INFANT GUT PEPTIDE RESPONSE TO FEEDING DIETS DIFFERING IN COMPOSITION

by

Stevi Anderson

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Approved: 

Jillian Trabulsi, Ph.D., R.D.
Professor in charge of thesis on behalf of the Advisory Committee

Approved: 

P. Michael Peterson, Ed.D.
Chair of the Department of Behavioral Health and Nutrition

Approved: 

Kathleen S. Matt, Ph.D.
Dean of the College of Health Sciences

Approved: 

James G. Richards, Ph.D.
Vice Provost for Graduate and Professional Education
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ABSTRACT

Background: Evidence indicates that the composition of an infant’s diet in early life affects their growth rate. Formula fed (FF) infants, the majority of whom are fed a cow milk (CMF) infant formula, have been shown to gain weight at a faster rate and weigh more than breast fed (BF) infants by the end first year of life.1-3 These early growth differences have important implications for future health; infants with accelerated growth rates and more rapid weight gain are at greater risk for obesity and related diseases in child- and adulthood.4-11 Interestingly however, a recent study of formula fed infants found that infants fed an extensive protein hydrolysate formula (ePHF) grew at a more normative rate and consumed less formula to satiation compared to infants fed CMF.12 That infants consumed significantly less volume per feeding and satiated earlier when feeding ePHF as compared to CMF suggested that the composition of formula, not bottle feeding alone, plays a role in energy intake and therefore growth. Energy intake is controlled by complex signals from the peripheral systems including the gut, which act on the central nervous system (brain) via neural and endocrine pathways. Gut and other peripheral hormones signal energy needs, fat stores, hunger, satiation and satiety.13 We postulate that a possible method to explain the differential growth between formula and breast fed infants and between some groups of formula fed infants, could be the gut peptide response to diet composition,
which in turns leads to differences in satiation, and in turn energy intake. Differential gut peptide response to diets of different macronutrient composition would impact feelings of fullness, meal termination, energy intake, and ultimately weight gain and growth. Gut peptide response to feedings of different composition in healthy infants is a relatively unexplored topic.

**Statement of Problem:**

A better understanding of the gut peptide response to diets of different composition in infants may provide insights into observed differences in satiation and growth. Peptide response to feeding has not been studied sufficiently in healthy, term infants, nor has the effect of diet composition (CMF, ePHF, breast milk) on gut peptides been explored.

**Aims:**

The purpose of the within-subject study was to 1) use a model system that experimentally manipulates diet by feeding formulas of different macronutrient composition to study biomarkers of satiation and satiety during a typical formula feeding and 2) study biomarkers of satiation and satiety during a typical breast milk feeding.

The **primary aim** of this study was to determine if gut peptide concentrations differ at the beginning compared to the end of a formula feeding and determine if the
patterning of gut peptide response (the change in concentration) differs based on the formula fed (the condition: CMF, ePHF).

The secondary aim of this study was to determine if gut peptide concentrations differ at the beginning compared to the end of a breast milk feeding and determine if the patterning of gut response is consistent within subjects.

An exploratory aim of this study was to determine if the concentrations of gut peptides prior to a feeding are stable within subjects, regardless of the mode of feeding (formula vs. breast).

**Design and Analysis:** This ongoing study is a within-subject, between-subject pilot study of healthy, term infants who are either exclusively formula-fed or exclusively breast-fed. Mother-infant pairs participate in two study visits within a 7-day period, during which intake, duration of feeding, and biomarkers of satiation and satiety are assessed using an infant-led feeding paradigm. Because this study is ongoing; the current data analysis mainly consisted of summary and descriptive statistics to assess means and variability as well as preliminary trends. Future analyses will be performed as sample size increases.

**Results:** In formula fed infants, gut peptide concentrations at the beginning of a feeding were significantly lower compared to the end of a feeding for neuropeptide YY (PYY), glucagon-like peptide-1 (GLP-1) and gastric inhibitory peptide (GIP). Ghrelin and leptin concentrations did not significantly differ from the beginning to the
end of a feeding. The relative change in peptide concentrations did not differ by the type of formula fed (CMF, ePHF). In breast fed infants, PYY, GLP-1, GIP and leptin concentrations were significantly lower at the beginning of a feeding compared to the end of a feeding. Ghrelin concentrations did not differ from the beginning to end of a feeding. The relative change in gut peptide concentrations was stable within breast fed infants for GLP-1, GIP, ghrelin and leptin. In all infants, the concentrations of PYY and leptin prior to feeding on day 1 were significantly correlated with concentrations prior to feeding on day 2.

Conclusions: Appetite peptides do respond during the course of feeding and likely act as satiation signals in healthy term infants, both formula and breast-fed. The concentrations of PYY and leptin prior to feeding appear to be consistent within an infant. The relative change in concentrations of these gut peptides did not differ when measured after the end of feeding CMF vs. ePHF, however feeding length and feeding volume varied, which likely impacted change; moreover a greater number of subjects are needed before final conclusions may be drawn. This study is ongoing and future work will assess gut peptide response to fixed volume and durations of feedings.
Chapter 1

INTRODUCTION

Growth in infancy and childhood is used as a marker of adequate nutritional status; it is imperative that children consume the nutrients required to sustain a healthy growth rate. Too slow a rate of growth or stunted growth is an indicator of malnutrition, which if not corrected may lead to developmental delays. Conversely, rapid or accelerated growth can increase future risk of obesity and its associated co-morbidities.\textsuperscript{6-11} Numerous prospective and epidemiologic studies demonstrate that rapid weight gain and greater weight-for-length during infancy continue into early childhood, and are risk factors for adult overweight and obesity.\textsuperscript{2,6-11} Energy balance, the relationship between energy intake and energy expenditure, is a key component that drives growth. In childhood, energy intake must exceed energy output to allow for energy deposition and growth. However in adults, energy input exceeding energy expenditure leads to energy deposition (excess body weight) and can be the etiology behind overweight or obesity.\textsuperscript{14}

One factor that can affect early growth in infancy is diet. Infants who are fed infant formula (the vast majority of whom are fed a cow’s milk formula; CMF) tend to weigh more and have a greater risk for later obesity than do infants who are breastfed.\textsuperscript{15-17} However, it has recently been found that formula fed infants may have differential
growth based on the type of formula they are fed. Infants fed an extensive protein hydrolysate formula (ePHF) gained weight at a rate similar to breastfed infants. ePHF formulas contain protein mainly in the form of free amino acids and small peptides, they are often referred to as ‘pre-digested’; conversely CMF formula contain intact proteins that must be cleaved into small peptides and free amino acids after ingestion. The mechanism or mechanisms by which diet composition affects energy balance is unknown, however it appears that infants satiate at lower intake volumes when feeding ePHF than when feeding CMF, which then results in slower weight gain. We hypothesize that the macronutrient composition of the diet differentially affects the gut peptide sensing systems that regulate meal termination and energy intake. We study infancy, as it is a time of early life growth, which can impact long-term health status. Additionally, infancy is a unique period of life where the environment has less of an impact on feeding; more so than any other life stage, feeding is based on physiological cues.
Chapter 2

REVIEW OF THE LITERATURE

The purpose of this review is to examine the relationship between infant diet composition and gut peptides in relation to appetite and feeding control. Infant diet composition has been shown to impact consumption and weight gain in infancy, but mechanisms are still poorly understood. As such we seek to gain a better understanding of the peripheral peptides and the role they play a role in energy balance.

Infant Diet Composition

Current Recommendations and Practices

Breast milk is considered the gold standard for infant nutrition providing the ideal concentrations of nutrients and other constituents to support growth and overall health. The American Academy of Pediatrics recommends infants be breastfed during the first year of life with complementary foods offered starting at 6 months of age. If an infant cannot be breastfed, infant formula is considered the next most suitable feeding alternative. In the United States, 62% of infants receive infant formula before 3 months of age either as a sole source of nutrition, or in conjunction with breast milk. The majority of infants (>80%) who receive infant formula are on a
cow’s milk protein formula (CMF), CMF contains intact casein and whey proteins. Alternative formulas do exist and primarily differ in the source and form of protein. Some other types of infant formulas include soy-protein based, rice protein based, and, hydrolyzed protein formulas. Extensively protein hydrolyzed formulas (ePHF) contain proteins mainly as free amino acids and small peptides; these formulas contain a diverse profile of amino acids and are often used for infants who are allergic to CMF. These differences in the form of protein, may affect infant intake, which in turn can affect growth and body composition.

**Diet Composition Affects Growth**

Early weight gain has been shown to relate to later life weight status. Accelerated growth (rapid weight gain) in infancy has been associated with increased risk for overweight or obesity in both childhood and adulthood. Risk of becoming overweight associated with rapid infant growth has been seen in children as young as 3 or 4 years old. Additionally, rapid growth in infancy can increase obesity-related metabolic disorders in adulthood.

Previous studies have found formula fed infants grow at a faster rate than breastfed infants. Growth has been found to differ significantly between formula fed and breast fed infants throughout multiple periods of infancy; 0-6 months, 3-6 months, 7-24 months. It is interesting to note that no studies have found differences in length gain of infants based on feeding (breast milk versus formula), which is consistent with the finding that formula fed infants tend to have
greater weight for length compared to breastfed infants.\textsuperscript{3,12,25} Additionally, Heinig et al. found that breast fed infants were found to have lower gains in fat mass during the first 12 months of life compared to formula fed infants.\textsuperscript{25} Consequently, these heavier formula fed infants were more susceptible to becoming overweight in childhood or adolescence.\textsuperscript{27,29} It should be noted however that the majority of research documenting growth patterns of formula fed versus breast fed infants has been conducted in infants who receive CMF, as it is the most common type of infant formula used today.\textsuperscript{23}

\textit{Differences between breast milk and CMF and ePHF}

Past studies have considered formula fed infants a homogenous group. However, a recent randomized control trial found that infants fed an extensively hydrolyzed protein formula (ePHF) have a more normative growth pattern (closer to breast fed infants) compared to infants fed a standard cow’s milk formula (CMF).\textsuperscript{12} Infants fed ePHF also consumed less formula to satiation than infants fed CMF.\textsuperscript{12,30} However, the number of feedings per day remained consistent between formula groups.\textsuperscript{12} Essentially, infants fed ePHF consumed less formula volume at each feeding and therefore throughout the day. This earlier satiation could act as a protection against overconsumption in infants.\textsuperscript{30} Several hypotheses exist as to why the intake differences were observed. Extensive protein hydrolysate formula and CMF have similar concentrations of vitamins, minerals and fat however; the major difference between the two formulas (ePHF and CMF) is the form of protein they contain,
hydrolyzed protein versus intact protein, respectively. ePHF formulas contain a greater concentration of free amino acids and small peptides as opposed to larger intact proteins in standard CMF. Interestingly, breast milk also contains a considerable proportion of protein as free amino acids, with the concentration of free amino acids in breast milk being not as high as that found in ePHF but greater than that found in standard CMF. The free amino acids of ePHF and breast milk transit the gastrointestinal tract and are absorbed more rapidly than the intact proteins present in CMF. Infants fed breast milk or ePHF have much higher concentrations of plasma amino acids post-feeding compared to infants fed CMF. Interestingly, the free amino acid in highest abundance in breast milk is glutamate, and researchers therefore hypothesized that perhaps this particular amino acid was important in signaling satiation. To test this hypothesis, Ventura, Mennella et al., conducted a within subject trial in which infants were fed either CMF or CMF with the added free amino acid, glutamate. In a counterbalanced cross-over design, when infants were fed CMF with added free glutamate they consumed less formula to satiation than when fed standard CMF. It is hypothesized the free glutamate may bind to amino acid receptors in the gut leading to earlier satiation and feeding termination.

**Gut Peptides**

Satiation and satiety biomarkers play a role in energy balance and feeding. Satiation signals are hormones secreted in response to food intake that signal fullness and trigger meal termination. In contrast, satiety signals peak at the end of a meal and
prevent further eating until hunger drives meal initiation; satiety signals impact energy balance and longer term intake control. These two signaling pathways are interconnected and work in relation to each other. For example, after energy restriction or food deprivation periods, levels of satiety peptides are lower and therefore the body in less sensitive to satiation signals. This mechanism protects against starvation and allows the body to consume a larger amount at a meal than it normally would in order to reach energy balance. Conversely, after excessive energy intake satiety markers are high in the body, increasing sensitivity to satiation signals. This encourages lower energy intake and smaller meals because of a lack of need for energy (food). The latter mechanism can be more easily overridden, contributing in part to overeating and obesity risk.

Criteria for hormones to be considered short-term satiation signals include that they must be secreted: in response to food, within the time of a meal and initiate meal termination, and be effective in natural doses. Satiation is generally studied through gut peptide response to food intake. Some of the peptides used as biomarkers of satiation include: glucagon-like peptide-1 (GLP-1), neuropeptide YY (PYY), and ghrelin. Satiety hormones include gastric inhibitory peptide (GIP) and leptin.

Satiation and satiety are controlled both centrally and peripherally to mediate energy homeostasis. Internal physiological hunger cues are important components to maintaining a healthy weight. However in adults, environmental cues can override physiological cues influencing eating behaviors like meal initiation and energy consumption. Such environmental cues can include habits, time of day, and social
environments, among other things. Interestingly however, these environmental influences are less existent to infants, increasing the importance of and reliance on physiological cues, and emphasizing the importance of infancy as a time to set the stage for life long eating habits.

Many studies have examined the response of gut peptides to food intake in normal weight, overweight or obese adults but few studies have examined these peptides in infants. It is important to study the interaction of diet composition with biomarkers of satiation and satiety in infants given the differences in diet composition and growth that may occur in the first year of life. Although much is known about these gut peptides in adults, their response to infant feeding is still a relatively unexplored area.

**Glucagon-like peptide-1**

Glucagon-like peptide-1 (GLP-1) is a hormone released by enteroendocrine cells in response to food intake. It is thought to play a role in satiation (meal termination). The most prevalent form of this peptide in the body is the 7-36 amide. In infants, basal GLP-1 is low and rises in response to food intake. The rise in GLP-1 plays a role in suppressing appetite, meal termination and satiation. In infants, GLP-1 infusion resulted in a decrease in energy intake. GLP-1 impacts satiation in part through the ileal brake mechanism, whereby it delays gastric emptying. It also acts on the hypothalamus, the area of the brain controlling appetite. A rise in GLP-1
concentrations appears to be related to meal termination but how diets of differing composition affect the GLP-1 rise is yet to be determined.

**Peptide YY**

The enteroendocrine cells in the ileum secrete peptide YY (PYY). PYY is thought to play a role in satiation. This gut hormone is low in the fasting state and rises in response to food causing an anorexigenic response. PYY levels remain elevated for several hours post meal and may exhibit control on subsequent meal size and timing. Fat causes a greater response of PYY when ingested compared to carbohydrate or protein in humans. A study found PYY concentrations in fasting blood samples to be significantly related to BMI and ghrelin levels in infants. This could suggest a role in energy control but no correlation was found between PYY and caloric intake or weight gain in the small sample size. Research in healthy infants is limited and the response of PYY to different macronutrient composition in the diet, as seen in adults, has not been explored in infants.

**Gastric Inhibitory Peptide**

Gastric inhibitory peptide (GIP) is a hormone secreted by the enteroendocrine K-cells of the duodenum that inhibits gastric motility and affects insulin response to a feeding. GIP is a satiety hormone. In humans, GIP levels are low during fasting and increase quickly post meal consumption. In a study of 2-month old infants, a rise in
GIP concentrations has been observed 30 minutes after a feeding. A study of 6-day old infants found that basal GIP concentrations were higher in formula fed infants (cow’s milk based infant formula) compared to breast-fed infants. This study also found that the postprandial rise in GIP concentrations after feeding appeared to be greater in formula versus breast-fed infants, however the difference did not reach significance. Another study of 9-month old infants however found no difference in pre-feeding GIP concentrations between breast-fed and formula-fed infants, and a significant difference in the post-prandial rise in GIP, with the response having shorter duration in breast-fed infants. The conflicting results and lack of additional studies suggests that further investigation is warranted.

**Ghrelin**

Ghrelin is a peptide produced primarily in the stomach and acts to stimulate appetite; as such it plays a role in satiety. Plasma ghrelin concentrations are high pre-meal and lower post-prandial. This relationship has been confirmed in infants. Research has found that ghrelin concentrations are inversely related to insulin levels in adults but there is a less solidified relationship in infants. Due to the relationship with insulin sensitivity, ghrelin may contribute to energy balance and obesity. A study found basal levels of ghrelin differ significantly in overweight compared to normal weight children; this relationship was mainly attributed to insulin sensitivity. Other research has found a positive correlation between ghrelin concentrations and age, weight, length and head circumference in infants 1-18 months old; suggesting a role in
energy intake control and consequently growth. We are interested in learning more about ghrelin concentrations before and after meals in healthy term infants and whether diet composition (human milk versus infant formula) has differing effects on this peptide.

**Leptin**

Leptin, a hormone produced in the body by adipose tissue, is considered a satiety signal. The amount of leptin produced is proportional to the amount of adiposity present within an individual. One role of leptin is to relay information to the hypothalamus region of the brain regarding the amount of fat stores. The circulating leptin concentration in humans does not seem to impact short-term energy intake, or meal initiation. However, research has shown leptin concentration’s influence on long-term energy intake. Leptin plasma concentration can be diminished after prolonged energy restriction. A recent study found higher serum leptin concentrations in breastfed infants compared to formula fed infants, these formula fed infants had higher BMI averages at follow up visits in later childhood. This inverse relationship between leptin levels in infancy and childhood BMI suggests a potential relationship in energy intake and growth.

**Inter-connections of Satiation and Satiety Signals**

Neither short-term nor long-term signals alone are enough to independently regulate energy balance. These signals work together through separate pathways in
order to exhibit control over the body’s energy consumption. The efficacy of short-term hormones is impacted by the presence of long-term hormones. A low leptin concentration in the blood due to fasting can lessen the influence of GLP-1 on meal termination. This results in increased meal size and energy consumption at meals to correct for the longer term decreased energy intake over time. Since breast milk contains leptin, and breast fed infants have higher serum leptin concentrations, breast fed infants may be more susceptible to satiation cues compared to formula fed infants, resulting in decreased intake and hence slower growth compared to formula fed infants. The response of these gut peptides to differing meal compositions in infants is a relatively unexplored topic.

**Statement of the Problem**

The composition of the infant diet can vary greatly between infants. Some infants are exclusively fed breast milk, others are fed infant formula exclusively, and others are fed both breast milk and infant formula. The varying compositions of the infant diet affect growth patterns. Cow’s milk formula-fed infants have a faster weight gain velocity; which increases risk of obesity in childhood and beyond. Infants fed extensively hydrolyzed protein formula have a weight gain velocity that more closely resembles breast-fed infants. This difference is thought to be due to the consumption of less formula per feed to satiation. The role of gut peptides in appetite and satiation in response to food intake has been extensively studied in adults. However, these studies fail to translate this to infants, whose diets differ greatly from
adults, as they are predominantly liquid for much of the first 6 to 9 months of life.

The changes of GLP-1, GIP, PYY, ghrelin and leptin in infants in response to different diet compositions, breast milk, CMF and ePHF fed could explain differences in satiation\textsuperscript{30} and intake during a feeding in the short term and growth in the longer term.
Chapter 3

AIMS

The purpose of this within-subject study was to use a model system that experimentally manipulates diet by feeding formulas of different macronutrient composition to study biomarkers of satiation and satiety.

The primary aim of this study is to investigate differences in gut peptide concentrations at the beginning when compared to the end of formula feeding (timing) and the differences in patterning of gut peptide response (change in concentration) based on the formula fed (condition: CMF, ePHF).

It is hypothesized that gut peptide concentrations will differ at the beginning when compared to the end of formula feeding (timing) and the patterning of gut peptide response (change in concentration) will differ based on the formula fed (condition: CMF, ePHF). Based on the literature in adult satiation,\textsuperscript{13,14,32,33,36,37} we hypothesize that relative to the end of feeding, concentration of ghrelin will be higher and concentrations of neuropeptide PYY, GIP, and GLP-1 will be lower at the beginning of a feeding in infants. We also hypothesize that the gut peptide response will differ based on the composition of the formula the infant feeds (condition: CMF vs ePHF). Because ePHF has higher levels of FAA and because of precedence in the
literature, we expect that infants will satiate on lower volumes when feeding ePHF compared to CMF.\textsuperscript{12,30}

The secondary aim of this study is to investigate differences gut peptide concentrations at the beginning when compared to the end of breastfeeding (timing) and the stability in patterning of gut peptide response between breastfeeding occasions.

\textit{It is hypothesized} that gut peptide concentrations will differ at the beginning when compared to the end of breastfeeding (timing) and the patterning of gut peptide response in breastfed infants will be consistent on the two days of testing. Based on adult literature, we hypothesize that relative to the end of feeding, concentration of ghrelin will be higher and concentrations of neuropeptide PYY, GIP, and GLP-1 will be lower at the beginning of feeding in infants. Further, we hypothesize that breastfeeding will result in similar changes in gut peptide concentrations from feeding to feeding (day 1 versus day 2), since the infant is fed the same diet (breast milk) on day 1 and day 2.

The exploratory aim of this study is to investigate the stability of gut peptide concentrations within subjects prior to a feeding.

\textit{It is hypothesized} that the concentrations of gut peptides prior to a feeding will be stable within subjects. Because of the design of the study such that all infants were tested on two separate days and the conditions at the beginning were identical, we will explore whether before feeding concentrations of each of the gut peptides correlate between the two days as a measure of reliability and stability. We will also explore
whether concentrations of the gut peptides just prior to a feeding correlate with each other to determine their relationships during the hunger state and whether concentrations of gut peptides at the start of a feeding differ by diet composition (formula, breast milk).
Chapter 4

METHODS

Institutional Review Board

The protocol, informed consent, data collections questionnaires were approved by the Institutional Review Board (IRB) at the University of Delaware prior to beginning any study activities. During the informed consent process, all subjects were informed about the purpose of the study, the risks and benefits associated with participating, the compensation provided, and the confidential and voluntary nature of the study and all mothers provided written consent prior to participation in the study.

Description of Participants

Mother-infant dyads were recruited from greater Wilmington, Delaware area using IRB-approved brochures, flyers and advertising in WIC offices, mailings to new mothers and Craigslist advertising. The desired sample size for this pilot study was 20 mother-infant dyads; 15 formula-fed infants, 5 breast-fed infants. The informed consent process was completed prior to beginning any study related activities.

Inclusion criteria:

Subjects must meet all of the following criteria to be enrolled in the study.
1. Infant must be a healthy, term (37-42 weeks gestation), singleton, appropriate for gestational age infant at birth.

2. Infant must be >30 <120 days old at enrollment

3. Mother must be ≥ 18 years of age.

4. Infant must not yet be receiving solid food.

5. If breast-fed, infants must be primarily breast-fed, not receiving >1 supplemental feeding of formula per day.

6. If formula-fed, infants must be primarily formula-fed and not receiving >1 supplemental feeding of breast milk per day.

7. Formula fed infants must have never received extensively hydrolyzed protein formula.

**Exclusion criteria:**

Meeting any of the following criteria will exclude subjects from participation.

1. Having a major congenital malformation (i.e. cleft palate, extremity malformation) or genetic disorders.

2. Have suspected or documented systemic or congenital infections (e.g. human immunodeficiency virus, cytomegalovirus).
3. Have evidence of significant cardiac, respiratory, endocrinologic, hematologic, gastrointestinal, or other systemic diseases.

4. Be receiving any prescription medication.

Mother-infant pairs who signed the informed consent, met the inclusion/exclusion criteria, then participated in two 3-hour study visits within a 7-day period, during which intake, duration of feeding, and biomarkers of satiation and satiety were assessed using an infant-led feeding paradigm. A more detailed description of study visits can be found in the next section of the protocol. Participants were compensated $75 at each visit in addition to parking expenses.

**Description of Variables**

The variables of interest were diet composition, feeding volume, feeding duration, gut peptide concentrations. The independent variable was diet composition. The dependent variables were the gut peptides concentrations; PYY, GLP-1, GIP, ghrelin, leptin and the change in these concentrations prior to and after a feeding. Feeding dynamics (volume of feed, duration of feed and rate of feeding) were also dependent variables of interest.

**Study Visit Procedures**

The informed consent process was completed at the beginning of the first study visit. After the informed consent was signed, study visit 1 procedures included:
• Completing Subject Master List with participant’s name, email address, date IC signed.

• Inclusion, exclusion screening

• Formula fed infants randomized to order of formula feeding

• Infant weight, height and head circumference measured

• Obtain saliva sample and heelstick sample prior to feeding

• Weigh bottle before and after feeding

• Observe and videotape 1 feeding

• Weigh infant after feeding

• Obtain saliva sample and heelstick sample post feeding

• Complete demographic, medications, general interview and feeding history forms.

Study visit 2 procedures included:

• Weigh infant before and after feeding

• Weigh bottle before and after feeding
• Obtain heelstick and saliva sample prior to feeding

• Observe and videotape 1 feeding

• Obtain saliva sample and heelstick sample post feeding

Formula fed infants, in random order, fed a cow’s milk formula at one study visit and an extensively hydrolyzed protein formula at the other study visit (counterbalanced design). The CMF used was Enfamil (Mead Johnson Nutrition, Evansville, IN), the ePHF used was Nutramigen (Mead Johnson Nutrition, Evansville, IN). Nutritional composition information for both test formulas can be found in Table 1. Mothers of formula fed infants were asked to bring 2 empty feeding bottles to each study visit and the infant formula was provided during the visit. Mothers of breastfed infants fed their infant breast milk from the breast.

Established methodologies were used to allow children to determine the pacing and duration of the feeding. To ensure that all feedings were child-led, mothers were instructed to (1) feed the infant at his or her customary pace until the infant signals fullness on three consecutive occasions, and (2) refrain from talking, to eliminate any potential influence of facial or verbal responses on infant behaviors. Infants were allowed to feed ad libitum.

**Description of Instruments**

Intake volume was measured via test weighing. All infants were weighed before and after feeding using a digital scale (Seca) accurate to 0.001kg. For formula
fed, the feeding bottle with the formula was also weighed before and after feeding. Duration of the feed was calculated as the time the feeding started, until the time the infant rejected the bottle for the third consecutive time.

Gut hormone concentrations were measured using blood samples. Blood was collected by heelstick into EDTA tubes containing protease inhibitors (25µL Aprotinin; Sigma-Aldrich, MO and 5µL dipeptidyl peptidase IV soluble form; Millipore, MA). Blood was immediately stored on ice until centrifuged at 3000rpm at 10° C for 15 minutes. Plasma was separated and stored at -80° C until it was ready to be assayed. Blood assays were completed in a batch analysis for pre and post feeding samples. An ELISA assay kit was used to determine gut hormone concentrations in plasma samples. The sensitivity of the assay was 0.05 pmole/L.

Anthropometrics including weight, recumbent length and head circumference were collected using standard techniques at the first study visit. Information about parental anthropometrics, ethnicity, race, smoking status and brief medical history regarding the pregnancy were collected by mother’s report using questionnaires.

**Statistical Analysis**

This feasibility study focused on: 1) whether feeding characteristics and gut peptide responses differed by diet condition (the type of formula fed; CMF, ePHF) within a group of formula fed infants; and 2) whether feeding characteristics and gut peptide responses were consistent within a group of breastfed infant (here the condition, breastfeeding, was the same on both test days). The primary outcome
measures included in this study were: concentrations of the following satiation and satiety peptides: neuropeptide PYY, glucagon-like peptide-1, gastric inhibitory peptide, ghrelin, and leptin as well as feeding dynamics [intake (mL), duration of feeding (min)]. Outcome measures were described using descriptive statistics (mean, minimum, maximum).

Before statistical analysis, normality of data was assessed using Shapiro Wilks W-test for each of the primary outcome measures. Significance for statistical tests was set at $\alpha = 0.10$, given the small sample size and the exploratory nature of the study. JMP Pro11 software (SAS Institute Inc., Cary, NC) was used to complete all statistical analyses.

To test hypothesis 1: Gut peptide concentrations will differ at the beginning when compared to the end of formula feeding (timing) and the patterning of gut peptide response (change in concentration) will differ based on the formula fed (condition: CMF, ePHF). A paired t-test of gut peptide concentrations prior to feeding versus after feeding was performed to determine the effect of a feeding (timing) on gut peptide concentrations. A paired t-test of the change in gut peptide concentration between each condition (CMF vs. ePHF) was performed (in formula fed infants only) to determine the effect of formula composition on gut peptide concentration response.

To test hypothesis 2: Gut peptide concentrations will differ at the beginning when compared to the end of breastfeeding (timing) and the patterning of gut peptide response will be consistent on the two days of testing. A paired t-test of beginning versus end concentrations of each gut peptide was performed to assess the effect of
timing. A paired t-test of the change in gut peptide response on day 1 versus day 2 was performed (in breast fed infants only) to test the consistency of the gut peptide response to a feeding.

**To test exploratory hypothesis 3:** The concentrations of satiation/satiety peptides prior to a feeding will be stable within subjects. Correlation analysis of gut peptide concentrations prior to a feeding (day 1 and day 2) was performed in all infants (formula fed and breast fed) to assess the stability of gut peptide concentrations prior to a feeding within subjects. Correlation analysis was be performed to determine whether gut peptide concentration prior to a feeding correlate with one another. A t-test was used to determine if concentrations of gut peptides prior to a feeding differ by diet composition (breast fed versus formula fed infants).
Chapter 5

RESULTS

Subject Characteristics

Table 2 describes demographic and anthropometric characteristics for the 10 mother-infant dyads that participated in the 2-day study. Six of the infants were exclusively formula fed and four were exclusively breastfed. None of the formula-fed infants had prior exposure to ePHF.

Formula-fed infants (3 girls, 3 boys) ranged in age from 66 to 115 days (mean: 92 days) and the breast-fed infants (3 boys, 1 girl) ranged in age from 35-121 days (mean: 76 days). Mothers of formula fed infants ranged in age from 19 to 41 years (mean: 28 years) and 33% were primiparous. Mothers of breast fed infants ranged in age from 28 to 35 years (mean: 30 years) and 25% were primiparous. Maternal BMI of formula fed infants ranged from 26.4 to 35.4 kg/m$^2$ (mean: 31.5 kg/m$^2$), and all of these mothers were either overweight or obese. Maternal BMI of breast-fed infants ranged from 22.2 to 41.3 kg/m$^2$ (mean: 30.9 kg/m$^2$) with 75% of these mothers being overweight or obese.
Completion of Task/Testing

While each dyad was tested on two separate days, intake and peptide data for one formula-fed infant was unavailable for one of the days because the infant regurgitated the formula and the test session was discontinued.

Feeding Dynamics

As shown in Table 3a, although formulas were isocaloric, formula fed infants had greater mean intake volume when feeding CMF (156 mL (± 61 mL) compared to ePHF 140 mL (±53 mL), representing a difference score of 16 mL. The difference in the mean volume consumed between CMF and ePHF did not reach statistical significance (mean difference: -24.86 mL; 90%CI: -73.5, 23.8; p=0.33). Examination at the individual level revealed that four of the five infants consumed less ePHF than CMF (Figure 1a). While there were no differences in the absolute length of the feeding (minutes), infants consumed the ePHF at a faster rate (mL/min) than the CMF with a difference score of 7 mL/min; again this difference did not reach statistical significance (mean difference: -8.12 mL/min; 90%CI: -20.55, 4.30; p=0.23). There was a significant difference in the time elapsed between the end of the lab feed and blood draw between the different feedings, with less time elapsing after ePHF feedings compared to CMF, difference score of 2 min (mean difference: -2.8 min; 90%CI: -5.07, -0.52; p=0.05).

As shown in Table 3b, mean breast milk intake at visit 1 was 121 mL (±62 mL) and 124 mL (±19 mL) at visit 2, with a difference score of 3 mL. The difference
in mean volume consumed between days was not significantly different (mean difference: -2.42; 90%CI: -92.40, 97.25; p=0.95)]. Individual differences in intake volumes between breast milk intake at visit 1 and visit 2 are shown in Figure 1b.

**Gut Peptides**

Table 4 provides descriptive statistics (means, minimum, maximum) for each gut peptide based on infant diet. Each gut peptide is described in further detail below.

*Neuropeptide YY (PYY)*

**Figure 2a** illustrates the blood concentration at the individual level of PYY before and after a CMF feeding and before and after an ePHF feeding, for each formula fed infant. As a group, the concentration of PYY in formula fed infants before feeding was significantly lower than concentrations of PYY after feeding (paired t-test, mean difference 180.7 pg/mL; 90% CI: 101.62, 259.77pg/mL; p=0.0028). The change in PYY concentration for each condition (CMF vs. ePHF feeding) did not significantly differ (paired t-test, mean difference: -174.16 pg/mL; 90% CI: -370.3, 21.97 pg/mL; p=0.1221).

**Figure 2b** illustrates the blood concentration at the individual level of PYY before and after the first breast milk feeding and before and after the second breast milk feeding for each breastfed infant. As a group, the concentration of PYY in breastfed infants before feeding was significantly lower than the concentration of PYY after feeding (paired t-test, mean difference: 181.33 pg/mL; 90% CI: 75.62, 287.04;
The change in PYY concentration did differ significantly for breast milk feed 1 versus breast milk feed 2 (paired t-test of the change in gut peptide response on day 1 versus day 2, mean difference: -160.79 pg/mL; 90% CI: -282.32, -39.25 pg/mL; p=0.0609).

**Glucagon-like peptide-1 (GLP-1)**

**Figure 2c** illustrates the blood concentration at the individual level of GLP-1 before and after a CMF feeding and before and after an ePHF feeding, for each formula fed infant. As a group, the concentration of GLP-1 in formula fed infants before feeding was significantly lower than concentrations of GLP-1 after feeding (paired t-test, mean difference 96.26 pg/mL; 90% CI: 48.89, 143.64 pg/mL; p=0.0054). The change in GLP-1 concentration for each condition (CMF vs. ePHF feeding) did not significantly differ (paired t-test, mean difference: -40.06 pg/mL; 90% CI: -181.12, 100.99 pg/mL; p=0.4941).

**Figure 2d** illustrates the blood concentration at the individual level of GLP-1 before and after the first breast milk feeding and before and after the second breast milk feeding for each breastfed infant. As a group, the concentration of GLP-1 in breastfed infants before feeding was significantly lower than the concentration of GLP-1 after feeding (paired t-test, mean difference: 77.25 pg/mL; 90% CI: 13.10, 141.40 pg/mL; p=0.0578). The change in GLP-1 concentration did not differ significantly for breast milk feed 1 versus breast milk feed 2 (paired t-test of the
change in gut peptide response on day 1 versus day 2, mean difference: -89.07 pg/mL; 90%CI: -284.16, 106.00 pg/mL; p=0.3140).

_Gastric Inhibitory Peptide (GIP)_

*Figure 2e* illustrates the blood concentration at the individual level of GIP before and after a CMF feeding and before and after an ePHF feeding, for each formula fed infant. As a group, the concentration of GIP in formula fed infants before feeding was significantly lower than concentrations of GIP after feeding (paired t-test, mean difference 288.95 pg/mL; 90% CI: 79.84, 498.05 pg/mL; p=0.0331). The change in GIP concentration for each condition (CMF vs. ePHF feeding) did not significantly differ (paired t-test, mean difference: 7.07 pg/mL; 90% CI: -953.57, 967.72 pg/mL; p=0.9848).

*Figure 2f* illustrates the blood concentration at the individual level of GIP before and after the first breast milk feeding and before and after the second breast milk feeding for each breastfed infant. As a group, the concentration of GIP in breastfed infants before feeding was significantly lower than the concentration of GIP after feeding (paired t-test, mean difference: 324.61 pg/mL; 90% CI: 178.07, 471.15; p=0.0051). The change in GIP concentration did not differ significantly for breast milk feed 1 versus breast milk feed 2 (paired t-test of the change in gut peptide response on day 1 versus day 2, mean difference: -223.83 pg/mL; 90%CI: -826.02, 378.36 pg/mL; p=0.3912).
Ghrelin

Figure 2g illustrates the blood concentration at the individual level of Ghrelin before and after a CMF feeding and before and after an ePHF feeding, for each formula fed infant. As a group, the concentration of Ghrelin in formula fed infants before feeding was higher than concentrations of Ghrelin after feeding, the difference did not reach statistical significance (paired t-test, mean difference: -7.63 pg/mL; 90% CI: -22.70, 7.43 pg/mL; p=0.3737). The change in Ghrelin concentration for each condition (CMF vs. ePHF feeding) did not significantly differ (paired t-test, mean difference: 35.63 pg/mL; 90% CI: -2.18, 73.45 pg/mL; p=0.1106).

Figure 2h illustrates the blood concentration at the individual level of Ghrelin before and after the first breast milk feeding and before and after the second breast milk feeding for each breastfed infant. As a group, the concentration of Ghrelin in breastfed infants before feeding was not significantly different than the concentration of Ghrelin after feeding (paired t-test, mean difference: 1.40 pg/mL; 90% CI: -22.83, 25.64 pg/mL; p=0.9140). The change in Ghrelin concentration did not differ significantly for breast milk feed 1 versus breast milk feed 2 (paired t-test of the change in gut peptide response on day 1 versus day 2, mean difference: -19.80 pg/mL; 90% CI: -86.29, 46.68 pg/mL; p=0.4761).

Leptin

Figure 2i illustrates the blood concentration at the individual level of Leptin before and after a CMF feeding and before and after an ePHF feeding, for each
formula fed infant. As a group, the concentration of Leptin in formula fed infants before feeding did not differ than concentrations of Leptin after feeding (paired t-test, mean difference 189.36 pg/mL; 90% CI: -154.96, 533.68 pg/mL; p=0.3364). The change in Leptin concentration for each condition (CMF vs. ePHF feeding) did not significantly differ (paired t-test, mean difference: -664.43 pg/mL; 90% CI: -1407.20, 78.32 pg/mL; p=0.1206).

Figure 2j illustrates the blood concentration at the individual level of Leptin before and after the first breast milk feeding and before and after the second breast milk feeding for each breastfed infant. As a group, the concentration of Leptin in breastfed infants before feeding was significantly lower than the concentration of Leptin after feeding (paired t-test, mean difference: 871.02 pg/mL; 90% CI: 275.28, 1466.77 pg/mL; p=0.0195). The change in Leptin concentration did not differ significantly for breast milk feed 1 versus breast milk feed 2 (paired t-test of the change in gut peptide response on day 1 versus day 2, mean difference: -310.45 pg/mL; 90%CI: -2074.90, 1454.03 pg/mL; p=0.6586).

**Hypothesis Testing**

**Hypothesis 1:** Gut peptide concentrations will differ at the beginning when compared to the end of formula feeding (timing) and the patterning of gut peptide response (change in concentration) will differ based on the formula fed (condition: CMF, ePHF).
In formula fed infants, gut peptide concentrations before a feeding were significantly different than gut peptide concentrations after a feeding (paired t-test) for the following peptides: PYY, GLP-1 and GIP; the concentration of each of these peptides was higher at the end of a feeding compared to before feeding. Ghrelin and leptin concentrations were not significantly different before compared to after a feeding.

In formula fed infants, the change in gut peptide concentration between each condition (CMF vs. ePHF) did not differ significantly for any peptides of interest: PYY, GLP-1, GIP, ghrelin and leptin.

**Hypothesis 2:** Gut peptide concentrations will differ at the beginning when compared to the end breastfeeding (timing) and the patterning of gut peptide response will be consistent on the two days of testing.

In breast fed infants, gut peptide concentrations at the beginning of a feeding were significantly different than gut peptide concentrations after a feeding (paired t-test) for the following peptides: PYY, GLP-1, GIP and leptin. Ghrelin concentrations were not significantly different at the beginning compared to after a feeding.

In breast fed infants, the change in gut peptide concentration did not differ significantly (paired t-test) between day 1 (breast milk feeding 1) and day 2 (breast milk feeding 2) for the following peptides: GLP-1, GIP, ghrelin and leptin. PYY concentrations did differ significantly between day 1 and day 2.

**Exploratory Hypothesis 3:** The concentrations of satiation/satiety peptides prior to a feeding will be stable within subjects.
In all infants, a significant correlation (Pearson) between gut peptide concentrations before feedings (day 1 versus day 2) were found for the following peptides: PYY (r=0.80; p=0.0279) and leptin (r=0.90; p=0.0126) (Table 5a).

The following peptides were also correlated with one another in all subjects (formula and breast-fed) prior to a feeding on day 1: GLP-1 was significantly correlated GIP (r=0.82; p=0.0060) and GLP-1 was significantly correlated with Leptin (r=0.74; p=0.0141) (Table 5b). Only the relationship between GLP-1 and leptin remained significantly correlated prior to feeding on day 2 (r=0.70; p=0.0503). Additionally, PYY and GLP-1 (r=0.64; p=0.0861), GIP and ghrelin (r=-0.71; p=0.0471) and GIP and leptin (r=0.89; p=0.0026) were all significantly correlated prior to feeding on day 2.

Before feeding gastric inhibitory peptide (GIP) concentrations were significantly lower (t-test) in breast-fed infants compared to formula-fed infants on day 1 (mean difference: 619.22 pg/mL; 90% CI: 159.04, 1079.40 pg/mL; p=0.0396). No other gut peptides of interest varied significantly between breast-fed and formula fed infants on day 1. On day 2 there were no significant differences in any gut peptide concentrations prior to feeding between breast-fed and formula fed infants.
Chapter 6

DISCUSSION

Similar to that reported for adults, PYY, GLP-1 and GIP concentrations were relatively higher at the end of feeding when compared to immediately before feeding in formula-fed infants. Additionally, there was a trend for lower intake when infants consumed ePHF compared to CMF. In this preliminary data set, the change in concentration from pre to post feed did not differ based on the type of formula fed (CMF or ePHF) for any of the peptides of interest (PYY, GLP-1, GIP, ghrelin and leptin), however the present study is not yet powered sufficiently to detect such differences. This study is ongoing and when 20 subjects have completed the study, we will use the data collected to conduct power calculations. Additional factors to consider are the differences in the length of the feeding, and differences in the time elapsed from the end of the feeding until the blood draw, which likely impact gut peptide response. The present analysis was unable to adjust for these variables at this time, due to the small sample size, however future analyses will consider these variables. Future studies may benefit from a design where both the feeding volume and length of feeding are fixed values.

The increase in peptide concentrations during feeding indicates that these peptides (PYY, GLP-1 and GIP) do respond to a feeding/the presence of nutrients in
the gut, and similar to adults, likely act as satiation signals in healthy, formula-fed infants. As hypothesized, PYY, GLP-1 and GIP concentrations were relatively higher post feed compared to before feeding in breast-fed infants. Additionally, leptin concentrations were also higher after a feeding compared to before a feeding in breast-fed infants. The change in concentration from pre to post feed did not differ from day 1 to day 2 for breast-fed infants for GLP-1, GIP, ghrelin and leptin, indicating stability in the response. The change in PYY concentration differed between day 1 and day 2 for breast-fed infants. This result was not anticipated and needs to be confirmed or refuted in a larger sample size. The increase in PYY, GLP-1 and GIP concentrations during a feed suggests that these peptides likely act as satiation signals in healthy, breast-fed infants as well. The increase in leptin concentrations that was seen in breast fed infants could be related to leptin naturally found in breast milk.

As hypothesized, the concentration of several gut peptides prior to a feeding, namely PYY and leptin, were stable within subjects from one visit to the next. The pre-feeding concentration of all other peptides of interest, GLP-1, GIP and ghrelin, were not stable within subjects. Interestingly, several gut peptides were related to one another prior to feedings. The strongest relationship was between pre-feeding concentration of GLP-1 and leptin; this association was present prior to feeding at both visits. The relationship between GLP-1 (satiation signal) and leptin (adiposity signal) suggests a system of interconnected signals working towards energy homeostasis.

One peptide of interest, GIP, varied significantly in baseline concentration between breast-fed and formula-fed infants at one visit. No other significant
differences were found in baseline concentrations between these groups. Therefore for the majority of gut peptides, concentrations prior to a feeding did not differ between breastfed and formula fed infants.

Other noteworthy findings from this pilot study relate to the feeding dynamics. Overall, breast-fed infants were stable in terms of volume of feed, volume of feed per kilogram bodyweight, duration of lab feed and rate of feeding, from one feed to the next. However there was variability in the feeding dynamics of formula-fed infants, most notably the lower volume of intake when ePHF was consumed compared to CMF.

Future studies should attempt to control the factors that we found varied between infants. Future work should consider providing a fixed volume of feeding, a fixed time of feeding, and blood sample collection at a fixed time after the feeding. This reduction in variability would allow for better assessment of the gut peptide response to diets of different composition. This study is on-going and the data analysis presented here will be repeated in a larger sample size upon completion.
### Table 1: Nutritional composition of the test formulas

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>CMF (Enfamil)</th>
<th>ePHF (Nutramigen)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Calories, kcal/100 ml</td>
<td>67.7</td>
<td>67.7</td>
</tr>
<tr>
<td>Protein, g/100 ml</td>
<td>1.4</td>
<td>1.9</td>
</tr>
<tr>
<td>Sources of protein</td>
<td>Nonfat milk (casein/whey), whey protein concentrate</td>
<td>Hydrolyzed casein protein</td>
</tr>
<tr>
<td>FAA (µmol/100 ml)</td>
<td>86.4</td>
<td>8037.5</td>
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<tr>
<td>Free glutamate (µmol/100 ml)</td>
<td>12.5</td>
<td>723.8</td>
</tr>
<tr>
<td>Carbohydrate, g/100 ml</td>
<td>7.4</td>
<td>7.0</td>
</tr>
<tr>
<td>Sources of carbohydrate</td>
<td>Lactose, galactooligosaccharides, polydextrose</td>
<td>Corn syrup solids, modified corn starch</td>
</tr>
<tr>
<td>Fat, g/100 ml</td>
<td>3.6</td>
<td>3.6</td>
</tr>
<tr>
<td>Sources of fat</td>
<td>4% palm olein, 19.5% coconut, 19.5% soy, 14.5% high-oleic sunflower oil</td>
<td>44% palm olein, 19.5% coconut, 19.5% soy, 14.5% high-oleic sunflower oil</td>
</tr>
<tr>
<td>DHA, mg/100 ml</td>
<td>0.23</td>
<td>0.23</td>
</tr>
<tr>
<td>ARA, mg/100 ml</td>
<td>0.11</td>
<td>0.11</td>
</tr>
</tbody>
</table>

Abbreviations: CMF, cow milk formula; ePHF, extensive protein hydrolysate formula; DHA, docosahexaenoic acid; ARA, arachidonic acid; FAA, free amino acid.
Table 2: Demographic and anthropometric characteristics of study participants by aim

<table>
<thead>
<tr>
<th></th>
<th>Aim 1: Formula fed dyads (n=6)</th>
<th>Aim 2: Breast fed dyads (n=4)</th>
<th>All infants (n=10)</th>
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<tbody>
<tr>
<td><strong>Infants</strong></td>
<td></td>
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<td></td>
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<tr>
<td>Gender (n)</td>
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<td>Male</td>
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<td>3</td>
<td>6</td>
</tr>
<tr>
<td>Female</td>
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<td>4</td>
</tr>
<tr>
<td><strong>Race (n)</strong></td>
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<td>White</td>
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<td>4</td>
</tr>
<tr>
<td>AA</td>
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<td>2</td>
<td>5</td>
</tr>
<tr>
<td>Other/Mixed</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td><strong>Age at visit 1 (days)</strong></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>92 (22)</td>
<td>76 (38)</td>
<td>84 (28)</td>
</tr>
<tr>
<td>Min</td>
<td>66</td>
<td>35</td>
<td>35</td>
</tr>
<tr>
<td>Max</td>
<td>115</td>
<td>121</td>
<td>121</td>
</tr>
<tr>
<td><strong>Weight at visit 1 (kg)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Mean (SD)</td>
<td>5.7 (0.8)</td>
<td>6.2 (1.7)</td>
<td>5.9 (1.2)</td>
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<tr>
<td>Min</td>
<td>4.8</td>
<td>4.7</td>
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<tr>
<td>Max</td>
<td>7.0</td>
<td>8.4</td>
<td>8.4</td>
</tr>
<tr>
<td><strong>Length at visit 1 (cm)</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>59 (3)</td>
<td>60 (5)</td>
<td>59 (3)</td>
</tr>
<tr>
<td>Min</td>
<td>54</td>
<td>56</td>
<td>54</td>
</tr>
<tr>
<td>Max</td>
<td>62</td>
<td>66</td>
<td>66</td>
</tr>
<tr>
<td><strong>Mothers</strong></td>
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</tr>
<tr>
<td>Maternal age (years)</td>
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</tr>
<tr>
<td>Mean (SD)</td>
<td>28 (8)</td>
<td>30 (3)</td>
<td>29 (6)</td>
</tr>
<tr>
<td>Min</td>
<td>19</td>
<td>28</td>
<td>19</td>
</tr>
<tr>
<td>Max</td>
<td>41</td>
<td>35</td>
<td>41</td>
</tr>
<tr>
<td>Parity (% primiparous)</td>
<td>33%</td>
<td>25%</td>
<td>30%</td>
</tr>
<tr>
<td><strong>Maternal BMI</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>32 (4)</td>
<td>31 (8)</td>
<td>31 (5)</td>
</tr>
<tr>
<td>Min</td>
<td>26</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>Max</td>
<td>35</td>
<td>41</td>
<td>41</td>
</tr>
<tr>
<td>% overweight/obese</td>
<td>100%</td>
<td>75%</td>
<td>90%</td>
</tr>
<tr>
<td>High school graduates n (%)</td>
<td>3 (50%)</td>
<td>4 (100%)</td>
<td>7 (70%)</td>
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</table>
Each formula fed infant was tested on 2 separate days. Days differed on the type of formula fed to the infant (CMF vs. ePHF) in the bottle. One of the 6 infants was not able to complete the ePHF due to spit up.

Volume was measured using bottle weight prior to feeding, bottle weight after feeding for formula fed infants.

<table>
<thead>
<tr>
<th></th>
<th>CMF feeding</th>
<th>ePHF feeding**</th>
<th>Difference score** (CMF-ePHF)</th>
<th>Relative Difference score (CMF-ePHF/CMF+ePHF)</th>
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<tr>
<td><strong>Volume of lab feed</strong>&lt;sup&gt;†&lt;/sup&gt; (ml)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>156 (61)</td>
<td>140 (53)</td>
<td>16</td>
<td>5.4%</td>
</tr>
<tr>
<td>Min</td>
<td>88</td>
<td>62</td>
<td></td>
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<tr>
<td>Max</td>
<td>233</td>
<td>204</td>
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<table>
<thead>
<tr>
<th></th>
<th>CMF feeding</th>
<th>ePHF feeding**</th>
<th>Difference score** (CMF-ePHF)</th>
<th>Relative Difference score (CMF-ePHF/CMF+ePHF)</th>
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<tbody>
<tr>
<td><strong>Volume of lab feed</strong>&lt;sup&gt;†&lt;/sup&gt; (mL/kg body weight)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>27 (11)</td>
<td>24 (9)</td>
<td>3</td>
<td>5.8%</td>
</tr>
<tr>
<td>Min</td>
<td>17</td>
<td>12</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td>41</td>
<td>36</td>
<td></td>
<td></td>
</tr>
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</table>

<table>
<thead>
<tr>
<th></th>
<th>CMF feeding</th>
<th>ePHF feeding**</th>
<th>Difference score** (CMF-ePHF)</th>
<th>Relative Difference score (CMF-ePHF/CMF+ePHF)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Duration of lab feed</strong> (min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>11 (6)</td>
<td>15 (6)</td>
<td>-4</td>
<td>15.3%</td>
</tr>
<tr>
<td>Min</td>
<td>5</td>
<td>10</td>
<td>-5</td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td>17</td>
<td>24</td>
<td>-7</td>
<td></td>
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<table>
<thead>
<tr>
<th></th>
<th>CMF feeding</th>
<th>ePHF feeding**</th>
<th>Difference score** (CMF-ePHF)</th>
<th>Relative Difference score (CMF-ePHF/CMF+ePHF)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Rate of Feeding</strong> (mL/min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>17 (11)</td>
<td>10 (5)</td>
<td>7</td>
<td>25.9%</td>
</tr>
<tr>
<td>Min</td>
<td>6</td>
<td>6</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td>38</td>
<td>17</td>
<td>21</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>CMF feeding</th>
<th>ePHF feeding**</th>
<th>Difference score** (CMF-ePHF)</th>
<th>Relative Difference score (CMF-ePHF/CMF+ePHF)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Elapsed time from last feed to lab feed</strong> (min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>204 (42)</td>
<td>218 (28)</td>
<td>-14</td>
<td>3.3%</td>
</tr>
<tr>
<td>Min</td>
<td>161</td>
<td>182</td>
<td>-21</td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td>270</td>
<td>250</td>
<td>20</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th></th>
<th>CMF feeding</th>
<th>ePHF feeding**</th>
<th>Difference score** (CMF-ePHF)</th>
<th>Relative Difference score (CMF-ePHF/CMF+ePHF)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Elapsed time from lab feed to blood draw</strong> (min)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>11 (5)</td>
<td>9 (4)</td>
<td>2</td>
<td>10%</td>
</tr>
<tr>
<td>Min</td>
<td>5</td>
<td>5</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td>19</td>
<td>13</td>
<td>6</td>
<td></td>
</tr>
</tbody>
</table>

*Each formula fed infant was tested on 2 separate days. Days differed on the type of formula fed to the infant (CMF vs. ePHF) in the bottle.

**One of the 6 infants was not able to complete the ePHF due to spit up.

†Volume was measured using bottle weight prior to feeding, bottle weight after feeding for formula fed infants.
Table 3b: Feeding Dynamics for Breast Fed Infants*: Stability between days

<table>
<thead>
<tr>
<th></th>
<th>BM feeding 1</th>
<th>BM feeding 2</th>
<th>Difference score** (BF1-BF2)</th>
<th>Relative Difference score (BF1-BF2/BF1+BF2)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Volume of lab feed (ml)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>121 (62)</td>
<td>124 (19)</td>
<td>-3</td>
<td>1.2%</td>
</tr>
<tr>
<td>Min</td>
<td>53</td>
<td>97</td>
<td>-44</td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td>204</td>
<td>141</td>
<td>63</td>
<td></td>
</tr>
<tr>
<td><strong>Volume of lab feed (mL/kg body weight)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>21 (10)</td>
<td>21 (7)</td>
<td>0</td>
<td>0%</td>
</tr>
<tr>
<td>Min</td>
<td>6</td>
<td>14</td>
<td>-8</td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td>30</td>
<td>27</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td><strong>Duration of lab feed (min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>17 (7)</td>
<td>18 (8)</td>
<td>-1</td>
<td>2.8%</td>
</tr>
<tr>
<td>Min</td>
<td>12</td>
<td>10</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td>27</td>
<td>29</td>
<td>-2</td>
<td></td>
</tr>
<tr>
<td><strong>Rate of Feeding (mL/min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>7 (2)</td>
<td>8 (4)</td>
<td>-1</td>
<td>6.6%</td>
</tr>
<tr>
<td>Min</td>
<td>4</td>
<td>5</td>
<td>-1</td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td>8</td>
<td>13</td>
<td>-5</td>
<td></td>
</tr>
<tr>
<td><strong>Elapsed time from last feed to lab feed (min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>200 (34)</td>
<td>144 (66)</td>
<td>56</td>
<td>16.2%</td>
</tr>
<tr>
<td>Min</td>
<td>164</td>
<td>74</td>
<td>90</td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td>242</td>
<td>230</td>
<td>12</td>
<td></td>
</tr>
<tr>
<td><strong>Elapsed time from lab feed to blood draw (min)</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>18 (12)</td>
<td>10 (4)</td>
<td>8</td>
<td>28.5%</td>
</tr>
<tr>
<td>Min</td>
<td>3</td>
<td>5</td>
<td>-2</td>
<td></td>
</tr>
<tr>
<td>Max</td>
<td>29</td>
<td>15</td>
<td>14</td>
<td></td>
</tr>
</tbody>
</table>

*Each breast fed infant was tested on 2 separate days.
†Volume was measured by infant test weighing (infant weight prior to feeding, infant weight after feeding.)
### Table 4: Descriptive statistics for each gut peptide by diet

<table>
<thead>
<tr>
<th></th>
<th>Formula Fed Infants (Aim 1)</th>
<th>Breast Fed Infants (Aim 2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CMF</td>
<td>ePHF</td>
</tr>
<tr>
<td></td>
<td>Before</td>
<td>After</td>
</tr>
<tr>
<td>n</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>PYY (pg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>439.1 (185.7)</td>
<td>712.2 (159.2)</td>
</tr>
<tr>
<td>Min</td>
<td>212.9</td>
<td>404.6</td>
</tr>
<tr>
<td>Max</td>
<td>690.5</td>
<td>852.5</td>
</tr>
<tr>
<td>GLP-1 (pg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>118.7 (77.0)</td>
<td>265.1 (90.8)</td>
</tr>
<tr>
<td>Min</td>
<td>58.8</td>
<td>203.5</td>
</tr>
<tr>
<td>Max</td>
<td>241.3</td>
<td>444.7</td>
</tr>
<tr>
<td>GIP (pg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>738.9 (423.4)</td>
<td>1033.7 (310.9)</td>
</tr>
<tr>
<td>Min</td>
<td>201.4</td>
<td>788.4</td>
</tr>
<tr>
<td>Max</td>
<td>1176.8</td>
<td>1509.4</td>
</tr>
<tr>
<td>Ghrelin (pg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>71.9 (18.7)</td>
<td>57.4 (26.8)</td>
</tr>
<tr>
<td>Min</td>
<td>47.78</td>
<td>25.8</td>
</tr>
<tr>
<td>Max</td>
<td>91.72</td>
<td>93.8</td>
</tr>
<tr>
<td>Leptin (pg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mean (SD)</td>
<td>3763.8 (2007.5)</td>
<td>3599.2 (2217.9)</td>
</tr>
<tr>
<td>Min</td>
<td>1114.2</td>
<td>1697.5</td>
</tr>
<tr>
<td>Max</td>
<td>6689.7</td>
<td>7719.7</td>
</tr>
</tbody>
</table>
Table 5a: Correlation matrix of gut peptide concentrations (n=7) prior to a feeding (Aim 3)

<table>
<thead>
<tr>
<th></th>
<th>PYY Day 1 Before Feeding</th>
<th>GLP-1 Day 1 Before Feeding</th>
<th>GIP Day 1 Before Feeding</th>
<th>Ghrelin Day 1 Before Feeding</th>
<th>Leptin Day 1 Before Feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYY Day 2 Before Feeding</td>
<td><strong>0.80</strong></td>
<td>0.18</td>
<td>0.20</td>
<td>0.21</td>
<td>0.33</td>
</tr>
<tr>
<td>GLP-1 Day 2 Before Feeding</td>
<td>0.35</td>
<td>0.43</td>
<td>0.006</td>
<td>0.47</td>
<td><strong>0.80</strong></td>
</tr>
<tr>
<td>GIP Day 2 Before Feeding</td>
<td>0.16</td>
<td><strong>0.69</strong></td>
<td>0.54</td>
<td>0.26</td>
<td>0.57</td>
</tr>
<tr>
<td>Ghrelin Day 2 Before Feeding</td>
<td>-0.21</td>
<td>-0.31</td>
<td>-0.19</td>
<td>0.02</td>
<td>-0.12</td>
</tr>
<tr>
<td>Leptin Day 2 Before Feeding</td>
<td>0.12</td>
<td><strong>0.78</strong></td>
<td>0.50</td>
<td>0.32</td>
<td><strong>0.90</strong></td>
</tr>
</tbody>
</table>

*Bold numbers indicate significant (p<0.10) correlations

Table 5b: Correlation matrix of gut peptide concentrations (n=8) prior to day 1 feeding (Aim 3)

<table>
<thead>
<tr>
<th></th>
<th>PYY Day 1 Before Feeding</th>
<th>GLP-1 Day 1 Before Feeding</th>
<th>GIP Day 1 Before Feeding</th>
<th>Ghrelin Day 1 Before Feeding</th>
<th>Leptin Day 1 Before Feeding</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYY Day 1 Before Feeding</td>
<td>1</td>
<td>0.28</td>
<td>0.34</td>
<td>0.24</td>
<td>0.32</td>
</tr>
<tr>
<td>GLP-1 Day 1 Before Feeding</td>
<td>0.28</td>
<td>1</td>
<td><strong>0.82</strong></td>
<td>0.12</td>
<td><strong>0.74</strong></td>
</tr>
<tr>
<td>GIP Day 1 Before Feeding</td>
<td>0.34</td>
<td><strong>0.82</strong></td>
<td>1</td>
<td>0.03</td>
<td>0.36</td>
</tr>
<tr>
<td>Ghrelin Day 1 Before Feeding</td>
<td>0.32</td>
<td>0.12</td>
<td>0.36</td>
<td>1</td>
<td>0.31</td>
</tr>
<tr>
<td>Leptin Day 1 Before Feeding</td>
<td>0.32</td>
<td><strong>0.74</strong></td>
<td>0.36</td>
<td>0.31</td>
<td>1</td>
</tr>
</tbody>
</table>

*Bold numbers indicate significant (p<0.10) correlations
FIGURES

Figure 1a: Intake volume of formula feedings (CMF, ePHF) at the subject level

Figure 1b: Intake volume of breastfeeding at the subject level
Figure 2a: PYY response to formula feedings

Figure 2b: PYY response to breast milk (BM) feedings
Figure 2c: GLP-1 response to formula feedings

Figure 2d: GLP-1 response to breast milk feedings
Figure 2e: GIP response to formula feedings

![GIP response to formula feedings graph]

Figure 2f: GIP response to breast milk feedings

![GIP response to breast milk feedings graph]
Figure 2g: Ghrelin response to formula feedings

Figure 2h: Ghrelin response to breast milk feedings
Figure 2i: Leptin response to formula feedings

Leptin (pg/mL)

CMF feeding  
Before  
After  
ePHF feeding  
Before  
After

Figure 2j: Leptin response to breast milk feedings

Leptin (pg/mL)

BM feeding 1  
Before  
After  
BM feeding 2  
Before  
After
REFERENCES


Appendix

IRB LETTER

DATE: June 14, 2013

TO: Jillian Trabulsi, PhD
FROM: University of Delaware IRB
STUDY TITLE: [407100-1] Infant feeding and biomarkers of satiation and satiety in healthy term infants.

SUBMISSION TYPE: New Project
ACTION: APPROVED APPROVAL DATE: June 14, 2013
EXPIRATION DATE: February 19, 2014
REVIEW TYPE: Full Committee Review

Thank you for your submission of New Project materials for this research study. The University of Delaware IRB has APPROVED your submission. This approval is based on an appropriate risk/benefit ratio and a study design wherein the risks have been minimized. All research must be conducted in accordance with this approved submission.

This submission has received Full Committee Review based on the applicable federal regulation.

Please remember that informed consent is a process beginning with a description of the study and insurance of participant understanding followed by a signed consent form. Informed consent must continue throughout the study via a dialogue between the researcher and research participant. Federal regulations require each participant receive a copy of the signed consent document.

Please note that any revision to previously approved materials must be approved by this office prior to initiation. Please use the appropriate revision forms for this procedure.
All SERIOUS and UNEXPECTED adverse events must be reported to this office. Please use the appropriate adverse event forms for this procedure. All sponsor reporting requirements should also be followed.

Please report all NON-COMPLIANCE issues or COMPLAINTS regarding this study to this office.

Please note that all research records must be retained for a minimum of three years.

Based on the risks, this project requires Continuing Review by this office on an annual basis. Please use the appropriate renewal forms for this procedure.

If you have any questions, please contact Jody-Lynn Berg at (302) 831-1119 or jlberg@udel.edu. Please include your study title and reference number in all correspondence with this office.