cota D. Regulation of food intake through hypothalamic signaling networks involving mTOR. *Annual Review of Nutrition*. 2008;28:295-311.

2.6 Tables and figures

Preference testing choices		**Breakfast**		
		Basal	**EW**	**WG**
Basal vs. WG	**Basal (g)**	2.7 ± 0.7	1.4 ± 0.3	2.8 ± 1.0
	WG (g)	2.8 ± 0.6	1.1 ±0.3	3.3 ± 0.9
	TOTAL (g)	*5.4 ± 0.4*	*2.4 ± 0.5*	*6.1 ± 1.0*
Basal vs. EW	**Basal (g)**	4.2 ± 0.6	1.4 ± 0.3	5.2 ± 1.3
	EW (g)	0.4 ±0.1	1.5 ± 0.4	0.2 ± 0.1
	TOTAL (g)	*4.6 ±0.6*	*2.9 ± 0.5*	*5.7 ± 1.4*
EW vs. WG	**EW (g)**	0.9 ± 0.5	0.8 ± 0.3	0.9 ± 0.4
	WG (g)	2.9 ± 0.8	3.1 ± 0.8	5.6 ± 0.9
	TOTAL (g)	*4.3 ± 0.5*	*4.2 ± 0.7*	*6.8 ± 0.7*

Table 1. Amount consumption between choices of testing diets, after being given breakfast treatment. Mean ± SEM.

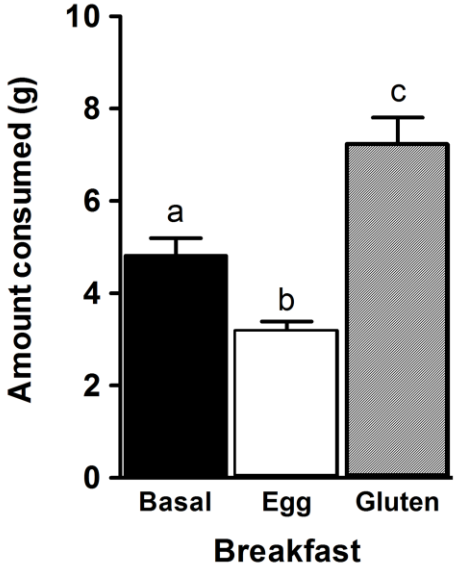

Figure 1. Effect of breakfast on the size of the subsequent meal of the basal diet in the first study (n = 8/treatment). Bars with different superscripts are different ($P < 0.05$).

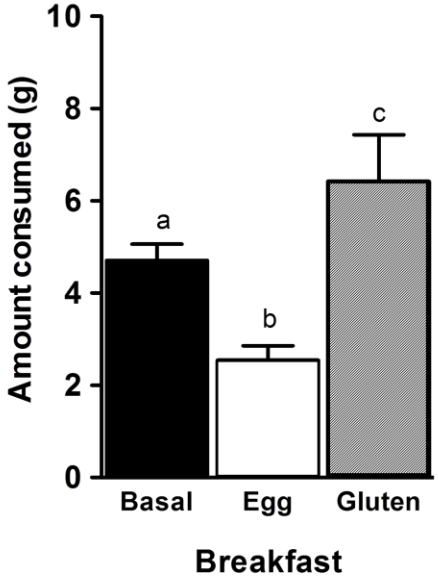

Figure 2. Effect of breakfast on total amount consumed in the preference testing period (n = 24/treatment). Bars with different superscripts are different ($P < 0.05$).

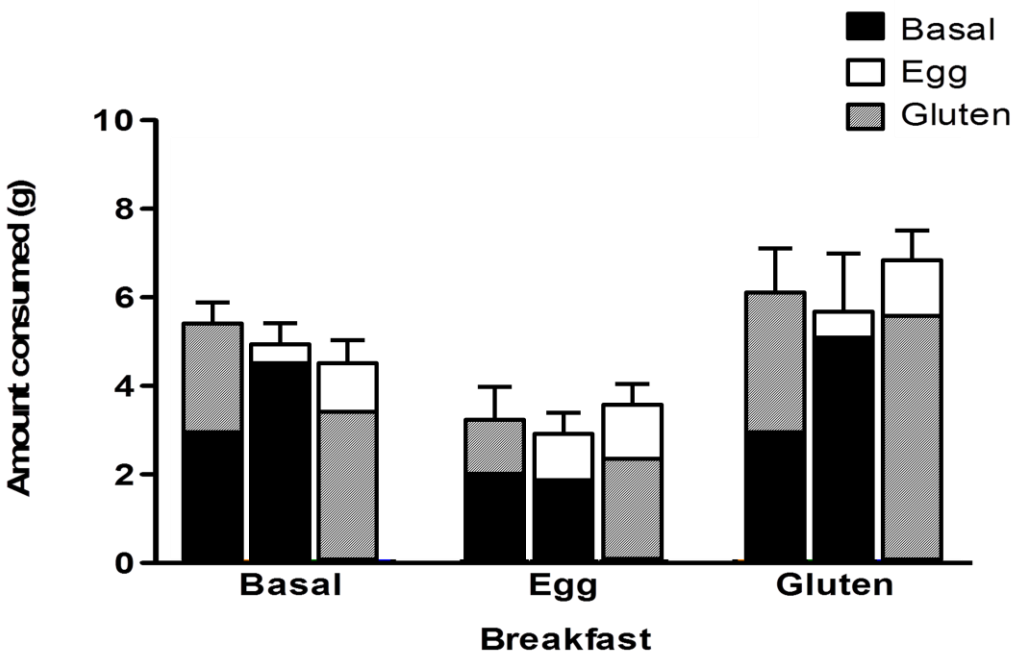

Figure 3. Amount of each diet consumed during preference testing period within choice pairs (n = 8/pairing/breakfast).

Chapter 3: The source of dietary protein for breakfast influences subsequent learning and memory performance

3.1 Introduction

Breakfast consumption has been associated with beneficial effects on memory, attention and self-reported energy, positive mood, increased interest and fullness (Cooper et al. 2011, Widenhorn-Muller et al. 2008). Omission of breakfast extends overnight fasts, leading to lower blood glucose and insulin concentrations, as well as potential changes in other metabolites (ie. neurotransmitters) that may disrupt fuel and nutrient availability involved in cognitive functioning processes. Educators, parents, and policy makers are invested in academic performance to increase scores in school, which has led to many areas implementing school breakfast programs (Pollitt and Mathews 1998).

Lesser understood is how breakfast components can be manipulated to maximize benefits from the "most important meal of the day." Fisher et al. (2002) conducted a study where young adult subjects were provided isoenergetic breakfast meals that varied in protein to carbohydrate ratios; higher carbohydrate (4:1 carbohydrate to protein), balanced (1:1), and higher protein (1:4). Subjects provided the test meal of balanced protein and carbohydrate ratio performed best on the central task, corresponding to the highest overall blood tyrosine to large neutral amino ratio. Decreased carbohydrate composition at breakfast created more stable postprandial profiles of glucose and glucagon to insulin ratio corresponded better cognitive performance. Their 'balanced' meal was considered to be the moderate treatment of the three; however, a 1670 kJ meal that is 50% protein can be considered to be high protein (Eisenstein et al 2002, Westerterp-Plantenga et al 2009). In their study, milk protein and egg white protein were used in

combination. This demonstrates protein level at breakfast influencing levels of hormones and metabolic products from the oxidation of dietary carbohydrates and proteins.

Dietary proteins can modulate available glucose (Layman and Baum 2004) and amino acid precursors to monoamines (Fernstrom and Fernstrom 2007) having behavioral effects. With the difference in satiety between protein sources discussed in Chapter 2, and macronutrients of diets effecting multiple brain functions, including mood, cognition, and arousal (Dye et al. 2000), we were interested in dietary protein influence on cognitive function. The objective of the present study was to determine whether breakfast, and especially the protein composition of breakfast, influenced cognitive performance in a rat model. We expected breakfast to positively impact performance in learning and memory tasks. Based on the higher level of tyrosine to large neutral amino acid in the egg white protein (Table 1), and its potential to provide precursors for catecholamine neurotransmitters, we hypothesize rats fed a breakfast containing egg white protein to perform better on the Barnes maze.

The Barnes maze tests for visual-spatial learning and memory performance, a task which is hippocampal dependent and requires visual cues in the testing room to successfully complete (Barnes 1979). The goal for the rat on the maze is to escape from the bright lights and open circular platform to a small dark tunnel located under one of the 20 holes around the perimeter of the platform. Rats that have better learning and memory capabilities would therefore find the correct hole leading to the escape tunnel more quickly, in shorter amount of time, and taking a shorter path length. This task is similar to that of the Morris water maze and the radial arm maze, which also test visual learning and memory functions. Proponents of the Barnes maze argue that it eliminates the stress induced by a strong aversive stimulus (i.e. water or shock) that could

potentially affect performance in the task. The radial arm maze requires food award for reinforcement, which would be a confounding factor with our study design.

3.2 Materials and Methods

Animals. Male Sprague-Dawley rats (125 g; Charles River Laboratories) were singly housed in plexiglass cages (30 x 30 x 38 cm) having corncob bedding in a temperature (26 ± 2 ˚C) controlled room and ad libitum access to water. Upon arrival to our research facility, animals were entrained to a reversed 12 h light:dark cycle (lights off at 1200) and to a meal-feeding schedule. Acclimation to the powdered diet and feeding schedule was designed to be a gradual process over 7-10 days. This started with pellet chow being replaced with powdered chow, food provided ad libitum for 10 hours of the dark cycle and progressed into being fed testing diet at restricted meal times. Food intake data was collected during this period to access efficiency of acclimation, and provided figures to based breakfast meal size off of. Food was removed one hour prior to the onset of the light cycle for an overnight fast. At one hour into the dark cycle, animal are given 30 minutes to consume a breakfast-like meal measured to 20% average daily intake of either one of the three testing diets. The basal diet was 60:20:20 (carbohydrate:protein:fat, as a % calories) with protein being provided equally by egg white protein and wheat gluten protein. The two remaining diets were 45:35:20 with protein provided by either egg white or wheat gluten. In addition, a fourth no breakfast (NB) group was included. All animal studies were approved by the University of Illinois' Institutional Animal Care and Use Committee (IACUC) and are in accordance with the Guide for Care and Use of Laboratory Animals (National Research Council).

Barnes maze. Learning and memory was assessed using the Barnes maze soon after the breakfast period (n=9/treatment). The maze platform is black, circular, 122 cm in diameter, and has 20 evenly spaced holes (10 cm diameter) around its edge. All holes are uniform in appearance, with only one hole leading to a removable black escape tunnel located at the underside of the hole. Visual cues, including intentional images placed on the walls and cues that are endogenous to the room, were not moved between trials of the same animal. Animals are brought into a darkened room. Bright lights and a standing fan are turned on during the task to encourage animals to find the escape tunnel and immediately turned off once the rat enters the target hole. The test paradigm consists of a habituation session on the first day, followed by 4 learning trial per day for 4 days, with a probe trial the day following the last learning day. For the habituation session, used to train the animal to the existence of the escape tunnel, animals were placed in the escape tunnel for one minute with the lights turned off before being introduced to the trial routine. During each of the trials, animals were placed in a black start chamber at one of the three start location on the maze and timing starts once the chamber has been lifted off and the lights turned on. Trials were recorded with a video camera attached to the ceiling, as TopScan software (Clever Sys Inc., Reston, VA) was used to measure latency and path length to target hole. Trial analysis ended when the front legs of the animal entered the escape tunnel or 180 seconds has elapsed. As soon as the animal successfully enters the escape tunnel, lights and fan are turned off and the animal remains in the escape tunnel for 60 seconds. Animals that are unsuccessful by the end of the 180 seconds are guided into the correct hole by the investigator before the lights and turned off with the animal in the escape tunnel for 60 seconds. Between each learning trial, there is approximately 15 minute intertrial interval as other animals undergo the same trial. The probe trial is conducted with the same procedure with the exception that the escape tunnel is removed

from the maze and the animal is given 90 seconds to search for the correct hole that previously led to the escape tunnel. Before and after each trial the maze is cleaned with 70% ethanol solution and rotated to eliminate olfactory cue.

Statistical analysis. Data were analyzed using SAS 9.2 (SAS Institute Inc., Cary, NC). Because measurements were taken from the same animals over multiple trials and day, maze acquisition data was analyzed using repeated measures analysis. Average latency (s), path length (mm), and speed (mm/s) to enter target hole during trials for learning days was analyzed by repeated measures analysis with Day as the within-subject factor and Treatment (Basal, NB, Egg, Gluten) as the between-subject factor. To assess working memory, the same measurements were analyzed by repeated measures analysis with trials as the within-subject factor. For the probe trial, latency (s), path length (mm), and speed (mm/s) in the target quadrant was analyzed using one-way ANOVA with treatment (Basal, NB, Egg, Gluten) as the factor. An alpha level of $P < 0.05$ was considered statistically significant. Because testing environment was altered after the first cohort of rats, to include brighter lights and a standing fan, the first cohort of three Basal-breakfast and three "no breakfast" rats were removed from statistical analysis.

3.3 Results

Rats in all 4 treatment groups learned to locate the target escape hole as indicated by decreased latency (Fig. 1; $F_{3,78}$=49.32; P<0.0001) and path length (Fig. 2, top; $F_{3,78}$=26.70; P<0.0001) to the target hole across the four training days. Speed increased across training days also (Fig. 3; $F_{3,78}$=23.70; P<0.0001). Main effects for latency and path length to the target hole were not significantly different across all four breakfast treatment groups. There was a significant main effect detected for speed (Fig. 3; $F_{3,78}$=3.17; P<0.05). Rats fed the gluten diet for

breakfast moved significantly more quickly on the maze than rats in the Basal breakfast and no breakfast group. There was also a trend ($P<0.1$) for a Treatment x Day interaction for path length. Rats provided the egg white diet for breakfast traveled a shorter path length to find the correct hole leading to the escape tunnel and animals not provided a breakfast took a longer path length (Fig. 2, top).

Analysis of the 4 trials on the first day showed a significant treatment main effect for path length to target hole across trials (Fig. 2, bottom; $F_{3,78}=3.83$; $P<0.05$), with the egg white and basal breakfasts showing reduced path length at each trial as compared to the gluten and no breakfast treatments.

In the probe trial, all treatment groups spent significantly more time in the target quadrant than would be expected by chance, indicating that the animals learned spatially where the hidden escape hole was located(Fig. 4; $P<0.0001$). A significant main effect (Fig. 4; $F_{3,24}=3.18$; $P<0.05$) indicated that rats provided the Basal and egg white diets spent more time in the target quadrant than rats in the other two treatment groups. Moreover, rats that had not been provided breakfast took longer (Fig. 5; $F_{3,24}=5.92$; $P<0.005$) to get to the hole where the escape tunnel had been on previous testing days as compared to the other three groups. Main effects for path length within the target quadrant were not significant. However, rats provided the wheat gluten diet for breakfast traveled a longer total path length (Fig. 6; $F_{3,26}=8.01$; $P<0.001$) than no breakfast and egg white diet breakfast rats. This translated to gluten fed rats also traveling faster (Fig. 7; $F_{3,26}=7.97$; $P<0.001$) during the probe trial as compared to the no breakfast and egg white diet groups.

3.4 Discussion

These results support the concept that having breakfast is important for improved cognitive functions in adolescents. Rats that were not provided breakfast were less active, as indicated by their slower speed throughout testing, and overall traveling a greater path length before finding the location of the correct hole. Longer path length during the first day of learning suggests compromised working memory, whereas longer latency to reach the location of the previously correct hole during the probe trial nearly 24 hours after the last learning trial suggests impaired long term memory. The present results support findings of a review conducted by Hoyland et al. (2009). Their group conducted a thorough review of breakfast consumption on cognitive performance of children and adolescent in which 28 studies were reviewed for acute effects of breakfast versus no breakfast. Most of these studies demonstrated breakfast having positive effects, and effects were most evident in memory and attention tasks and especially in the late morning period. In our study, rats in the no breakfast group tended to perform more poorly than rats in the Basal breakfast group.

Although breakfast proved to be beneficial in most of these studies, the greatest improvements in cognitive performance following breakfast consumption were most evident at-risk or undernourished children and adolescents (Pollitt and Mathews 1998, Bellisle 2004, Hoyland et al. 2009). Children and adolescents from families of lower socioeconomic status who skipped breakfast tended to have poorer attention and learning and memory performance. Breakfast omission didn’t impact performance as sharply in adequately nourished children, correlating to familial socioeconomic status. This causes difficulties in bringing about a conclusion to the amount of impact breakfast has to cognitive performance. In the present study, we developed a rat model replicating the negative effects of missing breakfast; poorer

performance on the Barnes maze. The use of this rat model may prove valuable to investigate the underlying neurobiology of memory and learning and eliminates many of the confounding factors commonly found in studies with human subjects.

The present results extend previous findings by providing some indication that a breakfast of moderately high levels of egg white protein being beneficial to learning and memory performance. On the first day of learning, rats in the egg white diet group traveled significantly shorter path lengths to reach the target holes indicating better working memory. Although rats in all treatment groups eventually learned to find the target hole, rats provide the egg white diet seemed to have learned more quickly. Studies comparing the effects of different macronutrient compositions in breakfast are even fewer, and we are unaware of any other study making comparisons between breakfasts of different protein sources. Fisher et al. (2002) varied a morning meal's protein to carbohydrate ratios and reported short term memory was best after a higher protein (1:4 carbohydrate to protein) testing meal. Better performance, as when compared to the other two treatment groups of lower protein levels, corresponded to more stable levels of glucose and glucagon to insulin ratio. Mahoney et al. (2005) reported oatmeal for breakfast, versus ready-to eat cereal, improved spatial memory for both boys and girls and improved short term memory in girls. They suggested increased protein and fiber, slower rate of digestion and more sustained glucose levels in the oatmeal breakfast to have been factors, which is supported by other studies finding breakfast of low glycemic index that slowly release glucose in the blood to have beneficial effects on learning and memory performance (Nabb and Benton 2006, Micha et al. 2011, Cooper et al. 2012). Benton and colleagues also demonstrated better memory and learning performance in humans and rats following low glycemic index breakfasts (Benton et al.,

2003). The involvement of protein in the results of the present study may be modulation of glucose availability, although we did not collect blood values for comparison in our study.

No differences in learning trial latency or path length were detected between protein sources. However, rats fed the wheat gluten diet moved faster on the maze both during learning and probe trial. Difference in speed between egg white and wheat gluten fed rats did not reach significance during training day, but wheat gluten fed rats did move significantly faster during the probe trial. Rats fed the egg white diet were also observed to have better working memory and spent more time in the target quadrant during the probe trial compared to rats provided the wheat gluten diet for breakfast. It is likely animals fed the wheat gluten diet for breakfast moved faster leading them to find the hole more quickly by chance. With protein sources being the only difference between the wheat gluten and egg white breakfast treatment group, differences in amino acid composition between the two diets may likely play a role in behavioral response following breakfast. Variations in amino acid composition of dietary proteins can change levels of uptake across the blood brain barrier with comparable levels of neurotransmitter synthesis and ultimately eliciting effects on mood and cognition (Fernstrom 2012). In our prior study, rats fed the wheat gluten diet for breakfast were less satiated than rats fed Basal or egg white diet breakfast, as indicated by increased subsequent meal size. The results from rats fed wheat gluten moving more quickly during this study, taken together prior observations of feeding behavior in this group, suggests there may be underlying biochemical or physiological differences in metabolism and that anxiety may be a factor in this group.

This study extends current understandings by strengthening the assertion that breakfast and the composition of the breakfast meal can affect cognitive function and should be considered in trying to maximize its benefits. Furthermore, this study demonstrated the use of rat model as

being a reliable model for this field of study where research with human subjects has left many unanswered questions. This study has provided promising data and can be strengthened by increasing sample sizes. Future investigation can use the rat model to evaluate underlying mechanisms of the effect of breakfast on learning and memory.

3.5 Literature Cited

1. Barnes CA. Memory deficits associated with senescence: A neurophysiological and behavioral study in the rat. *Journal of Comparative and Physiological Psychology*. 1979;93(1):74-104.

2. Bellisle F. Effects of diet on behaviour and cognition in children. *British Journal of Nutrition*. 2004;92(SUPPL. 2):S227-S232.

3. Benton D, Ruffin M-, Lassel T, et al. The delivery rate of dietary carbohydrates affects cognitive performance in both rats and humans. *Psychopharmacology (Berlin)*. 2003;166(1):86-90.

4. Cooper SB, Bandelow S, Nute ML, Morris JG, Nevill ME. Breakfast glycaemic index and cognitive function in adolescent school children. *British Journal of Nutrition*. 2012;107(12):1823-1832.

5. Cooper SB, Bandelow S, Nevill ME. Breakfast consumption and cognitive function in adolescent schoolchildren. *Physiology and Behavior*. 2011;103(5):431-439.

6. Dye L, Lluch A, Blundell JE. Macronutrients and mental performance. *Nutrition*. 2000;16(10):1021-1034.

7. Eisenstein J, Roberts SB, Dallal G, Saltzman E. High-protein weight-loss diets: Are they safe and do they work? A review of the experimental and epidemiologic data. *Nutrition Review*. 2002;60(7 I):189-200.

8. Fernstrom JD. Large neutral amino acids: Dietary effects on brain neurochemistry and function. *Amino Acids*. 2012:1-12.

9. Fernstrom JD, Fernstrom MH. Tyrosine, phenylalanine, and catecholamine synthesis and function in the brain. *Journal of Nutrition*. 2007;137(6):1539S-1547S.

10. Fischer K, Colombani PC, Langhans W, Wenk C. Cognitive performance and its relationship with postprandial metabolic changes after ingestion of different macronutrients in the morning. *British Journal of Nutrition*. 2001;85(3):393-405.

11. Hoyland A, Dye L, Lawton CL. A systematic review of the effect of breakfast on the cognitive performance of children and adolescents. *Nutrition Research Reviews*. 2009;22(2):220-243.

12. Layman DK, Baum JI. Dietary protein impact on glycemic control during weight loss. *Journal of Nutrition*. 2004;134(4):968S-973S.

13. Mahoney CR, Taylor HA, Kanarek RB, Samuel P. Effect of breakfast composition on cognitive processes in elementary school children. *Physiology and Behavior*. 2005;85(5):635-645.

14. Micha R, Rogers PJ, Nelson M. Glycaemic index and glycaemic load of breakfast predict cognitive function and mood in school children: A randomised controlled trial. *British Journal of Nutrition*. 2011;106(10):1552-1561.

15. Nabb S, Benton D. The influence on cognition of the interaction between the macro-nutrient content of breakfast and glucose tolerance. *Physiology and Behavior*. 2006;87(1):16-23.

16. Pollitt E, Mathews R. Breakfast and cognition: An integrative summary. *American Journal of Clinical Nutrition*. 1998;67(4):804S-813S.

17. Westerterp-Plantenga MS, Nieuwenhuizen A, Tomé D, Soenen S, Westerterp KR, eds. Dietary protein, weight loss, and weight maintenance. 2009; *Annual Review of Nutrition*; No. 29.

18. Widenhorn-Müller K, Hille K, Klenk J, Weiland U. Influence of having breakfast on cognitive performance and mood in 13-to 20-year-old high school students: Results of a crossover trial. *Pediatrics*. 2008;122(2):279-284.

3.6 Tables and figures

	Egg White Protein	**Wheat Gluten Protein**
Isoleucine (Lle)	5.9	3.5
Leucine (Leu)	8.4	6.9
Methionine (Met)	3.8	1.2
Phenylalanine (Phe)	6.1	4.7
Tryptophan (Trp)	1.5	1.1
Tyrosine (Tyr)	3.9	1.7
Valine (Val)	7.2	4.3
Tyr: LNAA	**0.11**	**0.07**

Table 2. Large neutral amino acid composition between protein sources expressed as grams/100 grams of protein.

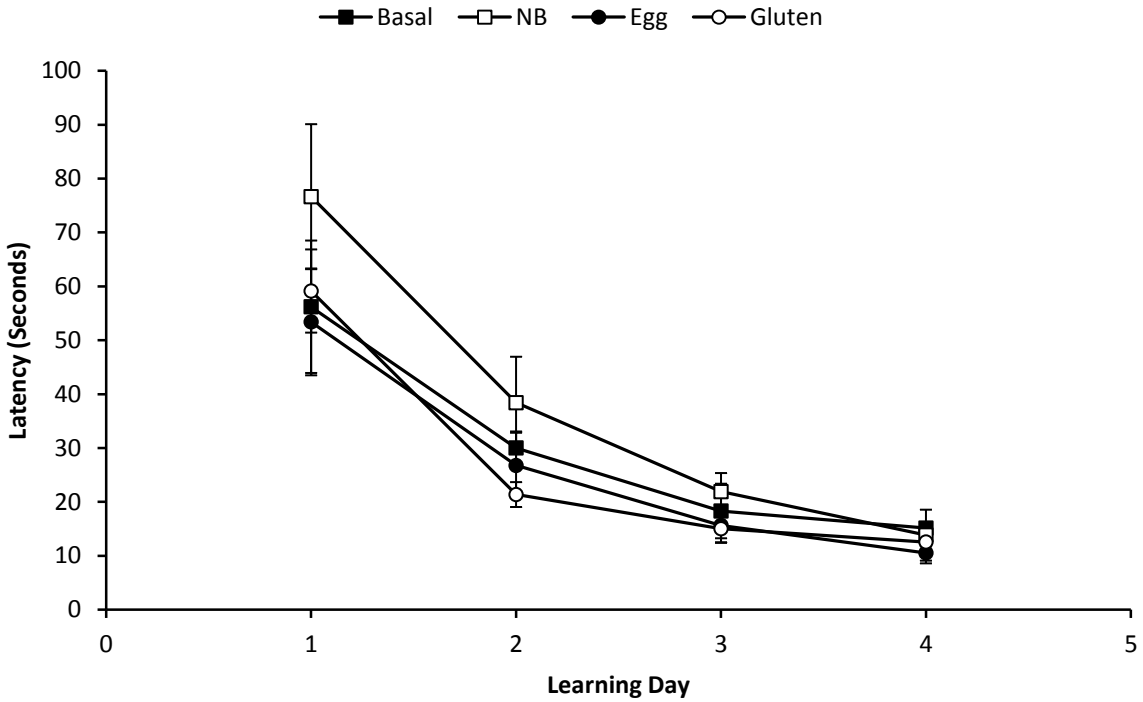

Figure 4. Average daily latency (sec) to target hole during across learning days (n = 6-9/treatment). Rats across all treatment groups had decreased ($P < 0.0001$) latency to the target hole across the four days of acquisition. Main effects were not significantly different across treatment groups. Bars represent mean ± SEM.

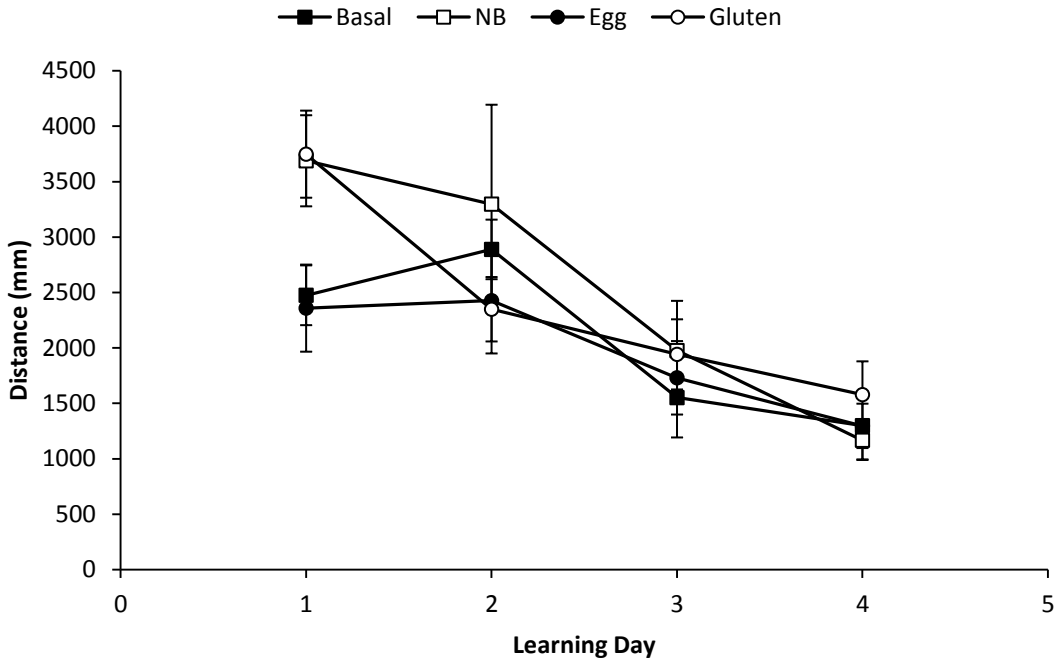

Figure 5. Average daily path length (mm) to target hole during across learning days (n = 6-9/treatment). Rats across all treatment groups had decreased ($P < 0.0001$) path length to the target hole across the four days of acquisition. Main effects were not significantly different across treatment groups. Bars represent mean ± SEM.

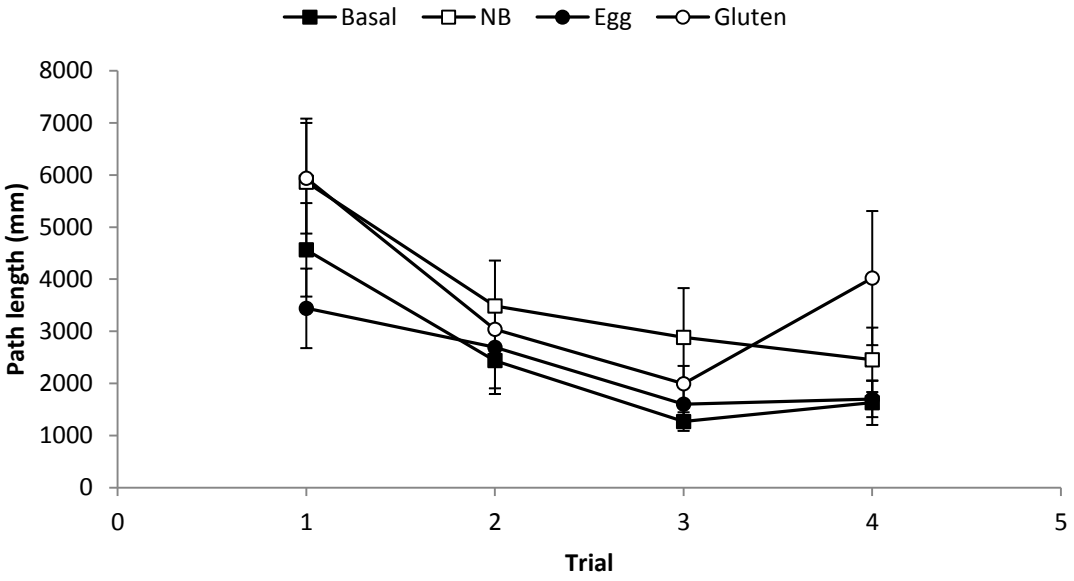

Figure 6. Average daily path length (mm) to target hole during across trials within the first learning day (n = 6-9/treatment). Repeated measures analysis showed significant main effect for path length to target hole across trials ($P < 0.05$). Egg white breakfasts showed reduced path length compared to the gluten and no breakfast treatments. Bars represent mean ± SEM.

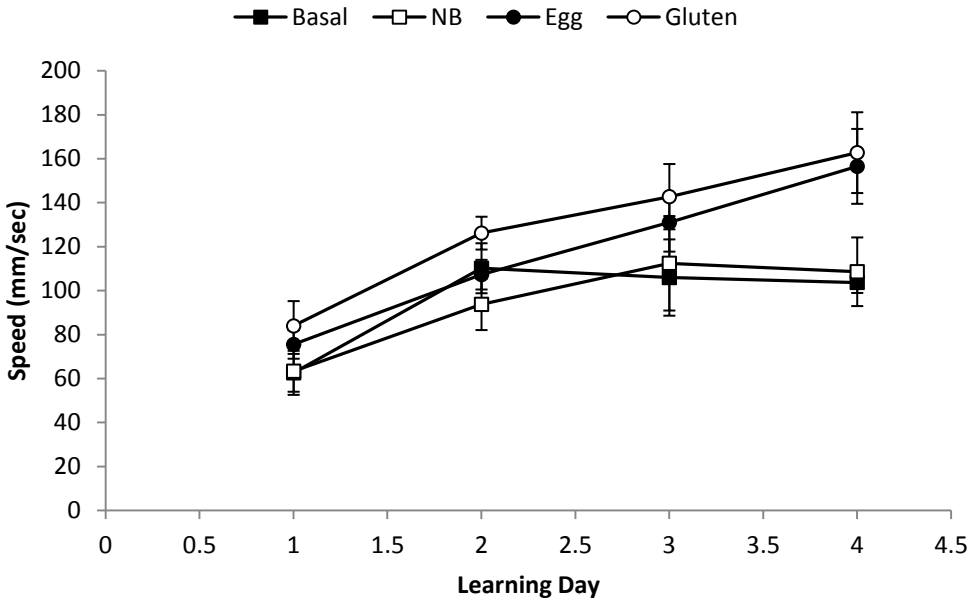

Figure 7. Average daily speed (mm/sec) to target hole during across learning days. Speed increased across training days ($P < 0.0001$). Rats fed the wheat gluten diet for breakfast moved more quickly than rats in the Basal and no breakfast groups ($P < 0.05$). Bars represent mean ± SEM.

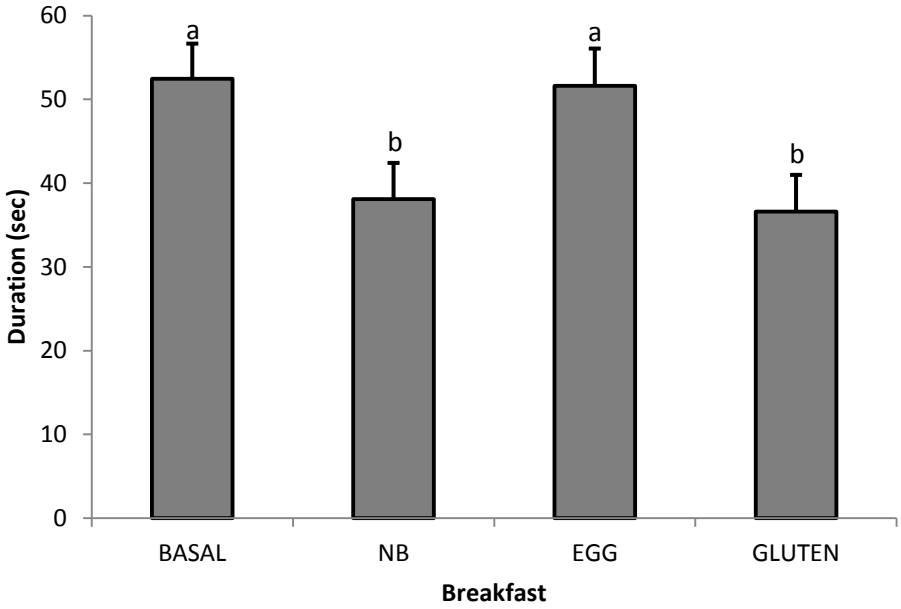

Figure 8. Duration (sec) spent in target quadrant during the 90 seconds probe trial (n = 6-9/treatment). All treatment groups spent more time in the target quadrant than would be expected by chance. Bars represent mean ± SEM. Bars with different superscripts are different ($P < 0.05$).

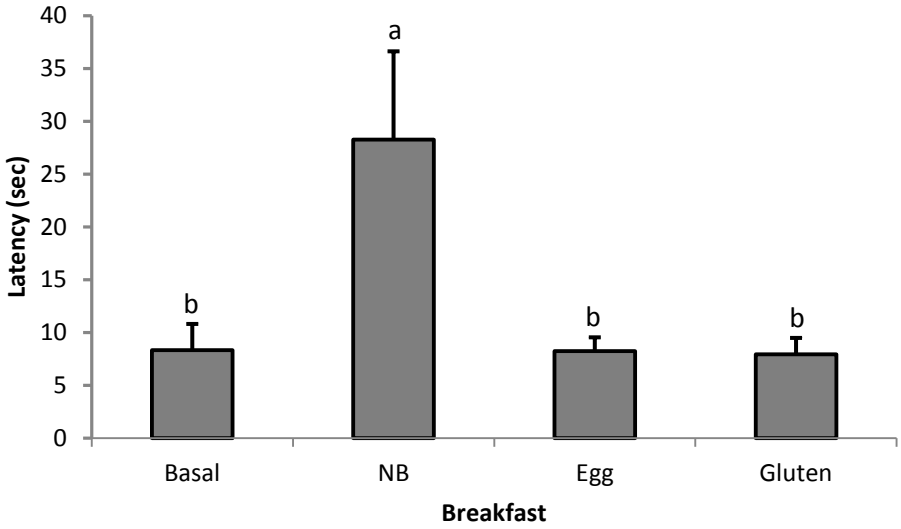

Figure 9. Latency (sec) to location or previously correct target hole during probe trial (n = 6-9/treatment). Rats not provided breakfast took longer to get to the hole where the escape tunnel had previously been ($P < 0.005$). Bars represent mean ± SEM. Bars with different superscripts are different ($P < 0.05$).

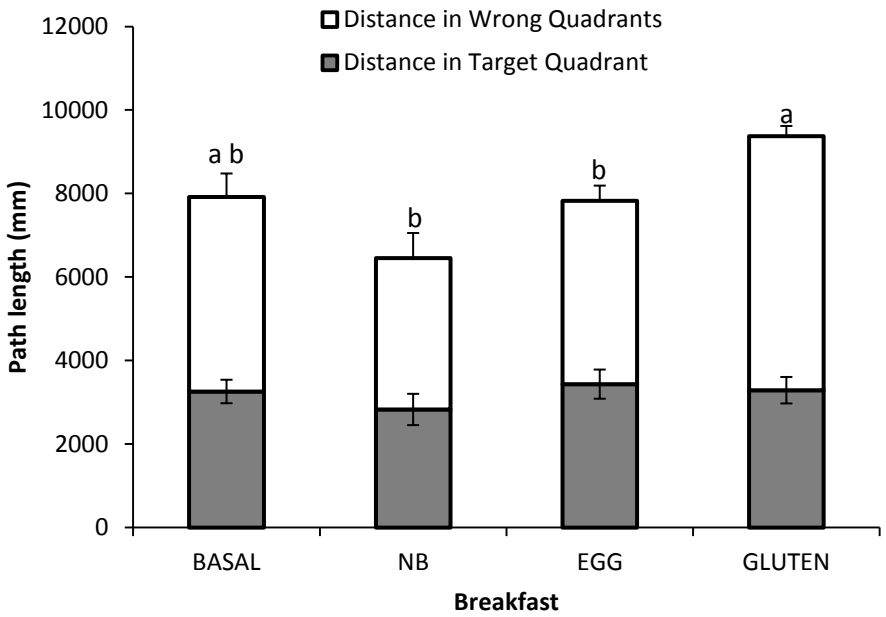

Figure 10. Path length (smm) traveled in target quadrant versus total path length traveled during the 90 seconds probe trial (n = 6-9/treatment). Main effects for path length within the target quadrant were not significant. Rats provided the wheat gluten breakfast traveled a longer total distance ($P < 0.001$). Bars represent mean ± SEM. Bars with different superscripts are different ($P < 0.05$).

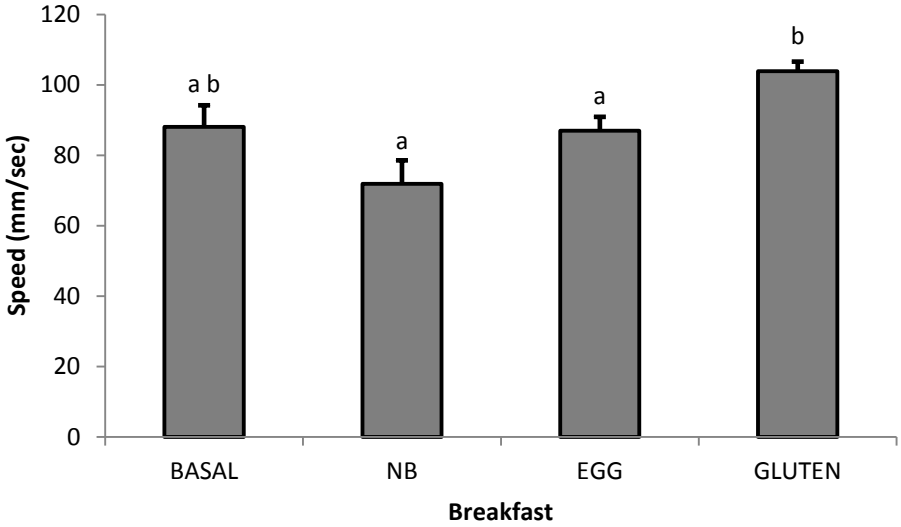

Figure 11. Speed (mm/sec) during the 90 seconds probe trial (n = 6-9/treatment). Rats provided the wheat gluten breakfast traveled faster ($P < 0.001$) than rats in the no breakfast and egg white treatment. Bars represent mean ± SEM. Bars with different superscripts are different ($P < 0.05$).

Conclusion

The present study evaluated two behaviors, satiety and cognitive performance, subsequent to a breakfast comprised of different protein sources: egg white and wheat gluten. After consuming equal amounts of the diets, rats provided egg white protein ate less food and rats provided the wheat gluten consumed more food during the subsequent period. During a preference test, egg white diet was less preferred, although we do not it was aversive. The results of our study also emphasized the cognitive benefits of consuming breakfast; rats not provided breakfast showed impaired working memory, were slower, and had decreased learning and memory performance. Increasing the level of protein from 20% to 35% of total energy was associated with increases in activity and improved working memory. Between the two protein sources, rats provided the egg white diet seemed to have increased attention during the probe trial.

The present research has further demonstrated protein source is an important factor in satiation provided by protein. Given the observed differences in satiety, the protein component of breakfast may need more careful consideration for the implication it may have for weight management, cognitive performance, mood, and productivity. Egg white was the more satiating of the two protein sources used in this study and induced better learning and memory performance of animals when provided as the sole source of protein at breakfast. It may be that greater satiety from egg white diet had a positive impact on behavior and cognitive performance, allowing rats to be more focused during a spatial learning task and learning more quickly.

One of the most interesting aspect of the present results occurred when rats were fed the wheat gluten as the sole protein source in breakfast. Even when fed a diet containing 35% energy from protein and given all the studies demonstrating the satiating properties of protein, rats

seemed less satiated than when fed the lower protein Basal diet (i.e. 20% of energy). Furthermore, rats fed the wheat gluten diet for breakfast moved much faster than rats in the other three treatment groups (egg white diet, Basal diet, and no breakfast) during the Barnes maze testing. One of the possibility for these behavioral observations may be anxiety following consumption of the wheat gluten diet. Anxiety and stress has been known to induce increased food intake and even hyperphagia (Polivy et al. 1994, Morley et al. 1983), explaining increased food intake following wheat gluten breakfast in our study. Anxiety may also be thought of to be induced by hunger. Rodgers et al. (2000) observed orexin-A induced increase in food intake, with analysis of behavioral satiety sequence showing increased locomotion. Behavioral satiety sequence assesses rodents for patterns of behavioral changes associated with the process of satiation; feeding, grooming and resting (Ishii et al. 2003). Orexin-A caused animals to display less of the activities associated with satiety, but increased locomotion duration. This is reflected in our study where rats fed the wheat gluten diet, consumed more in the subsequent period and moved faster when placed on the Barnes maze.

The underlying mechanism by which dietary protein induces satiety is still unclear. There are a number of future directions possible to extend on the findings of the present study. Studies comparing the satiating effects between protein sources are few in numbers and often times conflicting in their findings. It would be interesting and worthwhile to use this study design in evaluating the effects of a variety of other protein sources. In addition, post-prandial profiles of peptide hormones (for example, insulin, glucagon, ghrelin, and cholecystokinine), amino acids, and glucose will be neccessary to better understand the possible difference in digestion and absorption and how it may be signaling energy availability. Based on the observed increase in activity of rats provided the wheat gluten diet, evaluation of post meal behavior and activity may

also provide useful insight in understand the effects of these diets. The results of the Barnes testing were limited by the removal of 6 rats from analysis, which brought the sample size for the Basal and no breakfast treatment to 6 rats for each. This data can be strengthened by increased sample size that looks to have the potential for significant differences in acquisition to be detected.

Based on the difference between the protein sources used in this study on satiety and subsequent behavior, it appears that protein composition at breakfast deserves more consideration. The present study showed strong differences in the effects of egg white and wheat gluten proteins at breakfast, with egg white protein indicating increased satiety and enhanced cognitive performance.

Literature cited

1. E. Morley J, S. Levine A, E. Rowland N. Stress induced eating. *Life Sciences*. 1983;32(19):2169-2182.

2. Ishii Y, Blundell JE, Halford JCG, Rodgers RJ. Effects of systematic variation in presatiation and fasting on the behavioural satiety sequence in male rats. *Physiology and Behavior*. 2003;79(2):227-238.

3. Polivy J, Herman CP, McFarlane T. Effects of anxiety on eating: Does palatability moderate distress-induced overeating in dieters? *Journal of Abnormal Psychology*. 1994;103(3):505-510.

4. Rodgers RJ, Halford JCG, Nunes de Souza RL, et al. Dose–response effects of orexin-A on food intake and the behavioural satiety sequence in rats. *Regulatory Peptide*. 2000;96(1–2):71-84.

Appendix

Animals (n = 12) were placed in the Comprehensive Laboratory Animal Monitoring System (CLAMS) (Oxymax; Columbus Instruments International, Columbus, OH, USA) to quantify their oxygen consumption (VO2), carbon dioxide production (VCO2), and food intake. Rats were allowed three days to acclimate to the cages before data was collected. Gas measurements were taken every 13 minutes. The objective of this part of the study was to collect post-prandial respiratory exchange ratio (RER) following consumption of our three testing diets (20% energy protein basal diet, 35% energy egg white protein diet, and 35% energy wheat gluten protein diet) for breakfast. All rats received the basal diet for breakfast on the first three days. On days 4-6, half the rats were given the egg white diet for breakfast and the other half received the wheat gluten diet for breakfast. On days 7 and 8, the egg white and wheat gluten breakfast treatments were switched so that all animals received all three breakfast treatments by the end of the study. Mid-dark cycle, animals were given ad libitum access to the basal diet for 5.5 hours before food was removed one hour prior to the onset of light. Main effect of breakfast treatment diet and subsequent RER were analyzed by one-way analysis of variance (ANOVA) and Tukey's honest significance test. Values are presented as means ± SEM. Analysis were performed using SAS 9.2 (SAS Institute Inc., Cary, NC).

Table 3. Average respiratory exchange ratio (RER) during the three hours following the breakfast period.

	Basal	Egg	Gluten
Avg. RER of 1st hour after breakfast period	1.002 ± 0.004	0.905 ± 0.005	0.860 ± 0.006
Avg. RER of 2nd hour after breakfast period	1.038 ± 0.005	0.881 ± 0.008	0.873 ± 0.006
Avg. RER of 3rd hour after breakfast period	1.024 ± 0.004	0.844 ± 0.007	0.880 ± 0.005

Table 4. Average VO_2 (ml/kg/hr) consumed during the three hours following the breakfast period.

	Basal	Egg	Gluten
Avg. VO_2 of 1st hour after breakfast period	1871.77 ± 27.48	1828.00 ± 24.59	1731.06 ± 29.18
Avg. VO_2 of 2nd hour after breakfast period	1883.15 ± 34.67	1777.27 ± 35.86	1736.22 ± 26.55
Avg. VO_2 of 3rd hour after breakfast period	2083.50 ± 21.40	1932.96 ± 32.81	1888.95 ± 31.30

Table 5. Average metabolic rate (MR, kcal/kg/hr) during the three hours following the breakfast period.

	Basal	Egg	Gluten
Avg. MR of 1st hour after breakfast period	9.03 ± 0.13	8.82 ± 0.12	8.35 ± 0.14
Avg. MR of 2nd hour after breakfast period	9.09 ± 0.17	8.58 ± 0.17	8.38 ± 0.13
Avg. MR of 3rd hour after breakfast period	10.05 ± 0.10	9.33 ± 0.16	9.11 ± 0.15