Breast Milk Macronutrient Composition and Infant Growth in Rural West Africa

by
Margaret A. Gruca
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Written by: Margaret A. Gruca
has been approved for the Department of Anthropology

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Dr. Robin Bernstein (Committee Chair)

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Dr. Darna Dufour

_____
Dr. Joanna Lambert

Date _____

The final copy of this thesis has been examined by the signatories, and we find that both the content and the form meet acceptable presentation standards of scholarly work in the above mentioned discipline.

Margaret A. Gruca (M.A. Anthropology)

Breast Milk Macronutrient Composition and Infant Growth in Rural West Africa

Thesis directed by Associate Professor Robin Bernstein

Abstract

Infants in The Gambia often experience intense growth faltering within the first two years of life, which is strongly related to season of birth. Infants born in the wet season are significantly more likely to experience growth stunting and faltering and are ten times more likely to die in early adulthood than those infants born in the dry season. Women, who burden much of the agricultural workload, must increase physical labor during the wet season to prepare fields and plant the crops for the next season's harvest. The combined stress of caloric deprivation and intense physical labor during the wet season is energetically taxing on mothers and has been hypothesized to contribute to the differential individual short- and long-term outcomes dependent on season of birth. This study aims to assess different environmental, socioeconomic, and anthropometric variables associated with breast milk macronutrient composition and how these variables then affect infant growth outcomes. Results from this study demonstrate that breast milk is relatively buffered from changes in environment, socioeconomic factors, and maternal anthropometrics. However, examination of longitudinal patterns of milk macronutrients shows evidence of significant inter-individual variation. Significant variables in predicting milk macronutrients in this study include infant season of birth, number of livestock per household, and pre and postnatal infant dietary supplementation. In assessing the effect of these macronutrients on infant growth, results demonstrate that infant growth is significantly affected by fat (FAT), protein (PRO), lactose (LAC), and true protein (TRP) along with infant sex, maternal parity and socioeconomic factors

such as total number of livestock and number of members in the household. Analysis of Gambian milk samples demonstrate that mean values for LAC (6.58g/dL), FAT (3.62g/dL), and PRO (1.28g/dL) fall within the ranges of values reported from other population studies. In conclusion, this study demonstrates that while mean population breast milk composition is relatively buffered from many of the variables examined in this study, there is significant inter-individual variation in macronutrients that may contribute to infant growth outcomes that are dependent upon milk macronutrients, socioeconomic context, maternal anthropometrics, maternal parity, infant sex and season of birth in The Gambia.

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List of Acronyms

BMI – body mass index

BMR – basal metabolic rate

FAT – fat

FeFol – iron folate (prenatal supplement)

HAZ – height-for-age z-score (measure for growth stunting)

LAC – lactose

LNS – lipid-based nutrient supplement (postnatal supplement)

MMN – multiple-micronutrient (prenatal supplement)

MUAC – mid-upper arm circumference

NPN – non-protein nitrogen (nitrogenous compounds not amino acids/polypeptides; mostly urea)

PE – protein energy (prenatal supplement)

PEMMN – protein energy multiple-micronutrient (prenatal supplement)

PLAC – placebo

PRO – protein

TRP – true protein (total nitrogen content in milk minus the non-protein nitrogen fraction)

TSF – triceps skinfold

WAZ – weight-for-age z-score (measure for growth faltering)

WHZ – weight-for-height z-score (measure for nutritional status)

Introduction

Study Aims

Investigating the impact of early life nutrition, particularly breast milk composition, on infant growth is critical in predicting later-life health outcomes (Barker 1997). In The Gambia, a small country in West Africa, infants who are born in the wet season are ten times more likely to die in early adulthood compared to those infants born in the dry season (Moore et al. 1997). Women, who shoulder much of the agricultural workload, must increase physical labor during the wet season to prepare fields and plant the crops for the next season's harvest. The combined stress of caloric deprivation and intense physical labor during the wet season is energetically taxing on mothers and has been hypothesized to contribute to the differential individual short- and long-term infant growth and developmental outcomes dependent on season of birth (Moore et al. 2004; Prentice and Prentice 1988; Prentice and Prentice 1995).

Infants born in the "hungry", or wet, season are significantly more likely to experience infection and intense periods of diarrheal disease (Rowland et al. 1977). This high rate of infection is in large part due to the long duration (up to 8 hours) spent with alternate care-givers and away from their mother (and her breast milk) during the work day (Lowe et al. 2016; Moore et al. 1997; Prentice and Prentice 1995). Studies from both urban and rural Africa, as well as rural Bangladesh, have demonstrated the pronounced negative effect that increased intestinal permeability following intestinal infection has on infant growth and development (Goto et al. 2009; Lunn et al. 1991; Rowland et al. 1985). Lower maternal body weight and poor nutrition during pregnancy decreases average birth weight of Gambian infants by an average of 200-300g which in turn doubles the incidence of low-birth-weight infants (<2.5kg); however, this can be reversed through maternal prenatal supplementation (Ceesay et al. 1997). Despite mothers being marginally nourished and

undergoing intense physical demands during the wet season, milk output remains robust and comparable to mothers in other countries (Prentice et al. 1986; Prentice et al. 1994). Whitehead et al. have demonstrated that breast milk volume decreases at the peak of the wet season which is largely attributed to a decrease in maternal subcutaneous fat (Whitehead et al. 1978). Additionally, Prentice et al. demonstrated that average breast milk fat levels among mothers are at their lowest immediately preceding the beginning of the wet season (Prentice et al. 1981b). The combined effect of decreased breast milk volume and decreased overall energy content in breast milk due to the decrease in fat content exacerbates the seasonally induced nutritional deficiencies encountered during early infancy. However, this low-output, low-fat breast milk is not ubiquitous across all mothers in the population. The milk of many mothers (Prentice et al. 1981a, b) with low breast milk output actually has very high levels of fat thus providing their infants with sufficient dietary fat highlighting the importance of examining both individual and population wide differences.

Other studies of infant development in Gambia including measurements of thymic growth (Collinson et al. 2003; Ngom et al. 2004), the infant gut microbiome (Davis et al. 2017), and milk immunoglobulin A (IgA) in infant immune development and disease susceptibility (Turner et al. 2003; Weaver et al. 1998) show that there are several pathways through which infant growth and morbidity can be affected. The Gambia provides a unique ‘natural experiment’ for investigating the effects of the environment and early life nutrition on infant development, the role of maternal factors, and their potential long-term effects on infant health, growth, and life history.

In this study, I analyzed breast milk samples from 214 Gambian mothers across the first 52 weeks of lactation to determine how breast milk macronutrient composition contributes to early infant growth to examine whether these putative early life influencers can help explain the phenomenon observed between infant season of birth and early adult mortality rates. In the

following pages, I will describe factors that contribute to variability in breast milk composition which is hypothesized to be a significant determinant of infant growth outcomes. From there, study variables will be assessed in relation to breast milk composition and infant growth including pre and postnatal maternal and infant nutritional supplementation, parity (whether or not the mother has had previous children), socioeconomic, environmental, and anthropometric factors. These variables and their impact on milk composition will be assessed on three different levels: 1) differences in breast milk composition and infant growth between individuals within a population, 2) differences in breast milk composition across time within individual mothers, and 3) differences in mean breast milk composition between groups of individuals from different geographical regions. The three aims that will be evaluated across these levels are as follows:

Aim 1 – Intra-population analysis of factors that influence breast milk macronutrient composition and intra-individual milk macronutrient variation: I will assess which socioeconomic, environmental, and anthropometric factors significantly affect breast milk macronutrient composition between Gambian mothers (inter-individual analysis). I will also describe Gambian mother intra-individual variability in breast milk macronutrients.

Aim 2 – Intra-population analysis of factors that influence infant growth: I will explore the relative contribution of breast milk macronutrients, socioeconomic, environmental, and anthropometric measurements to infant growth outcomes in Gambia.

Aim 3 – Inter-population comparison of mean breast milk macronutrient composition: The final aim of this research is to compare the mean breast milk macronutrient values generated by this

study to eleven previous studies of other populations, including one older Gambian study, in order to consider how methodological differences in collection and analysis of samples may impact the ability to compare across studies.

Background

Feeding Practices and the Compositional Determinants of Breast Milk

Breast milk is the primary source for infant nutrition during the first year of life (Chung et al. 2007; Imdad et al. 2011; WHO 2003). Numerous studies have demonstrated the health benefits gained from breastfeeding for both the mother and infant over that of formula feeding or donor milk, especially if sustained for at least the first six months postpartum (Abu-Saad and Fraser 2010; Ballard and Morrow 2013; Boyd et al. 2007; Butte et al. 1984a; Chung et al. 2007; Prentice 1996). Frequently beginning between the age of four to six months, and even earlier in some populations, complementary foods, or easily digestible, nutrient-rich foods processed by the caregiver, are introduced to supplement the infant's diet (Sellen 2010; Sellen 2007). However, early cessation of lactation and reliance on complementary foods and/or alternative feeding methods in place of breastfeeding have been associated with numerous detrimental health outcomes including compromised immune development, diabetes, and childhood obesity (Armstrong and Reilly 2002; Koletzko et al. 2009). Additionally, other studies and reviews have suggested that foods introduced before six months of age have little impact on the overall nutritional health of the infant as they still possess an immature gut and are therefore unable to effectively digest and receive significant nutritional value from non-breast milk foods (Lartey et al. 1999; Sellen 2001b; Sellen 2007; Webb-Girard et al. 2012). Breast milk is uniquely suited to the infant containing numerous bioactive, immunological, and nutritional factors, all of which are variable in both presence and quantity

throughout lactation (Ballard and Morrow 2013; Prentice 1996). All of these factors contribute to the healthy growth and development of the infant immune and metabolic systems, organ development, and gut microbiome (Cabrera-Rubio et al. 2012).

Breast milk composition changes throughout lactation to meet the differing needs of the infant. During the first few days following delivery, the mother will produce colostrum which is unique from all other milk produced throughout lactation in composition and volume (Ballard and Morrow 2013). Unlike the milk produced throughout most of lactation, colostrum contains a very high concentration of many immunological and bioactive components that are essential to early infant immune development, and a lesser concentration of primary macronutrients including fat, lactose, and protein. Between five days and two weeks postpartum, mothers produce milk that is typically considered intermediate (transitional) breast milk that, as the name suggests, shares similar characteristics to both colostrum and mature breast milk. During this period prior to maturation of the milk, higher levels of fat and protein will be present in the milk to meet the growth needs of a rapidly developing infant. Between four to six weeks following birth, breast milk is considered fully mature and shifts from having a primarily immunologic and metabolic developmental function to that of a nutritional function necessary to support growth. From this point forward, the fat and protein levels will remain relatively constant while there will be a gradual decline in lactose. Breast milk is generally buffered from acute physiological (e.g. illness, extreme levels of physical activity) and environmental (e.g. climate change, varying caloric intake) stress, although long-term changes in either can cause significant variability in milk composition (Bravi et al. 2016; Butte et al. 1984b; Picciano 2003; Prentice et al. 1994).

Understanding what drives intra-individual, intra-population, and inter-population variability in breast milk macronutrient content is essential in understanding how changes in

environment or physiology contribute to breast milk macronutrient composition and subsequent infant development. Physiological cues received by the infant during the early stages of life can have a significant impact on metabolic and immune function observed throughout childhood, adolescence, and adulthood. Barker first described this phenomenon of early life influences having significant, and at times detrimental, effects on later life health as "the fetal origins of health and disease" (Barker 1994), which was later subsumed under the developmental origins of health and disease (DOHaD) hypothesis (Silveira et al. 2007). Studies from several populations have demonstrated the pronounced negative effect early life stressors (such as increased intestinal permeability, parasitic and bacterial infections, and chronic inflammation) have on infant growth and development as they impact the infant's ability to utilize nutrients from food sources (Goto et al. 2009; Lunn et al. 1991; Rowland et al. 1985). For example, inadequate early life nutrition can result in an immature immune system which can leave the infant more susceptible to later-life disease. Additionally, the introduction of complementary foods to an infant with increased intestinal permeability and a hyper-sensitized immune system can result in allergic responses observed in early childhood that may persist in adulthood (MacDonald and Monteleone 2005).

There are numerous maternal factors that also contribute to her offspring's early life development. For example, decreased maternal body weight resulting from sustained insufficient nutrition during pregnancy decreases average birth weight, however in some cases this can be reversed through maternal prenatal supplementation (Ceesay et al. 1997; Moore et al. 1999). Moore et al. (1999) demonstrated that early life effects manifest in long-term health outcomes beginning during puberty and increase through early adulthood in rural Gambian populations. These rural populations are ones in which early adult mortality is strongly associated with season of birth, in that individuals born in the wet season are ten times more likely to die than those born

in the dry season once they reach young adulthood with the odds ratio (wet:dry) of premature death before the age of 15 being 3.65 and increasing to 10.4 by the age of 35. (Moore et al. 1997). It has been proposed that the combined effect of compromised immune development and nutritional deficiency early in life both contribute to negative health outcomes observed late in life in Gambian children (Moore 1998; Moore et al. 1999). However, how these early-life challenges are acquired and subsequently manifest beyond early development is still incompletely understood. As such, ongoing studies in the rural Gambian villages of Keneba, Kantong Kunda, Manduar and several others continue to investigate the impacts of early life nutrition and environment on later-life health outcomes.

Variables Associated with Breast Milk Macronutrient Content

Breast milk macronutrient content, while relatively constant among different populations, shows significant inter-individual variation (Butte et al. 1988; de Halleux and Rigo 2013; Emmett and Rogers 1997; Prentice 1996; Quinn et al. 2016). Mean macronutrient values across the first year of lactation for mothers who carry infants to term are 0.9-1.2 mg/dL for protein, 3.2-3.6 mg/dL for fat, and 6.7-7.8 mg/dL for lactose (Nommsen et al. 1991). However, these values can range significantly higher or lower and are dependent on several environmental, socioeconomic, and anthropometric variables within mother-infant pairs. Fat is especially variable and highly dependent on time of day and amount of breast milk expressed when collecting a sample, with values ranging between 1.9-7.9 mg/dL (Butte et al. 1988; de Halleux and Rigo 2013; Khan et al. 2013). For example, foremilk, or the first volume of milk expressed during feeding, is substantially lower in fat and caloric content than hindmilk, or the last volume of milk expressed during feeding. Temporal changes, either short term (i.e. within feed) or long-term (i.e. across lactation), in

breastmilk composition can be influenced by a number of factors which include both physiological and ecological variability (Neville et al. 2001; Picciano 2003).

Environmental and Socioeconomic Variables

Examples of environmental and socioeconomic variables that account for the variation in macronutrient content include seasonality, food availability, household income, and cultural practices surrounding child-care, breastfeeding and milk expression (Prentice and Prentice 1995). Mothers from higher income households and greater food availability produce milk with higher mean macronutrients when comparing individuals within the same geographical region (Chung et al. 2007; Hickey et al. 1997; Jelliffe and Jelliffe 1978; Webb et al. 2009). Many of these variables are interconnected and drive feeding practices for both the mother and infant which in turn affect infant nutrient availability. For example, in some cultures mothers are responsible for a significant amount of agricultural labor which in turn affects their own body composition and frequency with which they can feed their infant (Singh et al. 1989). As such, if the mother can only feed her infant 2-3 times regularly each day, mean breast milk macronutrient content will increase at each feed. In contrast, mothers who feed their infant "on-demand" throughout the day will have lower mean breast milk macronutrient output per feed, however they both will have similar overall average energy output for the daily milk volume produced. Cohen et al. (1994) have also demonstrated that infants self-regulate their milk intake as complementary foods are introduced in that they consume less total breast milk once other foods or liquids are being consumed. As such, the demand on the mothers to produce milk decreases throughout lactation, which may subsequently affect overall breast milk macronutrient content.

Physiological and Anthropometric Variables

Several maternal physiological variables have been noted to correlate with breast milk macronutrient content such as maternal body weight, diet, triceps skinfold, parity, and mammary gland development (Dewey et al. 1991; Neville et al. 2001; Prentice 1996; Prentice et al. 1981b; Whitehead et al. 1978). Mothers with higher body mass indices (BMI) and triceps skinfold (TSF; used as a proxy measure for body fat percentage (Durnin and Womersley 1974)), have previously been shown to produce milk with higher fat, however there is no inter-population association between BMI and milk energy for mothers with a normal BMI (>18.5) (Prentice et al. 1994). Even comparing obese mothers (BMI >30) to those that fall within normal BMI range, only increased levels of insulin and leptin, not macronutrients, are observed in their breast milk (De Luca et al. 2016; Watkins et al. 2003). Studies have demonstrated that acute changes in maternal BMI have relatively little effect on breast milk macronutrient content (Butte et al. 1984b; Dewey et al. 1994; Emmett and Rogers 1997). This may in part be due to the fact that females have a natural physiological buffer, in part due to relatively high body fat stores, that may facilitate acute regulation of their basal metabolic rate (BMR) throughout pregnancy and lactation to adequately meet the demands of the infant even under chronic caloric deprivation or high physical stress (Dufour and Sauther 2002; Ellison 2008; Poppitt et al. 1993). Even those with very low body fat or receiving less than adequate nutrition are able to produce breast milk that is sufficient for healthy infant development. However, mothers under chronic nutritional or physical stress can produce milk lower in caloric content, and, in cases of extreme malnutrition breast milk volume may be significantly reduced or a complete cessation of lactation may occur which can ultimately be fatal for the infant in impoverished communities where alternative feeding may not be available (Allen 1994).

Maternal parity also influences breast milk macronutrient composition. Primiparous mothers (mothers with no previous births) typically produce milk with higher levels of macronutrients than multiparous mothers (mothers with previous births) (Prentice et al. 1989). Additionally, primiparous mothers are more likely to experience difficulties with lactation during the first few days postpartum (Dewey et al. 2003). These differences observed in breast milk composition between mothers of different parity is likely in part related to differences in mammary gland development. Mammary gland development occurs in different stages with significant cellular differentiation occurring during puberty and pregnancy, which is regulated tightly by hormones (Anderson et al. 2007; Neville et al. 2002). Both reproductive hormones (including estrogen, prolactin, progesterone, placental lactogen) and metabolic hormones (including growth hormone, insulin, and leptin) influence mammary gland development and cellular differentiation, gene expression, and milk output. While mammary gland development during pregnancy is nearly identical in both primiparous and multiparous mothers, transcriptional levels of gene expression in alveolar epithelial cells before conception and ductal morphogenesis, or the change in structure and function of ductal glands during pregnancy, are significantly different between the two (Anderson et al. 2007; Russo et al. 1992). Gene expression can be altered epigenetically in fetal and early infant development, result in modified tissue development in the fetus (Jaenisch and Bird 2003), and could potentially have critical implications for the function of different cell types such as glandular tissue in the breast. As these cells are tightly regulated by hormones, early alterations in metabolic pathways may dictate life-long changes in gene expression and subsequent function in adulthood such as those pathways responsible for milk production. Such factors likely contribute to individual variation in both output and mean macronutrient composition of breast milk across lactation in humans, however this direct relationship has only been explicitly demonstrated in other

mammals (Miller et al. 2006; Rudolph et al. 2007; Russo et al. 1992). Because of the influence of metabolic hormones on mammary gland programming, which begins within weeks of a woman's life, it is likely that the early environmental and physiological cues (e.g. body composition and diet) received by a woman as an infant have an effect on their milk composition and output in adulthood (Anderson et al. 2007; Farmer et al. 2014; Rezaei et al. 2016; Rudolph et al. 2007).

These aforementioned maternal physiological variables are further affected by environmental and socioeconomic variables such as food availability, household income, number of members within a household, and climate (e.g. season of conception and birth) in that scarcity of resources, either due to decreased food availability or increased household demand, would be expected to negatively impact either maternal milk production or macronutrients (Lawrence et al. 1987; Moore et al. 1997). All of these factors combined create a multi-layered interplay which

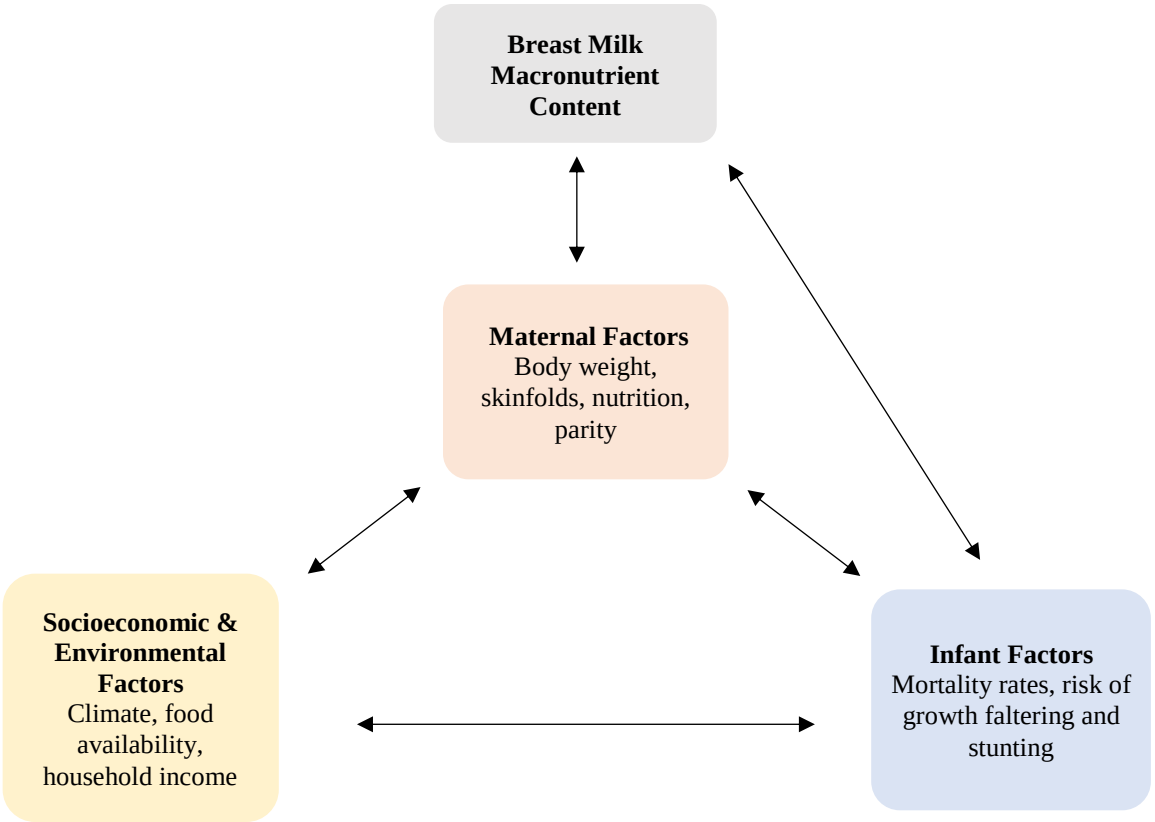

Figure 1. Flowchart showing relationship of breast milk macronutrient content to maternal, socioeconomic and environmental, and infant factors.

links breast milk macronutrient content to maternal variables that subsequently influence and are influenced by demographic and environmental factors and infant growth outcomes (**Figure 1**).

Impact of Environmental Cues on Early Infant Programming

Early life environment has a significant role in later-life health outcomes. Epigenetics, or non-DNA modes of inheritance and gene expression at both a cellular and organismal level, have introduced a new means by which to understand the impact of environment on micro-evolution and inheritance (Jablonka and Lamb 1999; Meier et al. 2006). Therefore, while acute changes in infant nutrition may not often have pronounced effects on early infant growth trajectories, there may be phenotypically unobservable pathologies that manifest later in life through immune and metabolic programming. Infants who experience chronic nutrient deprivation have an increased susceptibility to developmental growth faltering and later-life chronic disease (Barker 1994; Ellison 2005; Hanson and Gluckman 2008; Saffery and Novakovic 2014; Silveira et al. 2007). Barker (1997) describes critical stages of fetal development in which cells that are rapidly dividing are particularly susceptible to environmental influence. The way in which these early cells are programmed can have long-term systemic effects on infant health including negative long-term outcomes of common diseases such as diabetes, hypertension, stroke disorders and short-term negative outcomes such as lower infant birth weights and slower growth rates. Gut health, including the gut microbiome, also has an acute role in infant immune development which may be influenced by maternal nutrition and health, both of which are themselves influenced by early-life programming (Barker 1997; MacDonald and Monteleone 2005; Saffery and Novakovic 2014). As such, it is also important to consider the possibility of alternative modes of inheritance, such as transgenerational epigenetic inheritance, in which environmental effects alter gene expression and

such effects may subsequently be passed from mother to infant (Bjorklund 2006; Jablonka and Raz 2009). There are numerous mechanisms that have been proposed by which this may occur, particularly genomic imprinting during fetal development, however the mechanism and direct effect of such changes has yet to be further elucidated in humans (Heard and Martienssen 2014; Morgan et al. 1999; Probst et al. 2009; Whitelaw and Whitelaw 2008). Nevertheless, it is important to examine the relationship between maternal and infant variables to first determine if there is a causal relationship.

Components of the gut, oral, milk and skin microbiome can be transferred directly through contact between mother and child (e.g. during delivery and breastfeeding), which can in turn influence nutrient uptake and be further shaped by sampling the environment as the infant becomes mobile as well as subsequent changes in diet across infancy and childhood (Costello et al. 2012; Hooper and Gordon 2001; Kau et al. 2011; Mackie et al. 1999). Additionally, bacteria within the microbiome influence immune development and alter gene expression in metabolic pathways (Mueller et al. 2015; Renz et al. 2012; Sommer and Backhed 2013). These multitude of variables contribute to overall infant health and growth, both directly through exposure to environmental contaminants (Thompson 2012) and indirectly through seasonal effects on maternal physiology and breast milk composition/volume. Therefore, it is important to discern which might be most influential on later-life health (Grote et al. 2016; Prentice et al. 1981a; Prentice et al. 1981b).

Methodology in Assessing Breast Milk Macronutrient Content

Human breast milk analysis is used to answer questions regarding growth and development (Butte et al. 1984a; Ceesay et al. 1997; Chung et al. 2007; Dewey et al. 1992), evolution and life history (Ellison 2005; Ellison 2008; Hinde and Milligan 2011; Prentice and Prentice 1995), and

the early life programming that leads to altered immune function and later life disease (Hanson and Gluckman 2008). To address these questions, there has been a large increase in research examining intra- and inter-population variation in breast milk macronutrient composition, often requiring extensive field-based collections. Because breast milk collection is a sensitive process for the mother-infant pairs, appropriate methods must be considered that evaluate the ethical responsibilities to the subjects involved, resource availability, as well as laboratory limitations. A wide range of assays are employed in assessing breast milk macronutrient content, thereby making it difficult to discern whether population differences in fat, protein, and lactose values are the result of true population differences based on biology or ecology, or differences in methodology. In a recent review, Miller et al. (2013) examine the different methodologies commonly used in field research with regard to cost, availability of laboratory resources including samples, and sources of error. Assessing the reliability and repeatability of each method and collection protocol is critical in the comparative analysis of breast milk composition.

Collection protocol can significantly alter both mean and individual breast milk macronutrient values. Breast milk macronutrient content is highly variable diurnally, between each breast, and throughout expression (Ballard and Morrow 2013). Currently, there is no standard protocol for milk collection that is implemented by each study where milk is collected, and this has also historically been the case. Therefore, a variety of milk sample collection techniques are employed that vary in expression collected (i.e. fore vs. hind milk), time of day, and frequency of expression throughout the day. Due to fluctuation in breast milk macronutrient content throughout the day, samples are ideally collected from subjects at the same time of day. Additionally, mothers who feed less frequently will produce milk that is more concentrated and volume of milk produced is therefore often included in studies to accurately assess mean milk macronutrient composition

and infant caloric intake (Miller et al. 2013). Two primary methods are used to assess volume of a feed: 1) direct weighing of the infant before and after feeding (the 'test-weighing' method) and 2) the dose-to-mother deuterium dilution method. However, in cultures where the mother must work throughout the day, test-weighing is not always feasible, and the isotopic method is favored. The dose-to-mother deuterium method utilizes the stable deuterium isotope to measure water flux and subsequent breast milk consumption and, accounting for environmental water dilution, is accurate to within 1-2% of direct weighing (Butte et al. 1991; Coward et al. 1979). In order to control for differences in milk composition across the duration of a feed, some studies have the mother completely express the breast, pool the sample, and any excess may be returned to the mother and infant for normal feeding. While this gives an indirect measure of breast milk production, it is important to note that there are differences in the volume of milk that may be produced between infant suckling and manual expression in that the latter is typically less efficient (Becker et al. 2008; Jones et al. 2001; Meier et al. 2016). Despite this being a preferred method of analysis, complete emptying of the mammary gland is not acceptable in all populations due either to cultural practice or resource limitations, so researchers working in these contexts are generally only able to collect one sample during nursing (Miller et al. 2013). Because the fat levels decrease gradually across a nursing bout, it is best to collect a mid-feed sample rather than a fore or hind sample, however this can be difficult to gauge as the volume produced is variable between feedings and across lactation. Additionally, breast milk content can vary significantly between a mother's left and right breast (Kent et al. 2006; Neville et al. 1984), so in analysis it is best to pool samples from each breast. In such cases where it is not possible to collect samples from each breast, the researcher should strive to maintain consistency in the breast side collected throughout the study.

New methodologies in using dried milk spots (DMS) and mid-infrared analysis will hopefully provide a standard means for analysis which will reduce variability between studies (Miller et al. 2013; Smilowitz et al. 2014). Additionally, as an increasing number of studies assess and demonstrate the variability in milk composition both across lactation and throughout the day, there will hopefully be standardization in milk collection protocol for future studies that will include field-friendly techniques. The different methods of collection and analysis will be considered when examining both inter-population mean milk composition and inter-individual variation from this Gambian study.

Study Hypotheses: Milk Composition and Infant Growth

There are numerous variables that potentially influence breast milk composition including anthropometric, environmental, and socioeconomic factors. In the following study, I will model these factors against breast milk macronutrient composition across the first year of lactation to determine which significantly impact variation in fat (FAT), lactose (LAC), protein (PRO), and true protein (TRP). These factors, along with breast milk composition, will then be modeled in relation to infant growth to determine which are significant in affecting infant weight, height, triceps skinfold, weight-for-age (WAZ) z-scores, and height-for-age (HAZ) z-scores. I will also assess intra- and inter-individual variability in breast milk composition across the first year. Finally, I will assess how the mean macronutrient values obtained from this Gambian study compare to previous population studies examining breast milk composition. The following are the three primary aims of this study and subsequent hypotheses developed from the background literature review:

Aim 1 – Intra-population analysis of factors that influence breast milk macronutrient composition and intra-individual macronutrient analysis

H1: Because breast milk is relatively buffered from acute changes due to maternal diet and anthropometrics, I do not expect to see significant variance due in composition in either the intra-population or intra-individual analyses due to environmental, socioeconomic, or anthropometric variables. The effect of prenatal supplementation is expected to be most significant in the first few months following birth while postnatal supplementation is expected to be most significant after 6 months following birth.

Aim 2 – Intra-population analysis of factors that influence infant growth

H2: As breast milk is the primary source of infant nutrition for the first year of life in The Gambia, I expect that breast milk macronutrient composition will significantly affect infant growth while environment, socioeconomic, and maternal anthropometric variables are expected to have relatively little influence as the effect of these variables would be conveyed via the mother, and prior studies have shown these effects to be buffered by maternal compensatory mechanisms.

Aim 3 – Inter-population comparison of mean breast milk macronutrient composition

H3: I expect that the Gambian values for breast milk macronutrients obtained from this study will fall within the ranges of values reported from other studies as have demonstrated relatively little variance in breast milk between different populations.

Methods

Study Site

The Gambia is located in West Africa. Subjects were recruited from several villages in the West Kiang district, located in the lower river region (**Figure 2**). These populations rely heavily on agricultural work as a means of primary sustenance. Trade is limited in this region due to poor road access to outside major cities. Polygamy is practiced and the wives are ranked within the families by order of marriage to the husband. Higher ranking wives may delegate responsibilities to those of lower rank. All of the wives within the family contribute to child care, agricultural work, and household income (Jobe 2010).

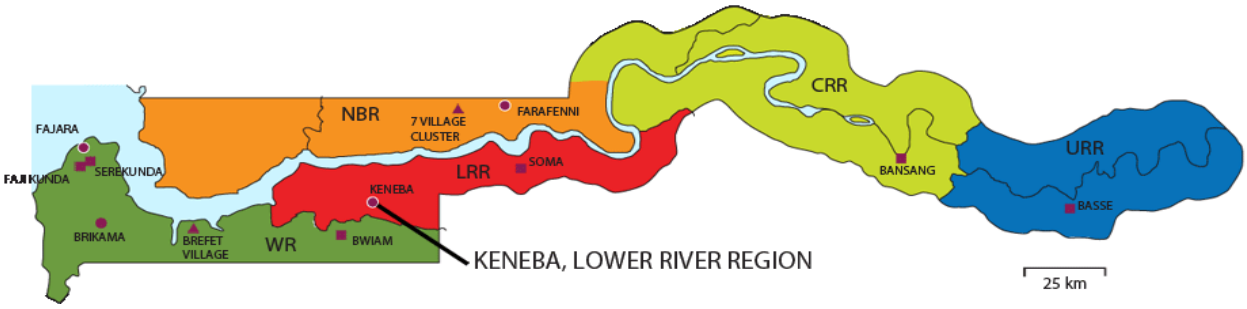

Figure 2..Image depicting the different regions in The Gambia, including the study site, Keneba. Image courtesy of MRC The Gambia.

Supplement Protocol

The Early Nutrition and Immune Development (ENID) Study (Early Nutrition and Immune Development: ISCRTN 49285450, PI S. Moore) is a randomized controlled trial focusing on nutritional contributors to infant immune outcomes. This study randomly assigned pregnant mothers to one of four prenatal supplements through use of an automated system upon enrollment: protein energy (PE), multiple micronutrient (MMN), protein energy plus multiple micronutrient (PEMMN), or iron folate (FeFol) (**Table 1**). FeFol is used as a control in this study as it is the standard of care for pregnant females in The Gambia. Infants were re-randomized to either a lipid

based nutritional supplement (LNS) or a placebo for 6 months following birth which was supplemented directly to the infant's diet (**Table 2**). ENID-Bioactives (PI R. Bernstein) was an add-on study to the ENID study, enrolling 285 mother-infant pairs from the 800 enrolled into ENID. Monthly milk samples, infant stool samples, and infant anthropometrics were collected from those enrolled in ENID-Bioactives. The present analysis is a sub-set of those carried out under the auspices of ENID-Bioactives (other analyses from ENID-Bioactives include milk adipokines, growth factors, and cytokines, milk oligosaccharides and gut microbiota). I measured milk macronutrients in all samples collected, and analyzed these data in combination with the collected anthropometric and socioeconomic data.

Prenatal Supplement	**FeFol**	**MMN**	**PE**	**PEMMN**
Number of Mothers	56	52	52	54

Table 1. Number of mothers assigned to iron folate (FeFol), multiple-micronutrient (MMN), protein energy (PE), and protein energy + multiple-micronutrient (PEMMN).

Postnatal Supplement	**PLAC**	**LNS**
Number of Infants	110	114

Table 2. Number of infants assigned to the placebo (PLAC) or lipid-based nutrient supplement (LNS).

Breast Milk Collection Protocol

Milk was collected mid-feed, typically in the morning before lunch, in 5mL volumes from both left and right breasts beginning at week 1, then monthly beginning at week 4 for one year following birth for a maximum of 13 collections per subject. While samples were collected at week 1, there was not enough sample volume for analysis in this study. Additionally, the number of samples tested are not uniform across all subjects (**Table 3**) or weeks (**Table 4**) as there were variable volumes of breast milk available for analysis which resulted in the exclusion of samples

for some subjects at different time points. For the current study, milk samples were included from 214 mothers with a median of 7 samples tested per subject with the majority of samples tested between 4 and 48 weeks postpartum. Samples were then stored at -80ºC, shipped on dry ice to Colorado, and further stored at -80ºC until analysis.

Demographic Information

Demographic data was collected for both mothers (**Table 5**) and infants (**Table 6**) born between January 2008 and ending in December 2013. Socioeconomic data including living status, education, income, and diet was also collected via survey (see **Appendix**) for each subject upon enrollment (**Table 7**). Number of livestock was used as an indirect measurement of income for subjects (McIntire et al. 2016).

Number of Samples Measured	Number of Mothers with this number of samples measured
1	10
2	3
3	9
4	9
5	13
6	24
7	58
8	41
9	31
10	20
11	7
12	1
Total Number of Milk Samples	**1512**

Table 3. Number of milk samples measured by number of total subjects for a total of n=214 subjects.

Week	1	4	8	12	16	20	24	28	32	36	40	44	48	52
Number of Samples	3	68	58	52	144	106	49	174	182	172	170	164	152	18

Table 4. Total number of milk samples tested by week.

Variable	
Mean Age in years (SD)	31.3 (7.1)
Maternal Season of Birth (Dry, Wet, Unknown)	43, 27, 146
Mean Weight in kg (SD)	57.5 (9.0)
Mean Triceps Skinfold in mm (SD)	13.7 (5.4)
Mean Mid-upper arm circumference in cm (SD)	26.3 (3.2)
Parity (primiparous, multiparious)	18, 198
Number of Child Deaths (0, 1, 2-6)	150, 41, 22
Number of miscarriages (0, 1, 2-3)	179, 29, 5

Table 5. Maternal anthropometric and demographic data (n=214). SD = Standard deviation

Variable	1 week	1 month	3 months	6 months	12 months
Sex (Male)	107	107	107	107	107
Season of Birth (Dry, Wet)	85, 129	85, 129	85, 129	85, 129	85, 129
Weight (g)	3289.9 (476.0)	4189.8 (644.2)	5838.6 (749.3)	6892.2 (894.5)	8176.2 (1013.5)
Length (cm)	50.6 (2.4)	53.9 (2.5)	60.2 (2.3)	64.5 (2.3)	72.3 (3.0)
Triceps Skinfold (mm)	5.7 (1.3)	7.8 (1.5)	8.7 (1.7)	8.6 (1.7)	8.0 (1.5)
Mid-upper arm circumference (mm)	109.7 (9.5)	121.2 (7.9)	133.2 (8.9)	136.5 (9.6)	137.9 (8.8)
Head circumference (cm)	35.2 (1.4)	36.7 (1.2)	39.7 (1.3)	41.7 (1.4)	44.3 (1.6)
Weight-for-height Z-score (WHZ)	-0.77 (1.22)	-0.22 (2.01)	-0.31 (1.24)	-0.46 (1.22)	-0.82 (1.38)
Weight-for-age Z-score (WAZ)	-0.31 (0.96)	-0.02 (1.30)	-0.20 (1.00)	-0.70 (1.04)	-1.18 (1.04)
Height-for-age Z-score (HAZ)	-0.06 (1.24)	-0.05 (1.53)	0.10 (1.07)	-0.45 (0.98)	-1.06 (1.14)

Table 6. Descriptive statistics for infant anthropometry over the first year of life (n=214). Z-scores are in reference to WHO anthropometric standards (WHO Anthro, Geneva, Switzerland). Values for anthropometric measurements are represented by mean (SD).

Variable	Mean (SD)
Father Education (years)	2.8 (4.7)
Number of Children, Father	8.6 (5.6)
Number of Wives (mean)	1.7 (0.9)
Mother Education (years)	1.9 (3.1)
Number of Children, Mother	4.0 (2.5)
Total Children	10.8 (7.8)
Total Members (household)	10.7 (7.4)
Fish eaten (times/week)	4.8 (1.8)
Meat eaten (times/week)	0.99 (0.97)
Sacks of Produce Produced (per month)	22.3 (20.9)
Number of Livestock (household)	7.2 (10.5)
Distance to Clinic (km)	9.4 (5.6)

Table 7. Demographic information collected through administration of a short survey upon study enrollment (n=214).

Anthropometry

Measurements were taken from infants every four weeks from birth and included weight, length, head circumference, triceps skinfold (TSF), abdominal circumference, and mid-upper arm circumference (MUAC). These measurements were then used to determine weight-for-age z-scores (WAZ), height-for-age z-scores (HAZ), and weight-for-height z-scores (WHZ). Z-scores were based on the WHO and Gambian reference values where z = (observed value - reference mean) / reference standard deviation. WHO anthropometric software was implemented to generate these data (WHO Anthro, Geneva, Switzerland). Both WHO and Gambian standardized z-scores were included in the analysis as Gambian infants are generally small for age and therefore WHO scores tend to overpredict growth stunting and faltering. When comparing Gambian infant data with infant data from other populations, WHO Z-scores are used. Maternal anthropometrics were taken during gestation at weeks 13, 20, and 30 and weeks 1 and 12 postpartum, and include weight, MUAC, and triceps skinfold (TSF) measurements.

Breast Milk Macronutrient Analysis

Stored milk samples were thawed at room temperature for 30 minutes. Samples were then pooled using 1mL from both the right and left breast whenever possible to create a 2mL sample. When there was not enough volume available from both breasts to create a pooled sample, samples were analyzed entirely using 2mL from one breast and the breast was noted on the sample spreadsheet. The 2mL sample was then diluted 10x by adding 18mL ddH_2O. Diluted samples were warmed to 40ºC using a water bath for no longer than 20 minutes prior to analysis. Each sample was run in duplicate using the Delta LactoScope which employs Fourier Transform Mid-Infrared Spectroscopy (FTIR) for rapid and reliable analysis of breast milk macronutrient content with a

high degree of accuracy (Delta Instruments B.V., Drachten, the Netherlands). This method shows < 3% variance in fat (FAT), total protein (PRO), true protein (TRP), and lactose (LAC) when tested against reference methods (Smilowitz et al. 2014). Pooled samples from subjects across multiple time points and sent to Eurofins for macronutrient analysis (Eurofins DQCI LLC, MN, USA). These reference values and samples were then used to calibrate the LactoScope.

Statistical Analysis

Analyses were conducted using JMP Pro 13.0 (SAS Institute, NC, USA). Milk macronutrients and maternal and infant growth measurements were $\log_{10}$ transformed prior to analysis. Preliminary stepwise regression models were used to identify significant variables from among the sets of data (**Tables 5-7**) to include in subsequent multivariate model-building. Because of the limited and heavily skewed data on variables such as agricultural productivity, meat and fish consumed per week, and total household income, these variables were not included in the models. Number of maternal and paternal children were also excluded from both models as they are likely collinear with parity and the total number of members in the household, and also did not show significance in the preliminary models. Results from these preliminary models are included in the appendix.

Following the preliminary analysis, a final summary least squares model was constructed to test the hypothesis H1 outlined in aim (1) to determine which variables (socioeconomic, maternal anthropometric, environmental) are significant predictors of breast milk macronutrient composition. The variables included in this model are: infant sex, infant birth season, pre and postnatal supplement group, maternal parity, maternal weight, maternal pre and postnatal weight change, total livestock, total number in the household, number of co-wives, total years of maternal

and paternal schooling (including traditional and Arabic schooling), and distance to the clinic. Two additional models were generated to examine the same variables and their effect on milk macronutrients over the 1) first six months and 2) last six months of the first year of lactation. All three models of milk macronutrients controlled for repeated measurements from the same mother-infant pairs with infant ID as a random factor.

To demonstrate the dynamics of milk composition across the first year of lactation and further assess hypothesis H1 under aim (1), each milk macronutrient (FAT, PRO, LAC, TRP) was plotted against the infant age in days for all subjects included in this analysis. Each macronutrient was also plotted against infant age in days and the mean change of each was assessed through a line of best fit. Five subjects were then selected as isolated illustrations of some of the different patterns of milk macronutrient composition over the first year postpartum. Subjects chosen for comparison had a minimum of 9 samples analyzed across the first year of lactation. Infants 0193S and 0980G were both born in the dry season while infants 1717V, 1750Z, and 2047S were born in the wet season. All infants had varied individual and maternal anthropometric data, health assessment records, and socioeconomic backgrounds.

An additional summary least squares model was constructed to test the hypothesis H2 outlined in aim (2) to determine which variables (socioeconomic, maternal anthropometric, environmental, and milk macronutrients) are significant predictors of infant growth (anthropometric measurements assessed in the model are summarized in **Table 5**). The variables included in this model are: infant sex, infant birth season, pre and postnatal supplements, milk macronutrients (FAT, PRO, TRP, LAC), maternal parity, maternal weight, maternal pre and postnatal weight changes, total livestock, total number in the household, number of co-wives, total years of maternal and paternal schooling, and distance to the clinic. Two additional models were

generated to examine the same variables and their effect on infant growth over the first six months and last six months postpartum. All three models of infant growth controlled for repeated measurements from the same mother-infant pairs with infant ID as a random factor.

Inter-population Study Selection

To assess whether significant inter-population differences in milk macronutrients exist addressing hypothesis H3 under aim (3), a survey of previous milk macronutrient studies was conducted. Using ScienceDirect, I ran a search for publications between 1970 to the present using key words including "breast milk macronutrient composition" which returned 1,222 search results. Of these results, eleven studies were identified which reported mean values for FAT, PRO, and LAC in breast milk across different populations with a variety of methodologies (**Table 11**). Mean macronutrient data from this study is also included for broad comparison, and will be discussed in further detail later in this document. Two studies were included despite only reporting FAT values: 1) one from the same populations in rural Gambia as the current study (Prentice et al., 1981), and 2) a study in Kenya that provides comparative data on methodology and associations with socioeconomic variables (Fujita et al., 2012). Studies that used more than one collection or analytical technique were excluded. Additionally, studies that pooled multiple samples from mothers at different time points were also excluded from this comparison. Studies that include data from mothers giving birth to low-birth-weight (LBW) and preterm infants were also excluded, since these conditions are associated with the production of milk higher in FAT and PRO (and thereby potentially confounding a comparison). Studies conducted before 1970 also were not included due to the reported unreliability of many of the collection and analytical techniques used at this time (Miller et al. 2013). Descriptive statistical reporting will be included in the results

section while an assessment of the methods used in each study will be further addressed in the discussion. Only mean values are included in the table as values were often given in ranges, across multiple group subsets, etc. which did not facilitate the inclusion of standard deviations.

Results

Aim 1: Variables Affecting Breast Milk Macronutrient Composition

Results from the summary model examining significant factors affecting breast milk macronutrient composition across the entire first year of lactation are detailed in **Table 8**. Infants born in the WET season received milk with significantly lower PRO (-0.021±SE 0.009, P=0.025) and TRP (-0.063±SE 0.022, P=0.0065) (**Table 8**). Infants who received the postnatal LNS supplement received milk with higher LAC (0.055±SE 0.0025, P=0.033) (**Table 8**). Mothers from households that had higher numbers of livestock produced milk with lower LAC (-0.00043±SE 0.00018, P=0.014) (**Table 8**).

Significant factors related to breast milk composition during the first 6 months of lactation are shown in **Table 9**. Mothers who received the prenatal FeFol supplement produced milk with lower FAT (-0.099±SE 0.036, P=0.034) while those who received MMN produced milk with lower LAC (-0.020±SE 0.0099, P=0.049) compared to those from all other supplement groups (**Table 9**). Mothers from households that had higher numbers of livestock produced milk with lower FAT (0.004±SE 0.0016, P=0.021), PRO (-0.0025±SE 0.0009, P=0.0092), LAC (-0.0016±SE 0.00043, P=0.0005), and TRP (-0.0042±SE 0.0018, P=0.026) (**Table 9**).

Significant factors related to breast milk composition between months 6-12 of lactation are shown in **Table 10**. Infants born during the DRY season received milk with lower levels of TRP (-0.11±SE 0.053, P=0.043) (**Table 10**).

Figure 3a-d shows the variability in each macronutrient (FAT, PRO, LAC, TRP) when plotted against infant age in days There was a significant linear decrease in mean macronutrient composition across the first year of life for all macronutrients except FAT (PRO: -1.86±SE 0.0057, P<0.0001; LAC: -1.16±SE 0.0029, P<0.0001; TRP: -1.89±SE 0.0131, P<0.0001) (**Figure 3e**). **Figure 4** shows the variability in macronutrients among individual subjects.

	FAT (g/mL)			***PRO (g/mL)***			***LAC(g/mL)***			***TRP(g/mL)***		
	Estimate (SE)	*t Ratio*	*P > | t |*	*Estimate (SE)*	*t Ratio*	*P > | t |*	*Estimate (SE)*	*t Ratio*	*P > | t |*	*Estimate (SE)*	*t Ratio*	*P > | t |*
Intercept	-2.00 (0.37)	-5.37	**<.0001**	-2.21 (0.52)	-8.30	**<.0001**	-1.17 (0.070)	-16.63	**<.0001**	-2.44 (0.64)	-3.82	**0.0006**
Infant Birth Season (DRY>WET)	-0.0091 (0.013)	-0.070	0.49	-0.021 (0.009)	-2.34	**0.025**	-0.0045 (0.0039)	-1.75	0.09	-0.063 (0.022)	-2.91	**0.0065**
Total Livestock	-0.0004 (0.0009)	0.49	0.63	-0.0009 (0.0007)	-1.37	0.18	-0.00043 (0.00018)	-2.63	**0.014**	-0.0014 (0.0016)	-0.91	0.37
Postnatal Supplement (LNS>PLC)	-0.013 (0.012)	-0.99	0.33	0.0019 (0.009)	0.21	0.84	0.0055 (0.0025)	2.23	**0.033**	0.0043 (0.022)	0.20	0.84

Table 8. Summary model including significant variables influencing breast milk macronutrient composition across the first year of lactation. In addition to the variables listed above, infant sex, prenatal supplement, maternal parity, number in the household, number of co-wives, total years of school for both mother and father (includes traditional and Arabic school), distance to the local clinic, and maternal average weight were also components of the best-fit model, but had no significant relationships with any of the four macronutrients. Significant results bolded.

	FAT (g/mL)			PRO (g/mL)			LAC(g/mL)			TRP(g/mL)		
	Estimate (SE)	*t Ratio*	*P > | t |*	*Estimate (SE)*	*t Ratio*	*P > | t |*	*Estimate (SE)*	*t Ratio*	*P > | t |*	*Estimate (SE)*	*t Ratio*	*P > | t |*
Intercept	-1.95 (0.40)	-4.84	**0.0026**	-1.95 (0.28)	-6.89	**<.0001**	-0.99 (0.15)	-6.50	**<.0001**	-2.44 (0.64)	-3.82	**0.0006**
Prenatal Supplement (FeFol)	-0.099 (0.036)	-2.75	**0.034**	-0.013 (0.024)	-0.53	0.60	0.010 (0.012)	0.82	0.42	-0.078 (0.053)	-1.48	0.15
Prenatal Supplement (MMN)	0.042 (0.032)	1.36	0.19	0.0078 (0.020)	0.40	0.70	-0.020 (0.0099)	-2.06	**0.049**	0.045 (0.042)	1.07	0.30
Total Livestock	0.004 (0.0016)	-2.47	**0.021**	-0.0025 (0.0009)	-2.76	**0.0092**	-0.0016 (0.00043)	-3.81	**0.0005**	-0.0042 (0.0018)	-2.35	**0.026**

Table 9. Significant variables affecting breast milk macronutrient composition between 0-6 months. In addition to the variables listed above, infant sex, maternal parity, number in the household, number of co-wives, total years of school for both mother and father (includes traditional and Arabic school), distance to the local clinic, and maternal average weight were also components of the best-fit model, but had no significant relationships with any of the four macronutrients. Significant results bolded.

	FAT (g/mL)			PRO (g/mL)			LAC(g/mL)			TRP(g/mL)		
	Estimate (SE)	*t Ratio*	*P > | t |*	*Estimate (SE)*	*t Ratio*	*P > | t |*	*Estimate (SE)*	*t Ratio*	*P > | t |*	*Estimate (SE)*	*t Ratio*	*P > | t |*
Intercept	-4.34 (0.94)	-4.61	**<.0001**	-4.59 (0.63)	-7.33	**<.0001**	-2.80 (0.19)	-14.46	**<.0001**	-4.56 (1.42)	-3.20	0.0028
Infant Birth Season (DRY>WET)	-0.019 (0.036)	-0.54	0.59	-0.042 (0.0023)	-1.79	0.081	-0.0022 (0.0075)	-0.31	0.76	-0.11 (0.053)	-2.10	**0.043**

Table 10. Significant variables affecting breast milk macronutrient composition between 6-12 months. Significant variables influencing breast milk macronutrient composition. In addition to the variables listed above, infant sex, pre and postnatal supplement, maternal parity, total livestock, number in the household, number of co-wives, total years of school for both mother and father (includes traditional and Arabic school), distance to the local clinic, and maternal average weight were also components of the best-fit model, but had no significant relationships with any of the four macronutrients. Significant results bolded.

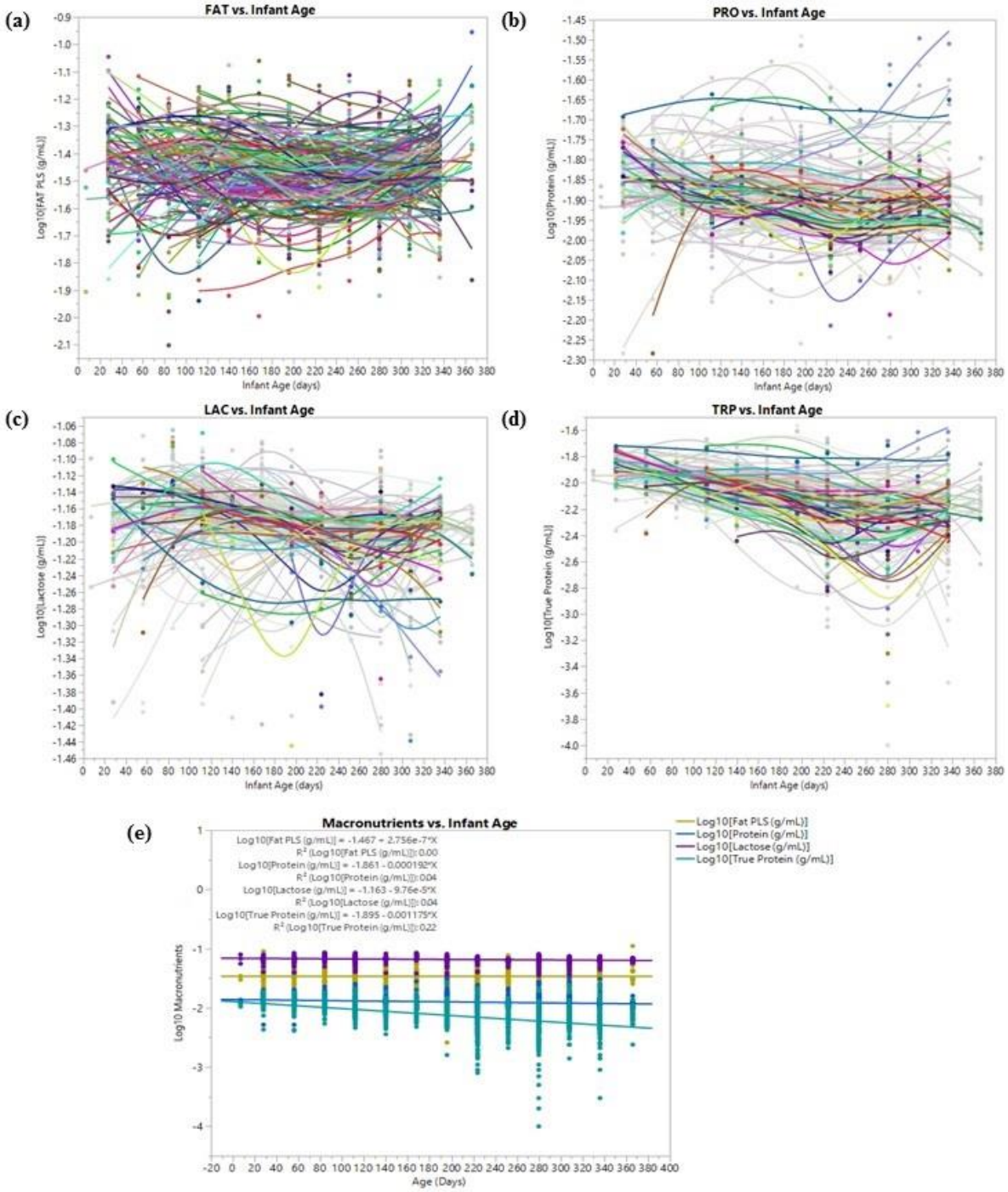

Figure 3. Change in breast milk macronutrient composition across lactation demonstrated through individuals and the population means. a) Log_{10} fat vs. infant age in days b) Log_{10} protein vs. infant age in days c) Log_{10} lactose vs. infant age in days d) Log_{10} true protein vs. infant age in days e) Mean macronutrient composition across all subjects vs. infant age in days.

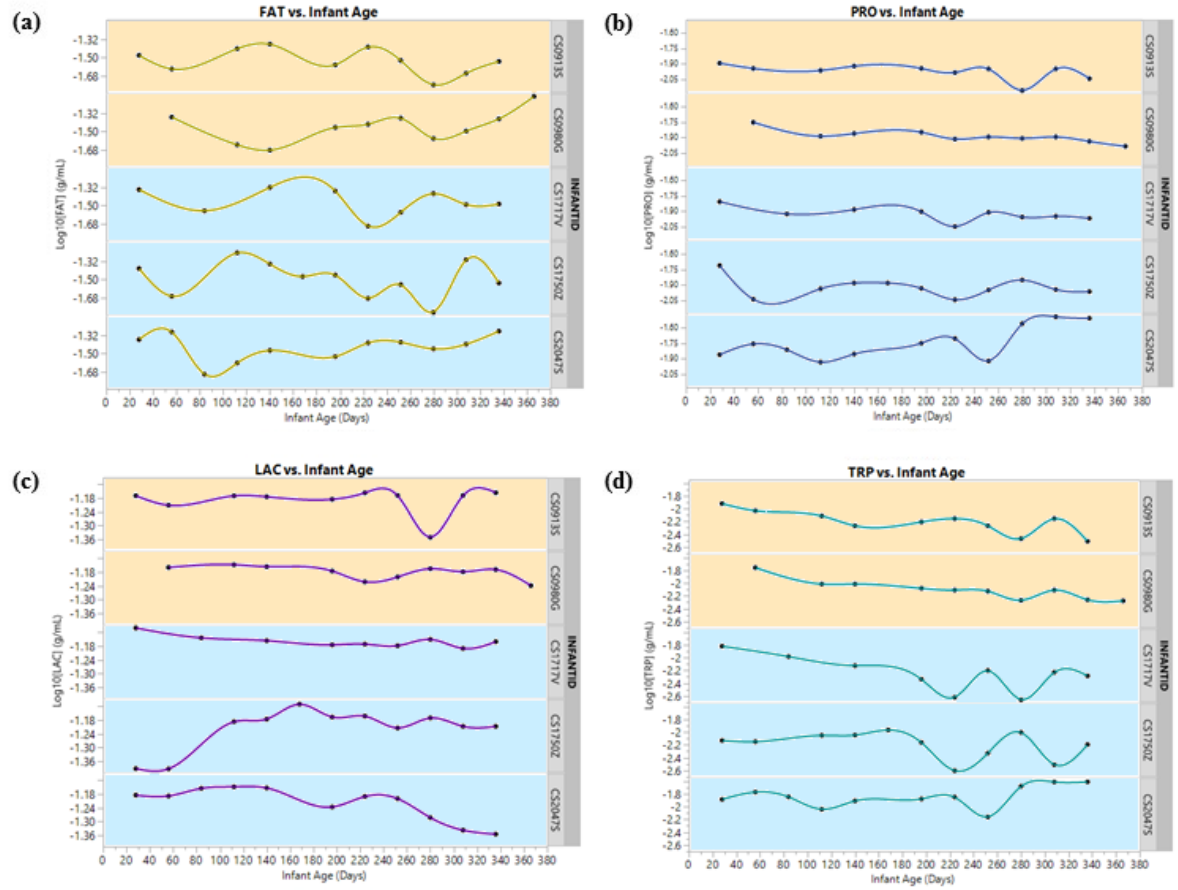

Figure 4. Macronutrients assessed against infant age in days for five different subjects. **a)** Log_{10} fat versus infant age in days **b)** Log_{10} protein vs. infant age in days **c)** Log_{10} lactose vs. infant age in days **d)** Log_{10} true protein vs. infant age in days. Subject 0913S was born in the DRY season and spent the first 6 months of life in the DRY season. 0980G was born in the DRY season and transitioned to the WET season two months following birth. Subject 1717V was born in the WET season and spent the first 6 months of life in the WET season. Subjects 1750Z, and 2047S were born in the WET season, but transitioned to the DRY season three and two months following birth respectively. Subjects 0980G and 2047S both experienced a fever late in the first year of life while subject 1750Z had a recurring eye infection.

Aim 2: Variables Affecting Infant Growth

Factors significant in predicting infant growth during the first year following birth are shown in **Table 11** with a list of variables included in the model but excluded from the table listed below. Female infants were significantly shorter than males (-0.0064±SE 0.0023, P=0.0083) (**Table 11**). Infants from primiparous mothers had higher WAZ scores (1.31±SE 0.52, P=0.017), weighed significantly more (0.075±SE 0.016, P<0.0001) and were taller (0.017±SE 0.008,

P=0.047) (**Table 11**). Infants from families with greater numbers of livestock had higher WAZ scores (0.034±SE 0.011, P=0.0045), higher HAZ scores (0.023±SE 0.010, P=0.027), weighed more (0.0018±SE 0.0003, P0.0001), and were taller (0.00045±SE 0.00017, P=0.013) (**Table 11**). Infants who received milk with higher FAT had lower HAZ scores (-0.47±SE 0.23, P=0.040) (**Table 11**). Infants who received milk with higher PRO weighed more (0.13±SE 0.064, P=0.049) and were taller (0.10±SE 0.027, P=0.0002). Infants who received milk with higher LAC were shorter (-0.079±SE 0.037, P=0.033), but had higher TSF (0.024±SE 0.009, P=0.005) (**Table 11**). Infants who received milk with higher TRP weighed less (-0.019±SE 0.025, P<0.0001) and were shorter (-0.097±SE 0.010, P<0.0001), but had higher TSF (0.056±SE 0.025, P=0.026) (**Table 11**).

Table 12 includes factors significant in predicting infant growth during the first 6 months following birth. Infants from primiparous mothers had higher WAZ scores (1.22±SE 0.53, P=0.028) and weighed more (0.098±SE 0.036, P=0.0095) (**Table 12**). Infants from households with more members had lower WAZ scores (-0.049±SE 0.021, P=0.027) (**Table 12**). Infants who received milk with higher LAC weighed more (0.54±SE 0.23, P=0.021) and were larger (0.17±SE 0.08, P=0.037) (**Table 12**). Infants who received milk with higher TRP weighed less (-0.25±SE 0.10, P=0.0015) and were shorter (-0.086±SE 0.036, P=0.017) (**Table 12**).

Factors significant in predicting infant growth in the latter half of the first year of life are listed in **Table 13**. Female infants were shorter than males (-0.0053±SE 0.0021, P=0.019) (**Table 13**). Infants from primiparous mothers had higher WAZ scores (1.38±SE 0.53, P=0.013) and weighed more (0.062±SE 0.029, P=0.036) (**Table 13**). Infants from families with more livestock had higher WAZ scores (0.035±SE 0.011, P=0.0038), higher HAZ scores (0.024±SE 0.010, P=0.028), weighed more (0.0017±SE 0.00061, P=0.0081), and were taller (0.00039±SE 0.00016,

P=0.022) (**Table 13**). Infants who received milk with higher levels of FAT had lower HAZ scores (-0.57±SE 0.29, P=0.049) (**Table 13**).

	WAZ GAM			HAZ GAM			Infant Weight (kg)			Infant Height (cm)			Infant TSF (mm)		
	Estimate (SE)	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*
Intercept	-3.96 (4.43)	-0.89	0.37	-2.84 (4.03)	-0.70	0.49	0.32 (0.18)	1.75	**0.082**	1.68 (0.085)	19.66	**<.0001**	1.01 (0.34)	2.92	**0.0053**
Infant Sex (F>M)	0.082 (0.14)	0.57	0.57	-0.081 (0.13)	-0.63	0.53	-0.0083 (0.0043)	-1.93	**0.054**	-0.0064 (0.0023)	-2.80	**0.0083**	0.017 (0.011)	1.55	0.13
Parity (Primiparous> Multiparous)	1.31 (0.52)	2.49	**0.017**	0.81 (0.47)	4.35	0.095	0.075 (0.016)	4.84	**<.0001**	0.017 (0.008)	2.05	**0.047**	0.058 (0.040)	1.47	0.15
Total Livestock	0.034 (0.011)	3.03	**0.0045**	0.023 (0.010)	2.31	**0.027**	0.0018 (0.0003)	-2.39	**<.0001**	0.00045 (0.00017)	2.63	**0.013**	0.00076 (0.00052)	0.89	0.38
Log10 FAT (g/mL)	-0.031 (0.20)	-0.15	0.87	-0.47 (0.23)	-2.06	**0.040**	-0.012 (0.023)	-0.55	0.58	-0.015 (0.010)	-1.52	0.13	0.00008 (0.22)	0.00	0.99
Log10 PRO (g/mL)	0.046 (0.58)	0.08	0.94	0.74 (0.64)	1.16	0.25	0.13 (0.064)	1.98	**0.049**	0.10 (0.027)	3.72	**0.0002**	-0.037 (0.063)	-0.60	0.55
Log10 LAC (g/mL)	1.08 (0.77)	1.40	0.16	0.17 (0.86)	0.20	0.84	-0.050 (0.086)	-0.59	0.56	-0.079 (0.037)	-2.14	**0.033**	0.024 (0.009)	2.82	**0.0050**
Log10 TRP (g/mL)	-0.29 (0.22)	-1.30	0.20	-0.20 (0.25)	-0.81	0.42	-0.19 (0.025)	-7.63	**<.0001**	-0.097 (0.010)	-9.08	**<.0001**	0.056 (0.025)	2.24	**0.026**

Table 11. Summary model examining effectors of infant growth across the first year of lactation. Variables also included in the model but not listed in the table above include infant birth season, prenatal supplement, postnatal supplement, total number in household, number of co-wives, total years of school for both mother and father (includes traditional and Arabic school), distance to the local clinic, and maternal anthropometrics were also components of the best-fit model but had no significant relationship in this model. Significant results bolded.

	WAZ GAM			HAZ GAM			Infant Weight (kg)			Infant Height (cm)			Infant TSF (mm)		
	Estimate (SE)	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*
Intercept	-6.76 (5.77)	-1.17	0.24	1.26 (5.56)	0.23	0.82	0.76 (0.42)	1.80	0.079	1.90 (0.15)	12.94	**<.0001**	0.38 (0.53)	0.72	0.4780
Parity (Primiparous> Multiparous)	1.22 (0.53)	2.31	**0.028**	0.40 (0.55)	0.74	0.47	**0.098 (0.036)**	2.75	**0.0095**	0.023 (0.012)	1.86	0.070	**0.083 (0.050)**	**1.68**	**0.10**
Total #Household	-0.049 (0.021)	-2.31	**0.027**	-0.015 (0.022)	-0.72	0.10	-0.0025 (0.0015)	-1.72	0.094	-0.0005 (0.0005)	-0.92	0.36	-0.00058 (0.0020)	-0.29	0.77
Log10 LAC (g/mL)	1.10 (2.57)	0.43	0.67	1.21 (1.92)	0.53	0.26	0.54 (0.23)	2.36	**0.021**	0.17 (0.08)	2.12	**0.037**	**0.066 (0.22)**	0.30	0.76
Log10 TRP (g/mL)	0.031 (1.17)	-0.99	0.98	1.27 (0.91)	1.40	0.17	-0.25 (0.10)	-2.47	**0.015**	-0.086 (0.036)	-2.43	**0.017**	-0.043 (0.10)	-0.42	**0.67**

Table 12. Model examining significant effectors of infant growth from 0-6 months. Variables also included in the model but not listed in the table above include infant sex, infant birth season, prenatal supplement, postnatal supplement, total livestock, number of co-wives, total years of school for both mother and father (includes traditional and Arabic school), distance to the local clinic, protein, fat, and maternal anthropometrics were also components of the best-fit model but had no significant relationship in this model. Significant results bolded.

	WAZ GAM			HAZ GAM			Infant Weight (kg)			Infant Height (cm)			Infant TSF (mm)		
	Estimate (SE)	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*
Intercept	-3.66 (4.45)	-0.82	0.41	-4.21 (4.26)	-0.99	0.33	0.69 (0.24)	2.85	**0.065**	1.81 (0.074)	24.61	**<.0001**	0.96 (0.37)	2.60	**0.012**
Sex (F>M)	0.095 (0.14)	0.66	0.51	-0.077 (0.13)	-0.57	0.57	-0.0072 (0.0078)	-0.93	0.36	-0.0053 (0.0021)	-2.46	**0.019**	0.019 (0.012)	1.64	0.10
Parity (Primiparous> Multiparous)	1.38 (0.53)	2.60	**0.013**	0.85 (0.49)	1.73	0.091	0.062 (0.029)	2.17	**0.036**	0.011 (0.0077)	1.42	0.16	0.064 (0.042)	1.52	0.14
Total Livestock	0.035 (0.011)	3.09	**0.0038**	0.024 (0.010)	2.29	**0.028**	0.0017 (0.00061)	2.80	**0.0081**	0.00039 (0.00016)	2.42	**0.022**	0.00063 (0.00090)	0.70	0.49
Log10 FAT (g/mL)	-0.34 (0.21)	-1.63	0.10	-0.57 (0.29)	-1.97	**0.049**	0.0057 (0.015)	0.38	0.70	0.00094 (0.0073)	0.13	0.90	-0.027 (0.028)	-0.97	0.34

Table 13. Model examining significant effectors of infant growth from 6-12 months. Variables also included in the model but not listed in the table above include infant birth season, prenatal supplement, postnatal supplement, total number in the household, number of co-wives, total years of school for both mother and father (includes traditional and Arabic school), distance to the local clinic, protein, lactose, true protein, and maternal anthropometrics were also components of the best-fit model but had no significant relationship in this model. Significant results bolded.

Aim 3: Inter-Population Comparison of Mean Macronutrient Composition

The studies included in this inter-population comparison of breast milk macronutrient content range in publication date over a period of 40 years and examine between 17-214 subjects (**Table 14**). A variety of methods were implemented both in collection techniques and macronutrient assays (detailed in **Table 14**). Collections were taken as early as immediately following birth through as late as three years postpartum. Reported macronutrient values across different populations for mean lactose (LAC), fat (FAT), and protein (PRO) vary between 6.14-8.03 g/dL, 2.04-5.24 g/dL, and 0.92-1.57 g/dL respectively.

Location	*Sample Size*	*Infant Age*	*Collection Technique*	*LAC (g/dL)*	*FAT (g/dL)*	*PRO (g/dL)*	*Method*	*Reference*
United States	92	0-12 months	24 h collection, pooled samples	7.41	3.77	1.11	Lowry assay, Folch extraction,	(Nommsen et al. 1991)
Australia	17	1-12 months	fore/hind collection, both breasts	6.14	3.74	0.92	Spectrophotometry, other (see reference)	(Mitoulas et al. 2002)
Bangladesh	58	0-9 months	24 hr collection, pooled samples	8.03	2.75	1.04	Graviometric, semimicro-Kjeldahl, colorimetry	(Brown et al. 1986)
China	436	0-8 months	Manual full expression, one breast, 9-11am	7.1	3.4	0.9	Mid-infrared assay	(Yang et al. 2013)
Philippines	124	<18 months	Manual expression, 6-10am	7.30	3.75	1.04	micro-rose Gottlieb, DuBois, Elmer-Perkins	(Quinn 2013)
Gambia (1981)	120	6 months	Small samples, manual expression	-	3.93	-	Creamatocrit	(Prentice et al. 1981b)
Gambia (2017)	214	0-12 months	Small samples, mid-feed, morning	6.58	3.62	1.28	Mid-infrared assay	This study
Italy	30	1-6 months	Manual expression, fore/hind expression	7.75	3.00	1.14	Chromatography, Kjeldahl, Folch	(Grote et al. 2016)
Kenya	83	0-20 months	Manual expression morning after fast, foremilk	-	2.08	-	Creamatocrit	(Fujita et al. 2012)
Singapore	50	1-4 months	Full expression, one breast	6.40	4.28	-	Mid-infrared assay	(Thakkar et al. 2013)
Sweden	50	0-6.5 months	Full expression, various times	6.98	-	1.57	Kjeldahl, Technocon Auto Analyzer	(Lönnerdal et al. 1976)
Tibet	83	7 days -3 years	Manual expression, 6am-10am	7.37	5.24	1.26	micro-rose Gottlieb, DuBois, Elmer-Perkins	(Quinn et al. 2016)

Table 14. Reported mean macronutrient content of breast milk across eleven populations for lactose (LAC), fat (FAT), and protein (PRO). Infant age, collection technique, and method for assessing macronutrients are included for comparison (see reference for complete details). Macronutrient values are typically reported in g/dL (same as %), however some conversions were necessary for uniform comparison.

Discussion

The first aim (**Aim 1)** of this study was to determine if any socioeconomic, anthropometric, or environmental variables affected breast milk macronutrient composition. There is significant evidence that breast milk is relatively buffered from changes in maternal diet and anthropometrics

(Butte et al. 1984b; Imdad and Bhutta 2012a; Lönnerdal 1986; Picciano 2003; Prentice et al. 1994; Rakicioğlu et al. 2006), so I therefore hypothesized (**H1**) that milk composition would be relatively buffered from any changes in maternal anthropometrics which may be linked to socioeconomic and environmental variables. Of the twelve variables included in the summary model, infant birth season, total number of livestock, and postnatal supplement were significant predictors of milk macronutrient composition. To account for the potential effect of complementary feeding on milk composition, which typically occurs between 3-6 months after the infant is born (Dewey 2001; Weaver 1994), two additional models were generated to assess variables affecting breast milk composition across the first 6 months of and between 6-12 months of lactation postpartum.

The results from these models support my hypothesis that breast milk is buffered from differences in maternal anthropometric and socioeconomic variables. While environment was not significant in predicting milk macronutrient composition in the 0-6 month model, it was significant during 6-12 month model and across the entire first year of lactation (0-12 month model). Infants born in the dry season received milk with significantly less protein and true protein than those born in the wet season. While this study did not document the timing of introduction of non-breast milk foods, it is possible that the lower concentrations of protein and true protein could be the result of complementary feeding. Previous studies have demonstrated that mothers with infants who receive outside nutrition are able to self-regulate energy intake from the mother to meet their caloric demands for growth (Cohen et al. 1994). Additionally, there are a number of maternal and infant factors that affect overall milk composition including infant sex, maternal body composition, maternal genes, and duration of lactation (Stam et al. 2013). While there has not been a detailed examination of the introduction of non-breast milk food sources and the influence on the macronutrient profiles of the milk received, milk protein does typically decline significantly during

and after the first 6 months of lactation (Saarela et al. 2005). The possible factors that may contribute to this have not been explored extensively. Alternatively, if mothers of infants born in the dry season have adequate nutrition for the first 6 months following birth, it is possible that the lower levels of protein and true protein observed from 6-12 months of lactation are the result of increased caloric stress and heavy workload induced by the transition into the wet season, which is the time when women farmers begin planting and growing crops. As stated in the introduction, Prentice et al. (1981b) demonstrated that infants born in the wet season received breast milk with lower fat content, even when accounting for volume of milk intake by the infant. While those results were not replicated here with regard to fat content (this effect may be volume dependent and volumes were not recorded in this current Gambian study), Prentice et al. (1981b) did not assess breast milk protein composition which may also be affected by seasonal stressors.

Additionally, prenatal supplement was only significant in predicting milk macronutrients during the first 6 months of lactation. Mothers who received the FeFol supplement, the standard of care for pregnant Gambian women, produced milk with significantly less fat (than mothers receiving all other supplements?) and those who received the MMN supplement produced milk with significantly less lactose than mothers receiving all other supplements. Studies have demonstrated that an FeFol supplement can reduce the incidence of low birthweight babies and decrease the incidence of anemia which thereby decreases infant risk of disease (Imdad and Bhutta 2012b; Mahomed 1997). If infants born from mothers receiving the higher FeFol have better birth outcomes, this result may be indicative of a caloric need at birth in that infants from mothers who did not receive the higher FeFol need milk with more energy due to their lower weight. Without milk volumes to assess overall energy received by the infant, this interpretation is speculative. A recent meta-analysis of studies in low-income countries demonstrated that mothers receiving the

prenatal MMN supplement over FeFol generally had infants with higher birthweights, so it is unclear why mothers who received the MMN supplement would produce milk with lower levels of lactose (Fall et al. 2009). As with the FeFol supplement, this may be indicative of differing caloric or compositional needs observed through milk composition based on infant nutritional status at birth.

Infants who received the postnatal lipid-based nutrient (LNS) received milk with significantly higher concentrations of lactose. A recent study in Bagladeshi women showed that mothers who received the LNS supplement during pregnancy had infants with more favorable birth outcomes (Dewey et al. 2016). Additionally, LNS supplementation has had positive outcomes for at-risk groups in emergency settings, including pregnant women and infants (Chaparro and Dewey 2010) While in this study the LNS was supplemented directly to the infant's diet, it not surprising that infants who receive the LNS might experience positive nutritional benefits. Again, a better understanding of the cues received by the mother from the infant are needed to elucidate this outcome.

Lastly, the results from this thesis showed that infants from families with greater numbers of livestock received breast milk with lower concentrations of all milk macronutrients. These results were more pronounced in the first 6 months of lactation compared to the last 6 months of lactation. In this study, livestock is used as a general indicator of household income. Milk from livestock, specifically cattle, is often used by the owners as a form of payment for herdsmen and therefore the milk produced is not necessarily used exclusively as a supplement to the owner's diet (Somda et al. 2005). Milk received by the herdsmen is either consumed or sent to the market for profit. Profit from milk sales is shared with the owner of the livestock (Hempen et al. 2004). As such, families with greater numbers of livestock may have additional food resources from the

animal milk itself and from the profit received from milk sales. Therefore, it may be that infants from families with greater numbers of livestock have greater nutritional supplementation, either through more nutrient dense foods or greater quantity of food, which may then influence the mother's milk composition through the infant's caloric and/or nutritional regulatory mechanisms.

An additional facet of **Aim 1** was to examine intra-individual variation in breast milk macronutrients across the first year of lactation. Evaluation of mean macronutrient values across the first year of lactation indicate FAT concentrations remained relatively constant, however PRO, LAC, and TRP concentrations all decreased linearly which superficially appears to support hypothesis **H1**. Previous studies have also demonstrated a general downward trend in milk macronutrient concentration across lactation (Mitoulas et al. 2002; Picciano 2001; Saarela et al. 2005; Weaver et al. 1998). However, when each macronutrient is plotted against infant age in days for each subject, significant inter-individual variation in breast milk composition outside of mean population values is present. Because of this, the intra-individual component of **Aim 1** that breast milk composition is relatively consistent between mothers is not supported. Instead, these data reveal that breast milk composition is dynamic at the level of the individual throughout the first year of lactation. Patterns of large fluctuations between milk composition by month were noted in numerous individuals. This individual variation may be due to a number of acute factors that occur during lactation including maternal workload, physiology due to mammary gland development (related to parity), seasonal variation in food resources, and socioeconomic factors (Barker 1997; Neville et al. 2001; Prentice et al. 1989; Prentice et al. 1983a; Prentice et al. 1983b; Prentice and Prentice 1988; Rakicioğlu et al. 2006). It is also likely that there are individual physiological differences that contribute to differential production of milk. While maternal season of birth was only known for a small subset of subjects in this study, analyses were still conducted for those

with data to see if there were significant differences in milk composition between mothers born in the wet season versus those born in the dry season (see **Appendix**). Mothers born in the wet season produced milk with significantly lower concentrations of all milk macronutrients than those born in the dry season. Additionally, mothers born in the wet season had much greater variance in milk macronutrient concentration than those born in the dry season assessed using Welch's test. Therefore, the negative later-life health outcomes of food scarcity inadequate nutrition early in life may persist and be perpetuated into future generations via the milk received from mothers born in the energetically taxing wet season. While the summary model and assessment of mean macronutrient values on an intra-population level illustrates key trends in factors that affect mean macronutrient composition across the first year of lactation, more detailed research approaches on an individual level (e.g., individual profiles that include milk volume, workload, nutrition, socioeconomic factors including rank among co-wives) may be necessary to understand the nuanced effect of environmental, socioeconomic, and maternal anthropometric variables on an individual basis.

The second aim of this study (**Aim 2**) was to examine how milk macronutrient, maternal anthropometric, socioeconomic, and environmental variables affect infant growth during the first year of life. As expected per hypothesis **H2**, milk macronutrients had a significant role in infant growth in their first year of life. More specifically, all four macronutrients examined here were statistically significant in predicting infant growth throughout the first year following birth. Infants who received milk with higher concentrations of protein were taller and weighed more while those who received milk with higher true protein were shorter and weighed less. It is unsurprising that protein is a significant predictor of infant growth, especially because several studies have already demonstrated its importance in early infant growth (Andreas et al. 2015; Ballard and Morrow 2013;

Saarela et al. 2005). Prenatal protein supplementation in Gambian mothers significantly decreased the incidence of low birthweight infants and increased overall birthweights further demonstrating the key role of protein in early infant growth (Ceesay et al. 1997). Increased amounts of protein received by the infant result in increased production of growth factors that are key in early somatic growth in the infant (Rajaram et al. 1997), and as such it is also commonly used in high concentrations to supplement infants born prematurely and at low birth weights (Cristofalo et al. 2013; Rezaei et al. 2011). A previous study has also suggested that feeding frequency, which influences the timing and amount of protein received by the infant, may play an important role in infant appetite control (Khan et al. 2013). True protein is determined by taking the entire nitrogen content in milk minus the non-protein nitrogen (NPN) fraction (Carratù et al. 2003) and consists of protein, free amino acids, and peptides (Feng et al. 2016). While non-protein nitrogen consists primarily of urea, the total protein measurement in milk is known to contain a number of bioactive factors that have been implicated in infant growth and immune development that are not present in the true protein fraction (Carratù et al. 2003; Lönnerdal 2013). A recent study demonstrated that total protein, true protein, and individual amino acid composition all decline steadily throughout lactation (Feng et al. 2016), but further studies are needed to assess the variability of these different components throughout lactation. As such, it may be the additional growth factors present in the total protein fraction that are critical to infant growth and may in part explain why infants who received higher levels of true protein had growth measurements that were lower than those who received less true protein.

Infants who received milk with more lactose were taller and weighed more from 0-6 months, however, they were shorter when assessed across the entire first year following birth. These results could suggest that infants require different milk macronutrients at different periods

following birth. These differences may also be indicative of the introduction of outside foods into the infant's diet as many first foods are simple carbohydrates either in the form of alternative dairy sources or cereals (e.g., greuls) (Dewey 2001; Sellen 2001a; Sellen 2001b; Weaver 1994).

In addition to macronutrients, infant sex was also a significant predictor of infant growth in that females were typically shorter and weighed less than males. This was not a significant predictor during the first 6 months following birth, but was from 6-12 months and across the entire first year. Because there are no differences in milk composition between mothers who gave birth to different sex infants, similar to findings in a previous study in the Philippines (Quinn 2013), this may be indicative of differences in infant care between female and male infants in The Gambia. It has been suggested that male infants have higher energy requirements than females which is reflected through breast size and subsequent increased energy content of milk produced by mothers of male infants (Powe et al. 2010). Additionally, a preference for sons over daughters may alter the mother's care for the infant including differences in interbirth interval and feeding practices (Sear et al. 2001), both of which could contribute to differences in growth between male and female infants. A recent anthropometric analysis of growth differences between male and female infants in Bangladesh demonstrates that female infants experience higher rates of growth faltering than males (Moestue 2009), which Choudhury et al. (2000) argue is linked to gender discrimination. Significant gender inequality has been demonstrated in Gambian women (Lowe et al. 2016; Mwangome et al. 2010) which contributes to the high rates of maternal morbidity, detriment to female reproductive health, and less than adequate infant care (Sundby et al. 1998). All of these factors combined likely contribute to female infant care practices and growth differences observed between male and female infants.

Infants from families with higher numbers of livestock had higher weight-for-age z-scores, height-for-age z-scores, weighed more, and were taller than those with lower numbers of livestock when assessed across the first year following birth. Livestock was not a significant predictor of infant growth in the first 6 months following birth, however it was significant from 6-12 months following birth. As stated previously, families with greater numbers of livestock may be better able to supplement the infants diet with nutrient-rich food sources thereby resulting in better infant growth outcomes.

Maternal parity also significantly predicted infant growth. Infants from primiparous mothers had higher weight-for-age z-scores and weighed more across all three time periods (0-6 month, 6-12 month, 0-12 month) modeled. When examining significant factors across the entire first year following birth, infants from primiparous mothers were also taller than those from multiparous mothers. Previous research has demonstrated that Gambian mothers with higher parity produce milk that is lower in fat (Prentice et al. 1989), but these results were not replicated here. However, when testing for significant variance in milk composition between primiparous and multiparous mothers using Welch's test (see **Appendix**), primiparous mothers showed significantly less variability in milk macronutrient composition compared to multiparous mothers. Therefore, infants from primiparous mothers may be receiving consistently richer milk than those infants from multiparous mothers. Additionally, while the direct effect of parity has not yet been clearly demonstrated in its relationship to breast milk macronutrient composition in humans, studies have demonstrated altered gene expression between primiparous and multiparous mothers, ultimately changing the physiology of the mammary gland in multiparous mothers (Anderson et al. 2007; Neville et al. 2002; Russo et al. 1992). However, additional studies are needed to understand key changes in metabolic pathway regulation that may explain the changes in

macronutrient composition in the breast milk produced from mothers of different parity, along with early environmental influence (e.g. nutrient availability) on mammary gland development and breast milk production. It is also possible that there are different patterns of complementary feeding or differential distribution of complementary food sources between mothers with only one child and mothers with multiple children, which is also indicated in that infants from households with more members have lower weight-for-age z-scores during the first 6 months following birth. However, this alone does not adequately explain the differences in infant growth due to maternal parity within the first 6 months following birth.

The results from the assessment for **Aim 1** demonstrate that milk composition is relatively buffered at the intra-population level. Intra-individual assessment of macronutrient composition across the first year of lactation in **Aim 1** shows significant individual-level variation that is not apparent when assessing population means. Results for **Aim 2** demonstrate that macronutrients significantly affect infant growth. It may therefore be these acute changes in milk composition across lactation that account for differential infant growth outcomes both in infancy and later in life (Moore et al. 1997). Additionally, as this study did not assess milk volume, there are no means to assess caloric intake of each infant and thereby how differences in milk macronutrients may affect overall infant nutrition.

Mean values of breast milk macronutrients obtained from this Gambian study fall within the range of values reported from previous studies (**Table 14)** which is consistent with hypothesis **H3** under **Aim 3**. The mean value for fat in this study was 3.62 g/dL which is comparable with the mean value for fat reported in a previous study by Prentice et al. (1981a) at 3.93 g/dL. The value reported from this previous study only assessed fat concentrations across the first 6 months of lactation whereas this study's mean was obtained from values across the entire first year of

lactation. As there is typically a gradual decline in macronutrient composition across lactation, this could account for this slight difference between studies (Ballard and Morrow 2013). As highlighted in the introduction, it is important to consider both collection and analytical techniques when comparing milk macronutrient values across different population studies. No specific time was reported for the 1981 study and samples were typically collected in the morning for this study. Because fat concentrations are highly variable diurnally and throughout milk expression within a feed (Daly et al. 1993; Grote et al. 2016; Khan et al. 2013), these subtle collection differences could account for the small observed differences between the two studies. Additionally, the previous Gambian study used the creamatocrit method to assess the fat composition of milk whereas this study used a mid-infrared assay; prior methodological studies have shown minimal difference between values obtained between these two methods (Fusch et al. 2015), and therefore the small differences observed between the two Gambian studies are not suggested to be a result of analytical technique.

While breast milk has been typically considered to be buffered from changes in maternal body composition, diet, and environment (Andreas et al. 2015; Bravi et al. 2016; Grote et al. 2016; Innis 2014; Quinn et al. 2016), **Figure 3** highlights the large degree of variability in milk composition both within the individual and between individuals in this study. However, without the raw data from previous studies, the standard deviations for the mean milk macronutrients cannot be derived and as such it is difficult to determine if these patterns can be repeated across other populations. These visualizations demonstrate that milk composition is not uniform across all points of lactation despite milk composition typically being considered relatively uniform both across lactation and between populations (Ballard and Morrow 2013; Bravi et al. 2016; Feng et al. 2016). While fat is highly variable and particularly subject to discrepancies in collection technique,

protein, lactose, and true protein concentrations are relatively constant across a 24-hour period (Khan et al. 2013). Additionally, differences in values obtained using different analytical techniques for protein, lactose, and true protein are minimal (Bradstreet 2015; Bremner et al. 1996; Fusch et al. 2015; Lucas et al. 1978; Miller et al. 2013; Sauer and Kim 2011; Smilowitz et al. 2014). Protein, lactose, and true protein levels gradually decline across the first year of lactation with the most significant decline occurring between 0-6 months (Andreas et al. 2015; Feng et al. 2016; Grote et al. 2016; Saarela et al. 2005), however there are large differences observed even between studies that assessed samples across similar periods of time. For example, the Swedish study reported mean milk protein at 1.57 g/dL when assessed between 0-6.5 months of lactation whereas the Chinese study reported 0.9 g/dL assessed between 0-8 months. The Tibetan study reported mean protein values at 1.26 g/dL with samples assessed across three years of lactation, which, excluding the Swedish study and this current Gambian study, is the highest reported mean protein value among the remaining nine studies, all of which assessed samples for a shorter duration following lactation. Mean lactose values between the populations do not seem to vary significantly, but examination of **Figure 3c** demonstrates how lactose may be dynamic throughout lactation when assessed on an individual basis.

Together, this assessment suggests that while methodology and collection technique may be important to obtain an accurate comparison for fat, these differences in protocol do not account for the differences observed between protein, lactose, and true protein between different populations. It is therefore important to reassess conclusions that milk composition is uniform across different populations and that its composition remains relatively constant throughout lactation. These acute fluctuations in milk macronutrient concentrations may help explain some of the differences in infant growth outcomes between infants who superficially appear to be receiving

the same or adequate nutrition (energetically and compositionally). Additionally, this variation observed both on an intra-individual level and inter-population level indicates that there are potential maternal and environmental variables that do affect milk composition. A recent Tibetan study (included in **Table 14**) examined the effect of altitude on milk composition and did not find that altitude was a significant predictor of milk composition (Quinn et al. 2016); however, this study also reported much higher fat values than any of the other studies included in **Table 14**. Understanding what drives both small and large scale differences observed may be key in elucidating the factors that are critical to maternal health and infant growth.

Conclusion

In this study, I use data from the ENID study to demonstrate that breast milk composition is relatively buffered from differences in socioeconomic, environmental, and anthropometric variables between mothers. As predicted, milk macronutrients are a significant predictor of infant growth across the first year following birth, however infant sex, maternal parity, and number of livestock were also significant factors which suggests that there are variables outside of milk composition that also affect infant growth. The outside factors that proved statistically significant may be the result of resource availability due to socioeconomic environment which would then affect feeding patterns (e.g., introduction and quality of outside food sources). Additionally, the variables that affected milk macronutrients and infant growth appear to be time dependent across the first year of lactation (demonstrated through the 0-6 month and 6-12 month models). Assessing intra-individual changes in milk composition across the first year of lactation highlights the importance of understanding variables that lead to these acute changes that could in turn affect early infant growth. An inter-population evaluation of mean macronutrient composition suggests

that breast milk is not uniform across different populations. This indicates that there are additional variables that may influence maternal health and physiology (e.g. mammary gland development) conveyed through milk composition that could help explain why some populations have higher levels of growth stunting and faltering, as well as poor later-life health outcomes. Future studies should include a detailed assessment of factors that produce these acute changes in milk composition both intra- and inter-individually including investigation of maternal variables such as workload, detailed dietary intake assessments, and physiological factors (e.g., hormone levels, genetic components of mammary gland development). Additionally, researchers should strive to reach a standardization in milk collection and analytical technique to minimalize variation in values due to study design to improve further inter-population comparisons, a component critical in understanding what drives differences between these populations.

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Appendix

	Mom Weight (kg)			Mom MUAC (cm)			Mom TSF (mm)			Mom Prenatal Wt Chg (kg)			Mom Postnatal Wt Chg (kg)		
	Estimate (SE)	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*
Intercept	1.73 (0.01)	120.7 7	**<.0001**	1.39 (0.01)	133.9 6	**<.0001**	1.08 (0.03)	35.01	**<.0001**	0.74 (0.01)	54.83	**<.0001**	0.27 (0.03)	8.39	**<.0001**
Number of Children Mom	0.0028 (0.0028)	0.97	0.3313	0.0050 (0.0021)	2.42	**0.0169**	0.012 (0.006)	2	**0.0475**	-0.019 (0.003)	-7.54	**<.0001**	0.019 (0.007)	2.83	**0.0047**
Number of Children Dad	-0.00031 (0.0017)	-0.19	0.8519	-0.00069 (0.001)	-0.57	0.5703	-0.0053 (0.0036)	-1.47	0.1427	-0.0064 (0.0016)	-4.1	**<.0001**	-0.0006 (0.003)	-0.19	0.8467
Number of Livestock	0.00062 (0.0005)	1.24	0.2173	0.00041 (0.0004)	1.14	0.2543	0.00043 (0.0011)	0.4	0.6899	-0.00043 (0.0005)	-0.92	0.3557	0.0029 (0.0009)	3.16	**0.0016**
Number in Household	0.00035 (0.0008)	0.45	0.6543	0.00037 (0.0006)	0.64	0.5213	-0.00016 (0.0017)	-0.1	0.924	-0.0012 (0.0007)	-1.62	0.1048	0.013 (0.002)	8.74	**<.0001**
Number of Co-Wives	0.0059 (0.0091)	0.64	0.5225	0.0053 (.0067)	0.79	0.4328	0.026 (0.020)	1.32	0.1898	0.075 (0.009)	8.82	**<.0001**	-0.18 (0.02)	-7.84	**<.0001**

Table 15. *Maternal socioeconomic data modeled against maternal anthropometrics using standard least squares. Significant results bolded.*

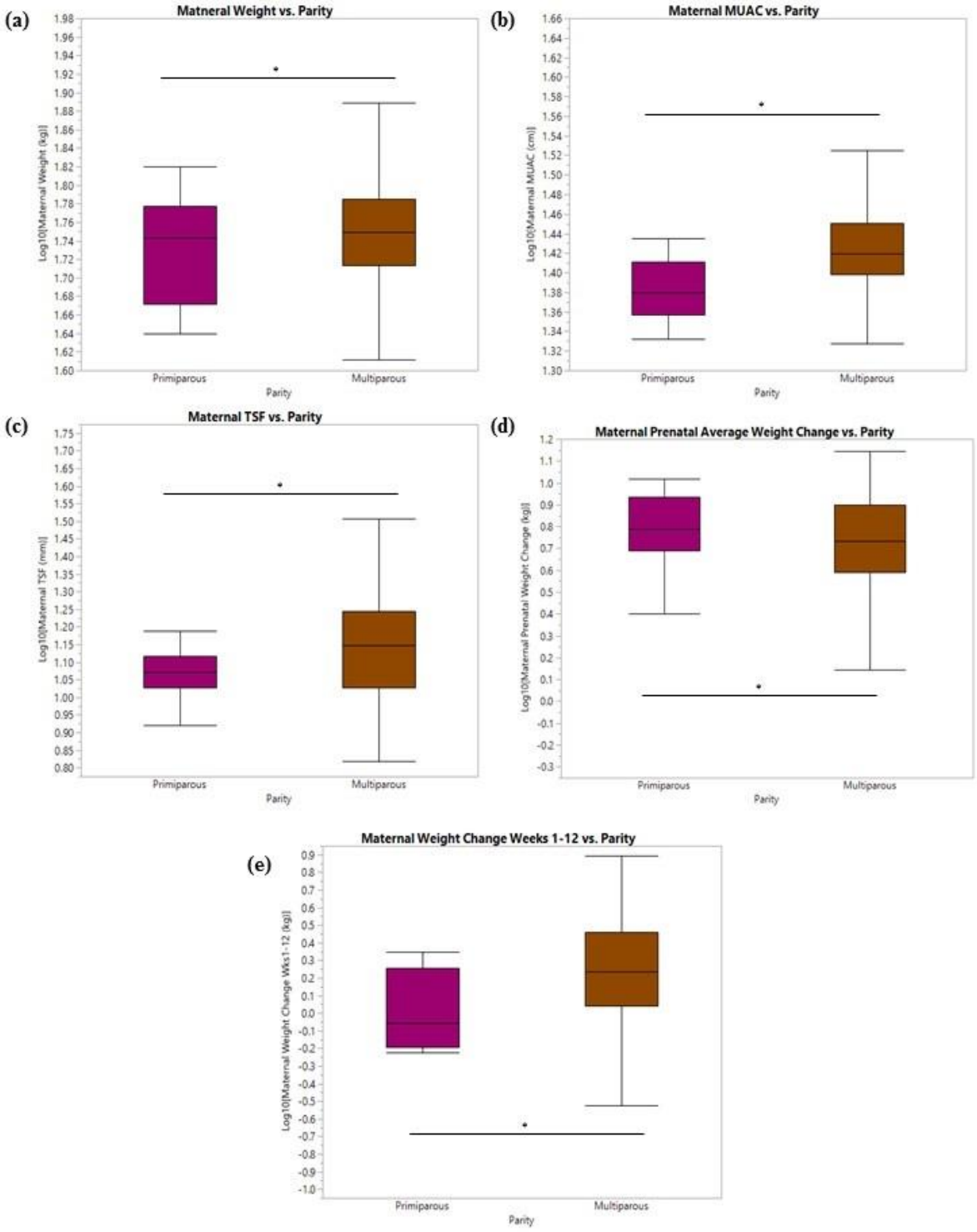

Figure 5. Average maternal anthropometrics measured against parity. a) Maternal average weight (kg) vs. parity b) maternal mid-upper arm circumference (cm) vs. parity c) maternal triceps skinfold (mm) vs. parity d) maternal prenatal average weight gain (kg) vs. parity e) maternal postnatal average weight gain weeks 1-12 (kg) vs. parity. *****Results significant using Wilcoxon's test ($P<0.0005$)

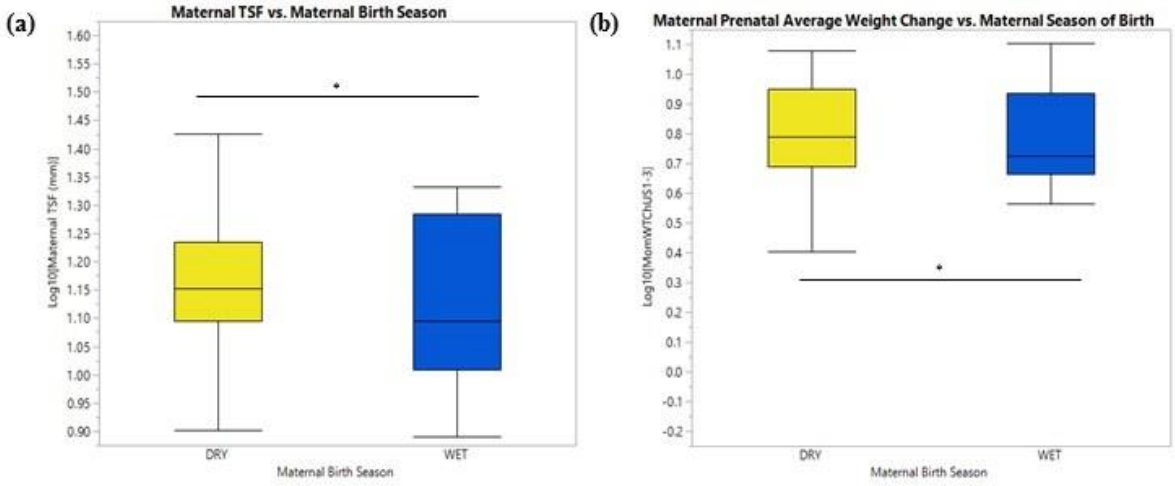

Figure 6. Differences in maternal anthropometrics were observed between mothers born in different seasons (DRY vs. WET). **a)** Log_{10} maternal triceps skinfold (mm) vs. maternal birth season **b)** Log_{10} maternal prenatal average weight change (kg) vs. maternal season of birth. ***** Significant using Wilcoxon's test ($P<0.0005$)

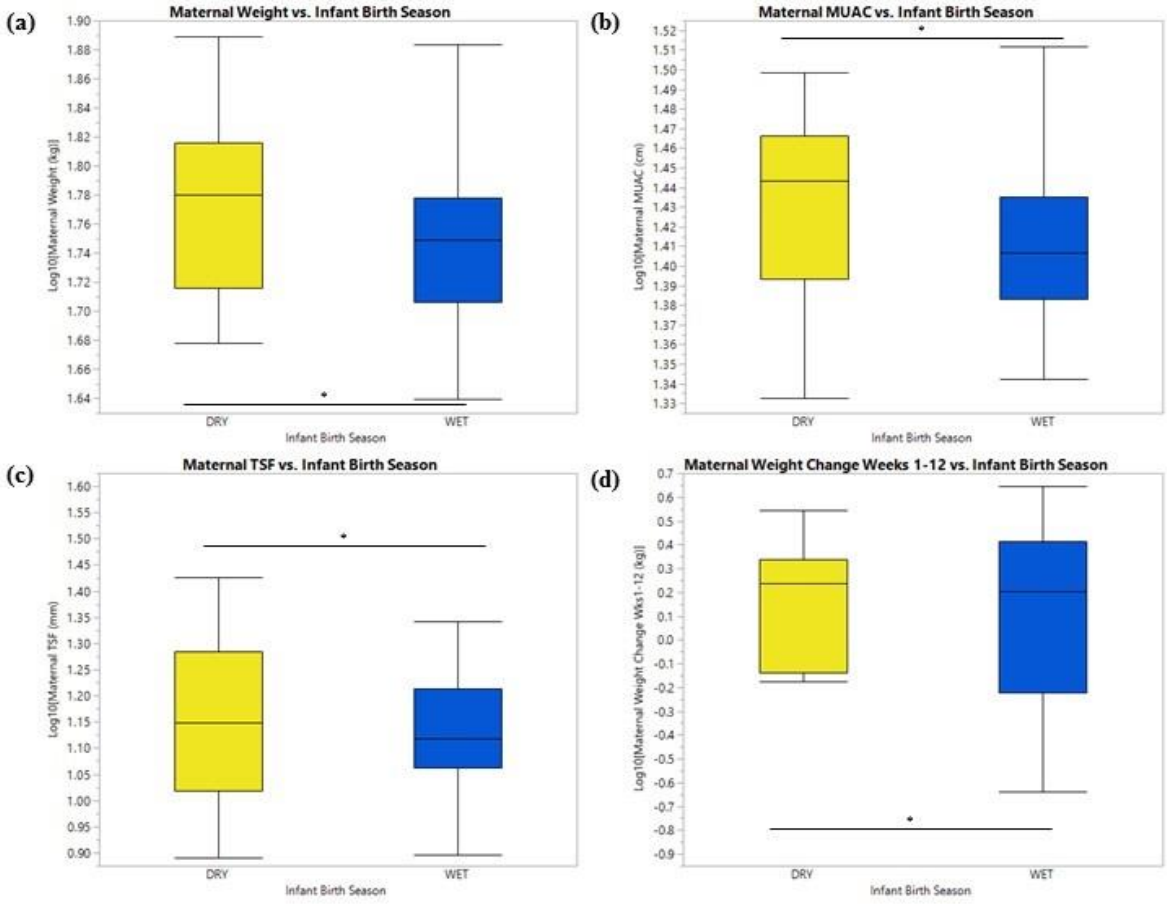

Figure 7. Maternal anthropometrics assessed against infant birth season (dry and wet). **a)** Log_{10} maternal weight (kg) vs. infant season of birth **b)** Log_{10} maternal mid-upper arm circumference (cm) vs. infant season of birth **c)** Log_{10} maternal triceps skinfold (mm) vs. infant season of birth **d)** Log_{10} maternal weight change (kg) weeks 1-12 postpartum vs. infant season of birth. *****Results significant assessed using Wilcoxon's test ($P<0.0001$)

	FAT (g/mL)			PRO (g/mL)			LAC(g/mL)			TRP(g/mL)		
	Estimate (SE)	t Ratio	P > | t |	Estimate (SE)	t Ratio	P > | t |	Estimate (SE)	t Ratio	P > | t |	Estimate (SE)	t Ratio	P > | t |
Intercept	-4.90 (0.79)	-6.16	**<.0001**	-3.67 (0.52)	-7.09	**<.0001**	-2.56 (0.19)	-13.74	**<.0001**	-2.31 (1.18)	-1.96	0.055
Mom Weight (kg)	-0.066 (0.25)	-0.27	0.79	0.35 (0.16)	2.19	**0.032**	-0.052 (0.057)	-0.9	0.37	0.49 (0.37)	1.33	0.19
Mom MUAC (cm)	0.67 (0.42)	1.62	0.11	-0.75 (0.27)	-2.74	**0.0079**	0.012 (0.098)	0.12	0.91	-1.55 (0.62)	-2.49	**0.015**
Mom TSF (mm)	-0.15 (0.10)	-1.53	0.13	0.12 (0.06)	1.86	0.067	0.0062 (0.023)	0.27	0.79	0.17 (0.14)	1.2	0.23
Mom Weight Change Wk 1-3 (kg)	-0.036 (0.035)	-1.03	0.31	-0.011 (0.023)	-0.47	0.64	-0.0047 (0.0081)	-0.59	0.56	-0.0084 (0.052)	-0.16	0.87
Mom Weight Change Wk 1-12 (kg)	0.0028 (0.021)	0.13	0.90	0.0081 (0.014)	0.57	0.57	0.0037 (0.0050)	0.75	0.45	0.025 (0.032)	0.78	0.44

Table 16. Maternal anthropometrics modeled against breast milk macronutrient composition using standard least squares. Significant results bolded.

	FAT (g/mL)			PRO (g/mL)			LAC (g/mL)			TRP (g/mL)		
	Estimate (SE)	t Ratio	P> | t |	Estimate (SE)	t Ratio	P> | t |	Estimate (SE)	t Ratio	P> | t |	Estimate (SE)	t Ratio	P> | t |
Intercept	-1.47 (0.012)	-124.6	**<.0001**	-1.89 (0.0065)	-293	**<.0001**	-1.19 (0.003)	-349.9	**<.0001**	-2.13 (0.02)	-120.9	**<.0001**
Number of Children Mom	0.0078 (0.027)	0.29	0.77	-0.033 (0.015)	-2.21	**0.027**	-0.0025 (0.0077)	-0.32	0.75	-0.048 (0.040)	-1.21	0.23
Number of Children Dad	0.012 (0.027)	0.46	0.65	-0.029 (0.015)	-1.95	0.051	-0.0040 (0.0077)	-0.51	0.61	-0.038 (0.040)	-0.94	0.35
Number of Livestock	-0.00047 (0.00040)	-1.19	0.24	-0.00033 (0.00022)	-1.5	0.13	-0.00022 (0.00011)	-1.93	**0.044**	-0.00090 (0.00059)	-1.52	0.13
Number in Household	-0.00084 (0.00067)	-1.26	0.21	0.00069 (0.00037)	1.87	0.062	0.00013 (0.00019)	0.69	0.49	-0.00023 (0.0010)	-0.23	0.82
Number of Wives	-0.0063 (0.0074)	-0.84	0.40	0.014 (0.004)	3.35	**0.0008**	0.00022 (0.0021)	0.1	0.92	0.030 (0.011)	2.71	**0.0068**

Table 17. Socioeconomic data modeled against breast milk macronutrient composition using standard least squares. Significant results bolded.

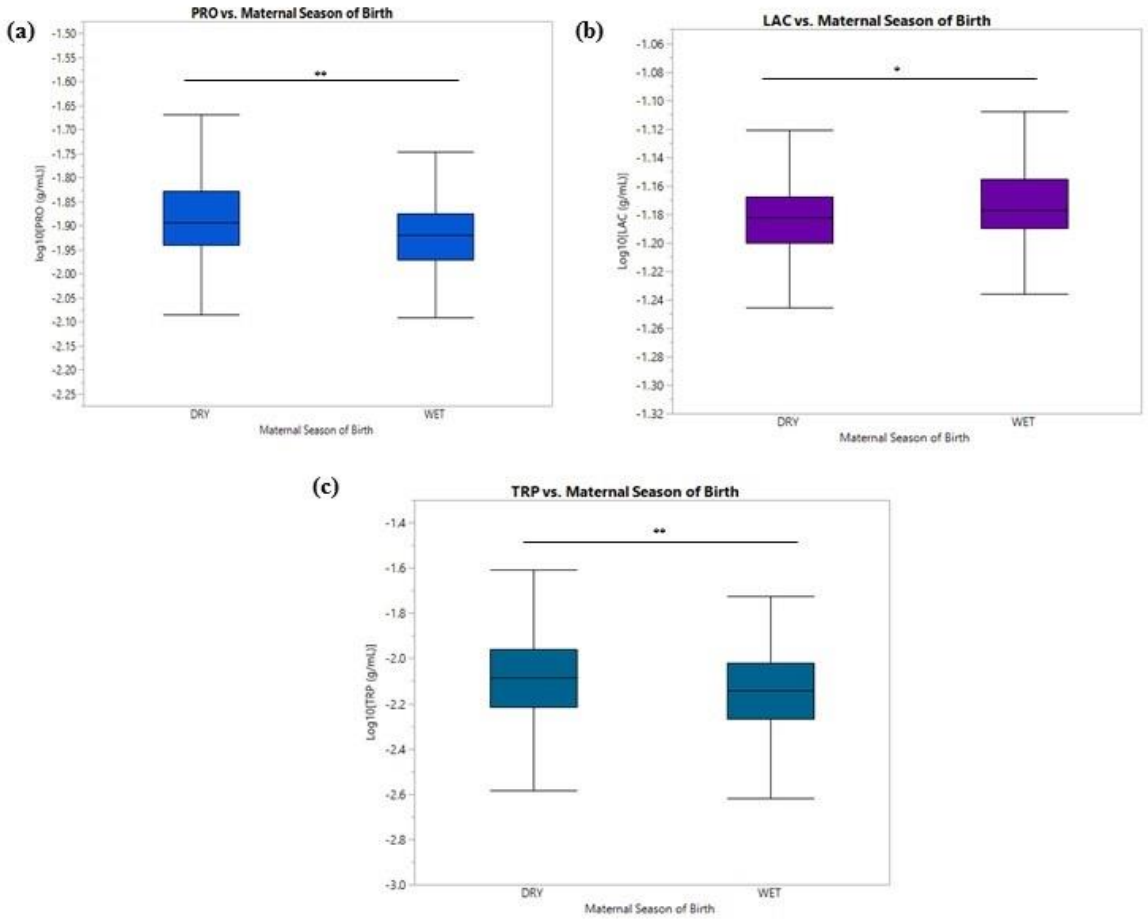

Figure 8. Breast milk macronutrient composition measured against maternal season of birth (wet and dry) to test for mean differences (Wilcoxon) and variance (Welch). **a)** Protein vs. maternal birth season **b)** Lactose vs. maternal birth season **c)** True protein vs. maternal birth season. ***** Results significant for Wilcoxon only ($P<0.005$). ****** Results significant for Wilcoxon ($P<0.001$) and Welch ($P<0.001$).

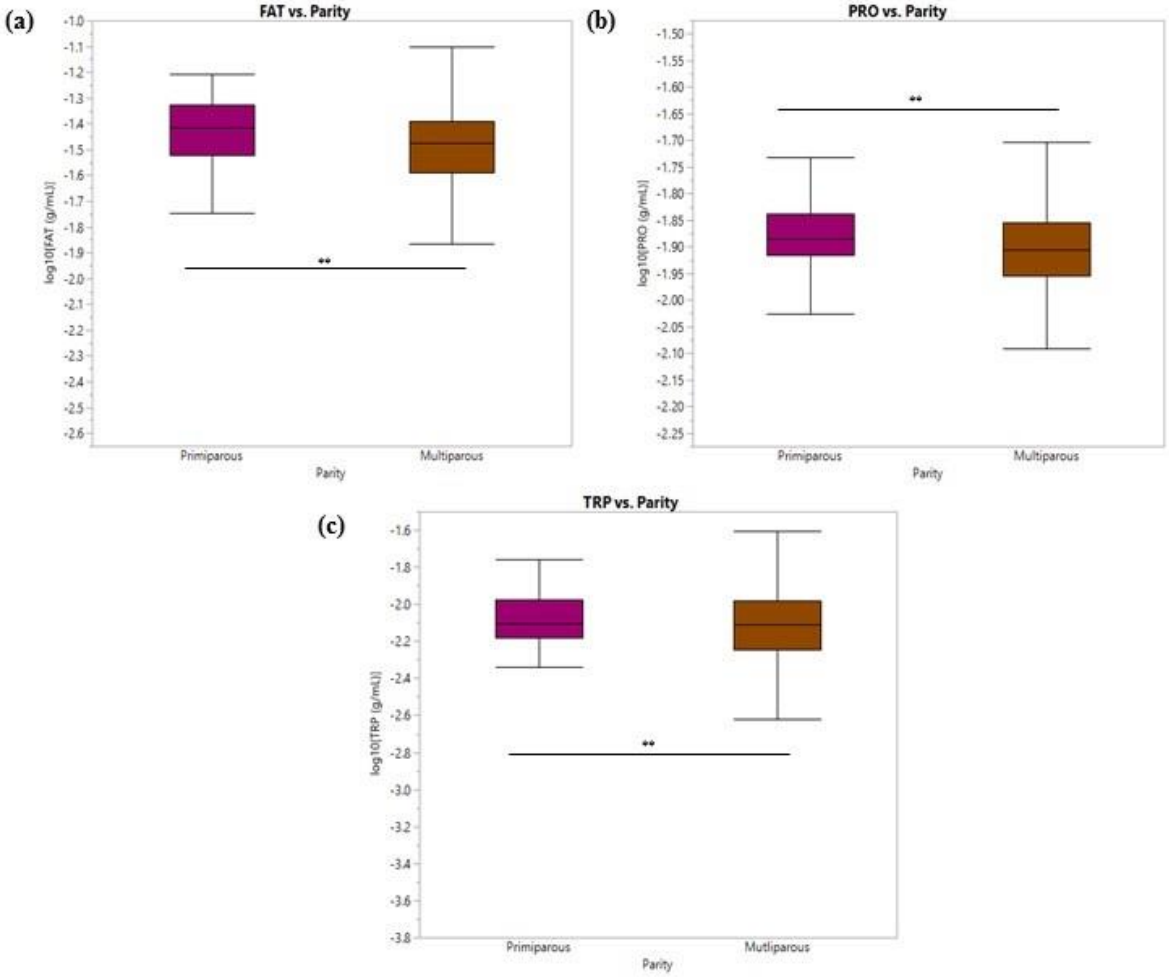

Figure 9. Breast milk macronutrient composition measures against maternal parity (primiparous and multiparous) measured for nonparametric mean differences (Wilcoxon) and variance (Welch). **a)** Fat vs. maternal parity **b)** Protein vs. maternal parity **c)** True protein vs. maternal parity. ***** Results significant for both Wilcoxon ($P<0.05$) and Welch ($P<0.05$).

	Intercept			FAT (g/mL)			PRO (g/mL)			LAC (g/mL)			TRP (g/mL)		
	Estimate (SE)	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*	*Estimate (SE)*	*t Ratio*	*P> | t |*
Infant Weight (g)	0.51 (0.06)	7.88	**<.0001**	-0.038 (0.013)	-3.0	**0.0028**	0.070 (0.030)	2.31	**0.0209**	-0.020 (0.042)	-0.46	0.65	-0.18 (0.01)	-17.2	**<.0001**
Infant Growth (g/day)	1.30 (0.42)	3.06	**0.0023**	0.20 (0.08)	2.66	**0.008**	-0.70 (0.17)	-3.97	**<.0001**	0.18 (0.27)	0.68	0.50	0.50 (0.07)	7.29	**<.0001**
Infant Height (cm)	1.64 (0.03)	57.76	**<.0001**	-0.020 (0.005)	-3.7	**0.0002**	0.058 (0.013)	4.47	**<.0001**	-0.060 (0.019)	-3.22	**0.0013**	-0.089 (0.005)	-19.13	**<.0001**
Infant TSF (mm)	1.14 (0.06)	17.63	**<.0001**	-0.0010 (0.013)	-0.08	0.93	-0.023 (0.030)	-0.74	0.46	0.19 (0.04)	4.46	**<.0001**	0.023 (0.010)	2.22	**0.027**
HAZ (WHO)	-0.39 (0.75)	-0.52	0.60	-0.016 (0.15)	-0.11	0.91	-1.14 (0.35)	-3.23	**0.0013**	0.59 (0.50)	1.2	0.23	0.67 (0.12)	5.55	**<.0001**
HAZ (Gambia)	-0.83 (0.83)	-1.00	0.32	0.01 (0.16)	0.06	0.95	0.46 (0.37)	1.25	0.21	-0.77 (0.53)	-1.45	0.15	-0.22 (0.13)	-1.72	0.086
WAZ (WHO)	0.71 (0.56)	1.25	0.21	0.24 (0.11)	2.15	**0.032**	-1.51 (0.27)	-5.67	**<.0001**	1.44 (0.38)	3.84	**0.0001**	1.09 (0.09)	11.97	**<.0001**
WAZ (Gambia)	-0.32 (0.46)	-0.72	0.47	-0.22 (0.08)	-2.64	**0.0084**	0.19 (0.20)	0.95	0.34	0.30 (0.30)	0.97	0.33	-0.17 (0.07)	-2.43	**0.015**
WHZ (WHO)	1.53 (0.85)	1.8	0.073	0.38 (0.17)	2.28	**0.023**	-0.88 (0.40)	-2.19	**0.028**	1.23 (0.57)	2.18	**0.029**	0.91 (0.14)	6.6	**<.0001**

Table 18. Stepwise model examining infant growth measures versus breast milk macronutrients including both WHO and Gambian z-scores. Significant results bolded.

ENID Growth

A randomized controlled trial to investigate the effect of Prenatal and early childhood nutritional supplementation on infant and early childhood body composition, growth and development

SOCIOECONOMIC QUESTIONNAIRE

STUDY NO: **#Name?**

Name: #Name?

Visit Date: |___|___|/|___|___|/|___|___|___|___| Interviewer's Initials: |___|___|

NO	QUESTIONS AND FILTERS	CODING
01	MAIN MATERIAL OF THE FLOOR OF THE HOUSE OF THE FATHER. *(OBSERVE FLOOR AND RECORD ACCORDINGLY)*	EARTH/SAND/MUD ..... 1 VINYL..... 2 CERAMIC TILES. ..... 3 CEMENT ..... 4 CARPET..... 5 OTHER..... 9 (S P
02	MAIN MATERIAL OF THE FLOOR OF THE HOUSE OF THE MOTHER. *(OBSERVE FLOOR AND RECORD ACCORDINGLY)*	EARTH/SAND/MUD ..... 1 VINYL..... 2 CERAMIC TILES. ..... 3 CEMENT ..... 4 CARPET..... 5 OTHER..... 9 (S P
03	MAIN MATERIAL OF THE WALLS OF THE HOUSE OF THE FATHER. *(OBSERVE WALLS AND RECORD ACCORDINGLY)*	CEMENT/BURNT BRICK. .... 1 MUD/KRINTING ..... 2 CORRUGATED IRON SHEETS..... 3 OTHER..... 9 (SPECIFY)
04	MAIN MATERIAL OF THE WALLS OF THE HOUSE OF THE MOTHER. *(OBSERVE WALLS AND RECORD ACCORDINGLY)*	CEMENT/BURNT BRICK. .... 1 MUD/KRINTING ..... 2 CORRUGATED IRON SHEETS..... 3 OTHER..... 9 (SPECIFY)
05	MAIN MATERIAL OF THE ROOF OF THE HOUSE OF THE FATHER. *(OBSERVE ROOF AND RECORD ACCORDINGLY)*	CEMENT ..... 1 IRON SHEETS. ..... 2 ASBESTOS..... 3 THATCH..... 4 OTHER..... 9 (SPECIFY)
		CEMENT ..... 1 IRON SHEETS. 2

06	MAIN MATERIAL OF THE ROOF OF THE HOUSE OF THE MOTHER. *(OBSERVE ROOF AND RECORD ACCORDINGLY)*	ASBESTOS..... 3 THATCH..... 4 OTHER..... 9 (SPECIFY)
07	MAIN MATERIAL USED FOR THE FENCE OF THE COMPOUND *(OBSERVE FENCE AND RECORD ACCORDINGLY)*	CEMENT..... 1 IRON SHEETS..... 2 KRINTING ..... 3 GRASS..... 5 NONE..... 6 OTHER..... 9 (SPECIFY)

NO:	QUESTIONS AND FILTERS	CODING
08	How many rooms in addition to the father's is the household occupying?	If none, state "00"
09	How many of these rooms have open roof eaves?	If none, state "00"
10	How many have closed roof eaves?	If none, state "00"
11	How many have ceilings?	If none, state "00"
12	How many wives has the father?	
13	How many children has the father including those from the other wives?	
14	How many children has the mother?	
15	How many people are in the household?	
16	Has the father a son/daughter/relative living abroad or elsewhere in the country?	Yes .....1 No 2
17	If yes, does this person assist by sending remittance?	Yes .....1 No 2
18	If yes, how much money does s/he sends you in a year?	LESS THAN D1,000 .....1 D1,000 – D2,500 .....2 D2,500 – D5,000
19	Has the mother a son/daughter/relative living abroad or elsewhere in the country?	Yes .....1 No 2
20	If yes, does this person assist by sending remittance?	Yes .....1 No 2

21	If yes, how much money does s/he sends you in a year?	LESS THAN D1,000 ..... 1 D1,000 – D2,500 ..... 2 D2,500 – D5,000 ..... 3 D5,000 – D10,000 ..... 4 D10,000 – D25,0000. ..... 5 OVER D25,000 6
22	What is the highest grade of schooling attained by the mother?	If none, state “00” |_|
23	What is the highest grade of schooling attained by the father?	If none, state “00” |_|
24	How many years of Arabic/Islamic education has the mother?	If none, state “00” |_|
25	How many years of Arabic/Islamic education has the mother?	If none, state “00” |_|
26	What is the main source of drinking water for members of your household?	Piped water in dwelling ..... 1 Piped water into compound. ..... 2 Public tap… ..... 3 Open well in compound ..... 4 Open public well ..... 5 Protected well in compound ..... 6 Protected public well. ..... 7 Other. ..... 9 (Specify)

NO:	QUESTIONS AND FILTERS	CODING CATEGORIES
27	What kind of toilet facilities does your household have?	Flush Toilet ..... 1 Traditional Pit Latrine ..... 2 Ventilated Improved Pit (VIP) Latrine. ..... 3 No Facility/Bush/Other Households ..... 4 Other. ..... 9 (Specify)
28	Do you share these facilities with other households?	Yes ..... 1 No 2
29	What kind of toilet facilities does your household have?	Firewood. ..... 1 Charcoal. ..... 2 Gas ..... 3 Kerosene.

30	How many times in a week does your household cook meat?	If none, state “00” ..... | ..... | ..... |
31	How many times in a week does your household cook fish?	If none, state “00” ..... | ..... | ..... |
32	Does your household have: Electricity? A television? A refrigerator? A cart? A bicycle? A motorcycle A car or truck or tractor?	Yes No Electricity..... 1 ..... 2 Television..... ..1 ..... 2 Refrigerator..... ..1 ..... 2 Cart..... 1 ..... 2 Bicycle..... 1 ..... 2 Motorcycle..... 1 ..... 2 Car/Tractor..... . 1 2

33	How many of the following animals does the mother has? (State "000" in any category reported "None") Cows? Sheep? Goats? Donkeys? Horses?	Cows | Sheep | Goats | Donkeys Horses |
34	How many of the following animals does the father has? (State "000" in any category reported "None") Cows? Sheep? Goats? Donkeys? Horses?	Cows | Sheep | Goats | Donkeys | Horses |

NO:	QUESTIONS AND FILTERS	CODING
35	What is the main occupation of the father?	Farmer.....1 Craftsman.....2 Tradesman. ..... Retired.....5 Other. .....9 (Specify)
36	If the father is a farmer, how many sacs of the following produce has he harvested last season? (Please note: 4 bundles of coos is equivalent to a sac)	Coos/Millet |___| | | Peanuts |___| | | Maize |___| | | Rice |___| | |
37	If the mother is a farmer, did he sell any of the produce from his farm for cash?	Yes .....1 No.....2
38	If yes, how many sacks of the produce did she sell in the just concluded trade season? (Please note: 4 bundles of coos is equivalent to a sac)	SACKS.....|___|___| _____|
39	What is the main occupation of the mother?	Farmer.....1 Craftsman.....2 Tradesman. ..... Retired.....5 Other. .....9 (Specify)
40	If the mother is a farmer, how many sacs of the following produce has he harvested last season?	Coos/Millet |___| | |

	(Please note: 4 bundles of coos is equivalent to a sac)	Peanuts |___| Maize |___| Rice |___|
41	If the father is a farmer, did she sell any of the produce from his farm for cash?	Yes .....1 No 2
42	If yes, how many sacks of the produce did she sell in the just concluded trade season? (Please note: 4 bundles of coos is equivalent to a sac)	SACKS.....|___|___| _____|
43	If the father is employed what is his annual pay? If not employed what is his annual earning?	Less Than D2,000. .....1 D2,000 – D2,500 .....2 D2,500 – D5,000 .....3 D5,000 – D10,000 .....4 D10,000 – D25,000. ...5 Over D25,000 6
44	If the mother is employed what is his annual pay? If not employed what is her annual earning?	Less Than D2,000. .....1 D2,000 – D2,500 .....2 D2,500 – D5,000 .....3 D5,000 – D10,000 .....4 D10,000 – D25,000. ...5 Over D25,000 6