The Life History Significance of Human Breast Milk: Immune and endocrine factors as indicators of maternal condition and predictors of infant health and growth

The Life History Significance of Human Breast Milk: Immune and endocrine factors as indicators of maternal condition and predictors of infant health and growth

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Abstract

This dissertation explores the relationships between maternal energetic condition, four bioactive compounds in milk, and infant health and growth outcomes through the lens of human life history theory. Research was conducted among the Toba of *Barrio* Namqom in Formosa, Argentina. This is among the first of studies to apply a life history biology lens to the dynamics of cortisol, insulin-like growth factor 1 (IGF-1), lactoferrin, and secretory immunoglobulin A (sIgA) in human milk. First, the role of maternal energetic condition as a predictor of the concentration of these four compounds in milk is explored. Several interesting relationships emerge, including significantly lower milk cortisol and IGF-1 among women with higher body mass index, and significantly higher milk cortisol among primiparous mothers. Next, the relationships between infant symptoms of illness and the two milk immunofactors, lactoferrin and sIgA, are investigated. Lactoferrin is found to exhibit a positive association with symptoms of illness, and sIgA shows a negative association. Finally, associations between concentration of milk bioactives and infant growth rate are tested, as well as associations between infant illness and growth rate, and maternal energetic condition and growth rate. Milk IGF-1 is found to positively associate with infant linear growth rate. Maternal parity is found to negatively associate with linear growth rate. First-born status is associated with significantly greater gains in

length and mass over a four-month period. These and other results are discussed through the lens of life history biology and avenues for future research at the intersection of life history biology and public health are identified.

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Chapter 1: The Life History Context of Milk

This dissertation explores the relationships between maternal energetic condition, four bioactive compounds in milk, and infant health and growth outcomes through the lens of human life history theory. While the nutritional parameters of milk are well-established, the dynamics of bioactive molecules in breast milk constitute a critical and underexplored area of human biology. Much of the research focusing on milk bioactives comes from the fields of public health and dairy husbandry. Application of a human life history framework changes the scope of inquiry from the *proximate* sources and effects of these compounds in milk to their *ultimate* explanations; how has evolution shaped the patterns we see?

Life history theory is an evolutionary framework that describes how species and individuals allocate finite resources like energy and time over the course of their lives (Stearns 1992). Investment in growth, maintenance, and reproduction varies with developmental stage and environmental conditions and is patterned to optimize lifetime reproductive fitness.

There are some important concepts described by life history theory that inform how we think about the way that time and resources are allocated by an individual over his or her lifetime. These concepts help guide specific life history hypotheses and predictions and the interpretation of results. The first of these concepts is the tradeoff. Tradeoffs are

allocation decisions made on the basis of relative scarcity; a tradeoff is a compromise. If an individual has 1000 calories to spend on two tasks—say, growing and fighting off a respiratory virus—but he needs 2000 calories to accomplish both tasks, he may shift all of the available resources to one process or the other, or he may invest some portion of these resources to both tasks, incompletely accomplishing both. In this case, we would say there was a tradeoff between immune function and growth; any investment in one process reduces investment in the other. Tradeoffs are often useful in explaining negative relationships or significant shifts in resource allocation. Tradeoff decisions are made on the basis of what is expected to result in the highest lifetime reproductive success.

In addition to tradeoffs made respect with to immediate physiological processes, mothers experience a tradeoff between investment in current offspring and future reproductive events. Investing in the growth and survival of her current offspring is a priority, but at some point her expected lifetime reproductive success is higher if she transitions some of these resources toward a new pregnancy.

On the other hand, the concept of phenotypic correlation is an allocation pattern observed during periods of relative abundance. Phenotypic correlation is useful in explaining positive relationships between two "desirable" outcomes or characteristics and is often used to explain allocation decisions among individuals with ample resources. For instance, say the above individual now has 2500 calories to spend on his two tasks. He is easily able to invest the amount required to complete them both. Investing in growth does not impair his ability to invest in immune function, and vice versa, so he does not experience a tradeoff with respect to these two processes.

Lactation is an interesting and complicated life stage from a life history perspective because the biologies of mother and infant interface and overlap. Understanding milk composition requires a consideration of the tradeoffs and investment decisions experienced by *both* individuals. With life history theory as a guiding framework, the following questions are explored in this dissertation:

•How does maternal energetic condition relate to the concentration of cortisol, IGF-1, lactoferrin, and sIgA in milk?
•How do concentrations of these four bioactive compounds associate with infant growth rate?
•Are symptoms of illness in infants related to the concentration of two immune proteins—lactoferrin and sIgA—received in milk?

It is necessary to consider the nature and strength of the relationships between maternal condition, milk, and infant outcomes alongside the underlying biology of the compounds. For instance, if elevated milk immune compounds are found to be associated positively with infant growth, it could indicate that the mother is investing in the growth of her infant by supplementing milk with helpful proteins; the compound may help him avoid an illness that might otherwise divert his resources preferentially to maintenance. It may also represent the mother's investment in her own reproductive effort, if immune support and/or increased growth rate are expected to help the child survive to reproductive age.

However, it could simply be reflective of investment by the mother in her own somatic maintenance, especially if the compound is transported into milk from her circulation.

This dissertation is among the first of studies to apply a life history biology lens to the dynamics of cortisol, IGF-1, lactoferrin, and sIgA in human milk. Milk bioactive compounds are explored in Chapter 2. In this chapter I present a brief overview of the life history biologies of mothers and infants, with a focus on milk. These are not exhaustive reviews, but serve to illustrate the ways in which we already know that milk is influenced by maternal biology and affects infant development. This interesting and dynamic biological fluid is a crucial component of the life history of mothers and infants.

Maternal Life History

Lactation is an extremely energetically expensive process, representing a large investment of resources by the mother in her current infant. The caloric cost of lactation in well-nourished populations has been estimated at 630 kcal/day, 640 kcal/day, and 670 kcal/day (van Raaij et al. 1991; Goldberg et al. 1991; Dewey 1997). Because milk production is a form of direct investment in reproductive effort and clearly increases a female's reproductive success, it may be expected to be fairly robust to energy budget cuts when allocation decisions are made. However, when energy intake is limited, or when maternal somatic energy reserves are depleted, a lactating mother may face tradeoffs between providing milk for her infant and investment in her own metabolism and maintenance. Even mothers for whom energy is abundant still face tradeoffs in the form of allocation of available resources to the current infant or a future reproductive

opportunity. In addition to these tradeoffs, juvenile mothers sometimes incur the energetic challenge of continued linear growth. Milk composition may represent a way that mothers can optimize the level of investment in the current offspring; mothers in better energetic condition may be able to invest more resources in the current infant without sacrificing their own health or their ability to conceive again. Mothers with limited resources are expected to experience one or more of these tradeoffs.

The energy density of milk seems to be buffered from maternal energy availability. Most studies investigating the effect of maternal weight loss on milk energy density have found no effect. Among presumably well-nourished American women, weight loss of around two kilograms per week associated with diet, exercise, or a combination of the two, has consistently been shown to have no negative impacts on the energetic quality of breast milk (Butte et al. 1984; Dewey et al. 1994; McCrory et al. 1999). A longer-term study of more severe and prolonged energy restriction was conducted among women in the Gambia (Prentice et al. 1981a). The average energy intake of Gambian women in the rainy season is around 50% of the recommended value (Whitehead et al. 1978), but energy content of the milk was similar to the dry season, when energetic circumstances are more favorable (Prentice et al. 1981a). Total energy content did not change when mothers were given an energetic supplement, although milk fat increased and lactose decreased (Prentice et al. 1983b). Similarly, the macronutrients contributing to the overall energy density of milk showed evidence of seasonality. Milk fat was found to correlate significantly with maternal subcutaneous fat stores, but not energy intake, in this population (Prentice et al. 1981b). As milk fat decreased, lactose increased and

contributed a greater proportion of the total energy in milk during the rainy season (Prentice 1980 as cited in Prentice et al. 1981a). A study of Otomi Indian women also found maternal body fat to be positively correlated with milk fat at three and six months postpartum (Barbosa et al. 1997).

In some instances, poor maternal condition in primates has been shown to reduce the energetic quality of milk produced. For example, heavily parasitized rhesus macaque mothers were shown to produce milk with lower fat content than non-parasitized mothers (Hinde 2007). Among common marmosets, small mothers rearing twins produced milk with a lower fat content than larger mothers who had twins or mothers with only one infant (Tardif et al. 2001). Smaller mothers of twins also lost more weight during lactation. In well-nourished humans, milk energy density was found to positively correlate with maternal percent of ideal body weight throughout lactation, reflecting higher concentration of lipids in milk of heavier women (Nommsen et al. 1991). The dietary quality of women in China was reflected in breast milk composition, with suburban women consuming a diet higher in carbohydrates and lower in lipids and proteins, and producing milk with the same characteristics (Qian et al. 2010). Despite being higher in carbohydrates, the milk of the suburban mothers had slightly but significantly lower energy density than milk of urban mothers.

The volume of milk produced has been demonstrated to vary more greatly than energy density in response to maternal condition in primates. For example, a study of caloric restriction in baboons found that energy density of the milk was unaffected by moderate

or severe restriction (Roberts et al. 1985). When mothers were fed at 80% of *ad libitum* intake, they were able to mobilize enough energy to sustain full yield, but at 60% of *ad lib.* intake, yield was reduced by 37%. Similarly, the studies of lactation performance among dieting American women found no effect of gradual weight loss on milk yield (Butte et al. 1984; Dewey et al. 1994; McCrory et al. 1999), but the more severe energy restrictions of the rainy season brought about a decrease of about 36% in the volume of milk produced by Gambian mothers (Prentice et al. 1981a; Whitehead et al. 1978). The positive correlation between maternal condition and milk yield has not been universally reported; a study of Otomi Indian women found that women in the lower BMI class produced a higher volume of milk with lower energy density (Barbosa et al. 1997).

Milk yield is known to increase with increasing parity in rhesus macaques (Hinde et al. 2009; Hinde and Capitanio 2010), dairy cows (Miller et al. 2006), horses (Doreau et al. 1991), and possibly in humans (Motil et al. 1997). This relationship was reversed in a study of Gambian women, where primiparous mothers were found to produce a higher volume of milk with a different trajectory than multiparous mothers (Whitehead et al. 1978).

The effect of parity on milk yield may have a life history explanation. There are two important ideas that address the relationship between parity and maternal investment. The terminal investment hypothesis posits that investment in any given offspring should increase with advancing parity, as the mother approaches reproductive senescence and her ultimate reproductive event. Another model is the maternal depletion hypothesis.

This states that, with advancing parity, repeated gestation and lactation events deplete a mother of her stores of fatty acids. If they are not adequately replenished between offspring, she is increasingly unable to invest in subsequent infants. These hypotheses are not necessarily mutually exclusive; a mother may find ways to compensate for depleted somatic reserves. For instance, she could produce a lower volume of milk or milk with lower fat content, but nurse for a few months longer.

There might also be a mechanistic biological explanation to the association between parity and milk yield. A study of rodent mammary cells found that some alveolar cells never experience apoptosis during involution and instead become alveolar progenitor cells in subsequent lactations (Wagner et al. 2002). This or a similar mechanism could increase milk yield with advancing parity.

Importantly, while most discussion focuses on maternal depletion with advancing parity, low-parity mothers may experience suboptimal energetic condition for a different reason. Particularly in natural fertility populations with a young age at first birth, women may initiate reproduction before full adult stature and body composition is achieved. For this reason, primiparity can reflect poor maternal energetic condition.

Milk composition is not the only way maternal condition influences lactation; many behavioral effects have been observed in primate mothers. Some low-condition primate mothers have been shown to participate in an increased number of suckling bouts, presumably to compensate for reduced milk quality (Japanese macaques: Tanaka 1997;

Rhesus macaques: Gomendio 1989). Captive vervet mothers characterized as having "marginal" reproductive condition spent less time in contact with their infants than mothers of average reproductive condition, probably limiting investment in their infants in an attempt to improve their own condition (Fairbanks and McGuire 1995). Another pattern was observed in free-ranging vervet monkeys; mothers who began rejecting their infants' suckling attempts earlier were more likely to conceive another infant (Lee 1987). After conception occurred, mothers allowed their infants to resume a more intensive suckling pattern, but only if they had access to high-quality food.

It is clear that maternal energetic condition can influence milk composition with respect to nutrition. The link between maternal condition and milk bioactive factors is a promising area for research to expand this body of literature. Should mothers in better condition send hormonal signals to their infants indicating that growth and immune function should be prioritized because resources are available to support it? Or should mothers in poor condition, who may be providing fewer total calories to their infants, provide a biochemical signal to influence what proportion of these calories is allocated to different functions? Hypotheses and predictions for the relationships between maternal condition variables and milk bioactives are presented at the end of Chapter 2.

Infant Life History

In infancy, the major competing demands for energy are growth and maintenance, of which immune function is a substantial component. Growth is obviously essential for attaining adult size and reproductive maturity, and adult height may be associated with a

reduced likelihood of childlessness in men (Nettle 2002a; Pawlowski et al. 2000), which the authors attribute to greater attractiveness for tall men. They argue that in noncontracepting populations, this trend would be associated with greater reproductive success. The findings are less clear for women; in a study of British women, maximum reproductive success was found at a height slightly below the mean (Nettle 2002b), but in a study of Gambian women, taller adult height was associated with a later age at maturation and first birth but a higher overall lifetime reproductive success (Sear et al. 2004). Regardless of optimal adult height, growth is clearly a critical function for juvenile animals, and is therefore subject to a variety of selective pressures.

Infants can flourish for many months on breast milk alone. Even after supplemental foods are introduced, infants receive a significant portion of their total calories through breast milk. The caloric content of milk is therefore a hugely important determinant of infant growth dynamics. Available milk energy is the product of two variable qualities in milk: milk yield volume and milk energy density (Hinde 2009). Infants who consume a higher volume of milk consume more calories and therefore have a larger energy budget to allocate to growth and maintenance. Milk can also vary in its nutrient density. Of the three macronutrients (fats, proteins, and carbohydrates), fat is most likely to vary significantly between mothers and across time (Mitoulas et al. 2002). As fat contains the highest number of calories per gram of the macronutrients, this variation can result in substantial differences in milk energy density and therefore total milk energy.

Milk volume exerts a strong effect on infant growth in humans and other primates. In a study of energy restriction in baboon mothers, infants whose mothers were fed at 60% of *ad libitum* intake were receiving 37% less milk (of similar quality) by the end of the study than infants of mothers fed *ad lib.*, and their growth rate was reduced by 10% (Roberts et al. 1985). A more complicated relationship between milk yield and infant growth was uncovered in rhesus macaque infants by Hinde's group. They found that the *increase* in milk yield volume between 1 month and 3-4 months postpartum was positively correlated with the rate of infant growth (Hinde et al. 2009). Though data are not presented, Prentice's group reports that human infants born during the dry season in Gambia, when maternal energetic conditions are more favorable, experience faster growth relative to standard weight-for-age growth curves in the immediate postnatal period than infants born during the wet season (Prentice et al. 1981a). In this study, milk volume was reduced by 36% during the wet season while energy density of the milk was essentially unaffected, suggesting that volume of milk consumed is a significant contributor to growth in human infants. A study of American infants likewise found growth rate to be positively associated to volume, but not energy density, of milk consumed (Butte et al. 1984). In this study, milk volume accounted for up to 30% of the variability in weight gain of infants.

There is mixed evidence about the role of milk energy density on infant growth in primates, but most seems to suggest that it does not contribute as powerfully to variation in infant growth as milk volume. For instance, a study of Australian infants found no relationship between infant growth rate and the amount or concentration of fat, protein, or

lactose in milk (Mitoulas et al. 2002). In one study of rhesus macaques, gross energy of milk was not associated with infant weight (Hinde et al. 2009), though in another study, available milk energy (the product of energy density and milk yield volume) was a better predictor of infant mass than yield alone (Hinde 2009), suggesting some contribution of energy density to variability in infant mass. Human infants receiving milk that was relatively high in carbohydrates and low in lipids and protein grew less during the first 6 months of life than those receiving milk of higher quality and higher energy density (Qian et al. 2010).

While growth is critical for future reproductive success, an acute infection will demand that priority is given to investment in immune function, leaving less energy for growth. Similarly, chronic immune activation such as inflammation (even in the absence of an acute infectious episode) is costly. The relationship between immune activity and growth in infants has been studied in several populations where children experience high pathogen exposure. Many of these studies have found that immune activation is associated with reduced growth. Early work in Guatemala found infants experiencing infectious disease were more likely to lose weight and stop growing in height as a result of anorexia and the cost of immune function (Mata et al. 1977). A recent study by Panter-Brick's group found elevated immunoglobulin G (IgG) in Nepalese children to be associated with a reduced weight-for-age Z score (Panter-Brick et al. 2009).

One particularly interesting way immune activation interacts with nutrition to influence growth is through intestinal enteropathy, damage of the intestines. Damage to the

intestinal wall can limit growth in two ways (Lunn 2000). First, the increased permeability of the intestine permits large proteins and other intestinal contents to be released into the bloodstream, where they are unavailable for digestion and may induce an immune response. Secondly, many important digestive enzymes are found on the intestinal wall, and damage to the structure can greatly reduce their concentrations in the gut. Most studies suggest that intestinal enteropathy arises as a result of immune activation (Lunn 2000; Campbell et al. 2003a). As Solomons points out, in addition to acute infection, chronic immunostimulation resulting from exposure to an unhygienic environment can lead to activation of immune responses, and may contribute importantly to barrier defects in the intestines and resulting growth impairments (Solomons 2003). A review by Calder and Jackson illuminates the many ways in which undernutrition and infection are interrelated and contribute to growth faltering in children (Calder and Jackson 2000).

Permeability of the intestines is simple to measure in exclusively breastfeeding infants; the lactose: creatinine ratio (termed the Mucosal Damage Index) in urine is a very good indicator of the degree of damage, with higher urinary lactose resulting from enteropathy and subsequent impairment of lactose digestion (Panter-Brick et al. 2009). Panter-Brick's group found that mucosal damage was negatively associated with growth in Nepalese infants (Panter-Brick et al. 2009). In this population, 9% of the deficit in height-for-age and 19% of the deficit in weight-for-age was explained by MDI. A study by Campbell and colleagues similarly found that a high degree of intestinal permeability was associated with impaired growth in Gambian infants (Campbell et al. 2003b). In this

study, infants who experienced a high degree of mucosal enteropathy also displayed high levels of plasma endotoxins, and higher levels of immunoglobulins. The authors advance these interrelationships as evidence that all three markers are indicative of the same pathway of growth retardation.

Maternal investment in milk production is known to support infant growth and immune function. Traditionally, arguments about life history tradeoffs in infants rely on the infants "deciding" how to allocate the calories received through milk to different physiological functions. In addition to this, it is possible that bioactive factors in milk influence the tradeoffs, and perhaps some of the maintenance costs can actually be defrayed by the bioactives (e.g. immune compounds preventing illness and subsequent costs). One important consequence of a reduction in energy spent on maintenance during infancy may be an increase in the relative energy budget available to support growth. In Chapter 2, I present hypotheses and predictions for the relationships between milk composition and infant health and growth outcomes.

Chapter 2: Bioactive Compounds in Milk

Milk is a dynamic biological fluid that contains much more than just the nutrition required by infants. A number of functional compounds are secreted into milk, including hormones, T and B lymphocytes, antibodies, cytokines, adipokines, interleukins, bacteria, and oligosaccharides, all theoretically capable of exerting a biological effect on the infant. Some of these factors, such as antibodies, have a clear role in supporting the infant's developing immune system. Others, such as oligosaccharides and bacteria, may promote a healthy gut microbiome that is appropriate for the developmental stage and ecology of the infant (Sela and Mills 2010). Still others may signal some aspect of maternal biology or environment to the infant and influence development, such as the effects of milk-borne corticosterone on rodent offspring anxiety and spatial memory (Angelucci et al. 1985; Catalani et al. 2000; Catalani et al. 2002).

It is clear that maternal energetic condition affects certain aspects of milk biology, such as milk yield volume (see Chapter 1). There is also some evidence that maternal condition may influence the composition of milk with respect to bioactive compounds. For instance, a meta-analysis reports that most studies find a positive correlation between maternal BMI and milk leptin (Andreas et al. 2014). A study of Gambian women found that women produced milk with lower concentration of immune proteins during the hunger season (Prentice et al. 1983a).

Bioactive compounds have also been demonstrated to affect infant development, including behavioral development and growth. Macrophages in milk can release factors that ultimately stimulate the development of the infant's own T lymphocytes (Ichikawa et al. 2003), and cytokines can promote or reduce inflammation and promote tolerance of dietary antigens (Ando et al. 2007). Glucocorticoids have been shown to exert or associate with a wide variety of effects on behavioral development (Angelucci et al. 1985; Catalani et al. 2000; Catalani et al. 2002; Sullivan et al. 2011; Glynn et al. 2007; Hinde et al. 2015), which are discussed in greater detail later in this chapter. Milk leptin may be involved in the regulation of meal size by infants (Casabiell et al. 1997), resulting in a lower BMI-for-age z-score among infants who ingest more leptin (Fields and Demerath 2012).

The study of bioactive compounds in milk is currently a very exciting area of biology. Hundreds of compounds remain understudied with respect to cellular mechanisms, associations with maternal and infant physiology and behavior, and interaction with milk nutritional factors (Neville et al. 2012). This dissertation will focus on four bioactive compounds that are predicted to have associations with maternal energetic variables, infant health outcomes, and infant growth. These compounds—cortisol, IGF-1, lactoferrin, and sIgA—are described in this chapter. Background is given for each compound regarding effects in circulating adults, mechanisms of transfer or secretion into milk in humans, a description of the compound in milk, known effects on infant development, and maternal energetic factors known to associate with the compound. A

brief overview is found in Table 2.1. This chapter concludes with the hypotheses to be tested in this dissertation and consideration of alternative hypotheses.

Table 2.1: A brief overview of the background of milk bioactives presented in Chapter 2

	Effect on mother (circulating)	How does it get into milk?	Role in mammary/ milk synthesis	Effect on infant
Cortisol	• Energy mobilization, lipid metabolism • Anti-inflammatory, immunosuppressive	• Passive diffusion from blood	• Casein production (with PRL) • Lipogenesis	• Nervous temperament (rhesus), learning (rat) • Promotes growth (rhesus, red squirrel)
IGF-1	• Glucose uptake in muscle • Promotes cellular anabolism (glucose transport, amino acid uptake, protein synthesis) • Adipocyte glucose uptake (via insulin receptor) • Stimulates mitosis	• Most probably of hepatic origin, transferred from blood via transcytotic pathway • Some produced in mammary stroma	• In pregnancy, growth/differentiation of mammary epithelial cells • In lactation, increase blood flow • Increase milk yield • Increase glucose transport	• gut development • nutrient absorption • programming of IGF-1 axis
Lactoferrin	• Innate nonspecific immunity	• Transcription by mammary epithelial cells (responds to prolactin)	• Anti-infection	• Anti-bacterial, -fungal, -viral effects in respiratory and gastrointestinal tracts
sIgA	• Acquired immunity	• B cells from gut/respiratory tract "home" through lymph to mammary • IgA dimers secreted into extracellular matrix • Bind to pIgR on epithelial cell, which dissociates from membrane, becomes secretory component • Transcytosis into lumen	• Anti-infection	• Prevents symptoms of illness

Cortisol

Cortisol in circulation

Cortisol, a steroid hormone in the glucocorticoid family, is one of the most important endocrine regulators of glucose availability during the fasting state (Nelson 2005; Tempel and Leibowitz 1994). It promotes gluconeogenesis (Khani and Tayek 2001) lipolysis, lipid metabolism and glucose transport (Wang 2005). Glucocorticoids also have an anti-inflammatory effect through the post-translational expression of pro-inflammatory genes (Barnes 1998) and a variety of other immunosuppressive effects (Cupps and Fauci 1982). Normal cortisol function is also essential for a number of behavioral processes, such as prefrontal cortex cognitive function and working memory (Mizoguchi et al. 2004). In addition to its most famous designation as "the stress hormone," disregulation of the hypothalamic-pituitary-adrenal axis is associated with poor sleep quality (Steiger 2002) and mood disorders (Young 2004).

Cortisol is found in three states in circulation: free (unbound), loosely bound to proteins such as albumin, or tightly bound to corticosteroid-binding globulin (CBG). Traditional understanding of the bioavailability of these hormones is that only free steroids are capable of exerting a biological effect. A recent review by Levine and colleages (Levine et al. 2007) challenges this framework. They claim that albumin binds cortisol very weakly, making the cortisol bound to it functionally free in many respects. Another important consideration the authors raise is that free cortisol is more likely to be cleared from circulation entirely than bound cortisol, meaning that the ratio of biological activity to clearance is also important. The amount of free cortisol cleared by the liver is three times the amount of circulating free cortisol at any time, suggesting that cortisol

dissociates from albumin and CBG very rapidly to maintain the pool of free cortisol. Finally, it was recently discovered that the cortisol-CBG complex can be taken up whole by some cells, meaning that bound cortisol might actually be capable of exerting a biological effect *without* dissociating from CBG (reviewed by Willnow and Nykjaer 2010). For these reasons, and because the effect of binding proteins in the infant digestive tract is unknown, this study measures *total* cortisol in milk.

Cortisol in the mammary gland

It is likely that cortisol plays a role in the synthesis of casein within human mammary epithelial cells. Both cortisol and prolactin were required to induce casein mRNA expression in mammary cells of mice (Ganguly et al. 1980). In another study using mouse mammary tissue, prolactin induced the synthesis of casein but the effect was amplified in a dose-dependent manner by addition of cortisol (Ono and Oka 1980). In goat mammary cells, prolactin was sufficient to sustain casein production, but cortisol and insulin both sensitized the cells to the stimulus of prolactin (Skarda et al. 1982). The amplification of casein production by cortisol is achieved by upregulating the amount of casein mRNA available for translation; it does not appear that translation itself is influenced by cortisol (Devinoy et al. 1978).

Glucocorticoids also potentiate lipogenesis. Incubation of rabbit mammary explants with corticosterone, insulin, and prolactin resulted in a massive increase (up to 42-fold) in fatty acid synthesis compared to hormone-free culture (Forsyth et al. 1972). Prolactin alone initiated a 15-fold increase, and insulin and prolactin biased synthesis toward

medium-chain fatty acids, whereas the addition of corticosterone biased synthesis toward long-chain fatty acids.

Cortisol in milk

Cortisol is highest in prepartum secretions and colostrum, and rapidly declines within a few days of parturition (Kulski and Hartmann 1981). It passively diffuses into milk, maintaining equilibrium with the concentration in blood. In a study of dairy cows, injection with adrenocorticotropin (ACTH) stimulated a rise in circulating cortisol that was reflected in the milk at first milking, four hours after treatment (Fox et al. 1981). Without continued ACTH treatment, both plasma and milk cortisol returned to baseline by twelve hours post-treatment. The study design was clever because only half the udder was milked four hours after treatment. The finding that both udder halves had milk with similar and relatively low concentrations of cortisol at twelve hours allows for two interesting conclusions about the dynamics of cortisol entry into milk. Because half the udder had additional time for milk to accumulate, the volume of milk in that half was much higher by twelve hours. However, cortisol concentration in both halves was identical, meaning that cortisol concentration in milk was not diluted proportionally with milk volume. Secondly, since it can safely be assumed that cortisol *had* entered and increased in concentration in the udder half that was not milked, that means that cortisol is free to diffuse both ways between blood and milk.

More evidence for passive diffusion between blood and milk comes from a similar study in which dairy cows were injected with ACTH or saline (Termeulen et al. 1981). Plasma

and milk sampled from the cows receiving ACTH showed an increase in cortisol by 15 minutes after treatment. This increase peaked 1 hour after treatment and declined thereafter. Figure 2.1 shows the similarity between the profiles of milk and plasma cortisol. Across samples, plasma and milk cortisol were very strongly correlated (R^2=0.94). These findings are similar to another study of dairy cows by Bremel and Gangwer, who went on to propose that passive diffusion is the model that best fits the equilibrium between blood and milk cortisol (Bremel and Gangwer 1978).

Figure 2.1: Effect of ACTH injection on blood and milk cortisol concentrations

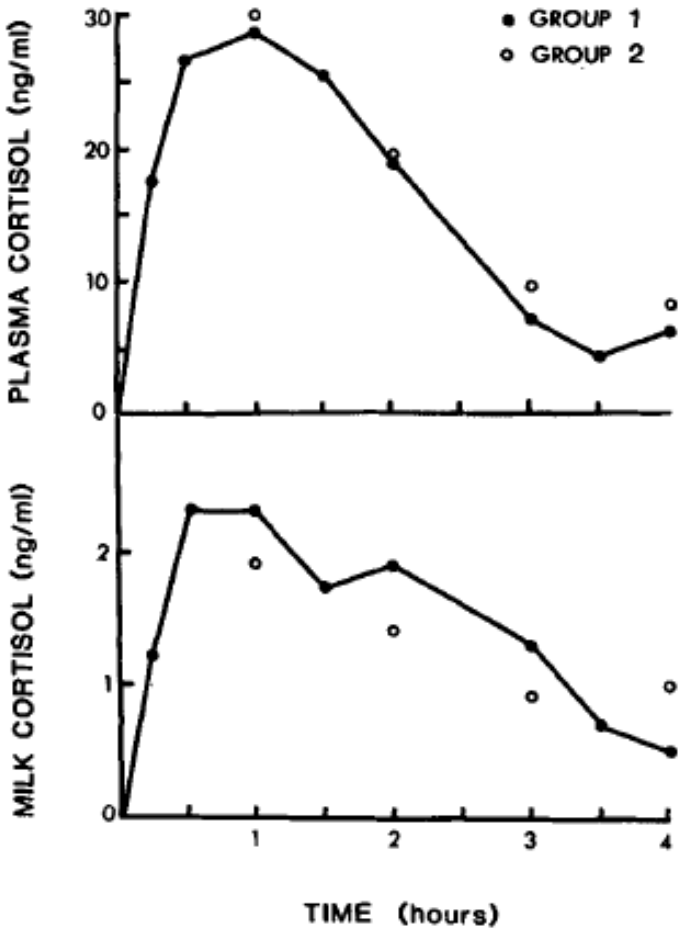


Figure 2.1 (continued): *Effect of ACTH injection on blood and milk cortisol concentrations. Means for each sampling interval for controls were set to zero; thus, deviations from the X-axis represent differences from treatment. Standard error associated with plama means = 4.9 ng/ml, with milk means = 0.4 ng/ml. (Group 2 differs from Group 1 only by sampling frequency). Reprinted from "Rapidity of Cortisol Transfer Between Blood and Milk Following Adrenocorticotropin Injection," by S.B. Termeulen et al., 1981, Journal of Dairy Science, 64, p. 2197. Copyright 1981 by Elsevier Science Ltd. Reprinted with permission.*

Corticosteroid binding globulin (CBG) is present in milk, and a study of guinea pigs showed that the source of CBG in milk is maternal circulation (Kato et al. 1985). These authors did not report what fraction of the cortisol in milk was bound, or what fraction of the CBG entering milk was occupied by glucocorticoids.

Because it is a steroid, cortisol cannot be digested by the infant but because it passes easily through cell membranes and into circulation, it may be cleared by the infant's liver or kidneys. It is unknown whether CBG is resistant to degradation in the infant gut.

The relationship between human milk and serum cortisol values has not been well-established. One study found a moderate positive correlation of r=0.6-0.7 (Patacchioli et al. 1992). Another study found a similar correlation of r=0.52 (Kulski and Hartmann 1981). However, there has been some investigation into maternal effects on milk cortisol. Researchers to date have overwhelmingly adopted the lens that cortisol is a "stress" hormone, largely interested in how variables like maternal mood state and life events affect milk cortisol, and how milk cortisol affects infant temperament and personality (see discussion below). Unfortunately, existing studies are few and do not contribute to a unified understanding of this relationship. For instance, one study found that greater satisfaction with breastfeeding was associated with higher milk cortisol

(Groer et al. 1994), another study found milk cortisol positively associated with higher self-reported hostility (Hart et al. 2004), and yet another found no relationship between negative mood states and milk cortisol (Groer et al. 1999).

Cortisol may be better understood as a metabolic hormone, first and foremost, with important secondary effects such as its role in the stress response (itself a metabolic role: to mobilize energy to cope with the stressor). The narrow focus of research on the effects of stress and mood on milk cortisol, combined with the fact that relatively few studies have investigated milk cortisol at all, have resulted in a piecemeal and rather confusing picture of what influences the amount of cortisol that gets expressed in milk. The effects of maternal metabolic state on milk cortisol have been completely ignored by most researchers, and have the potential to serve as a more unifying platform from which researchers can examine the ways that changes in maternal psychological state can alter the expected pattern of cortisol expression in milk.

Roles of cortisol in infant development

Many studies of cortisol provide excellent examples of how mothers and infants coordinate their physiology through breast milk. The early finding that infant rats express have more glucocorticoid receptors in their intestines than weaned rats (Henning et al. 1975) has been interpreted by many as evidence of the importance of cortisol cues in milk for infant behavior and physiology. Several studies in rats provide a foundation for interesting behavioral research in this area. When rat mothers' water is supplemented with corticosterone (the rodent equivalent of cortisol), their offspring exhibit fewer

anxious behaviors in response to a stressor (Angelucci et al. 1985; Catalani et al. 2000; Catalani et al. 2002). Maternal corticosterone supplementation is associated with improved performance of offspring on a conditioned learning test (Catalani et al. 2000; Catalani et al. 2002). Corticosterone enrichment of dams is also linked to an increase in the expression of glucocorticoid receptors in the hippocampus of male (Casolini et al. 1997; Catalani et al. 2000) but not female offspring (Catalani et al. 2002). Researchers have begun to explore the relationships between milk cortisol and infant behavior in primates. Cortisol in milk was associated with a confident temperament in infant rhesus macaque males, but not females (Sullivan et al. 2011). However, after controlling for available milk energy, this relationship was reversed (Hinde et al. 2015). Both male and female infants displayed more nervous and less confident behavioral phenotypes if they received higher milk cortisol. For female infants, the important cortisol measure was total concentration early in lactation, but males seemed to be more sensitive to cortisol dynamics, specifically the increase in cortisol between early and peak lactation. In humans, maternal plasma cortisol was positively associated with the amount of fearfulness exhibited by the infant, though milk cortisol was not directly measured (Glynn et al. 2007).

In addition to the fascinating research about the role of milk cortisol on infant behavioral development, recent studies have focused on the role of milk cortisol on infant growth. As previously described, metabolic state can affect the quantity, and in some cases, the quality of milk transferred to the infant. Additional information may be transmitted through non-nutritive bioactive compounds, and cortisol is a promising candidate. One

study of rhesus macaque infants found that higher milk cortisol concentration at peak lactation was positively associated with infant growth between early and peak lactation (Hinde et al. 2015). A positive relationship between maternal cortisol and growth was found among red squirrels (Dantzer et al. 2013); although milk cortisol was not measured, it is assumed to reflect circulating levels. In this study, maternal cortisol was experimentally elevated by increasing signals of population density in her environment. Because population density is known to influence optimal growth rate in this species, the authors present the idea that cortisol is the signal by which mothers modulate infant growth rate.

Cortisol and maternal condition

Because milk cortisol reflects circulating concentration so closely, factors affecting maternal circulating cortisol will largely determine milk cortisol concentration. Fasting is known to increase circulating cortisol, increase its half-life in plasma, and reduce HPA sensitivity to negative feedback (Fichter et al. 1986). A study of rhesus macaque mothers found that lower-parity mothers produced milk with a higher cortisol concentration (Hinde et al. 2015).

Surgical weight loss by obese women (from average BMI of 42.57 to average BMI of 31.78) induced a 30% decrease in corticosterone binding globulin and a corresponding rise in free cortisol (Manco et al. 2007). The authors speculate that increased free cortisol might be a mechanism by which hypoglycemia is avoided during weight loss, and the regulation of "post-obese" women's cortisol supply is reprogrammed by this experience.

Less CBG was produced by insulin-resistant obese patients compared to insulin-sensitive obese patients (Fernández-Real et al. 1999). This may be a mechanism to promote cortisol clearance, as free cortisol has a shorter half-life than bound (Manco et al. 2007).

Insulin-like growth factor-1

IGF-1 in circulation

Insulin-like growth factor-1 (IGF-1, formerly called somatomedin C) is used by nearly every cell in the body. As its name implies, IGF-1 has two major roles, a metabolic role in which it behaves similarly to insulin, and a growth-promoting role. Accordingly, IGF-1 has the ability to affect both proliferative growth by inducing mitosis, and hypertrophic growth by stimulating anabolic processes such as glucose transport, amino acid uptake, and protein synthesis (Sara and Hall 1990).

Growth hormone (GH) has been established as a primary regulator of IGF-1 gene expression in the adult in a variety of tissues (Hynes et al. 1987; Mathews et al. 1986; Roberts et al. 1986; reviewed by Sara and Hall 1990). IGF-1 is produced in most tissue types for paracrine action within tissues, and in large quantities by the liver (along with binding proteins) for its endocrine functions (reviewed in Sara and Hall 1990). A primary role of IGF-1 (in response to growth hormone) is to stimulate cell division at the epiphyseal plates of long bones, leading to linear growth (Ernst and Froesch 1988; Schlechter et al. 1986). Even after an individual's linear growth is complete, IGF-1 is involved in the proliferation of many tissue types, including mammary tissue.

IGF-1 in the mammary gland

Starting in pregnancy, IGF-1 has a number of effects on mammary gland development and milk production. IGF-1 concentration in mammary fluids is highest in late pregnancy and early lactation and declines thereafter (Eriksson et al. 1993; Milsom et al. 2008; Marcotty et al. 1994). This, along with the greater number of functional IGF-1 receptors in early pregnancy compared to later pregnancy and lactation (Baumrucker and Erondu 2000) suggests that IGF-1 acts to help develop the mammary gland in preparation for lactation. Indeed, *in vitro* studies have shown that incubating mammary epithelial cells from pregnant dairy cows with IGF-1 enhances their proliferation (McGrath et al. 1991**;** Collier et al. 1993).

Once lactation is established, IGF-1 and growth hormone (acting through the IGF-1 pathway), promote lactation in a number of ways. IGF-1 stimulation increases blood flow to the mammary gland and increases glucose and amino acid availability for maintenance and milk production (Breier et al. 1991; Prosser et al. 1987). Milk yield is higher in animals (Prosser et al. 1991b**;** Prosser et al. 1989; Zhao et al. 1994) and humans (Breier et al. 1993) given exogenous GH. The 6-fold increase in IGF-1 experienced by the cows in at least one of these studies (Prosser et al. 1989) was enough to increase total IGF-1 concentration in milk despite the concomitant increase in volume, thus outweighing the effect of dilution. IGF-1 also enhances the role of prolactin on casein synthesis in rabbits (Duclos et al. 1989) and mice (Prosser et al. 1987).

IGF-1 in milk

IGF-1 is highest in prepartum mammary secretions and colostrum (Prosser 1996) and then declines rapidly with the onset of mature milk production. One study found that levels remain relatively stable after four days of age for about two weeks and then experience a moderate decline for one to three months, and then remain stable until at least nine months after birth (Milsom et al. 2008). Another study found very high levels of IGF-1 in prepartum mammary secretions and colostrum, followed by the expected decrease between colostrum and mature milk concentrations, but then a rise in concentration between early and peak lactation (Corps et al. 1988). These authors reported a concomitant increase in milk volume over this period and estimate that the increase in total IGF-1 production is about four-fold.

After transfer to the mammary gland, some IGF-1 is excreted into milk. IGF-1 is resistant to gastric proteolysis but begins to be degraded in the small intestine (Rao et al. 1990). Casein appears to protect IGF-1 from degradation and a significant proportion remains intact in the intestine, confirming the potential for bioactivity.

A multi-species review of IGF and IGFBPs in milk (Grosvenor et al. 1993) outlined the possible generic pathways for secretion of IGF and binding proteins into milk (Figure 2.2).

Figure 2.2: Model of conceivable mechanisms whereby IGFs and their binding proteins (IGFBP) appear in milk

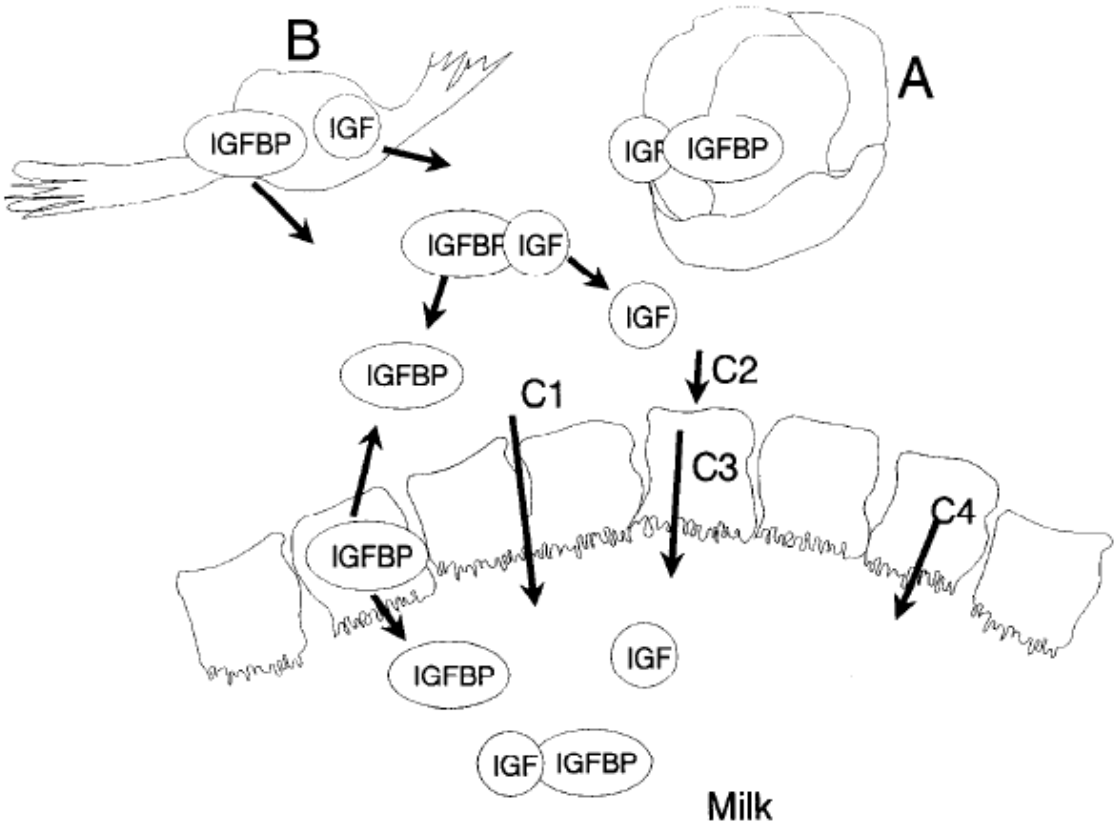


Figure 2.2: *Model of conceivable mechanisms whereby IGFs and their binding proteins (IGFBP) appear in milk. A, Capillary bed; B, nonepithelial cells (fibroblasts); C1 to C4, possible routes for movement of IGFs and IGFBPs into milk. Reprinted from "Hormones and Growth Factors in Milk," by C.E. Grosvenor et al., 1992, Endocrine Reviews, 14(6), p. 710. Copyright 1992 by the Endocrine Society. Reprinted with permission.*

This diagram displays the complicated nature of how IGF-1 and its binding proteins may be secreted into human milk. IGFs are either produced by distal tissues such as the liver and travel to the mammary through the bloodstream (A) or are produced in mammary stromal (non-epithelial) cells (B). IGFBPs are either transported to the mammary via the bloodstream bound to IGFs (A), produced in mammary stromal (non-epithelial) cells (B), or produced by mammary epithelial cells (not numbered, to the left of C1; C4). C1

indicates a paracellular pathway, whereby IGFBP, IGF, or both together as a complex pass through gaps between mammary epithelial cells. C2/C3 represents uptake of free IGF-1 through a receptor into a cell and subsequent secretion into milk, where it may bind to a binding protein in the lumen. C4 represents de novo synthesis of either protein by mammary epithelial cells (but there is very little evidence that IGF-1 is produced by mammary epithelial cells in any species). Not only are there a variety of pathways for transfer and synthesis of the various proteins, but regulation of these pathways also varies by species and by stage of mammary development and lactation. Nearly all of these pathways have been demonstrated to exist in a variety of species. The questions then become: what *proportions* of secreted IGF-1 and binding proteins are derived from which pathway in humans, and which factors in those dominant pathways respond appreciably in response to changes in maternal physiology?

Because these experimental studies on transfer and clearance of IGFs and their binding proteins involve injection with radiolabeled proteins to study clearance and transfer or sacrifice of the animal to investigate mRNA production in various tissues, all are animal studies. Unfortunately for our ability to generalize these studies to humans, the pathways of synthesis and transfer differ—often entirely—in rodents and ruminants, the two main experimental animal models. One consistent finding across species is that mammary stromal cells do produce at least some mRNA for IGF-1 (Glimm et al. 1992; Marcotty et al. 1994; Lee et al. 1993), but the majority of IGF-1 that is secreted into milk is transferred from blood, and not synthesized in the mammary (goats: Prosser et al. 1991a; rats: Donovan et al. 1995, Marcotty et al. 1994). One study measured the lag between

peripheral injection and appearance of $^{(125)}$I-labeled IGF-1 in the milk of dairy goats (Prosser et al. 1991a). The delay of approximately 80-120 minutes was similar to the amount of time required for $^{(14)}$C-labeled amino acids, administered to the same goats, to be incorporated into and secreted as milk proteins. This suggests that IGF-1 needs to be taken up by secretory cells and secreted through the transcytotic pathway, rather than directly secreted via the paracellular pathway. Administration of excess (non-labeled) IGF-1 to the goats significantly reduced the transfer of radiolabeled IGF-1 into milk. The authors suggest a "competitive and saturable mechanism" of IGF-1 transfer. This probably means that IGF-1 is taken up by secretory cells via an IGF receptor, but to the best of my knowledge, this has not been confirmed. This hypothesis is complicated by the fact that IGF-1 binding to its most common receptors, IGF-1R and the insulin receptor, result in a mitogenic effect in the cell. A separate receptor-mediated signaling pathway for transcytosis would be necessary to reconcile these facts.

IGFBP-3 is the dominant circulating binding protein in human serum (Baxter 1993), but IGFBP-2 is by far the most common binding protein in human milk (Donovan et al. 1991; Milsom et al. 2008), with concentrations more than an order of magnitude greater than IGFBP-3 and IGFBP-1 (Milsom et al. 2008). Surprisingly, there are no human studies that investigate IGFBP-2 concentration in serum and milk in the same individuals, but comparing across studies it seems clear that IGFBP-2 is present at much higher concentrations in milk (7±1.3 ug/mL) than in serum (0.15±0.061 ug/mL) (Milk samples from 23 New Zealand mothers: Milsom et al. 2008; serum samples from 38 healthy adults; Clemmons and Snyder 1991).

These two facts taken together, that IGFBP-3 is much more common in serum than milk, and IGFBP-2 is much more common in milk than serum, leave two reasonable contenders for the human-typical pattern of IGF-1 and binding protein secretion into milk. One possibility is that circulating IGFBP-2 acts as a "shuttle" for IGF-1 to the mammary gland, so that circulating IGFBP-2-bound IGF-1 is preferentially taken up by the mammary and transported into milk. This targeted transport hypothesis for the role of different binding proteins proposes that larger proteins such as IGFBP-3 (forming a 150 kDa unit) protect circulating IGFs from degradation, but prevent entry into capillary spaces, whereas smaller binding proteins such as IGFBP-2 (40 kDa) extend the half-life of circulating IGFs to a lesser degree but may permit their entry to different tissues through the capillary barrier, effectively acting as shuttles to these tissues (Sara and Hall 1990). The mammary shuttle hypothesis for IGFBP-2 does not seem to be upheld in goats. Injection of IGFBP-2 into the circulation of lactating goats actually *reduced* transfer of IGF-1 to milk (Prosser and Schwander 1996). Overall clearance from plasma was increased, suggesting that IGFBP-2 was preferentially targeting circulating IGFs to some non-mammary tissues. This finding is puzzling because most IGFBP-2 in lactating sheep (and probably therefore goats) is derived from circulation—almost no IGFBP-2 mRNA is found in the mammary tissue of lactating sheep (Klempt et al. 1993). In contrast to the pattern found in sheep, mammary tissue in lactating rats expressed IGFBP-2 mRNA, but not IGFBP-3 mRNA, suggesting mammary synthesis of IGFBP-2 and maternal hepatic origin of IGFBP-3 in rats (Donovan et al. 1995).

The other possibility is that IGF-1 dissociates from its binding protein (likely IGFBP-3) in the mammary, binds to an IGF receptor on a mammary epithelial cell, is taken up and secreted into the lumen, where it binds with mammary-synthesized IGFBP-2. GH stimulation of lactating women resulted in an increase in plasma and milk IGF-1, and an increase in plasma IGFBP-3 (Breier et al. 1993). In the same study, plasma IGFBP-2 was reduced but milk IGFBP-2 concentration stayed the same. The authors suggest that IGFBP-3 is the primary delivery protein of IGF-1 to the mammary.

With the information available, it seems likely that IGF-1 in human milk is derived from maternal circulation, transported to the mammary by binding proteins, and secreted into milk via the transcytotic pathway, but the source of the high concentration of IGFBP-2 (mammary versus liver) in human milk is unknown.

Roles of IGF-1 in infant development

Once consumed by the infant, IGF-1 has important effects on development. The primary role of orally-ingested IGF-1 appears to be support for development of the gut. Formula-fed newborn pigs supplemented with IGF-1 had greater small intestinal weight, protein, and DNA content, and greater jejunal and ileal villus height than controls (Burrin et al. 1996). Supplementing milk replacer formula with IGF-1 had no effect on the weight of the gastrointestinal tract of neonatal calves, but xylose uptake increased, indicating an improvement in intestinal absorption (Baumrucker et al. 1994). Oral IGF-1 supplementation upregulated the expression of IGF-1 receptors on calf intestinal cells (Baumrucker and Blum 1993). IGF-1 also has a strong trophic effect on calf intestinal

cells *in vivo* (Baumrucker and Blum 1993) and on human infant intestinal cells *in vitro* (Hirai et al. 2002). Premature human infants fed formula supplemented with IGF-1 displayed reduced gut permeability compared to unsupplemented infants (Corpeleijn et al. 2008).

In addition to maturation of the gut and stimulation of intestinal cell division, oral administration of physiological doses of IGF-1 in rats and pigs increases the specific activity of lactase and other brush-border enzymes in the intestine (Young et al. 1990; Donovan et al. 1996).

It is unlikely that IGF-1 consumed orally enters infant circulation in any appreciable quantities. Studies of calves, neonatal rats, mice, and piglets find the amount entering circulation to be less than 1% (Vacher et al. 1995; Hammon and Blum 1997**;** Philipps et al. 1995; Burrin 1997; Donovan et al. 1997; Burrin et al. 1996). It is possible that the gut of newborn animals, especially those born prematurely, have incompletely formed guts that allow the passage of larger proteins such as IGF-1 (Burrin 1997) and the animals in these studies had passed this phase. Another unlikely possibility is that orally-consumed IGF-1 does pass into the circulation in its free state and is immediately taken up by nearby cells; it could be absorbed through the gut and exerting biological effects, but it would not show up in a plasma sample because of rapid uptake.

Despite the probable impermeability of the gut to IGF-1, orally-consumed IGF-1 may enhance endogenous production of IGF-1 such that circulating levels are increased, albeit

by a different mechanism. A study of calves fed colostrum, milk replacer, subcutaneous or oral IGF-1, or subcutaneous growth hormone found that colostrum-fed calves had higher endogenous IGF-1 production that those fed milk replacer (Hammon and Blum 1997). Oral IGF-1 administration did not increase endogenous levels, suggesting that other compounds in colostrum in addition to IGF-1 are necessary to induce endogenous production.

Breastfed infants tend to be smaller and have lower endogenous production of IGF-1 than formula-fed infants, a finding that has been attributed to the higher protein content of formula (Socha et al. 2011). However, this relationship reverses as childhood progresses. At age 17, young adults who had been breastfed had higher IGF-1 than their formula-fed peers (Larnkjaer et al. 2009). It has been proposed that this is due to a "programming" effect on the IGF-1 pathway by recalibrating the pituitary sensitivity to IGF-1; if this is true, then high levels of IGF-1 in infancy reduce pituitary sensitivity, resulting in a blunting of negative feedback and lower production of growth hormone and IGF-1 later in life (Martin et al. 2012). Oral IGF-1 could also affect systemic receptor production (it is known to affect intestinal receptor expression in calves: Baumrucker et al. 1994) or systemic binding protein expression.

IGF-1 and maternal condition

Because IGF-1 is transported into the mammary from the circulation, factors affecting circulating IGF-1 may help to guide predictions about concentrations in milk. Circulating IGF-1 is primarily produced in the liver in response to stimulation by growth

hormone. Growth hormone secretion decreases with increasing BMI, particularly with increasing visceral adiposity (Veldhuis and Iranmanesh 1996). IGF-1 shows no relationship with BMI, but is negatively associated with visceral adiposity (Rasmussen et al. 1994). However, these and most other studies investigating the effect of body composition on growth hormone and IGF-1 focus on normal circulating levels obese subjects and therefore may only reveal part of the story. Fasting in rats led to a decrease in hepatic mRNA expression of IGF-1 (Emler and Schalch 1987), and a ten-day fast resulted in a highly significant reduction in plasma IGF-1 in obese men (Clemmons et al. 1981). Estradiol positively associates with growth hormone secretion (Veldhuis and Iranmanesh 1996; Ho et al. 1987), suggesting the possibility for a positive relationship between BMI and growth hormone (and therefore IGF-1) for women at the underweight and lower range of normal BMI.

Lactoferrin

Lactoferrin in circulation

Lactoferrin is a multifunctional innate immune protein found at mucosal surfaces. It is primarily synthesized by neutrophils, and it is capable of restricting the proliferation of infectious organisms in a variety of ways. Perhaps the best-known role of lactoferrin is to bind iron in the gastrointestinal tract, which sequesters it from pathogenic bacteria that need iron to proliferate (Bishop et al. 1976; Bullen et al. 1972). Lactoferrin also contains a highly basic n-terminal domain called lactoferricin, which is capable of lysing bacterial cell membranes (reviewed by Gifford et al. 2005). There are many effects of lactoferrin on the action of other immune cells, including increasing motility of granulocytes,

regulation of lymphocyte maturation, upregulating production of natural killer cells and influencing production of both pro- and anti-inflammatory cytokines (reviewed in Legrand et al. 2004). Finally, lactoferrin can bind to host epithelial cells via the lactoferrin receptor and increase the resistance of those cells to intracellular invasion by pathogens by interfering with pathogen adhesion mechanisms (Longhi et al. 1993; Marchetti et al. 1996). While the antibacterial role of lactoferrin is widely known, lactoferrin is also capable of interfering with viruses, fungi, and protozoa (reviewed in Vorland 1999).

Lactoferrin in the mammary gland

Milk lactoferrin is elevated about a week after an episode of mastitis (Prentice et al. 1985), suggesting a protective antimicrobial role of lactoferrin within the breast. This is further supported by the finding from the same study that women who went on to develop mastitis had lower average concentration of milk lactoferrin at baseline than women who did not develop mastitis.

Lactoferrin in milk

Maternal-origin lactoferrin is produced in mammary epithelial cells (Neville et al. 1998) and secreted into milk. Expression of mammary-origin lactoferrin is prolactin-dependent (Green and Pastewka 1978). Lactoferrin is at least partially resistant to digestion and degradation within the infant digestive system; at least 2% of ingested lactoferrin is excreted intact in infant feces, confirming that some lactoferrin is capable of maintaining function in the gut (Prentice et al. 1989).

Most lactoferrin in milk is not bound to iron, meaning that the binding site is still free and active. However, there seems to be substantial inter-individual variation in the proportion of lactoferrin secreted in the unsaturated vs. saturated state. One study presented individual values from three human milk samples, in which the proportion of saturated lactoferrin in the samples ranged from 11 to 43% (Bullen et al. 1972). In another study with 15 samples from infants of a large range of ages, the maximum saturation rate was only 4% (Fransson and Lönnerdal 1980). A substantial fraction of iron found in milk (20-40%) is bound to lactoferrin (Fransson and Lönnerdal 1980).

Lactoferrin is highest in colostrum and decreases with the onset of mature milk production (Reddy et al. 1977). Lactoferrin gradually decreased over the first 15 months of lactation in the milk Zaïrean mothers (Hennart et al. 1991) and over the first 4 months of lactation in American mothers (Butte et al. 1984). After 15 months, levels steeply increased. Total amount of lactoferrin (concentration multiplied by milk volume) decreased significantly between 1.5 and 17 months in infants from the Gambia and between 1.5 and 3 months in the UK (Prentice et al. 1989).

Roles of lactoferrin in infant development

Once ingested by the infant, maternal-origin lactoferrin provides immune protection during early life while the infant's immune system develops immunological competence. Lactoferrin in human milk inhibits the growth of pathogenic *E. coli* in vitro, and saturation with iron removed this effect (Bullen et al. 1972). The same study reported

that infant guinea pigs dosed with E. coli experienced lower bacterial loads if they were also fed guinea pig milk instead of formula. Addition of human recombinant lactoferrin and lysozyme to an oral rehydration solution was found to shorten the duration of illness among infants with diarrhea and dehydration in a randomized controlled trial (Zavaleta et al. 1995). Similarly, a study of formula-fed infants found that supplementation with bovine lactoferrin was associated with lower risk of illness, particularly wheeze and lower respiratory tract infections in the first year of life (King et al. 2007).

Hassiotou and colleagues published a study of milk composition across lactation in urban Australian women that found that lactoferrin concentration remains unchanged in the event of maternal or infant illness (Hassiotou et al. 2013). With the exception of this single study, infant outcomes in relation to natural variation in milk lactoferrin concentration have not been previously published.

Lactoferrin and maternal condition

There is some evidence that maternal metabolic state has the ability to influence the expression of non-nutritive compounds in milk. Malnutrition is associated with a lower concentration of milk lactoferrin. In the Gambian rainy season, when energy expenditure is high and intake is low, all tested immune compounds in milk, including lactoferrin, were found to decrease, despite the fact that infections were more common among mothers and infants during this time (Prentice et al. 1983a). Aboriginal and white Australian women greater than 90% of their suggested weight for height (WFH) had higher levels of lactoferrin than women under 90% WFH (Houghton et al. 1985).

A mother's parity may be another important contributor to her condition. Among Gambian mothers, those with lower parity had higher expression of all protective factors, including sIgA and lactoferrin, in milk, suggesting that maternal depletion with advancing parity may influence milk composition (Prentice et al. 1983a). Because these authors found that milk volume did not differ by parity until the ninth offspring, maternal depletion with advancing parity seems likely to have directly influenced milk immunofactors.

Secretory Immunoglobulin A

IgA and sIgA in circulation

IgA is a protein of the acquired immune system produced by B cells. B cells are exposed to antigens in mucosa-associated lymph tissue, primarily in the gut but also in the respiratory tract (reviewed in Brandtzaeg 2003). The B cells then secrete immunoglobulins (Igs, also called antibodies), including immunoglobulin A that are specifically targeted against the pathogen to which the B cell was exposed. About 90% of terminally differentiated B cells produce IgA in dimeric or polymeric form, where two or more IgA molecules are joined by a protein called a J chain (Brandtzaeg 1974; reviewed by Brandtzaeg et al. 1999). The J chain allows the molecule to bind to a receptor called pIgR, which becomes the secretory component and protects circulating sIgA from proteolysis (Crottet and Corthésy 1998).

The most common mechanism by which sIgA protects against infection is a process called immune exclusion (reviewed in Brandtzaeg 2003). Essentially, sIgA prevents pathogens from binding to and entering epithelial cells and agglutinates and neutralizes bacteria. It also traps pathogens in mucus and stimulates peristalsis and ciliary activity to remove them, reduces the virulence of bacteria, and reduces certain inflammatory responses (Mantis et al. 2011).

sIgA in the mammary gland

Maternal B cells migrate from gut and respiratory mucosa to the mammary gland through lymph in a process called "homing" (reviewed in Van de Perre 2003). The B cells become plasma cells in the interstitial space and stay located within the mammary (Hayward 1983). Once the B cells are settled, they secrete dimers of IgA (two IgA molecules joined by a J chain) that bind to a receptor called pIgR located on mammary epithelial cells. Once bound, this receptor detaches from the cell membrane and becomes the secretory component. This complex—dimeric IgA, J chain and secretory component—is then secreted into the lumen via transcytosis. IgA is the most common immunoglobulin in milk (Telemo and Hanson 1996). The IgA molecules are specific to pathogens encountered by the mother; due to their origin, they protect against a variety of gut- and respiratory-associated pathogens.

Milk sIgA is elevated about a week after an episode of mastitis (Prentice et al. 1985), suggesting a protective antimicrobial role of sIgA within the breast. This is further supported by the finding from the same study that women who went on to develop

mastitis had lower average concentration of milk sIgA at baseline than women who did not develop mastitis.

Figure 2.3: Model for local generation of secretory IgA (SIgA) and secretory IgM (SIgM)

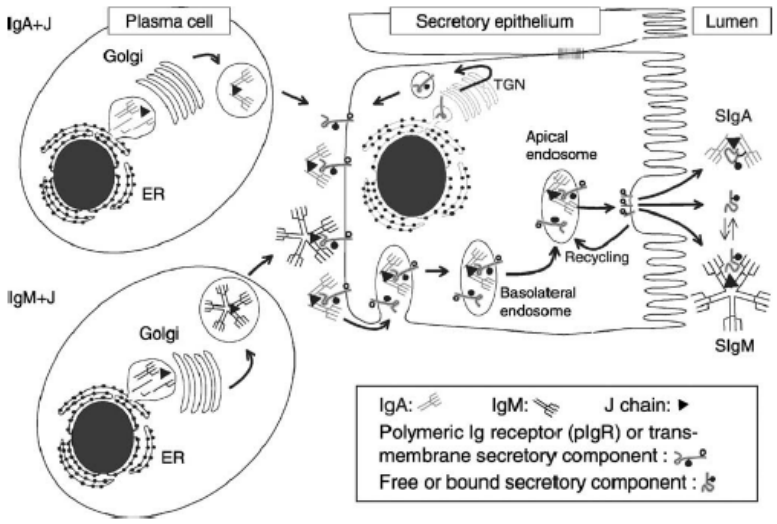


Fig. 2. Model for local generation of secretory IgA (SIgA) and secretory IgM (SIgM). J chain-containing dimeric IgA (IgA + J) and pentameric IgM (IgM + J) are produced by local plasma cells (left). Polymeric Ig receptor (pIgR), or membrane secretory component (SC), is synthesized by secretory epithelial cell in the rough endoplasmic reticulum and matures in the Golgi complex by terminal glycosylation (●). In the trans-Golgi network (TGN), pIgR is sorted for delivery to the basolateral plasma membrane. The receptor becomes phosphorylated (Ⓟ) on a serine residue in its cytoplasmic tail. After endocytosis, ligand-complexed and unoccupied pIgR is delivered to basolateral endosomes and sorted for transcytosis to apical endosomes. Some recycling from basolateral endosomes to the basolateral surface may occur for unoccupied pIgR (not shown). Receptor recycling also takes place at the apical cell surface as indicated, although most pIgR is cleaved to allow extrusion of SIgA, SIgM and free SC to the lumen. During epithelial translocation, covalent stabilization of SIgA regularly occurs (disulfide bond between bound SC and one IgA subunit indicated), whereas free SC in secretions stabilizes the non-covalently bound SC in SIgM (dynamic equilibrium indicated). Modified from Brandtzaeg et al. [2].

Figure 2.3: *Model for local generation of secretory IgA (sIgA) and secretory IgM (sIgM). Reprinted from "Mucosal immunity: integration between mother and the breast-fed infant", by P. Brandtzaeg, 2003, Vaccine, 21, p. 3382. Copyright 2003 by Elsevier Science Ltd. Reprinted with permission.*

sIgA in milk

SIgA enters the milk through transcytosis out of the secretory epithelial cell and into the lumen of the mammary gland (Brandtzaeg 2003). This process is shown in Figure 2.3. The secretory component protects the molecule from digestion by proteolytic enzymes, so sIgA reaches the newborn gut intact and functional (Crottet and Corthésy 1998). sIgA

is at least partially resistant to digestion and degradation within the infant digestive system; approximately 30% of sIgA ingested by the infant is excreted in infant feces, confirming that lactoferrin is capable of maintaining function in the gut (Prentice et al. 1989).

Roles of sIgA in infant development

Concentration of sIgA is highest in colostrum and decreases with the onset of mature milk production (Reddy et al. 1977). The total amount of sIgA (concentration multiplied by milk volume) decreased significantly between 1.5 and 17 months in infants from the Gambia (Prentice et al. 1989) and over the first 4 months of lactation in American mothers (Butte et al. 1984). In another study, the concentration and total amount of sIgA did not change significantly change over the first 18 months of lactation (Hennart et al. 1991)

Infants, particularly newborns, do not have a functioning adaptive immune system (Brandtzaeg 1998; Brandtzaeg et al. 1991; Holt 1995), and are therefore vulnerable to infection by many pathogens that favor mucosal entry, including most respiratory and gastrointestinal pathogens. Transfer of maternal sIgA in milk provides a critical form of immune support for breastfeeding infants. Experiments in neonatal rabbits and mice show that sIgA promotes gut integrity (Johansen et al. 1999) and protects against certain epithelial infections (Dickinson et al. 1998; Lycke et al. 1999; Asahi et al. 2002**).** The protective action of sIgA in the infant gut occurs via many processes, including intracellular neutralization and excretion of viral particles; immune exclusion, whereby sIgA prevents the binding of a pathogen to a mucosal surface; agglutination of bacteria

and viruses; and interference with bacterial motility (reviewed in van Egmond et al. 2001; Mantis et al. 2011; Brandtzaeg 2003).

Previous studies have shown that higher levels of pathogen-specific secretory immunoglobulin A (sIgA) in milk have been associated with lower incidence of diarrhea in infants infected with *Giardia lamblia* (Walterspiel et al. 1994) and *Campylobacter jejuni* (Ruiz-Palacios et al. 1990). Another study found sIgA concentration in milk to rise following maternal or infant illness (Hassiotou et al. 2013).

Infant B-cells become activated a few weeks after birth. This activation is a result of external pathogen exposure that begins sIgA production, and occurs faster in populations with higher pathogen exposure (reviewed in Brandtzaeg et al. 1991). Similarly, neonatal mice nursed by immunodeficient mothers underwent a more rapid development of endogenous IgA production than mice that received sIgA through milk (Kramer and Cebra 1995).

Milk sIgA may also play a role in the development of oral tolerance to dietary proteins. Breastfeeding is protective against the development of food allergies (Greco et al. 1988) and sIgA with specific antibodies to dietary antigens may protect against development of food allergies, at least cow's milk allergy (Savilahti et al. 1991). One possible mechanism for this effect is that sIgA may reduce contact with developing lymphoid tissue and B cells in the gut, the mechanism proposed to explain the accelerated

development experienced by mice receiving IgA-depleted milk (Kramer and Cebra 1995).

Only one study has investigated the relationship between milk sIgA on infant growth, among Ariaal infants in Kenya (Miller 2001). When milk IgA was regressed against standardized infant growth measurements, including height-for-age, weight-for-age, weight-for-height, upper arm fat area, and triceps skinfold-for-age, only upper arm fat was significant. When this regression controlled for the amount of fat in the milk sample, this effect disappeared, but new associations surfaced. Fat-corrected milk sIgA concentration correlated positively with height- and weight-for age, meaning that larger infants received more sIgA per gram of fat from their mothers. However, these results do not control for milk volume.

sIgA and maternal condition

There is some evidence that maternal metabolic state has the ability to influence the concentration of sIgA in milk. Previous studies have found that inadequate diet (Weaver et al. 1998) and high levels of exercise (Gregory et al. 1997) reduce the amount of sIgA expressed in breast milk. In the Gambian rainy season, when energy expenditure is high and intake is low, all tested immune compounds in milk were found to decrease, despite the fact that infections were more common among mothers and infants during this time (Prentice et al. 1983a). Malnourished Colombian mothers had significantly lower sIgA in colostrum than the well-nourished group, but this difference disappeared by 2 weeks postpartum (Miranda et al. 1983). However, most other studies have been unsuccessful

in extending this relationship to natural variation in maternal state and milk immunity. One such study, primarily interested in the effect of protein malnutrition on milk immunofactors in Zaire, found no relationship between maternal BMI or arm circumference and sIgA and lactoferrin present in milk (Hennart et al. 1991). Another study of the Ariaal of Kenya also reported no relationships between an extensive list of reproductive, health, nutrition, and socioeconomic factors of mothers and sIgA concentration in milk (Miller 2011). In both studies, the measures of maternal condition were limited to height, weight, and arm circumference at a single study visit.

A mother's parity may be another important contributor to her condition. The same study of Gambian mothers and infants found that low parity was associated with higher expression of sIgA in milk, with no parity-associated change in milk volume until the ninth offspring (Prentice et al. 1983a). Miller also investigated the relationship between infant age, maternal parity and milk sIgA among the Ariaal but found that concentration of sIgA in milk rises with parity until a mother's 4th infant, followed by a decrease with advancing parity, which she attributes to maternal depletion (Miller 2011). Again, Miller's findings did not account for milk volume, which may have varied with parity. Multiparous women from Zaïre had a significantly higher concentration of sIgA in their milk than did primiparous women; when this was multiplied by milk volume to calculate the total amount transferred to infants, only rural multiparous women still had significantly higher milk sIgA transfer than their primiparous counterparts (Hennart et al. 1991).

Hypotheses and predictions

1. Maternal condition and milk composition

In Chapter 4, I explore the relationship between several maternal energetic variables (BMI, monthly change in BMI, C-peptide of insulin dynamics, and parity) and the concentration of cortisol, IGF-1, lactoferrin, and sIgA in milk. Table 2.2 contains the hypotheses to be tested in Chapter 4, along with some alternative hypotheses informed by the background presented in this chapter.

Table 2.2: Hypotheses and predictions relating maternal energetic condition to milk bioactives

All compounds	
A **negative association** between maternal energetic condition and the concentration of all milk compounds…	…would be predicted if maternal energetic condition positively associates with milk volume. *[However, IGF-1 may positively correlate with milk volume as it does in dairy cows (Prosser et al. 1989), and milk cortisol does not appear to be subject to the effect of dilution, at least in dairy cows (Fox et al. 1981)].*
Cortisol	
A **positive association** between maternal energetic condition and milk cortisol concentration…	…may be predicted if cortisol is a signal from mother to infant aimed at increasing costly behavioral activity, such as a confident and bold behavioral phenotype (as in rhesus macaques; Sullivan et al. 2011).
A **negative association** between maternal energetic condition and milk cortisol concentration…	…may be predicted because poor maternal condition, particularly weight loss, may increase circulating cortisol, and cortisol passively diffuses into milk (Fox et al. 1981; Termeulen et al. 1981).
A **negative association** between maternal parity and milk cortisol…	…is predicted because this pattern has been demonstrated in rhesus macaques (Hinde et al. 2015). This has been proposed as a mechanism whereby mothers with fewer energetic resources program a "cheap" infant who reduces costly exploratory behaviors and grows more quickly.

Table 2.2 (continued)

Cortisol (continued)	
No association between maternal energetic condition and milk cortisol…	…may indicate that maternal and milk cortisol is predominately mediated by psychosocial stress rather than metabolic condition.
IGF-1	
A **positive association** between maternal energetic condition and milk IGF-1 concentration…	…may be predicted because IGF-1 is expected to directly affect infant digestive function and growth, and mothers in good condition are better able to afford a fast-growing infant.
	…may be predicted if mothers in good condition produce a higher volume of milk, because IGF-1 in the mammary increases milk yield.
	…may be predicted because IGF-1 is transferred from maternal circulation and some maternal energetic factors are known to associate with circulating IGF-1. IGF-1 is reduced during fasting (Emler and Schalch 1987; Clemmons et al. 1981) and positively associated with estradiol (Veldhuis and Iranmanesh 1996; Ho et al. 1987).
	…may be predicted if IGF-1 is somehow costly to produce (either in terms of the calories required for its production or because it exerts some negative fitness impact on the mother).
A **negative association** between maternal energetic condition and milk IGF-1 concentration…	…may be predicted if milk IGF-1 concentration communicates a signal to infants about the proportion of calories from milk they should invest in growth relative to other functions (particularly if mothers in poor condition are producing milk with less available energy). If infants are receiving fewer calories, they may need more biochemical encouragement to invest what they can in growth.
No association between maternal energetic condition and milk IGF-1 concentration…	…may be predicted if there is a U-shaped relationship between IGF-1 and BMI which is reflected in milk concentration. Because subjects range widely in BMI, there may be no significant linear relationship between IGF-1 and maternal condition as presently defined.

Table 2.2 (continued)

Immune factors (lactoferrin and sIgA)	
A **positive association** between maternal energetic condition and milk immunofactor concentration…	…may be predicted if production of these proteins is somehow costly to the mother (either in terms of the calories required for their production or because it exerts some negative fitness impact on the mother). If there were no cost, we would expect production of these compounds to be canalized to a uniformly high concentration. This hypothesis, that good maternal energetic condition promotes an active, symptom-prevention approach toward infant illness, is supported by findings that extreme energy restriction is associated with a reduction in milk immunofactors (Prentice et al. 1983a).
A **negative association** between maternal energetic condition and milk immunofactor concentration…	…may be predicted if mothers in poor energetic condition are more susceptible to illness (or, conversely, a long bout of illness has impaired maternal condition). In this case, mothers may experience upregulation of immune factors to fight infection. Because lactoferrin production is independently regulated in the mammary, this relationship may be absent or less strong with lactoferrin compared to sIgA.
A **positive association** between maternal parity and milk immunofactor concentration…	…may be predicted because mothers who interact with more children may be exposed to a greater number and variety of pathogens, and may protect themselves or their infants with higher production of immunofactors.
Lactoferrin	
A **negative association** between maternal energetic condition and milk lactoferrin concentration…	…may be predicted because lactoferrin is a form of nonspecific immunity, and may not represent the most effective or efficient approach to fighting infection. If it is cheaper in some way to produce than a variety of specific antibodies, we might see a negative association between maternal condition and concentration of lactoferrin.
	…may be predicted because transcription of mammary lactoferrin mRNA is stimulated by prolactin (PRL). Nutritional supplementation of malnourished lactating women is associated with a significant decrease in PRL (Lunn et al. 1984). Furthermore, obese women have been shown to have a reduced PRL response to suckling (Rasmussen et al. 2004), so the relationship between BMI and PRL may be truly linear across the full range of BMI values.

2. Milk composition and infant illness

In Chapter 5, I explore the relationship between concentration of the milk immune proteins, lactoferrin and sIgA, and symptoms of illness in infants. Hypotheses and predictions are presented in Table 2.3; the first two hypotheses presented below will be the main hypotheses I test between.

Table 2.3: Hypotheses and predictions relating milk immunofactors to symptoms of illness experienced by infants

A **positive association** between concentration of milk immunofactors and illness…	…may be predicted if lactoferrin and sIgA respond to illness, only being elevated when infection is present and they are necessary.
A **negative association** between concentration of milk immunofactors and illness…	…may be predicted if lactoferrin and sIgA prevent illness.
	…may indicate that the total *amount* of immunofactors consumed is more important for predicting illness than the *concentration* of those factors (depending on variability in milk volume and immunofactor concentration).

3. Milk composition and infant growth

Chapter 6 will focus on the relationships between the four milk compounds and infant growth rates (linear growth, growth in mass, head circumference, and arm circumference). Hypotheses are presented in Table 2.4 and in the discussion that follows.

Table 2.4: Hypotheses and predictions relating milk bioactives to infant growth rate

All compounds	
A **negative association** between concentration of milk immunofactors and infant growth rate…	…may be predicted if milk volume is the primary factor driving growth, and these compounds are diluted by high milk volume. *[However, IGF-1 may positively correlate with milk volume as it does in dairy cows (Prosser et al. 1989), and milk cortisol does not appear to be subject to the effect of dilution, at least in dairy cows (Fox et al. 1981)].*
Cortisol	
A **positive association** between concentration of cortisol in milk and infant growth rate…	…is predicted based on findings in rhesus macaque (Hinde et al. 2015) and red squirrel (Dantzer et al. 2013) infants.
IGF-1	
A **positive association** between concentration of IGF-1 in milk and infant growth rate…	…may be predicted because of IGF-1's role in promoting infant intestinal development and improved nutrient absorption (Burrin et al. 1996; Baumrucker et al. 1994; Baumrucker and Blum 1993; Hirai et al. 2002; Corpeleijn et al. 2008).
A **negative association** between concentration of IGF-1 in milk and infant growth rate…	…may be predicted because breastfed children, who tend to grow more slowly than their formula-fed peers, consume more oral IGF-1 but have lower circulating IGF-1 (Larnkjaer et al. 2012).
Immune factors (lactoferrin and sIgA)	
A **positive association** between concentration of immunofactors in milk and infant growth rate…	…may be predicted if these compounds are negatively associated with symptoms of illness, because avoiding illness might allow more calories to be allocated to growth.
A **negative association** between concentration of immunofactors in milk and infant growth rate…	…may be predicted if these compounds are positively associated with symptoms of illness (indicating a responsive pattern of immunofactors and illness), implying that infant growth was impaired by illness.
No association between concentration of immunofactors in milk and infant growth rate…	…may indicate that immunofactors are able to "rescue" growth in the event of an illness.
	…may indicate that the energetic savings of avoiding illness are not substantial enough to contribute to growth, or that the calories saved are spent on functions other than growth.

It is also possible that infants are sensitive to *changes* in the amounts or concentrations of bioactive compounds they ingest. This may be a way that mothers are able to signal changing environmental conditions to the infants to influence growth rate.

Milk cortisol has been shown to impact infant behavior in animal studies (Hinde et al. 2015; Sullivan et al. 2011; Angelucci et al. 1985; Catalani et al. 2000; Catalani et al. 2002). It is expected that variation in milk cortisol will also influence the behavior of human infants, with possible effects on growth. This prediction is complicated because behavior data is not collected in the present study, and because is very difficult to consider the effect of hormones on behavior without information about milk energy as well (Hinde et al. 2015). Without consideration of available milk energy, higher milk cortisol predicted a confident temperament in infant male rhesus macaques (Sullivan et al. 2011); controlling for available milk energy revealed that this relationship was actually reversed (Hinde et al. 2015). Because available milk energy is not estimable in the present study, we might expect a finding similar to the earlier, cortisol-only relationship. If cortisol (confounded with available milk energy) promotes increased exploratory behavior in human infants, this might be observed through its effects on growth rate; high cortisol, and high-cost behavior may be reflected in a reduced rate of growth. On the other hand, perhaps mothers would only signal infants to exhibit a more costly behavioral phenotype if growth and maintenance costs were already met. Adequate growth may be signaled by another mechanism, and cortisol (and resulting high-cost behavioral activity) would positively associate with rate of growth. These

predictions presume that cortisol would have the same general effects on human infant behavior as it has on rats and rhesus monkeys, which is not known.

Predicting relationships between milk IGF-1 and infant growth is similarly complicated because oral ingestion of milk IGF-1 may influence endogenous IGF-1 production (Martin et al. 2012). The mechanism by which this process occurs in humans is unknown, but may include the upregulation of receptor expression, changes in binding protein concentration, ratio, or function, and/or programming of pituitary sensitivity to feedback. Given the paucity of information available, a variety of predictions could be made. Since breastfed children tend to grow more slowly than their formula fed peers and have lower circulating IGF-1 (Larnkjaer et al. 2012), it may be that oral IGF-1 is negatively associated with growth. However, since breastfed infants grow up to have higher circulating IGF-1 than their peers, the process of pituitary sensitization to IGF-1 may begin in infancy such that oral IGF-1 is positively associated with growth.

Finally, it is important to remember that hormone levels are only part of what determines hormonal action. For a complete understanding of the pathways discussed, it would also be necessary to measure receptor density on the infant tissues of interest. In addition, almost all of the infants in the present study received supplemental dairy foods. It is unknown how this may have affected their growth or endogenous hormone production, particularly with regard to IGF-1. Finally, the ages of these infants span a very large range. There may be a critical window for any of these compounds to affect growth that is missed by this diverse sample.

Chapter 3: Study Methodology

This chapter explains the methodology common to all results in the subsequent chapters, including the study population, field methods, laboratory methods, and statistical methods. Any methodology pertaining to only one set of questions is presented in the relevant chapter.

Study population and demographics

This study was conducted among the indigenous Toba people of Namqom, Formosa Province in northeastern Argentina. Namqom is a village of approximately 3500 Toba located 11 km. northwest of the city of Formosa. The Toba, who have historically been hunter-gatherers, are experiencing dramatic changes in their lifestyle (Braunstein and Miller 1999). During the last century, disruptions to their traditional livelihood and ecological deterioration of the habitat have forced massive migrations to urban centers. While rural communities still use the forest as a source of food and shelter, families like the ones in Namqom live in poor peri-urban *barrios*. This population has free access to health services, mainly provided by the local health center and the city's hospitals. The provincial government offers pre- and postnatal care programs.

We conducted this study in an environment with greater exposure to a variety of pathogens than generally found in the US or other regions of the developed world,

reasoning that greater exposure to pathogens would highlight the relationships being tested. None of the homes in the community have indoor toilets, and there is standing water in ditches close to most homes containing sewage runoff. About half of homes have dirt floors, and infants are routinely placed on uncovered soil, both inside and outside the home. Drinking water is available through shared taps. Most homes do not have refrigerators, and much of the cooking is done over outdoor fires. These environmental conditions are known to increase risk of exposure to pathogens, and appreciably elevate the exposure risk above the typical environmental conditions found in the West.

Toba women typically breastfeed their children on demand for 2 or 3 years or until the next pregnancy is noticeable (Valeggia and Ellison 2004). Co-sleeping also allows for on-demand nighttime nursing. Semisolid and solid supplements are usually introduced around 6 months of age (Olmedo and Valeggia 2014). Exclusive bottle-feeding is uncommon, though supplementing breastfeeding with cow's milk is fairly common. For a more detailed report of the demographic profile of this population see (Valeggia and Ellison 2004). This population has been studied extensively since 1997 as part of the Chaco Area Reproductive Ecology (C.A.R.E.) Program under the direction of Dr. Claudia Valeggia.

Study dyads

Thirty breastfeeding infants and their mothers were recruited for the present study. Study protocol was approved by the University of Pennsylvania Institutional Review Board

(#811200), and subjects provided verbal consent at enrollment and each study visit. All infants recruited into the study had been born in the local hospital. Each mother-infant pair was visited approximately once a month for 4-5 months, until they weaned their infant, or indicated they wished to cease participation (Of 30 participating dyads: 4 women participated in 5 visits; 18 women had 4 visits; 4 women had 3 visits; 2 women had 2 visits; and 2 women had 1 visit). Data collection took place over a six-month period in 2012 and 2013.

Descriptive statistics of maternal characteristics at the time of recruitment are displayed in Table 3.1. Nine women (30%) were primiparous and 21 (70%) were multiparous.

Table 3.1: Maternal characteristics at intake (n=30)

	Range	Median	Mean	Std. Dev.
Age at recruitment (years)	15-37	23.50	24.43	6.45
Starting BMI	18.2 - 40.8	26.2	27.6	4.96
Parity	1-10	2	3.20	2.46
Days enrolled	1-155	114.50	105.23	37.21

Infant characteristics at intake are summarized in Table 3.2. Of the recruited infants, 18 (60%) were female and 12 (40%) were male.

Table 3.2: Infant characteristics at intake (n=30)

	Range	Median	Mean	Std. Dev.
Age at recruitment (days)	35-448	240	244	125.7
Starting length (cm.)	55-77	68.75	67.82	5.87
Starting mass (g.)	4241-11800	8766	8558	1813

Collection of biological samples

Milk collection

At each study visit, a hand-expressed mid-feed milk sample was collected using a test-weigh protocol (Neville et al. 1984). Mid-feed sampling produces a milk sample representative of a full mammary evacuation and only results in a minimal loss of milk to the infant, making it a more ethical method in a nutritionally-stressed population (Miller et al. 2013). Hand expression is preferable to using a breast pump due to logistical constraints in the field (such as keeping pump parts sterile), and was more culturally appropriate for our population, who do not typically use pumps. When the interviewer arrived, the mother was asked to nurse the infant from the breast opposite her dominant hand. Then nursing was restricted from that breast for the subsequent two hours (the infant was free to feed from the other breast during those two hours). At the end of the two-hour milk collection period, the infant nursed for two minutes from the study breast, and then a sample of 10 mL of breast milk was expressed into a collection tube and immediately mixed by gentle inversion and aliquotted into 1 mL screw-top storage vials. Samples were transported to the field station on ice and frozen within 2 hours of collection. Samples were stored frozen until they shipped to the US on dry ice, where they were stored at -20°C.

Urine collection

A urine sample was collected from mothers and infants at each study visit. Mothers were provided with a sterile wipe and asked to collect a clean-catch specimen into a plastic cup. They were given a disposable pipette and a 1 mL screw-top vial, and transferred 1

mL of urine to the vial. Samples were collected in the morning, but they were not first morning voids. Infant urine samples were collected following the procedure parents preferred in (Liaw et al. 2000), and similar to the method outlined in (Panter-Brick et al. 2009). A sterile urine collection pad (*Uricol, Ontex* Healthcare) was affixed to a clean diaper. The pad was checked every 10 minutes until the infant urinated; then, urine was collected using a disposable pipette and transferred to a 1 mL screw-top vial. Urine samples were transported to the field station on ice and frozen within 3 hours of collection. Samples were stored frozen until they shipped to the US on dry ice, where they were stored at -20°C.

Anthropometric measurements

Maternal height (cm.) was measured at the first study visit using a portable SECA 214 stadiometer. Maternal mass (kg.) and percent body fat was measured at each visit using a portable Omron HBF-510 body composition monitor scale. This scale automatically calculated body mass index (kg/m^2) at each visit.

Once a month, the following measures of infant growth were conducted following the standardized procedures of the collaborative Bones and Behavior project (www.bonesandbehavior.org):

- body mass (g.) was measured with a Medela Baby Weigh II Pediatric Portable Scale
- length (cm.) was measured with a portable SECA 210 infantometer
- head circumference and arm circumference (cm.) were measured with a sliding pediatric tape

Interview

During the two-hour milk collection period, an interview was conducted. The infant's mother was asked to describe the infant's diet (if no longer exclusively breastfeeding). Infant health at the time of the interview and over the month prior to the interview was assessed, with questions targeted at symptoms of gastrointestinal illness (diarrhea and vomiting) and respiratory illness (cough, cold, mucus). The interview also included questions about maternal health, maternal diet, parity, and household demographics.

Laboratory methods

All laboratory analyses were conducted in the Harvard University Reproductive Ecology Laboratory by the author. Histograms are provided for each compound to show the distribution of untransformed and log-transformed values. All untransformed distributions show a strong right skew, so log-transformed values were used in statistical analyses.

Adjustment for urinary concentration

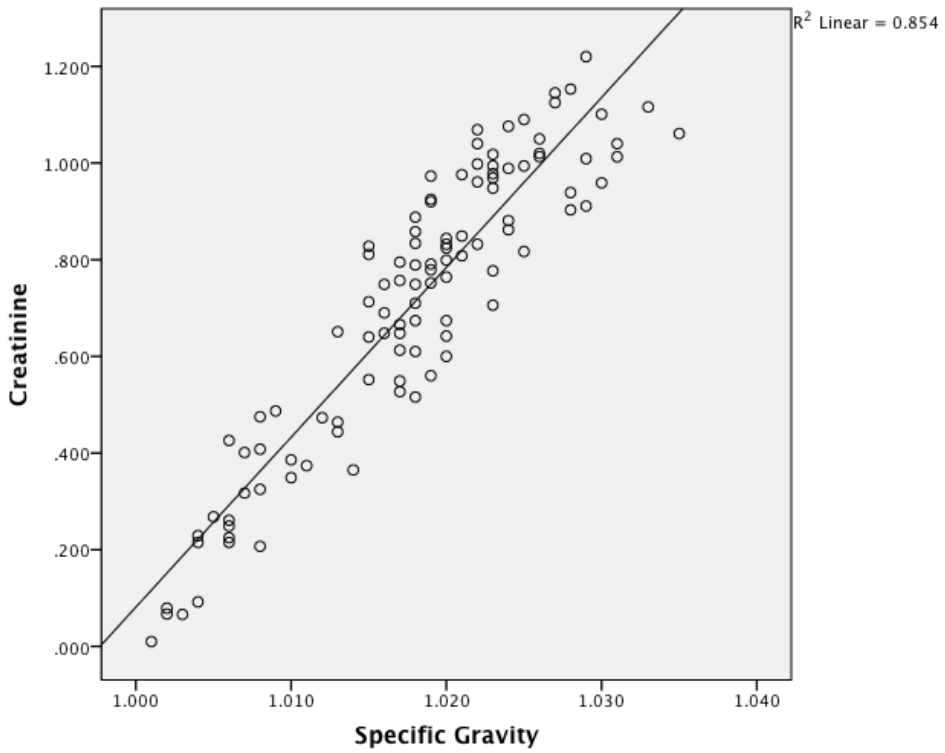
Urine concentration was assessed using two methods: creatinine and specific gravity. Urinary creatinine was measured using the Jaffe method (Taussky 1954). Samples were diluted 1:10 with ultrapure water and run in quadruplicate. The median of the 4 values is reported.

Specific gravity was measured using a hand-held refractometer (Atago). Specific gravity is the ratio of the optical density of a sample compared to that of water (1.000). Specific

gravity of infant urine samples ranged from 1.001-1.036 and specific gravity of maternal urine samples ranged from 1.001-1.035.

Specific gravity and creatinine were very highly correlated (R^2=0.854). Urinary cortisol and C-peptide values presented in this study were adjusted by specific gravity. Specific gravity is unitless, so the formula to correct a urine sample for specific gravity is [sample concentration x (SG_{mean} -1.000/SG_{sample} -1.000)], where SG_{mean} is the mean of all specific gravity values in study samples, and SG_{sample} is the specific gravity of the sample (Miller et al. 2004).

Figure 3.1: Relationship between creatinine and specific gravity of maternal urine samples



Urinary cortisol

Free urinary cortisol was analyzed using in-house enzyme immunoassay (EIA). NUNC Maxi-sorp (Thermo Scientific) plates were coated with 50 uL anti-cortisol antibody (Coralie Munro, UC Davis) diluted to 1:20,000 and allowed to absorb at least overnight. Urine samples from mothers and infants were diluted 1:100 with assay buffer and run in duplicate. If samples ran out of range at 1:100, dilutions were adjusted to 1:50 or 1:200 and rerun. Cortisol enzyme conjugate (Coralie Munro, UC Davis) was added to the plate at a concentration of 1:200,000 and competitive binding occurred during a 2-hour incubation. TMB (BioFX) was used as a substrate. Plate development with substrate occurred during a 25-minute development period, then stop solution (BioFX) was added and the plate was read at 450 nm. All samples from a mother-infant pair were run in the same plate to minimize the effect of interassay variation. Intraassay variability was 6.1%. Using the midpoint of the standard curve as a control, interassay variablility was calculated at 5.2%.

Figure 3.2: Distribution of untransformed urinary cortisol concentration values

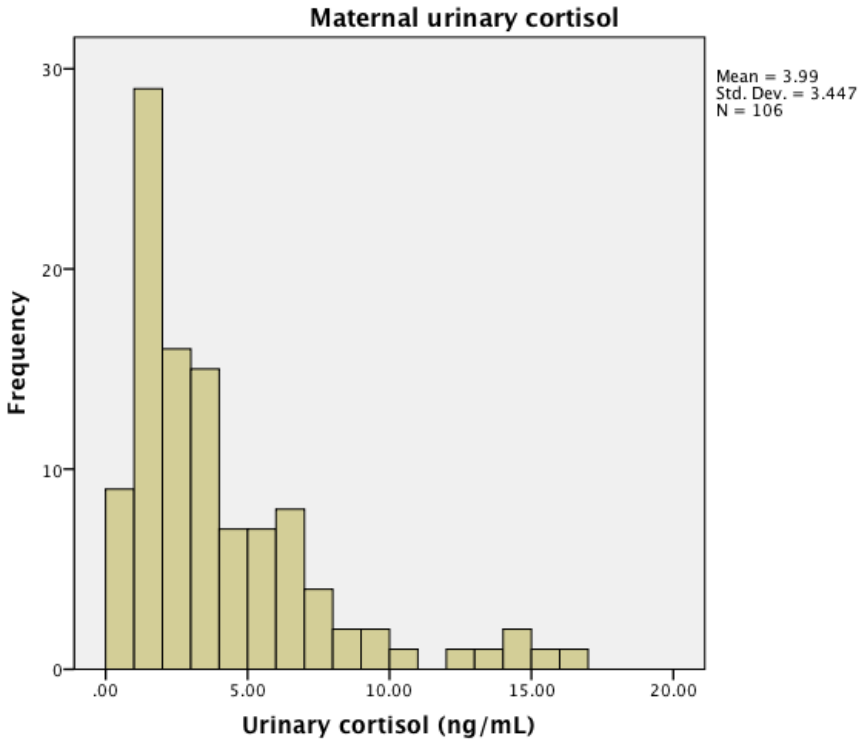
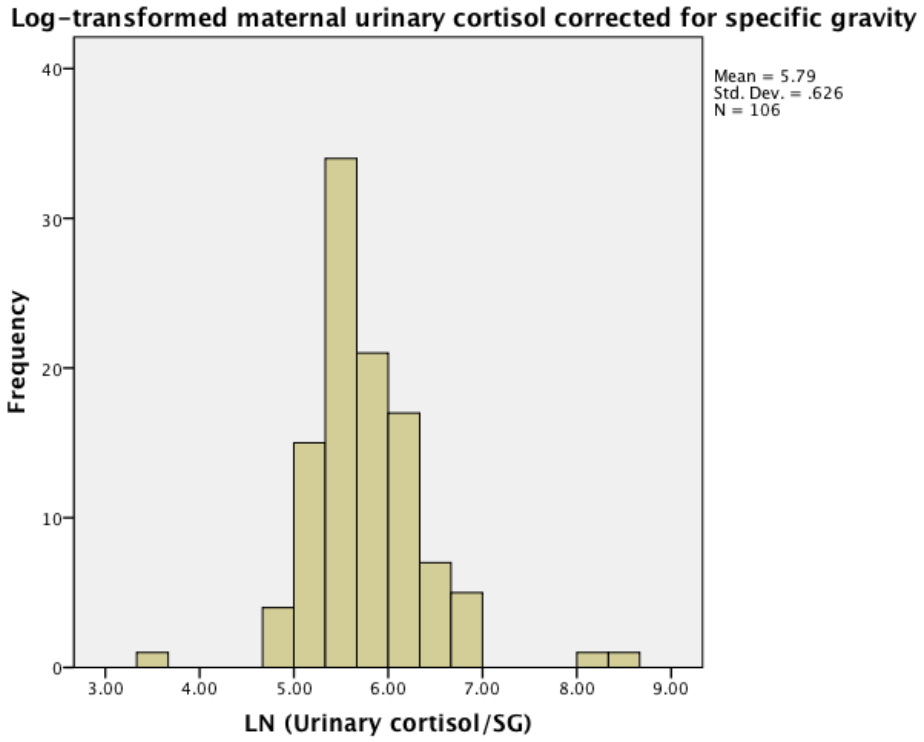


Figure 3.3: Distribution of log-transformed urinary cortisol concentration values



Urinary C-peptide of insulin

Urinary c-peptide of insulin was analyzed using a radioimmunoassay (RIA) kit from Millipore (catalog number HCP-20K). Urine samples were diluted 1:20 with assay buffer and then assay procedure followed kit instructions. Intraassay variability was 4.0% and interassay variability was 5.9%.

Figure 3.4: Distribution of untransformed urinary C-peptide concentration values

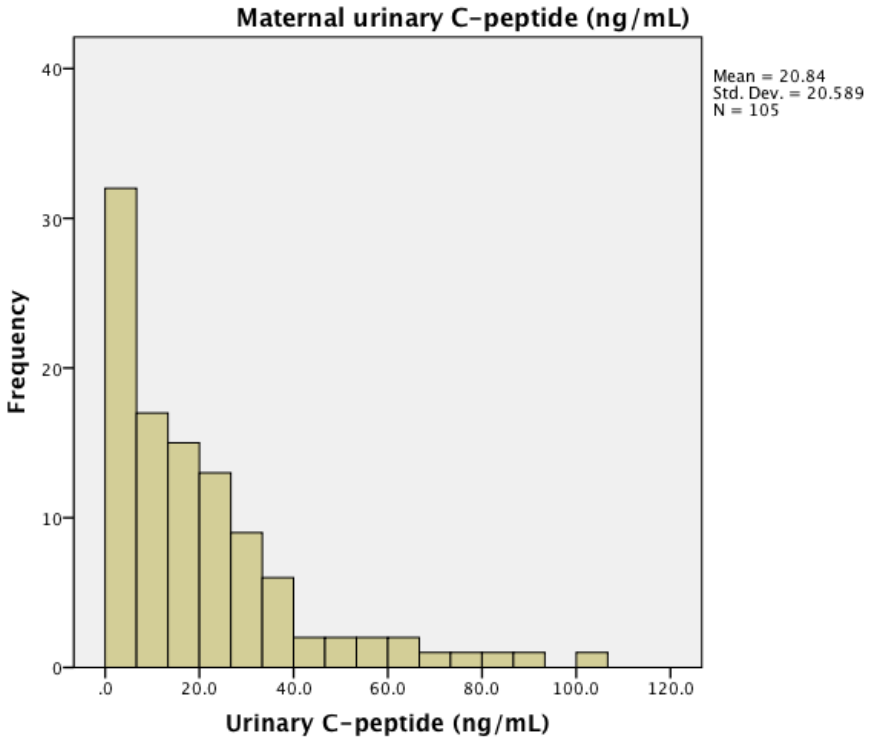
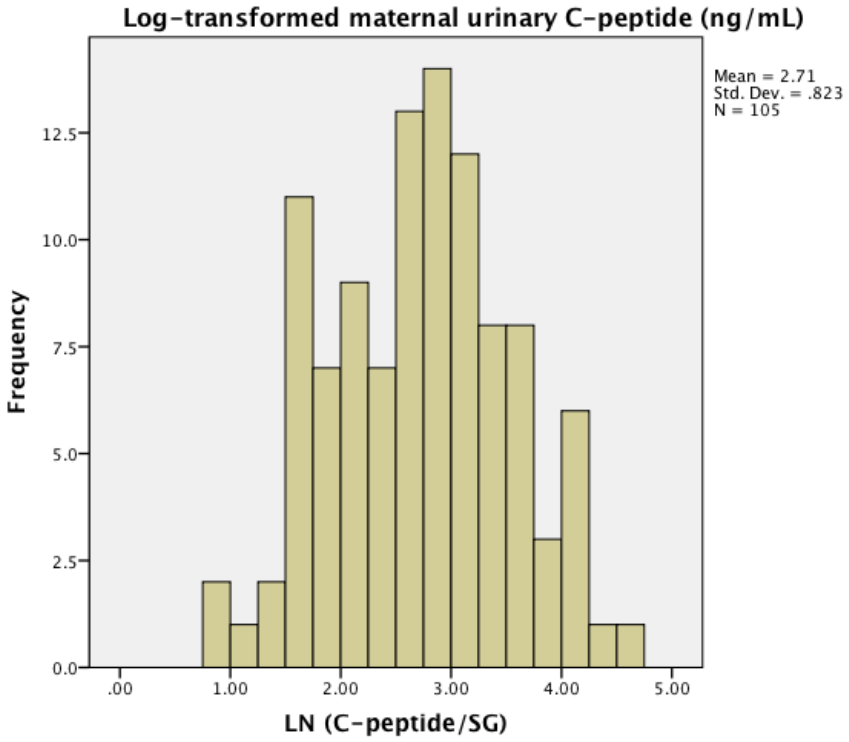


Figure 3.5: Distribution of log-transformed urinary C-peptide concentration values



Defatting of milk samples

Some milk assays were performed using defatted milk. Whole milk was inverted several times and briefly vortexed to mix the sample. A portion was aliquotted into a new screw-top vial and centrifuged at 3000 RPM for 30 minutes. The aqueous portion of the sample was pipetted from under the fat layer and centrifuged again.

Milk cortisol

Cortisol was measured using a radioimmunoassay kit from Siemens (#TKCO5). Whole milk samples were diluted 1:2 with deionized water; all samples ran in range at this dilution. Samples were run in 2 batches; all samples from an individual woman were included in the same batch. The kit manufacturers report an interassay variability of 4.0-

6.4%. No controls were provided for use with this kit, but standard curve values were consistent with values published on the kit insert. Intraassay variability was 17.1%. The high coefficients of variation in this assay result from the very small absolute values obtained; the average concentration was .13 ug/dL, so small absolute differences between replicates led to very high CVs.

Figure 3.6: Distribution of untransformed milk cortisol concentration values

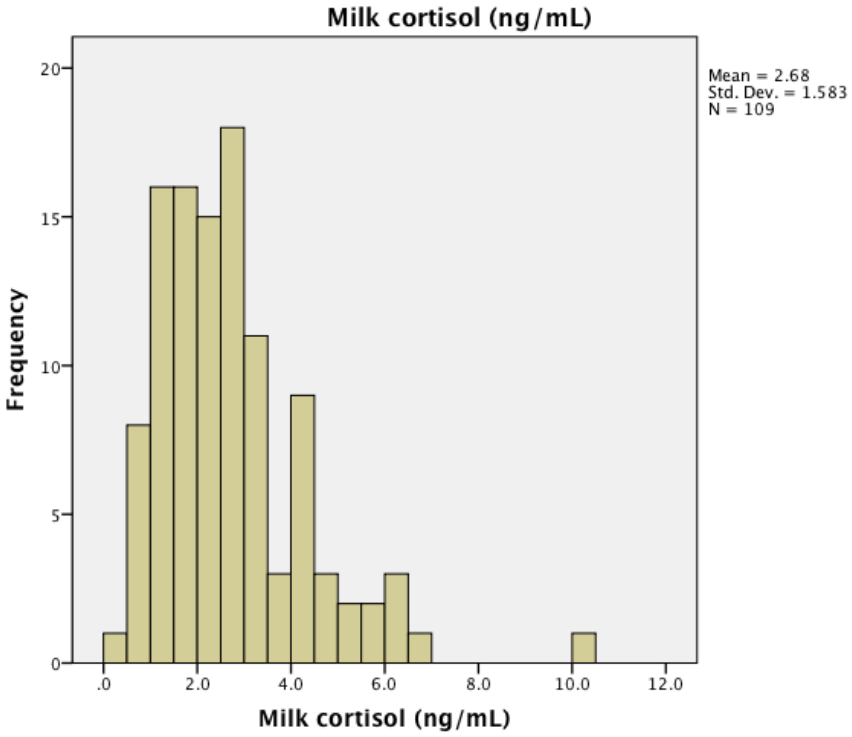
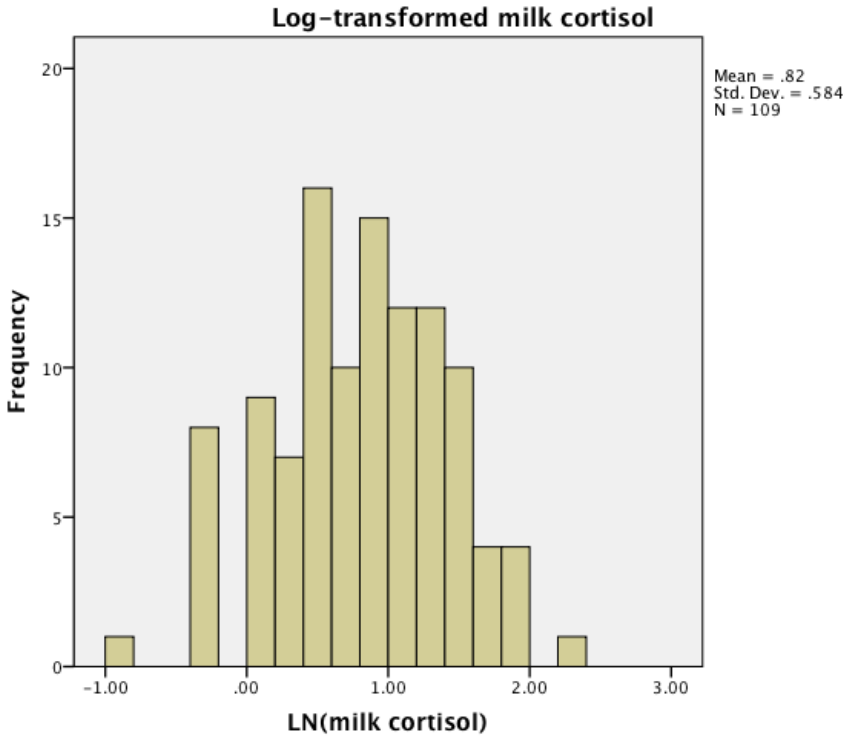


Figure 3.7: Distribution of log-transformed cortisol values



Milk IGF-1

Milk IGF-1 was measured using a radioimmunoassay kit from Mediagnost (#IGF-R21). Milk samples were diluted 1:3 in assay buffer. Samples that ran out of range (and all other samples from the same individual) were rerun at 1:2. An acidic substrate was added to the buffer to encourage separation of IGF-1 from binding proteins (Ratio for 1:3 dilution was 100 μL sample, 170 μL assay buffer, 30 μL acid buffer). A relatively large amount of IGF-2 is added after incubation to occupy the binding sites and prevent the binding proteins from re-binding the IGF-1. After dilution, samples were run according to the kit instructions. Intrassay variability was 8.3% and interassay variability was 4.1%.

Figure 3.8: Distribution of untransformed milk IGF-1 concentration values

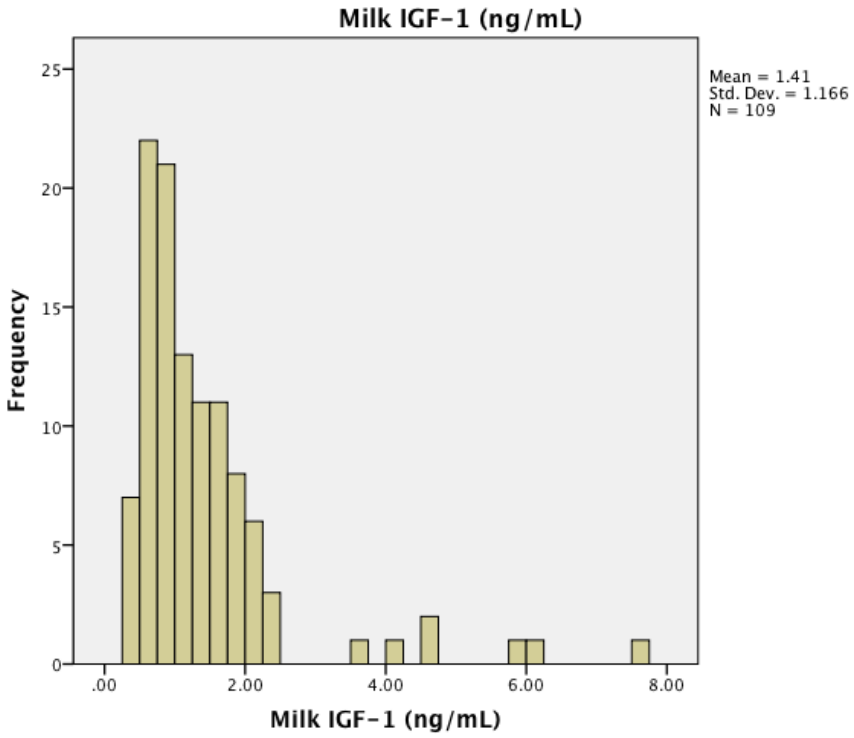
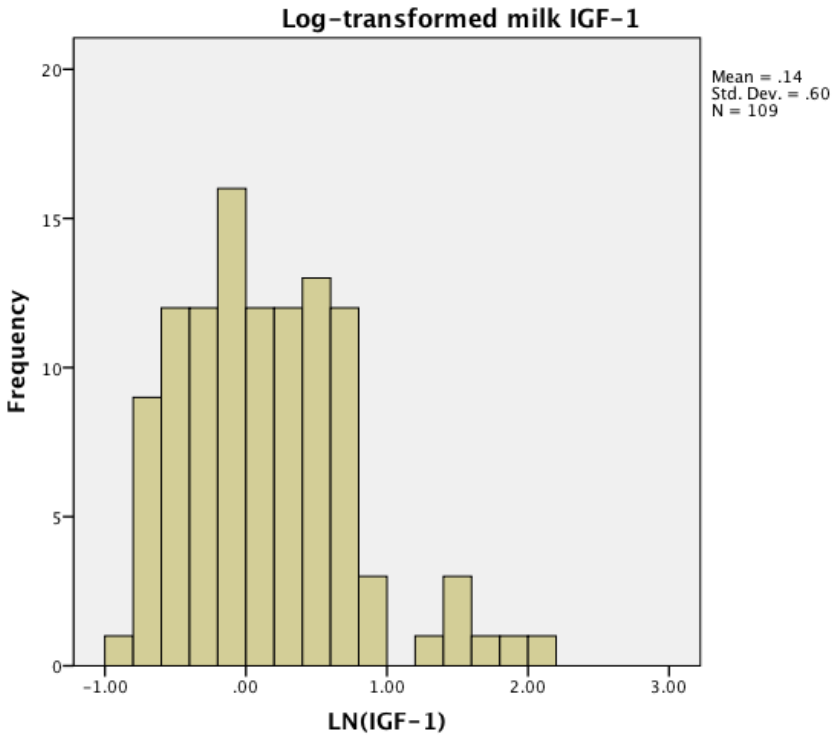


Figure 3.9: Distribution of log-transformed IGF-1 values



Milk lactoferrin

Lactoferrin in milk was assayed using an enzyme immunoassay kit from ALPCO (#41-LACHU-E01). Following kit instructions, whole milk samples were diluted 1:50,000 with diluent. Samples that ran out of range were rerun at 1:100,000. All samples from an individual woman were run in the same plate to minimize the effect of interassay variation. Intraassay variability was 2.2%. The kit manufacturers report an interassay variability of <10%. No controls were provided for use with this kit, but standard curve values were consistent with values published on the kit insert. One sample with extreme outlying values in both lactoferrin and sIgA was excluded from analyses for suspected contamination.

Figure 3.10: Distribution of untransformed milk lactoferrin concentration values

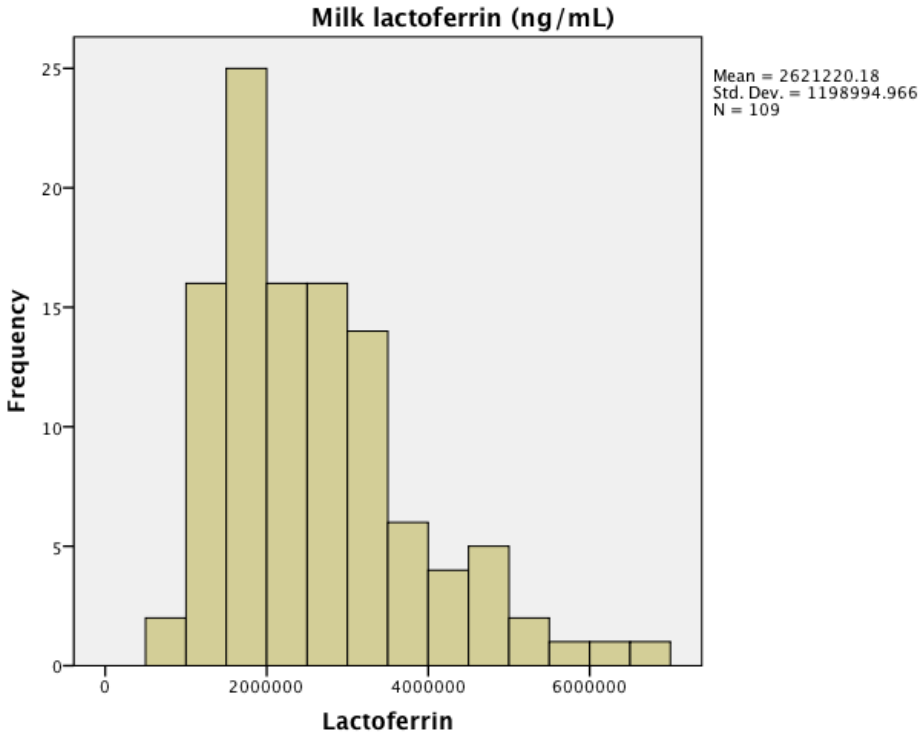
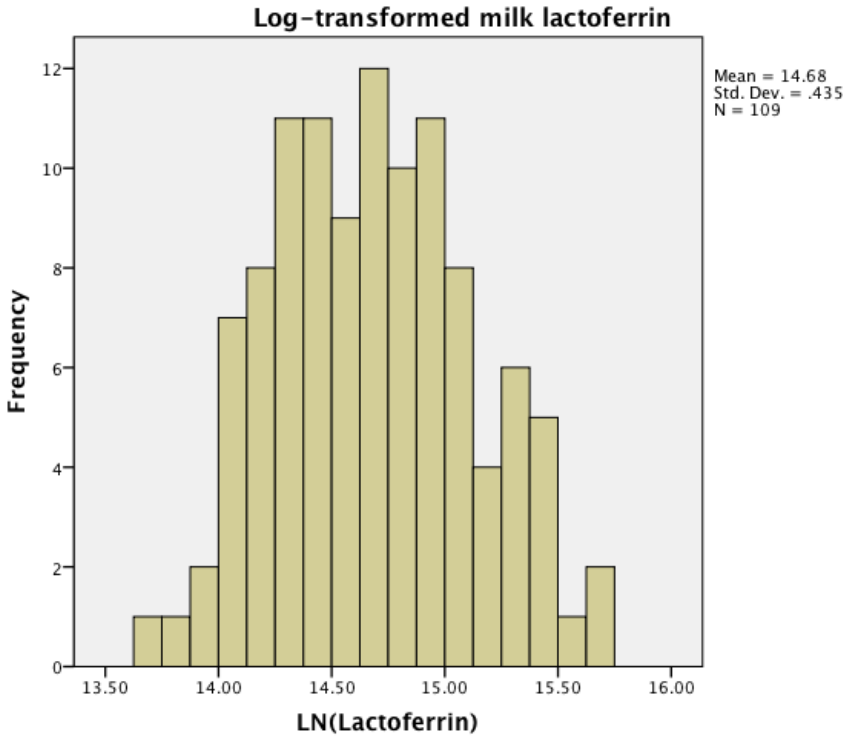


Figure 3.11: Distribution of log-transformed lactoferrin values



Milk sIgA

Secretory IgA in milk was assayed using an enzyme immunoassay kit from Salimetrics (#1-1602) intended for use with saliva. The kit protocol was followed exactly, with the exception that milk samples were diluted 1:5 with diluent. No samples ran out of range. All samples from an individual woman were run in the same plate to minimize the effect of interassay variation. Interassay variability was 11.7% and intraassay variability was 4.8%.

Figure 3.12: Distribution of untransformed milk sIgA concentration values

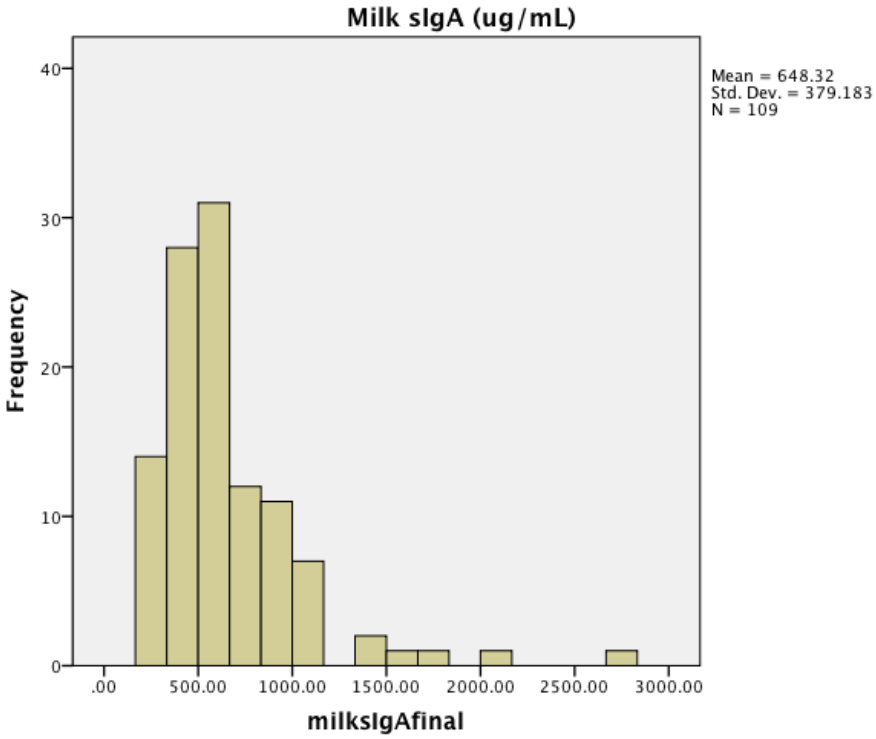
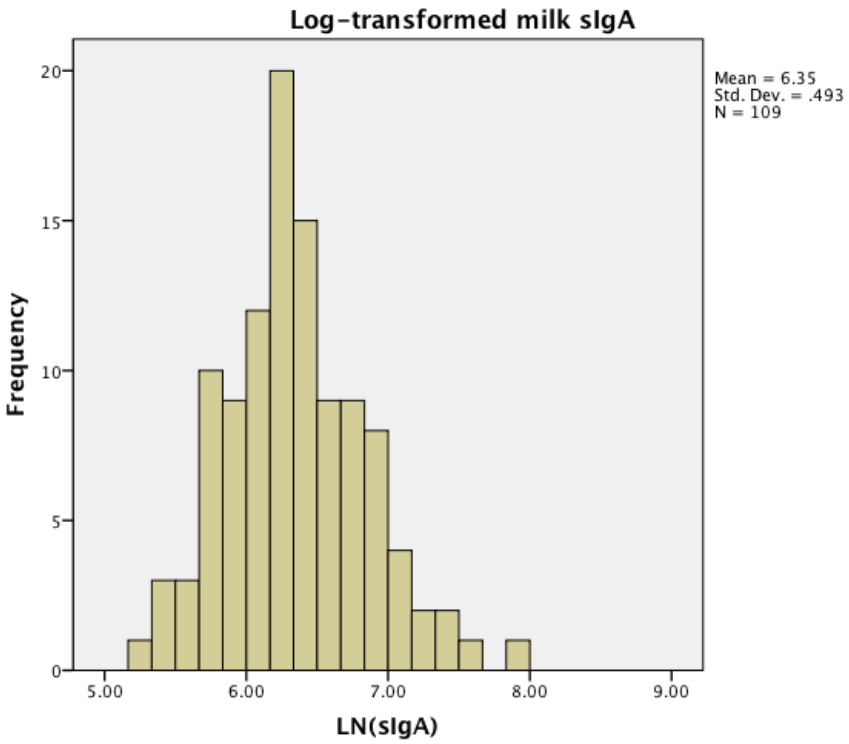


Figure 3.13: Distribution of log-transformed sIgA values



Milk volume

Because milk volume is such an important contributor to infant growth, I tried to estimate the amount of milk consumed during a study visit as a proxy for 24-hour intake. At the study visit, infants were weighed before and after each feed and the change in mass between the weighs was taken as milk consumption in grams (the weigh-test-weigh protocol). All feeds were added for total milk consumed. However, in 110 study visits, only 33 of these values were highly reliable. Infants did not always cooperate with the weighing procedure, particularly if they fell asleep in the middle of a feed. The on-demand nursing pattern of Toba infants meant that mothers or infants initiated several feeds during the two-hour study visit, and the probability of a missing weight at the start or end of at least one feed was fairly high.

To try to rescue these data, I attempted to calculate an average rate of insensible water loss (evaporative water loss through respiration and perspiration) for Toba infants so I could create a mathematical formula to estimate milk consumption based on change in mass over the entire study visit. That formula is:

Estimated milk volume = End mass – Beginning mass – Mass of supplemental food consumed + Voided (diaper) mass + clothing change + water loss

Study visits were identified where infants had a period of at least 30 minutes where no nursing occurred, with a reliable weight from the beginning and end of the time period (these weights were usually the end of one feed and the beginning of another). I calculated the loss in mass (g.) and how many minutes had passed, so I was able to calculate an individual-specific rate of water loss in g/kg/hr for that time period. There were 45 visits from 22 individuals that met these criteria. The average rate of insensible water loss from these 45 visits was 5.43 g/kg/hr. Because this included multiple values from the same infants, I calculated an average rate of water loss from each infant, and then averaged those values, resulting in a very similar rate of 5.88 g/kg/hr.

I was then able to use the population-level estimate of 5.43 g/kg/hr in the formula to estimate milk volume. However, when I took the absolute value of the difference between the high-confidence test weigh values and the estimates obtained using the formula with population average water loss, mean difference between the values was 57

grams, with a standard deviation of 54 grams. This unfortunately does not improve the accuracy with which milk volume can be estimated, so this approach was abandoned.

Statistical methods

Statistical analyses were conducted using SPSS 21. For most analyses, generalized estimating equations (GEE) with an exchangeable correlation structure were used. GEEs were chosen because they correct for the non-independence associated with repeat measures and allow for a binary outcome variable. More detail about the specific variables and model-building strategies used to address each set of questions can be found in the relevant chapters.

Chapter 4: Maternal energetic condition and milk composition

Introduction

Background and hypotheses

This chapter focuses on the relationship between maternal energetic condition and milk composition. These relationships were explored with the overarching hypothesis that mothers in better energetic condition (briefly: higher BMI, positive energy balance, higher insulin, lower parity; defined in greater detail later in this chapter) would produce milk with greater concentrations of IGF-1, lactoferrin and sIgA, all factors predicted to associate positively with infant growth rate and lower concentrations of cortisol, which is predicted to reflect maternal energy mobilization. This fits the simple framework presented in Chapter 2 that predicts that moms in better condition will make milk that results in healthier, faster-growing infants.

Most of the existing research about maternal condition and milk composition focuses on nutritional quality of the milk. Most studies investigating the effect of maternal weight loss on milk energy density, in well-nourished and marginally nourished populations, have found no effect (Butte et al. 1984; Dewey et al. 1994; McCrory et al. 1999; Prentice et al. 1981a; Whitehead et al. 1978). Milk yield volume is more sensitive to severe

energy restriction in primates (Roberts et al. 1985), but not moderate energy restriction (Butte et al. 1984; Dewey et al. 1994; McCrory et al. 1999).

The associations between maternal energetic condition and concentration of the bioactive compounds of interest (cortisol, IGF-1, lactoferrin, and sIgA) are less well studied. There is some evidence that milk sIgA concentration decreases in response to nutritional stress. Both inadequate diet and high levels of exercise reduce the amount of sIgA secreted into breast milk (diet: Weaver et al. 1998; exercise: Gregory et al. 1997). In the Gambian rainy season, when energy expenditure is high and intake is low, concentrations of all tested immune factors in milk were found to decrease, despite the fact that infections were more common among mothers and infants during this time (Prentice et al. 1983a). This decrease in concentration was accompanied by a decrease in milk volume, meaning that the total amount of immune factors transferred to infants was drastically reduced. A mother's parity may be another important contributor to her condition. The same study of Gambian mothers and infants found that lower parity was associated with higher concentration of all protective factors, including sIgA and lactoferrin, in milk. This, in combination with the finding that milk volume did not differ by parity until the ninth offspring, suggests that maternal depletion with advancing parity may influence milk composition (Prentice et al. 1983a). Based on these findings, better maternal condition is predicted to associate positively with concentrations of sIgA, and lactoferrin.

IGF-1 is also predicted to show a positive association with maternal condition. Previous studies in rats (Emler and Schalch 1987) and humans (Clemmons et al. 1981) found that

circulating IGF-1 is reduced during in the fasting state. Estradiol also positively associates with growth hormone secretion (Veldhuis and Iranmanesh 1996; Ho et al. 1987), suggesting the possibility for a positive relationship between BMI and growth hormone (and therefore IGF-1) for women in the underweight and lower range of normal BMI.

Cortisol passively diffuses into milk, reflecting circulating concentration (Fox et al. 1981). As a result, milk cortisol may simply reflect maternal condition. Because cortisol is required for energy mobilization, nutritional stress is predicted to result in elevated circulating cortisol, and consequently, elevated milk cortisol. Therefore, cortisol is predicted to follow a different pattern from the other compounds. Mothers in better energetic condition are predicted to have *lower* concentrations of milk cortisol.

Maternal condition variables

Body mass index (BMI) was chosen to represent current maternal energetic state. Percent body fat and BMI correlated very strongly (R^2=.919), but BMI was selected because it was presumed that the values obtained for BMI were likely to be more accurate than those for percent body fat, and that body fat distribution (visceral vs. gluteofemoral, for instance) may have influenced the reading given by the electroimpedance scale or may have important biological consequences. Generally speaking, an individual with a higher BMI has more stored energy available to draw upon, and thus higher BMI was chosen to indicate "better" maternal condition. However, none of the women in the present study were underweight, and many would be

considered overweight or obese by standard BMI cutoffs. The relationships presented here do not represent all of the important variation in BMI. BMI was predicted to associate positively with concentrations of lactoferrin, IGF-1, and sIgA in milk, and negatively with cortisol.

In addition to the previous analyses, which use BMI at the time of the study visit to investigate current maternal energetic *status*, monthly change in BMI was included as a variable of interest because it represents maternal energy *balance*. Energy balance is more important than energy status in predicting ovarian function (Ellison 2003) so it was predicted that energy balance would have the capacity to influence the hormonal composition of milk as well. Positive energy balance (a positive change in BMI) was predicted to associate with higher concentrations of lactoferrin, IGF-1, and sIgA in milk, and negatively with cortisol.

The interaction between BMI and monthly change in BMI on milk composition was also investigated. A significant interaction would indicate that women who are heavier for their height respond to changes in weight differently than thinner women. It was predicted that there would be a negative interaction between BMI and ΔBMI on milk proteins: women with a lower starting BMI would be more sensitive to weight fluctuations and experience a larger increase in the concentration of lactoferrin, IGF-1, and sIgA in milk with weight gain than women with a higher starting BMI.

Insulin dynamics are another way to probe maternal energetic condition. After the birth of a child, maternal insulin levels are relatively low, and remain low as long as the energetic burden of lactation continues to outpace maternal caloric intake and resumption of fat storage (Valeggia and Ellison 2004). C-peptide of insulin is a molecule cleaved from the proinsulin molecule during the production of insulin. It is biologically inert and produced in a 1:1 ratio with insulin (Rubenstein et al. 1969), making it an ideal biomarker to investigate the activity of insulin, a hormone primarily responsible for energy storage and therefore associated with good somatic condition (Sherry and Ellison 2007). However, static C-peptide could be highly variable between mothers due to differences in insulin sensitivity and stage of lactation. Therefore, urinary C-peptide concentration was transformed in two ways that have more biological significance. The first is the magnitude of monthly change in C-peptide (expressed as a percentage of initial value). Women whose C-peptide increased can be thought of as increasing investment in energy storage that month, and women whose C-peptide decreased were reducing their investment in energy storage. The other transformation of C-peptide concentration should correct for differences in baseline insulin sensitivity between women by comparing the C-peptide value at the study visit of interest and the woman's own average concentration over all study visits. If a mother has higher C-peptide than she usually does, it indicates relatively high investment in energy storage and therefore better energetic condition. Higher values of C-peptide for both of these transformations were predicted to positively associate with concentrations of lactoferrin, IGF-1, and sIgA in milk, and negatively with cortisol.

The interaction between BMI and insulin was tested to see whether women of different body mass indices have different relationships between insulin (C-peptide) and the milk compounds of interest. For this interaction, the static log-transformed urinary C-peptide concentration from the study visit of interest was used. It was hypothesized that high concentrations of lactoferrin, sIgA, and IGF-1 would be associated with a negative interaction between these two variables. Low-BMI women (who have fewer bodily resources to draw upon) were predicted to respond strongly to a signal of increased energy availability (high insulin production) by producing "better" milk with more of these proteins. High-BMI women are predicted to respond less strongly to high insulin levels (because stored energy may act as a buffer) and increase production or transport of these bioactives to a lesser degree.

Parity was the final maternal condition variable of interest. Lactation is very energetically costly and is associated with depletion of maternal fat stores (Prentice 1980; Miller et al. 1994). Increasing parity is associated with long-term depletion of maternal energy reserves in some energy-limited populations (Shell-Duncan and Yung 2004; Tracer 1991; Little et al. 1992). In adequately-nourished populations, BMI tends to increase with increasing parity. Importantly, though, fat is redistributed away from the gluteofemoral region (where it is most accessible to support infant growth during late pregnancy and lactation) and increases in central fat depots—a phenomenon termed "covert maternal depletion" (Lassek and Gaulin 2006). If a woman has sustained the great energetic burden of gestating and lactating for many infants, she may have different resources available to invest in her current offspring, and may adjust milk composition

accordingly. This is supported by the finding that low-parity Gambian mothers produced milk with higher concentration of sIgA and lactoferrin (Prentice et al. 1983a). However, according to the terminal investment hypothesis, investment in individual offspring is predicted to increase toward the end of a woman's reproductive career, as the potential for future reproduction diminishes. Older mothers who are closer to their ultimate reproductive event may invest more heavily in the current offspring. Parity is also a proxy for household composition. In the event that all of a mother's previous offspring are in the same household, high parity might represent a very different pathogenic environment for the mother and infant that may affect milk composition, particularly with respect to the immune compounds of interest. It was predicted that parity will be negatively associated with concentrations of lactoferrin, sIgA, and IGF-1 in milk, and positively with cortisol. However, this relationship may be tempered or eliminated as a result of increasing investment in later offspring and the possibility of a more pathogenic environment with increasing parity.

While advancing parity contributes to maternal depletion and therefore poor energetic condition, primiparity represents another challenging energetic state. Younger primiparous mothers often have not yet reached prime reproductive condition, especially if linear growth has not been completed. Therefore, the role of parity as an indicator of maternal energetic condition is complex and should be considered from multiple angles.

In addition to the relationships described above, a variety of relationships between maternal condition and the *dynamics* of milk composition were tested. Associations

between BMI, ΔBMI, ΔC-peptide, relative C-peptide, and parity and monthly changes in milk cortisol, IGF-1, lactoferrin, and sIgA were investigated. None of the interactions were tested in these models.

Caveats

There are some limitations to the data that could affect the interpretation of the results. First of all, the values for the milk compounds are the concentrations of those compounds taken in a mid-feed sample. Concentration may not always be the most relevant way to describe the consumption of these bioactive compounds by infants; it is possible that the total amount consumed in a 24-hour period would be more useful to know. In addition, concentration is highly dependent on the volume of fluid, and cannot fully be understood in isolation. For instance, when discussing results such as age-related changes in the concentration of compounds, it may be tempting to interpret the findings as, "mothers alter the amount of lactoferrin in the milk to give older infants more," when it really may be that milk volume is decreasing as the infant gets older, and mothers are not altering production or secretion of the compound at all. Similarly, milk volume and milk nutritional composition are known to have important effects on infant growth and development. While I am not explicitly considering infant outcomes in this chapter, it is important to keep in mind that the relationship between maternal condition and secretion of these four compounds in milk may interact in important ways with milk nutritional composition. For instance, signaling to the infant to invest in growth by increasing concentration of IGF-1 in the milk may be most effective if the mother also increases milk volume and/or milk energy density to support that growth. On the other hand, if

IGF-1 signals the *proportion* of energy to be allocated to infant growth, it may vary inversely with the amount of energy the mother supplies.

To streamline analysis and approach these questions with the most evolutionarily appropriate lens, maternal condition variables were interpreted in a strictly "more is better" framework—higher BMI, weight gain, and higher insulin were all taken as indications of good maternal condition. This neglects the possibility the benefit of improving maternal condition may plateau at an intermediate value, or even that these systems may become disregulated with diseases of the metabolic syndrome. The median BMI of women enrolled in the present study was 26.8, with ten of the thirty women classified as overweight by Western standards (BMI>25) and nine more as obese (BMI>30). It is important to note that Toba women are probably much shorter than the people for whom these cutoffs were created, and there is no indication that these cutoffs are associated with biologically meaningful health consequences. Future studies limited to only women of healthy BMI, studies including more underweight women, or studies incorporating more sophisticated statistical modeling may be useful to expand upon the relationships being investigated in the present study. In addition, future research should focus directly on the way that milk bioactives are impacted by maternal overweight and obesity. If there are obesity-related functional signals present in milk, or even changes indicating dysfunction or disregulation, it is likely that they would involve different compounds than those presently under investigation, perhaps adipokines or inflammatory molecules.

Methods and variables

Milk variables

Concentrations of compounds in milk were log-transformed before analysis. Monthly changes in these compounds are expressed as the percent change incurred in the non-log transformed concentration of the initial value. Full laboratory methods are described in Chapter 3.

Maternal condition variables

Monthly change in C-peptide was expressed as the percent change incurred in the non-log transformed concentration of the initial value. Difference from maternal average C-peptide value was calculated by adding each month's log-transformed C-peptide value, dividing by the number of study visits, and subtracting each month's value. Full laboratory methods are described in Chapter 3. BMI was calculated at each study visit and monthly change in BMI was calculated by subtracting each month's BMI from the subsequent month's BMI. Parity was self-reported by mothers and is assumed to represent the number of live-born offspring.

Statistical methods

Results were calculated using Generalized Estimating Equations (GEEs) in SPSS v. 22. GEEs allow for the testing of relationships in a manner similar to a regression model, but adjust the values for the data non-independence that occurs with repeated measures from

the same individuals. Models were constructed using an exchangeable correlation structure.

Continuous variables were mean- or median-centered to reduce variable collinearity and aid in the interpretation of interaction effects.

The effect of each maternal condition variable (and both interactions between two maternal condition variables) was tested on each milk compound for a total of 28 final models. However, different combinations of covariates (control variables) and interactions between these control variables and the maternal condition variable of interest were included to maximize the predictive power of each model. These variables included were infant age, age^2, and sex. The best model for each relationship was chosen using a combination of p-values (significant terms were always retained in the "best" model) and QICc (assuring that the removal of any non-significant term improved the predictive power of the model). This stepwise procedure allowed for the control of relevant infant variables when appropriate.

After selection of the best model, histograms of residuals were examined to check for outliers, and scatter plots of these residuals versus the mean predicted value from each model were examined for outliers and heterogeneity. No outliers were found in the models using static values of milk compounds, so the tests used were appropriate for the data.

The relationship between each maternal condition variable and monthly change in each milk constituent was performed, for a total of 20 more models. The methods for model selection were similar, but simpler because no interactions were tested. Age and sex were initially included in the model, and were almost always not significant. They were removed (if non-significant) and the effect of the maternal condition variable on the monthly percent change in the milk compound was assessed.

Once the best model was selected, residuals were examined for outliers. Each of the monthly change models contained at least one outlier. Outliers were removed and models rerun. This had the effect of eliminating most of the significant results. The results presented in the final section of this document are these, with outliers removed.

P-values were not adjusted for multiple comparisons. One reason this was not possible was because a "family" of tests was difficult to define for this set of questions. For example, it could be instructive to compare the relationships between BMI and each of the four milk compounds. It may also be useful to compare the relationship between each of the maternal condition variables and just one of the milk compounds. Another reason p-values were not adjusted is because each model had different combinations of variables, including age, sex, and interactions between different variables. Because 48 models were tested in this chapter, it is likely that some Type I errors were committed.

Results

Infant age was significantly positively associated with concentration of cortisol and IGF-1 in milk, marginally positively associated with lactoferrin, and positively but not significantly associated with sIgA concentration (Table 4.1).

Table 4.1: Relationships between infant age and milk compounds

	B	p
Cortisol	.062	.000
IGF-1	.041	.004
Lactoferrin	.023	.066
sIgA	.020	.259

Table 4.1*: Relationships between infant age and concentrations of the four milk compounds from a GEE (adjusting for repeated measures). All relationships are positive, indicating that concentrations increase with age of the infant. B (x100) can be interpreted as the average percent increase in each milk compound per one-month increase in infant age.*

Sex of the infant was not significantly associated with the concentration of any compound (Table 4.2).

Table 4.2: Relationships between infant sex and milk compounds

	B	p
Cortisol	-.100	.465
IGF-1	-.184	.210
Lactoferrin	-.156	.278
sIgA	-.142	.344

Table 4.2*: Relationships between infant sex and concentrations of the four milk compounds from a GEE (adjusting for repeated measures). Infant age was included as a covariate in all models. Males were the reference sex; B can be interpreted as the difference in average log-transformed concentration of each milk compound for female infants compared to males. There were no significant relationships between infant sex and concentration of milk compounds.*

Descriptive statistics of the milk compounds are shown in Table 4.3 for the convenience of the reader. More about the milk compounds, including histograms of their distributions and discussion of log-transformation can be found in Chapter 3.

Table 4.3: Descriptive statistics of concentrations of milk compounds (untransformed and log-transformed)

	Cortisol (ng/mL)		IGF-1 (ng/mL)		Lactoferrin (ng/mL)		sIgA (ug/mL)	
	Raw	**LN**	**Raw**	**LN**	**Raw**	**LN**	**Raw**	**LN**
Min.	0.4	-3.22	0.42	-0.87	0.86	13.67	183.3	5.21
25th	1.6	-1.83	0.72	-0.33	1.68	14.33	417.3	6.03
Median	2.4	-1.43	1.08	0.08	2.38	14.68	556.1	6.32
Mean	2.7	-1.47	1.41	0.14	2.62	14.68	648.3	6.35
75th	6.7	-1.08	1.70	0.53	3.25	14.99	786.6	6.67
Max.	7.9	0.04	7.50	2.01	6.58	15.70	2723.7	7.91
S.D.	0.5	0.59	1.17	0.60	1.20	0.44	379.2	0.49

Table 4.3*: Descriptive distribution statistics for raw and log-transformed values of all milk compounds. One sample had extreme outlying values for IGF-1, lactoferrin, and sIgA and was not included in these descriptive statistics or in any analyses.*

Table 4.4: Descriptive statistics of maternal condition variables

	BMI	ΔBMI	Relative C-peptide (LN)	Percent ΔC-peptide	Parity
Min.	18.2	-2.3	-1.24	-94.30	1.00
25th	24.2	-0.6	-0.36	-47.65	1.00
Median	26.8	-0.1	-0.08	12.70	3.00
Mean	28.0	-0.1	0.00	34.80	3.33
75th	31.2	0.5	0.38	80.05	4.25
Max.	40.8	2.1	1.71	327.20	10.00
S.D.	4.78	0.84	0.53	102.39	2.465

Table 4.5: Slopes, confidence intervals, and p-values of all maternal condition models

	Static		Magnitude of monthly change (Percent Δ compound)	
	B [95% CI]	**p**	**B [95% CI]**	**p**
BMI				
Cortisol	-.027 [-.052, -.001]	.041	-1.448 [-2.932, .036]	.056
IGF-1	-.029 [-.050, -.008]	.007	-1.146 [-3.141, .848]	.260
Lactoferrin*	.000 [-.023, .022]	.988	-.297 [-1.093, .499]	.465
sIgA*	.002 [-.027, .032]	.873	-.683 [-1.690, .324]	.184
ΔBMI				
Cortisol	-.063 [-.175, .050]	.275	-8.856 [-25.753, 8.012]	.303
IGF-1	.015 [-.139, .169]	.850	13.040 [-.2998 29.078]	.111
Lactoferrin	.069 [-.023, .160]	.143	5.644 [-1.511, 12.798]	.122
sIgA	.066 [-.038, .171]	.212	.681 [-5.720, 7.082]	.835
BMI*ΔBMI				
Cortisol	-.028 [-.057, .000]	.051	-	
IGF-1	-.017 [-.043, .009]	.203	-	
Lactoferrin	-.013 [-.026, .001]	.072	-	
sIgA	-.013 [-.030, .003]	.122	-	
BMI*C-peptide				
Cortisol	.003 [-.023, .030]	.801	-	
IGF-1	-.021 [-.044, .003]	.088	-	
Lactoferrin	-.008 [-.018, .001]	.095	-	
sIgA	-.015 [-.026, -.004]	.007	-	
ΔC-peptide				
Cortisol	.000 [-.001, .001]	.725	-.058 [-.183, .066]	.359
IGF-1	.000 [-.001, .000]	.187	-.070 [-.195, .054]	.269
Lactoferrin	.000 [.000, .001]	.321	.009 [-.038, .056]	.701
sIgA	.000 [.000, .000]	.858	-.010 [-.079, .060]	.784
Relative C-peptide				
Cortisol	-.127 [-.258, .004]	.058	-1.716 [-20.245, 16.812]	.856
IGF-1	-.002 [-.157, .153]	.980	1.616 [-33.977, 7.241]	.204
Lactoferrin	-.022 [-.081, .037]	.465	-9.751 [-19.863, .362]	.059
sIgA	-.042 [-.126, .042]	.332	-6.879 [-20.182, 6.424]	.311
Parity				
Cortisol	-.038 [-.097, .021]	.211	-1.118 [-2.989, 754]	.242
IGF-1	.003 [-.075, .080]	.948	-1.399 [-4.555, 1.758]	.385
Lactoferrin**	.010 [-.067, .086]	.805	.560 [-2.443, 3.563]	.715
sIgA	.039 [-.022, .101]	.206	-1.870 [-5.626, 1.886]	.096

*significant interaction with age
**significant interaction with sex

Table 4.5*: B [95% confidence interval] is the slope of the model (interpretation differs by variable). Models with p values <0.1 are highlighted.*

A summary of the results of all maternal condition models is presented in Table 4.5, followed by discussion of significant relationships. Where illustrative, interaction plots are displayed to aid in the interpretation of significant interaction effects. To demonstrate the interaction, variables were divided into tertiles. In these plots, each value is treated as an independent data point; the interaction plots do *not* account for the non-independence of repeat measures. Not all plots clearly displayed the true statistical relationships derived from GEE models; only appropriate plots are included below.

Body mass index (BMI)

BMI and cortisol

BMI and milk cortisol were negatively related (B= -.027 [-.052, -.001], p=.041). As maternal BMI increases one unit, milk cortisol decreases 2.7%, on average. Infant age had a significant main effect in this model (B=.060 [.034, .085], p=.000).

Maternal urinary cortisol shows almost exactly the same relationship (B= -.030 [-.051, -.009], p=.005); as maternal BMI increases one unit, urinary cortisol decreases 3%. Infant age was not significant in this model. This fits with the understanding that cortisol passively diffuses into milk from the bloodstream (Fox et al. 1981).

In addition to predicting lower cortisol overall, high maternal BMI also associated with a greater likelihood that cortisol would experience a decrease in any given month (B= -1.448 [-2.932, .036], p=.056). For each additional unit that maternal BMI increases, the

monthly change in cortisol was reduced by 1.45% (for reference, the mean change was 12.44%; the median change was 0%; the standard deviation was 51.40%).

There were no significant interactions between BMI and sex or age of the infant on milk cortisol.

BMI and IGF-1

BMI was negatively related to milk IGF-1 (B= -.029 [-.050, -.008], p=.007). As maternal BMI increases one unit, milk IGF-1 decreases 2.9%, on average. Infant age had a significant main effect in this model (B=.041 [.017, .065], p=.001).

There were no significant interactions between BMI and sex or age of the infant on milk IGF-1.

There relationship between maternal BMI and monthly change in IGF-1 was not significant.

BMI and lactoferrin

There was no relationship between maternal BMI and lactoferrin in milk. There was no interaction between BMI and infant sex on lactoferrin.

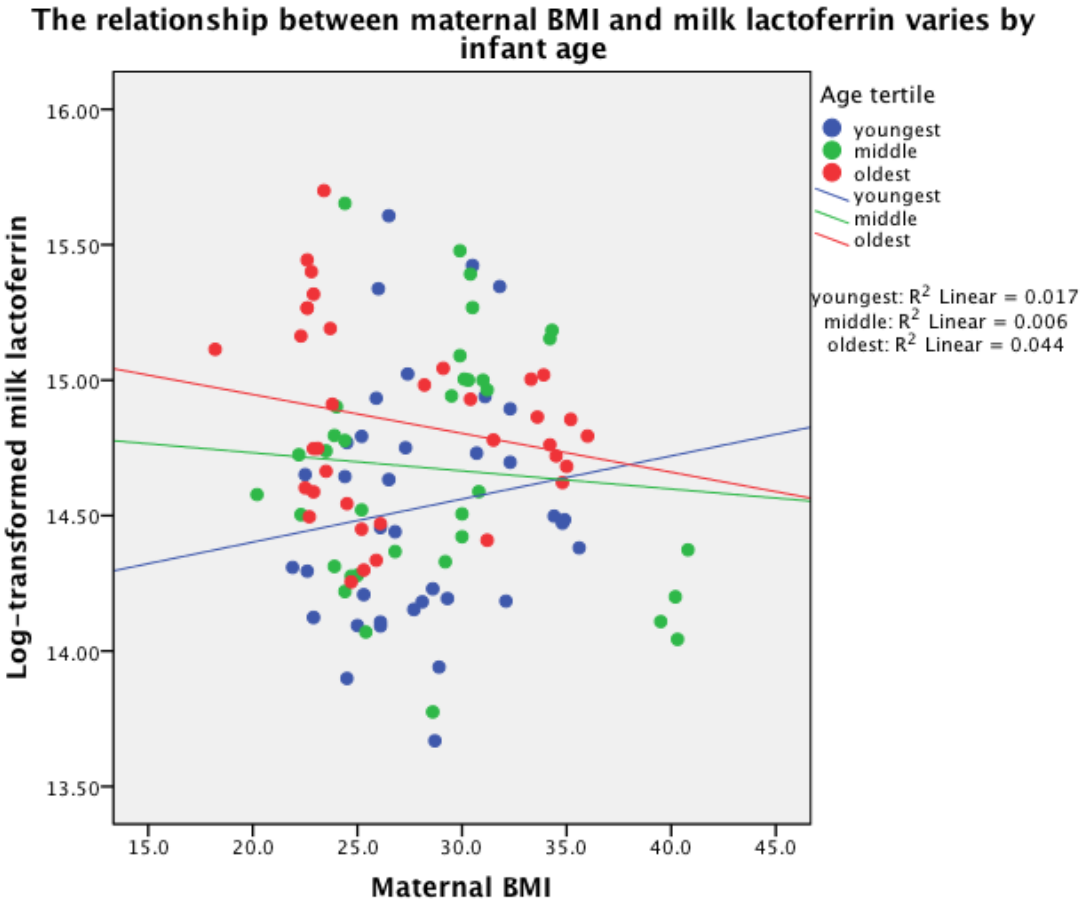
There was a nearly significant interaction between BMI and age of the infant on lactoferrin (B=-.004, [-.009, .000], p=.054). Mean-centered BMI did not have a

significant simple effect in this model (B=.004 [-.022, .030], p=.773). Both mean-centered age of the infant (B=.022 [.001, .043], p=.039) and mean-centered age squared (B=.004, [.000, .008], p=.039) did have significant simple effects in this model.

As infants get older, concentration of lactoferrin in milk generally increases (see Table 4.1). However, when mothers have higher BMI, the slope of this relationship is less steep. Thinner mothers experience a steeper increase in lactoferrin across lactation whereas heavier mothers experience a slightly less steep increase in lactoferrin across lactation. As BMI increases by 1 unit, there is a 0.4% reduction in the monthly age-related increase in milk lactoferrin concentration (Figure 4.1, Figure 4.2).

Figure 4.1: Infant age and lactoferrin by maternal BMI

Figure 4.2: Maternal BMI and lactoferrin by infant age



There was no significant relationship between maternal BMI and monthly change in lactoferrin.

BMI and sIgA

There was no relationship between maternal BMI and sIgA in milk. There was no interaction between BMI and infant sex on sIgA.

There was a significant interaction between BMI and age of the infant (B=-.006, [-.012, .000], p=.043). Mean-centered BMI did not have a significant simple effect in this model (B=.008 [-.018, .034], p=.543). Mean-centered age of the infant did not have a significant simple effect (B=.019 [-.011, .049], p=.206) but mean-centered age^2 did (B=.006, [.001, .010], p=.019).

As infants get older, milk sIgA generally increases (although this relationship is not significant; see Table 4.1). However, when mothers have higher BMI, this slope is less steep. Thinner mothers experience a steeper increase in sIgA across lactation whereas heavier mothers experience a slightly less steep increase in sIgA across lactation (Figure 4.3, Figure 4.4). As BMI increases by 1 unit, there is a 0.6% reduction in the monthly age-related increase in milk sIgA concentration (for reference, the mean change was 13.05%; the median change was -1.59%; the standard deviation was 61.42%).

Figure 4.3: Infant age and sIgA by maternal BMI

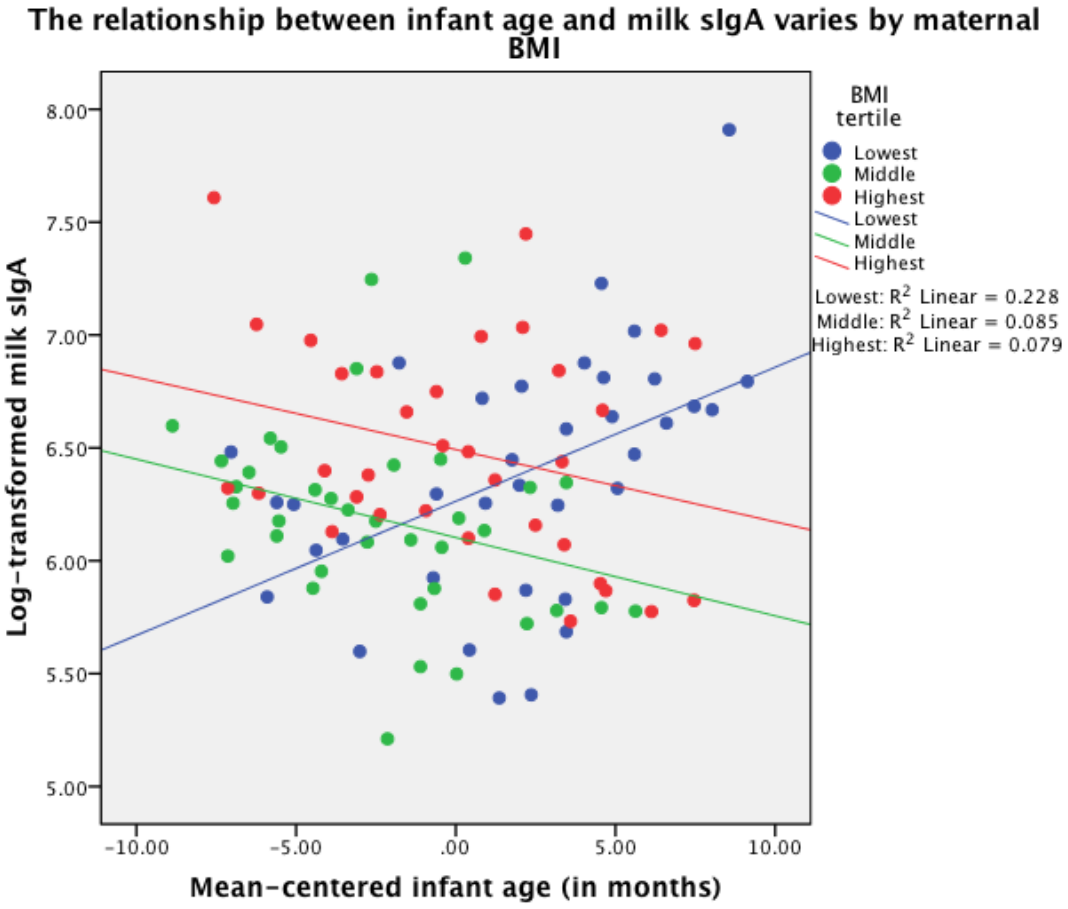
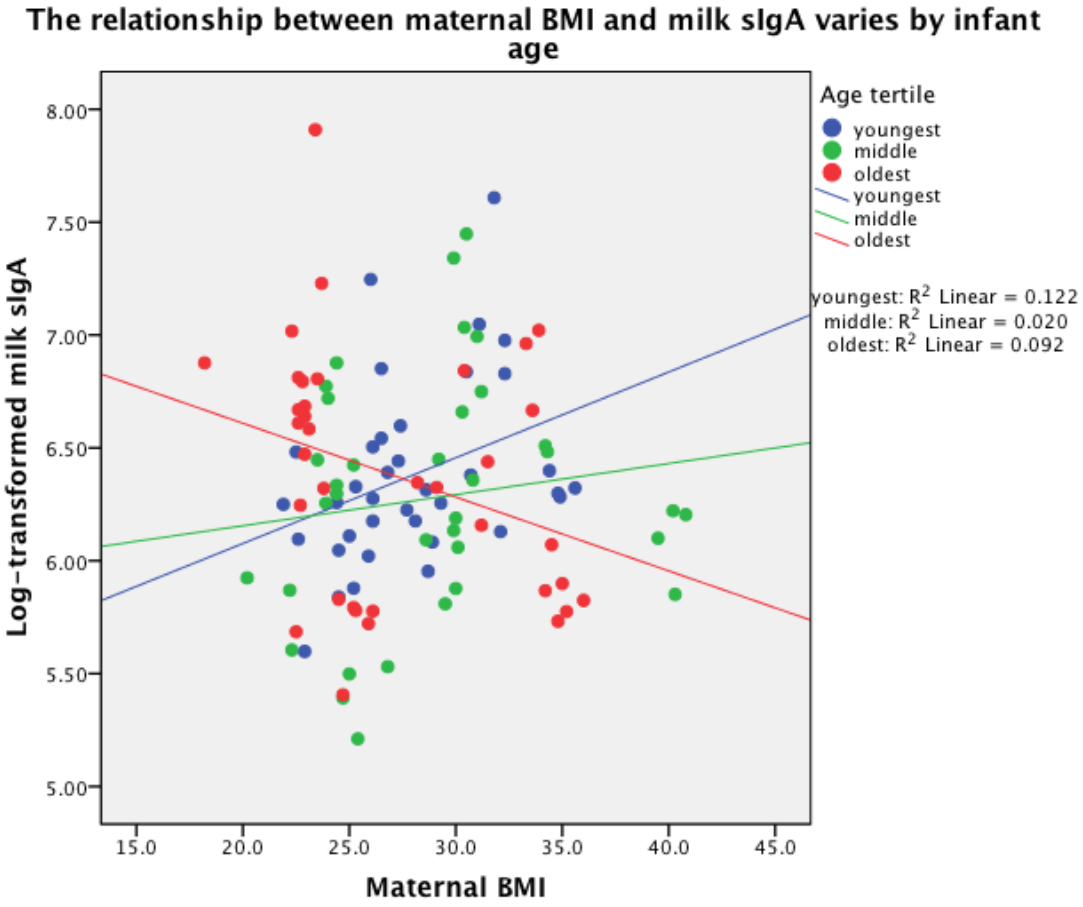


Figure 4.4: Maternal BMI and sIgA by infant age



There was no significant relationship between maternal BMI and monthly change in sIgA.

Monthly change in body mass index (ΔBMI)

There was no significant association between monthly change in BMI and milk cortisol, IGF-1, lactoferrin, or sIgA. There were no significant interactions between ΔBMI and sex or age of the infant on any milk compound.

There was no significant relationship between change in maternal BMI and monthly change in any of the compounds tested.

Interaction between BMI and ΔBMI

*BMI*ΔBMI and cortisol*

There was a nearly significant negative interaction between maternal BMI and monthly change in BMI on milk cortisol (B=-.028 [-.057, .000], p=.051). There was a significant simple effect of mean-centered BMI in this model (B=-.031 [-.059, -.003], p=.028), but the simple effect of ΔBMI was not significant (B=-.058 [-.156, .041], p=.252). Mean-centered infant age had a significant simple effect (B=.076 [.049, .102], p=.000).

The relationship between starting BMI and milk cortisol is more steeply negative among women who are gaining more weight. For each unit increase in a woman's starting BMI, the amount of weight gain associated with a 1-unit increase in BMI is associated with an additional 2.8% reduction in milk cortisol, on average (Figure 4.5).

The relationship between weight gain and milk cortisol is more steeply negative among women who are heavier to begin with. With the amount of weight gain that causes a 1-unit increase in BMI, having a 1-unit higher BMI at the start is associated with an additional 2.8% reduction in milk cortisol, on average (Figure 4.6).

This analysis was also performed with ΔBMI instead modeled as a binary variable representing significant weight gain or loss. Data were coded for weight loss or gain

greater than 0.5 standard deviations from the mean ΔBMI (mean= -.077). Modeled this way, there was a significant negative relationship between BMI and milk cortisol for women who were gaining weight (B=-.069, p=.003), but there was no significant relationship between BMI and milk cortisol for women who were losing weight (B=-.003, p=.854).

Figure 4.5: Maternal BMI and cortisol by change in BMI

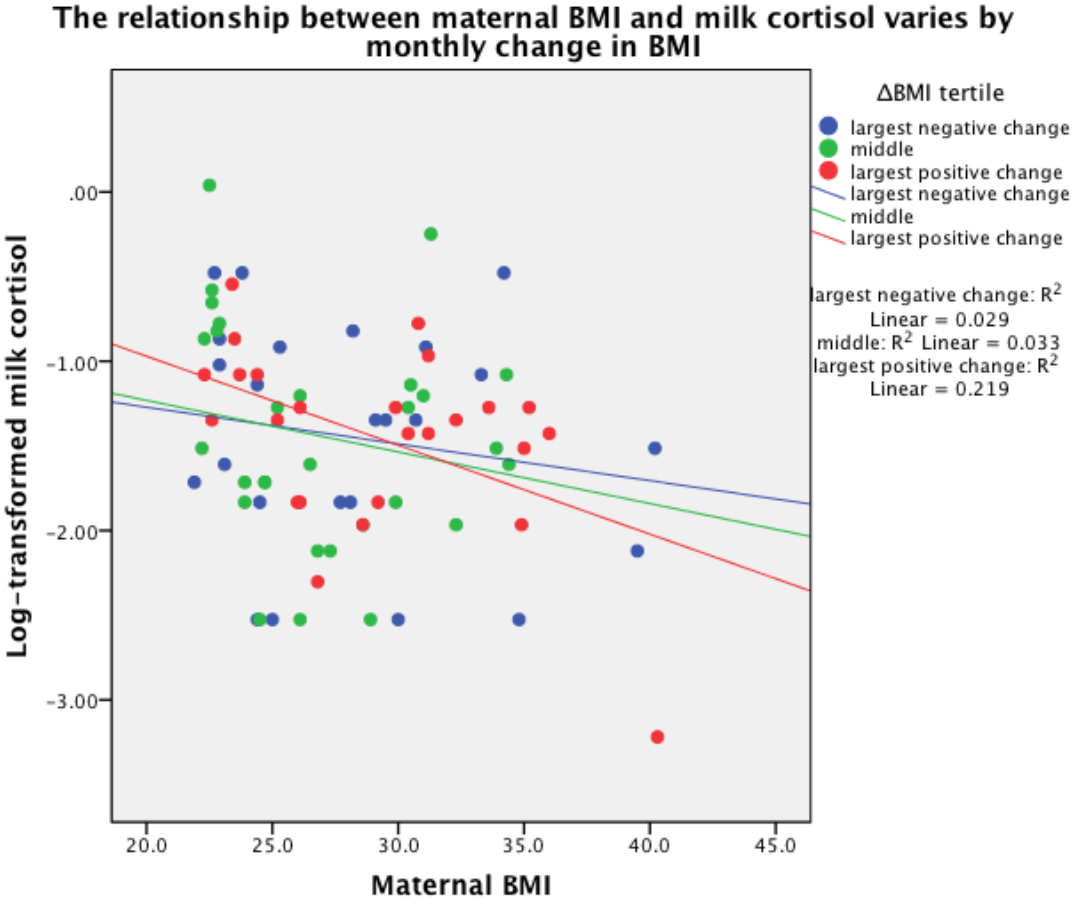
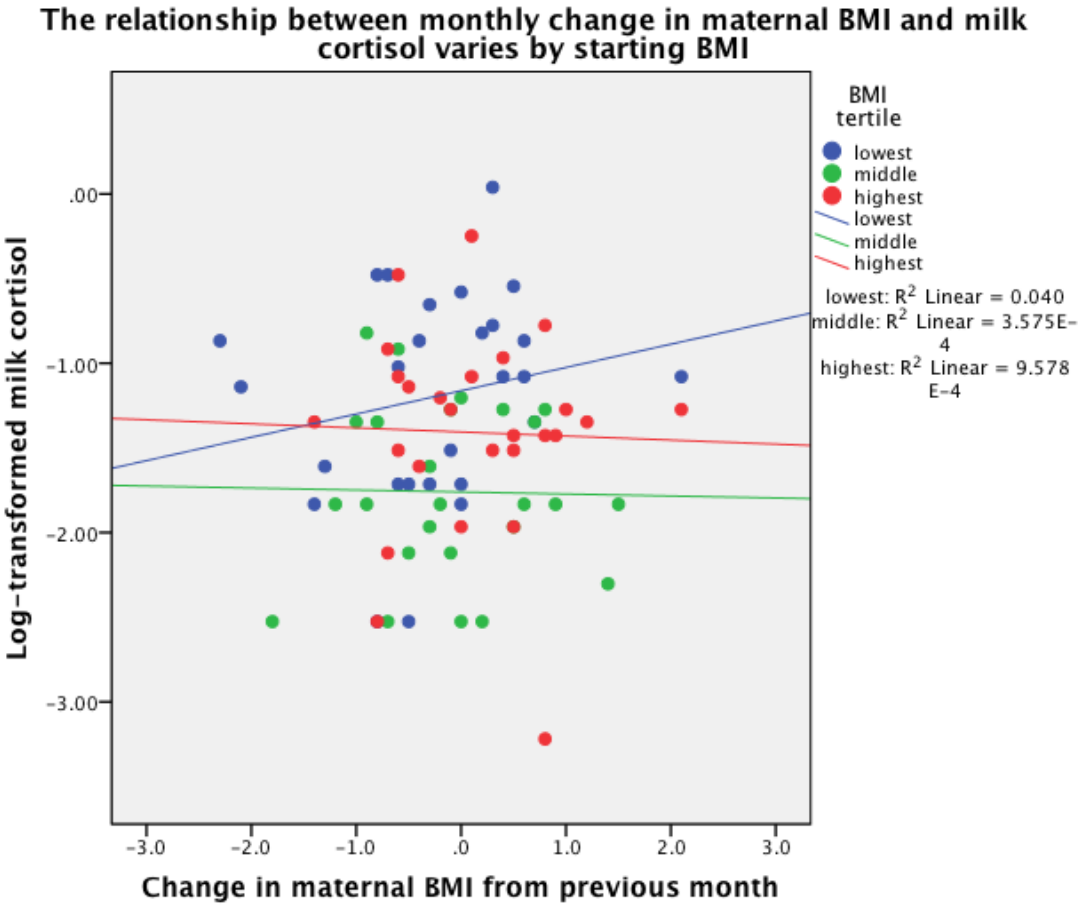


Figure 4.6: Maternal change in BMI and cortisol by BMI



*BMI*ΔBMI and IGF-1*

There was no significant interaction between maternal BMI and change in BMI on milk IGF-1.

This analysis was also performed with ΔBMI instead modeled as a binary variable representing significant weight gain or loss. Data were coded for weight loss or gain greater than 0.5 standard deviations from the mean ΔBMI (-.077). Modeled this way, there was a significant negative relationship between BMI and milk IGF-1 for women

who were gaining weight (B=-.082, p=.000), but there was no significant relationship between BMI and milk IGF-1 for women who were losing weight (B=-.007 p=.545).

*BMI*ΔBMI and lactoferrin*

There was a marginally significant negative interaction between maternal BMI and monthly change in BMI on milk lactoferrin (B=-.013 [-.026, .001], p=.072). There was no significant simple effect of mean-centered BMI (B=-.005 [-.032, .021], p=.708), or ΔBMI (B=.067 [-.017, .152], p=.117) in this model. Mean-centered infant age had a significant simple effect (B=.037 [.015, .059], p=.001).

The relationship between weight gain and milk lactoferrin is more steeply negative among women who are heavier to begin with. With the amount of weight gain that causes a 1-unit increase in BMI, having a 1-unit higher BMI at the start is associated with an additional 1.3% reduction in milk lactoferrin, on average.

The relationship between starting BMI and milk lactoferrin is more steeply negative among women who are gaining more weight. For each unit increase in a woman's starting BMI, the amount of weight gain associated with a 1-unit increase in BMI is associated with an additional 1.3% reduction in milk lactoferrin, on average.

This analysis was also performed with ΔBMI instead modeled as a binary variable representing significant weight gain or loss. Data were coded for weight loss or gain greater than 0.5 standard deviations from the mean ΔBMI (-.077). In this analysis, there

was a significant interaction effect between BMI and the binary weight change variable, but the slope of the relationship between BMI and lactoferrin did not significantly differ from 0 in either category.

*BMI*ΔBMI and sIgA*

There was no significant interaction between maternal BMI and change in BMI on milk sIgA.

This analysis was also performed with ΔBMI instead modeled as a binary variable representing significant weight gain or loss. Data were coded for weight loss or gain greater than 0.5 standard deviations from the mean ΔBMI (-.077). In this analysis, there was a significant interaction effect between BMI and the binary weight change variable, but the slope of the relationship between BMI and sIgA did not significantly differ from 0 in either category.

Monthly change in maternal urinary C-peptide (ΔC-peptide)

There was no significant association between monthly change in C-peptide and milk cortisol, IGF-1, lactoferrin, or sIgA. There were no significant interactions between ΔC-peptide and sex or age of the infant on any of the milk compounds.

There was no significant relationship between change in maternal urinary C-peptide and monthly change in any of the compounds tested.

Maternal urinary C-peptide relative to average (Relative C-peptide)

Relative C-peptide and cortisol

There was a nearly significant negative association between maternal urinary C-peptide relative to average and milk cortisol (B=-.127 [-.258, .004], p=.058). As the difference between current urinary C-peptide and a woman's average value increased by 1%, cortisol decreased by 0.127%, on average. Infant age was also a significant main effect in this model (B=.064 [.041, .086], p=.000).

There was no significant interaction between relative C-peptide and infant age or sex.

There was no significant relationship between relative C-peptide and monthly change in cortisol.

Relative C-peptide and IGF-1

There was no significant relationship between maternal urinary C-peptide relative to average and milk IGF-1. There was no significant interaction between relative C-peptide and infant age or sex.

There was no significant relationship between relative C-peptide and monthly change in IGF-1.

Relative C-peptide and lactoferrin

There was no significant relationship between maternal urinary C-peptide relative to average and milk lactoferrin. There was no significant interaction between relative C-peptide and infant age or sex.

Having higher urinary C-peptide than average predicted a decrease in lactoferrin over the subsequent month, though this relationship is marginally significant (B=-9.751 [-19.863, .362], .059). As the difference between current urinary C-peptide and a woman's average value increased by 1%, lactoferrin experienced a subsequent monthly decrease of -0.98%, on average.

Relative C-peptide and sIgA

There was no significant relationship between maternal urinary C-peptide relative to average and milk sIgA. There was no significant interaction between relative C-peptide and infant age or sex.

There was no significant relationship between relative C-peptide and monthly change in sIgA.

Interaction between BMI and maternal urinary C-peptide of insulin

*BMI*C-peptide and cortisol*

There was no significant interaction between maternal BMI and maternal urinary C-peptide concentration on milk cortisol.

*BMI*C-peptide and IGF-1*

There was a marginally significant negative interaction between maternal BMI and maternal urinary C-peptide on milk IGF-1 concentration (B= -.021 [-.044, .003], p=.088). Mean-centered BMI had a significant simple effect in this model (B=-.031 [-.056, -.007], p=.012) but mean-centered C-peptide concentration did not (B= .079 [-.075, .233], p=.314). Mean-centered infant age had a significant simple effect (B= .039 [.010, .067], p=.008).

The slope of the relationship between BMI and IGF-1 is different depending on value of C-peptide. Similarly, the slope of the relationship between C-peptide and IGF-1 is different depending on BMI. Compared to thinner women, heavier women have a sharper decrease in milk IGF-1 concentration associated with a rise in C-peptide. Concentrations of IGF-1 in milk are highest for women with lower BMIs. As BMI increases, IGF-1 goes down, on average (B= -.029 [.050, -.008], p=.007). This decrease happens faster for women with higher urinary C-peptide concentration (and therefore higher insulin production).

*BMI*C-peptide and lactoferrin*

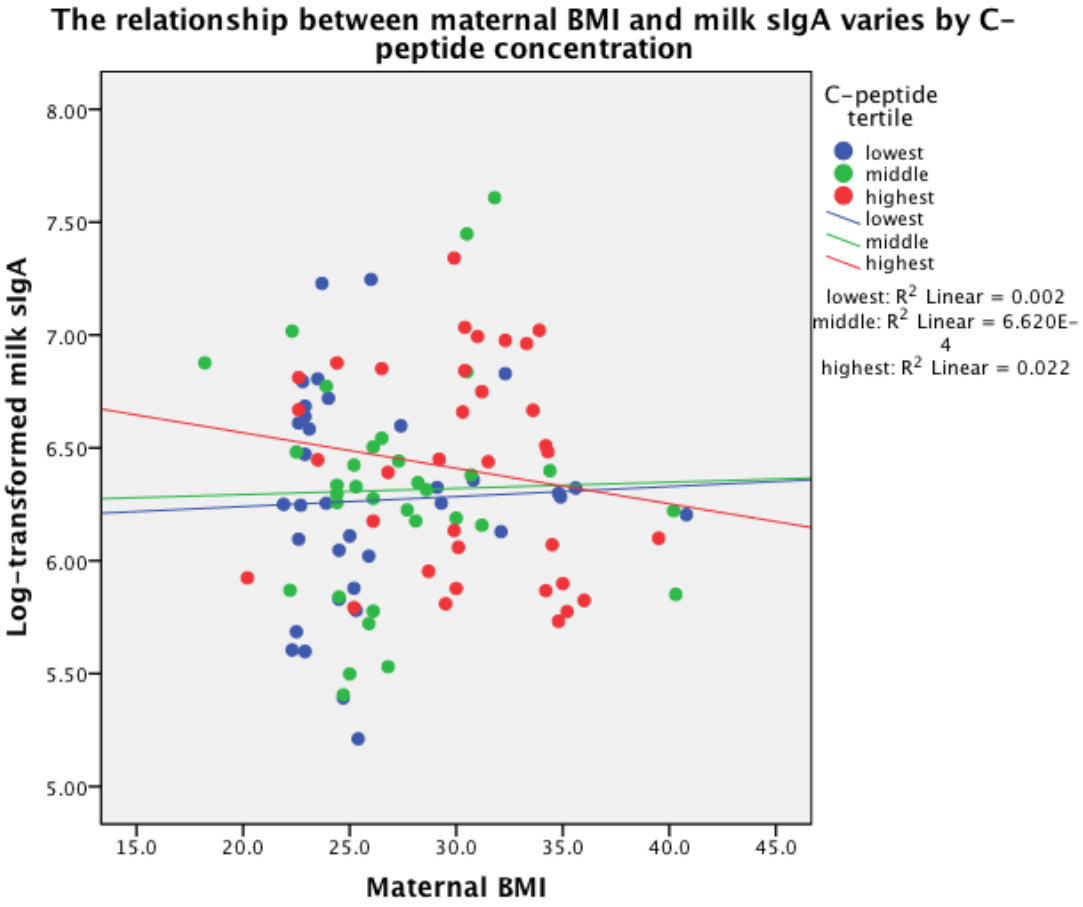
There was no significant interaction between maternal BMI and maternal urinary C-peptide concentration on milk lactoferrin.

*BMI*C-peptide and sIgA*

There was a significant negative interaction between maternal BMI and maternal urinary C-peptide on milk sIgA concentration (B= -.015 [-.026, -.004], p=.007). Neither mean-centered BMI (B=.007 [-.025, .039], p=.663) nor mean-centered C-peptide concentration (B=-.042 [-.109, .025], p=.217) had a significant simple effect on this model. Infant age did not have a significant main effect on the model before the interaction term was added, and was therefore not included in this model.

The slope of the relationship between BMI and sIgA is different depending on value of C-peptide (Figure 4.7). Similarly, the slope of the relationship between C-peptide and sIgA is different depending on BMI. Compared to thinner women, heavier women have a sharper decrease in milk sIgA concentration associated with a rise in C-peptide. Overall, there is no significant relationship between maternal BMI and sIgA concentration in milk. However, the slope of this relationship is significantly different at different concentrations of C-peptide. At higher BMI, high C-peptide (and therefore higher insulin production) is associated with lower sIgA than low C-peptide. Similarly, with higher insulin production, higher BMI predicts lower sIgA than does low BMI.

Figure 4.7: Maternal BMI and sIgA by C-peptide



Parity

Parity and cortisol

There was no significant relationship between maternal parity and milk cortisol when parity was modeled as a continuous variable, and there were no significant interactions between parity and infant age or sex. However, when parity was modeled as a binary variable (primiparous mothers compared to multiparous mothers), there was a significant and large effect of parity on cortisol (with multiparous women as the reference group, B

for primiparous women = .386, p=.029). This translates to 38.6% higher milk cortisol among primiparous mothers.

When milk volume (restricted to high-confidence two-hour test-weigh values as described in Chapter 3), is included in the model, the effect of parity on milk cortisol is eliminated. This could indicate an effect of dilution, but this is unlikely because the effect of milk volume on cortisol was not significant (B=.000, p=.765). It is more probable that the sample size of 45 visits where high-quality estimates of milk volume were available was not large enough to detect the effect.

There was no significant relationship between maternal parity and monthly change in cortisol.

Parity and IGF-1

There was no significant relationship between maternal parity and milk IGF-1. There was no significant interaction between parity and infant age or sex. There was no difference in milk IGF-1 concentration between primiparous and multiparous mothers.

There was no significant relationship between maternal parity and monthly change in IGF-1.

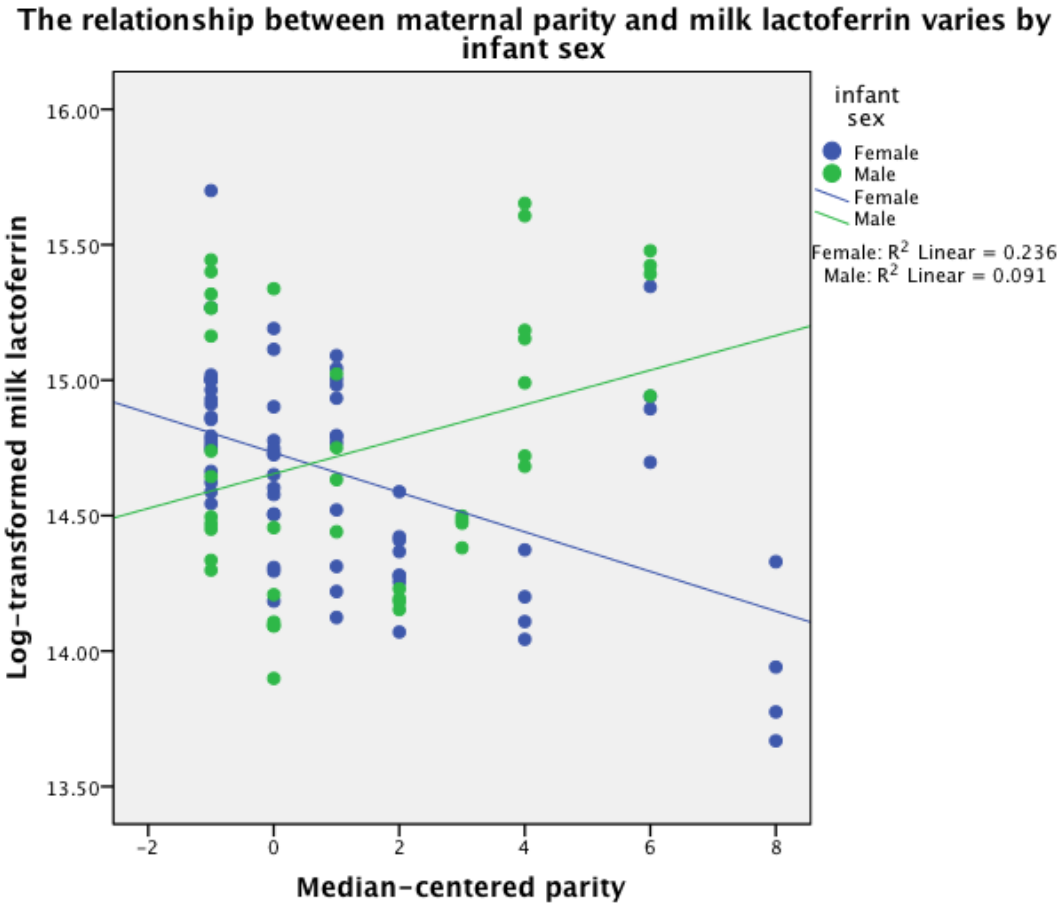
Parity and lactoferrin

There was no significant relationship between maternal parity and milk lactoferrin. There was no interaction between parity and age of the infant on milk lactoferrin concentration. There was no difference in milk lactoferrin concentration between primiparous and multiparous mothers.

There was a significant interaction between maternal parity and infant sex on milk lactoferrin (with males as the reference sex, B for female infants = -.151, [-.253, -.048]), p=.004). Median-centered parity had a significant simple effect in this model (B for males=.105 [.025, .185]), p=.010), but infant sex did not have a significant simple effect (B for females = .083 [-.172, .339]), p=.522). Mean-centered infant age (B=.024 [.001, .047], p=.039) and mean-centered age squared (B=.005 [.001, .009], p=.022) both had significant simple effects.

The significant interaction term means that the slope is significantly different for male and female infants (Figure 4.8). The slope of the relationship between parity and milk lactoferrin for male infants is significant and positive (B=.105, p=.010). For female infants, the slope is not significantly different from zero (B= -.046, p=.189). As the parity of his mother increases, a male infant receives a higher concentration of lactoferrin in milk. On average, each additional older sibling is associated with a 10.5% increase in milk lactoferrin.

Figure 4.8: Maternal parity and lactoferrin by infant sex



There was no significant relationship between maternal parity and monthly change in lactoferrin.

Parity and sIgA

There was no significant relationship between maternal parity and milk sIgA. There was no significant interaction between parity and infant age or sex. There was no difference in milk lactoferrin concentration between primiparous and multiparous mothers.

There was no significant relationship between maternal parity and monthly change in sIgA.

Conclusions and discussion

This chapter explores the relationships between several maternal energetic variables (BMI, monthly change in BMI, C-peptide of insulin dynamics, and parity) and the concentration of cortisol, IGF-1, lactoferrin, and sIgA in milk. This discussion summarizes the results of these investigations and addresses the viability of the primary and alternative hypotheses in light of the results. Promising directions for future research are identified.

All significant results indicate a negative relationship between maternal energetic condition and concentration of milk compounds. These findings are contrary to most of the initial predictions set forth in the introduction, those involving cortisol notwithstanding. The most parsimonious speculation is that mothers in better energetic condition are producing a higher volume of milk, thereby diluting the concentration of each of these compounds. Generally, milk volume is expected to positively correlate with maternal energetic condition. The expectation of a positive relationship between energetic condition and milk volume might be more applicable to a marginally nourished population. However, if this relationship does hold in our study population, we might expect a negative association between maternal condition and concentrations of all milk compounds simply because of dilution. This hypothesis is still viable in light of the

results, and is indeed the simplest explanation for the ubiquity of negative associations uncovered between maternal condition and concentrations of milk bioactive factors.

However, there are some mechanistic considerations specifically about cortisol and IGF-1 that undermine this simple explanation. The process of passive diffusion by which cortisol enters milk is more consistent with a model of constant equilibration with circulating concentration than an accumulation in the mammary that would be subject to dilution (Fox et al. 1981). IGF-1 typically positively associates with milk volume, probably due to its direct positive effect on milk production (Breier et al. 1993; Prosser et al. 1989).

No significant relationships were detected between milk volume and any of the milk compounds, using high-confidence test weigh values (n=45, see Chapter 3). The lack of any relationship could easily be because of the small sample size and the fact that these values only represent two hours of milk accumulation. The dilution explanation is still possible but it is puzzling that the only compounds directly displaying simple negative relationships with maternal condition, cortisol and IGF-1, were predicted to be the *least* susceptible to the effects of dilution. A study integrating 24-hour milk volume with the concentrations of these compounds would easily resolve whether this dilution is a viable explanation for these findings.

One alternative explanation is that, if mothers in worse physical condition are producing less milk, or milk of lower energetic quality, they might be signaling to their infants to

invest a greater proportion of available calories to growth with a higher concentration of milk bioactive factors. This explanation, henceforth referred to as the "Calorie Push" hypothesis, is addressed in detail in Chapter 7. Additional alternative hypotheses are addressed in this section.

Cortisol

Poor maternal condition, specifically in the form of weight loss, was predicted to associate with higher circulating cortisol levels. This would be reflected directly in milk cortisol concentration, resulting in a negative association between maternal condition and milk cortisol. This was the primary hypothesis for cortisol and maternal condition and was ultimately supported by the data. In fact, all significant relationships involving cortisol are consistent with this framework.

While monthly change in BMI did not relate to cortisol as predicted, BMI itself was negatively related to milk cortisol concentration. Higher BMI was also associated with a greater likelihood of experiencing a reduction in milk cortisol in any given month. Given that higher BMI predicts lower cortisol concentration overall, this finding is somewhat surprising; simply due to the nature of these variables, one might expect that having a lower baseline cortisol value would be associated with a higher likelihood of experiencing a rise over the following month, but this does not seem to be the case. Perhaps heavier individuals need less cortisol to mobilize sufficient energy to support lactation and other functions.

BMI and monthly change in BMI significantly interacted to predict cortisol concentration such that, at higher BMIs, weight gain resulted in a greater reduction in cortisol than losing or maintaining weight. The exact nature of this relationship was clarified by the creation of a binary variable to explicitly compare weight loss to weight gain. There was a significant negative relationship between BMI and milk cortisol among women who were gaining weight, but no significant relationship among women who were losing weight.

Having higher C-peptide than average was also negatively associated with milk cortisol; this is an intuitive finding, as insulin and cortisol have opposing actions in the body.

A final hypothesis involving parity and milk cortisol was particularly well-supported. Based on a study of rhesus macaque mothers (Hinde et al. 2015), it was predicted that poor maternal condition (particularly as indexed by low parity reflecting subadult status) would associate with higher milk cortisol. On average, primiparous mothers in the present study had 40% higher milk cortisol than multiparous mothers. This effect was not driven by BMI. In this sample, BMI and parity are positively correlated (R^2=.202), but there was not a significant difference in BMI between primiparous and multiparous women (t=-.662, df=28, p=.513). This finding nicely echoes the rhesus study, which found that lower-parity mothers had higher milk cortisol (Hinde et al. 2015). Those authors proposed that low-parity mothers may be programming a behaviorally "cheap" infant through elevated cortisol. This idea is explored further in Chapter 7.

Hinde's group also presents an interesting idea about the physiological importance of cortisol as a mechanism by which first-time mothers compensate for their relatively underdeveloped mammary gland architecture. Glucocorticoids are known to prevent apoptosis of epithelial cells, including mammary epithelial cells (Berg et al. 2002; Feng et al. 1995; Green and Streuli 2004). Across species, the capacity for milk synthesis increases with each additional parity (Miller et al. 2006; Lang et al. 2012; Anderson and Sheffield 1983), so prolonging the life of mammary cells might help primiparous or low-parity mothers sustain higher milk yield.

One alternative hypothesis proposed in Chapter 2 led to the prediction that maternal condition would positively associate with cortisol. This idea, that cortisol might signal the infants of mothers in good condition to exhibit costly behaviors, was not supported.

Overall, milk cortisol consistently displays negative associations with many indices of maternal energetic condition.

IGF-1

IGF-1 was predicted to be positively associated with maternal condition for many reasons. Fasting is known to be associated with reduced circulating IGF-1, so better energetic condition should be associated with higher circulating levels. Since IGF-1 is transferred to milk from the circulation, this should be reflected in milk IGF-1 concentration as well. Also, because IGF-1 has beneficial effects on infant intestinal development and growth, these functions may be better supported by mothers who are

better able to invest in them. The role of IGF-1 in increasing milk yield also leads to the prediction that mothers in better energetic condition will produce a higher volume of milk with higher concentrations of IGF-1.

Despite all of the reasons to expect a positive relationship between IGF-1 and maternal condition, these hypotheses were not supported by the current data. High BMI actually predicted lower concentrations of milk IGF-1. There was also a (marginally significant) negative interaction between BMI and C-peptide of insulin on milk IGF-1. Put simply, the slope of the relationship between insulin (C-peptide) and IGF-1 depends on BMI. At a high level of insulin production, thinner women produced higher milk IGF-1 than heavier women. This may stem from differences in systemic insulin dynamics between women of different body compositions; because many women in the sample were overweight or obese, they could be experiencing insulin resistance. Higher insulin production in a diabetic or insulin-resistant state would not necessarily indicate improved nutritional status or increased investment in energy storage the way it might in healthy, insulin-sensitive individuals. It might also be that thinner women alter milk production more readily in response to cues of current energy availability. If indications of improving energy status, such as increased insulin production, result in an increase in circulating IGF-1, this should be reflected in both increased milk production and more IGF-1 in milk. Alternatively, women with more body fat may be less sensitive to changes in energy availability because their stored fact acts a buffer. The full biological significance of this relationship is difficult to parse with the current data but fits with the overall pattern of a negative relationship between maternal condition and IGF-1.

Overall, milk IGF-1 concentration was negatively associated with maternal condition, but there were not many significant relationships contributing to this pattern. Reflecting on the *a priori* prediction that the overall trend would be positive, it now seems likely that the majority of the women enrolled in the present study fell outside the range of energetic condition where an increase in adiposity would be expected to significantly increase IGF-1 concentration. The *a priori* predictions were based on data from fasting subjects, but the positive association between energy status and IGF-1 may not be significant once a minimum energetic status is attained. In fact, combining observations from the studies presented in Chapter 2 suggests that the true relationship between BMI and IGF-1 is probably parabolic, with maximal levels seen at an intermediate BMI. If this is true, the negative relationship seen in the current study population is expected, since the majority of the subjects are overweight or obese.

Milk immunofactors (lactoferrin and sIgA)

Good maternal condition was predicted to promote an active, symptom prevention approach toward infant illness. A positive association between maternal condition and concentrations of lactoferrin and sIgA would lend support to this hypothesis. However, this framework was not supported by the data. The significant relationships between maternal condition and milk immunofactors were negative.

BMI and infant age had significant negative interactions in the models predicting both immune proteins. Overall, lactoferrin is positively related to infant age in this sample,

but as maternal BMI increases, the relationship becomes less steep. For older infants, thinner mothers produce milk that is more concentrated in lactoferrin than do heavier mothers. Again, the interpretation of this relationship would be better informed if reliable estimates of milk volume were available. As supplementary foods are introduced and infants approach weaning, the volume of milk produced generally decreases, which could be driving the increased concentration of lactoferrin in the milk. Similarly, there might be differences in milk yield between high- and low-BMI mothers that explain this interaction. It may also be that thinner mothers are actively fortifying the milk with more lactoferrin, regardless of volume. Since lactoferrin was found in to rise in response to infant illness (Chapter 5), this may indicate an increased propensity for the lighter mothers or their older infants to fall ill. It may also indicate a different response to illness by heavier mothers or their infants.

The interaction between BMI and age on sIgA is more complicated to interpret because the main effect of infant age on sIgA is positive, but not significant. Regardless, the pattern is the same as for lactoferrin; the slope of the relationship between infant age and sIgA is less steep for mothers with higher BMI.

The interaction between BMI and C-peptide was significantly negatively associated with sIgA and negatively but marginally significantly associated with lactoferrin. At a given high level of insulin, heavier women have, on average, lower sIgA and lactoferrin than thinner women. At a given high BMI, women with high insulin have lower sIgA and lactoferrin than women with low insulin.

Some of the alternative hypotheses presented in Chapter 2 were supported by the negative relationships uncovered between maternal condition and milk immunofactors. One likely explanation that unifies all of these findings is that mothers in poor energetic condition are more susceptible to illness (or, alternatively, that long bouts of illness impair maternal condition). Illness might elevate expression of immune factors and impair maternal condition, resulting in the observed negative association. I predicted that this relationship would be weaker or absent for lactoferrin compared to sIgA because lactoferrin production is independently regulated in the mammary and therefore less likely to reflect circulating concentration. There were more significant negative relationships for lactoferrin than sIgA, so this part of the hypothesis was not upheld. Future studies would benefit from inclusion of maternal infection status to test this idea.

Lactoferrin

Additional associations between maternal and lactoferrin were uncovered. Although the immune factors were generally expected to show similar relationships, the fact that two entirely distinct processes govern their secretion into milk provides a simple explanation for these divergent results.

The interaction between BMI and monthly change in BMI was significantly negatively associated with lactoferrin in milk. Compared to thinner women, as heavier women gain weight, they are expected to experience a larger decrease in lactoferrin. This interaction is perhaps better interpreted through the lens of weight *loss* rather than weight gain.

Women who are thinner experience a greater rise in lactoferrin as they lose weight. Women who are losing weight experience a greater rise in lactoferrin if they are thin. One explanation of this effect may be that the mothers who are losing weight and experiencing a rise in lactoferrin have been ill. Maternal illness would then be a causal variable that explains both increased lactoferrin and weight loss.

Having higher C-peptide than usual was also negatively associated with future change in lactoferrin. Women who have higher C-peptide than usual are more likely to experience a drop and less likely to experience a rise in lactoferrin in the following month. If insulin is high and a woman is storing fat, it is more likely that lactoferrin will decrease over the following month. This could be the other side of the speculative explanation above, that weight loss and increased lactoferrin are both due to illness. In this case, if a woman and her infant are healthy, she is more likely to store fat *and* experience a reduction in milk lactoferrin.

A final set of predictions specifically focused on the relationship between maternal parity and milk immune factors. Parity was found to be positively associated with milk lactoferrin among the mothers of male infants, but not females. While parity was predicted *a priori* to be negatively associated with lactoferrin, there were several possible scenarios presented in Chapter 2 to explain the positive relationship. One reason is that low and high parity are associated with different pathogenic environments because of the increasing number of children in the household. Another explanation relies on the

terminal investment hypothesis, whereby mothers invest more in their current infants as they approach their ultimate reproductive event.

The finding that this relationship was significant and positive for male infants may indicate an additional element of sex-biased investment. Lactoferrin appears to rise in response to illness (Chapter 5), but it is not clear why an increase in lactoferrin would be useful to male, but not female infants if household pathogen load were the explanation. Perhaps male infants are more susceptible to infection and this susceptibility is exaggerated in males with more older siblings. It could also be that mothers who are later in their reproductive careers alter their investment in male offspring to maximize total reproductive success, but increased lactoferrin alone provides minimal evidence to support this claim.

Overall, maternal condition showed a negative association with concentration of milk immunofactors. These findings suggest that the production of these proteins is not costly to the mother, or that other influences on their expression are stronger.

The role of prolactin as a transcription factor for lactoferrin might explain a mechanism for the negative relationship between lactoferrin and maternal condition. Malnourished women are known to have higher prolactin levels (Lunn et al. 1984) so high-parity women experiencing the effects of maternal depletion may have higher prolactin and higher lactoferrin as a result. This hypothesis would be simple to test by integrating plasma prolactin samples into a future study.

Discussion

All significant relationships between maternal condition and milk bioactives were negative. The simplest speculation offered to explain this finding is that mothers in better energetic condition are producing a higher volume of milk, and the concentrations of each of these compounds are more dilute as a result.

I looked for evidence of this effect by running a GEE with BMI and 2-hour milk volume, limited to the estimates in which I had the highest confidence in accuracy (described in Chapter 3). BMI was not a significant predictor of 2-hour milk volume in this model. However, due to the strict inclusion criteria, fewer than half of the total samples were included (45/110). Furthermore, 2-hour milk volume is not as meaningful as 24-hour milk volume would be for this analysis. Although no relationship between BMI and milk volume was detected, that does not mean that such a relationship does not exist.

It might also be that the *a priori* prediction about maternal energetic condition associating with an increase in the transfer of these proteins is simply wrong. Infant growth is a function of *both* caloric intake and hormonal stimulus, and milk sits at the intersection. IGF-1 provides an illustrative example. An infant receiving a high volume of milk, and therefore ample calories to support both growth and maintenance, may need less IGF-1 to stimulate linear growth than an infant who receives fewer calories. Rather than my initial prediction in which mothers in good condition effectively "double down" and use hormones or other bioactive molecules to amplify the strength of an already strong signal to invest in growth, IGF-1 may instead be a mechanism by which mothers who are

producing less milk, or milk of lower quality, can signal to their infants to devote a greater *proportion* of their available calories to growth. This "Calorie Push" Hypothesis is explored in greater detail in Chapter 7.

Unfortunately, the interpretation of the results presented in this chapter will remain unresolved until the data can be collected alongside a reliable estimate of milk volume.

Chapter 5: Milk immunofactors and infant illness[1]

Introduction

Background and hypotheses

Breast milk is known to provide several health and developmental benefits to infants in a variety of environments. These benefits include lower rates of infectious illness (reviewed in Heinig and Dewey 1996), especially with regards to infant respiratory and gastrointestinal illness. In affluent developed nations, breastfeeding is associated with a reduced incidence and shorter duration of lower respiratory infections (Cushing et al. 1998). In this setting, breastfed children also benefit from a reduced risk of allergies (reviewed in van Odijk et al. 2003) and asthma (Dogaru et al. 2014). The protective role of breastfeeding is even more evident in developing countries, where infectious disease mortality is a greater threat to infants. For instance, one study of infants in a slum in Bangladesh found that infants who were not exclusively breastfed experienced a higher risk of death by respiratory infection and diarrheal illness (Arifeen et al. 2001). The latter finding was reinforced by a recent meta-analysis of infant deaths attributable to diarrheal illness in the developing world which found a much higher relative risk of death to infants who were not exclusively breastfed during the first 6 months of life and at least

[1] A version of this chapter has been published as Breakey AA, Hinde K, Valeggia CR, Sinofsky A, Ellison PT. 2015. Illness in breastfeeding infants relates to concentration of lactoferrin and secretory Immunoglobulin A in mother's milk. *Evolution, Medicine, and Public Health* 2015(1): 21-31. Copyright 2015, The Authors. Reprinted with permission.

partially breastfed until two years of age (Lamberti et al. 2011). The mechanisms underlying these effects are not completely categorized, but bioactive immunofactors in breast milk are clearly involved.

A handful of studies have investigated the protective role of milk immune factors against infant diarrheal illness *in vivo*. Walterspiel and colleagues reported that, among infants infected with *Giardia lamblia*, those infants who received higher concentrations of anti-*Giardia* specific sIgA from milk experienced fewer episodes of diarrhea, though there was no difference in the total concentration of sIgA between the milk of infants who were infected and those who were not (Walterspiel et al. 1994). A similar finding was reported by Ruiz-Palacios' group: breastfed infants who developed diarrhea specifically as the result of infection by *Campylobacter jejuni* were found to lack sIgA molecules specific to *C. jejuni* antigens, although there was no difference in total (non-specific) sIgA in the milk between breastfed infants who developed *C. jejuni* diarrhea and those who did not (Ruiz-Palacios et al. 1990). Addition of human recombinant lactoferrin and lysozyme to an oral rehydration solution was found to shorten the duration of illness among infants with diarrhea and dehydration in a randomized controlled trial (Zavaleta et al. 1995). Similarly, a study of formula-fed infants found that supplementation with bovine lactoferrin was associated with lower risk of illness, particularly wheeze and lower respiratory tract infections in the first year of life (King et al. 2007). Hassiotou and colleagues published a study of milk composition across lactation in urban Australian women that found that lactoferrin concentration remains unchanged in the event of maternal or infant illness, but sIgA slightly rises (Hassiotou et al. 2013). With the

exception of this single study, infant outcomes in relation to natural variation in milk lactoferrin concentration have not been previously published.

This chapter explores the relationships between the concentration of lactoferrin and sIgA in milk and the experience of gastrointestinal and respiratory symptoms in infants. The immune composition of milk is complicated from a life history perspective, because understanding milk composition requires a consideration of the tradeoffs and investment decisions experienced by both individuals. For instance, if immune compounds in milk are elevated, it could be reflective of investment by the mother in her own somatic maintenance (especially if the compound is passively transported into milk), investment in reproductive effort (if it is expected to help the child survive to reproductive age), and perhaps even investment in the growth of her infant (if the compound helps the infant avoid an illness that might divert resources preferentially to maintenance).

This study was designed to address some of the deficits in our understanding of individual variation in milk bioactive compounds and related infant outcomes. Here we test the hypothesis that natural variation in the concentration of the immune compounds lactoferrin and sIgA in human milk relates to the development of symptoms of illness in infants in an environment with relatively high exposure to pathogens, including gastrointestinal symptoms like diarrhea and vomiting, and respiratory symptoms like cough and mucus. We propose two theoretical frameworks to understand the relationship between immune compounds in milk and symptoms of infection. The **protective** paradigm posits that an initial elevation of immune compounds in milk will serve to fight

infection, resulting in fewer symptoms of illness experienced by the infant. In this paradigm, symptoms of illness and concentration of immune compounds will have a negative relationship. Between mothers, higher levels of a preventive compound could be thought of as a biomarker of a healthy infant, as its elevated concentration in the milk is more likely to predict an infant with no symptoms. In contrast, the **responsive** paradigm posits that infection in the infant will be detected by the mother (through increased environmental exposure to the pathogen, or perhaps via immunological changes in the infant's saliva detected by breast tissue), who will increase the transfer or production of immune compounds in the milk to the infant. In this framework, symptoms of illness and concentration of immune compounds will be positively related. Higher levels of a responsive compound may be thought of as a biomarker of an ill infant, as its elevated concentration in the milk would be more likely to predict an infant experiencing symptoms of illness.

We present these paradigms as logical frameworks rather than strictly temporal explanations, because the timeframe of milk sampling with respect to individual bouts of illness may obscure the temporal dimension of the relationship. Logically, then, a preventive compound is one that is upstream of infant health status, and a responsive compound is downstream. It is possible, and even likely, that the same compound may serve both a protective and a responsive role, especially when considered over an extended period of time. Ultimately, the effectiveness of elevating any individual compound in either a protective or responsive capacity will determine where along the protective-responsive continuum it is primarily categorized.

Methods and variables

Interview and sample collection

Milk samples were collected as described in Chapter 3. Symptoms of illness were assessed in an interview. Mothers were asked if the infant was currently in good health or experiencing any symptoms of illness, and whether he had been ill over the month prior to the interview. Questions were targeted at symptoms of gastrointestinal illness (diarrhea and vomiting) and respiratory illness (cough, cold, mucus). The interview also included questions about maternal health, maternal diet, household demographics, and supplementary feeding of the infant (see Chapter 3).

Statistical analyses

Statistical analyses were conducted using SPSS 21. Generalized estimating equations (GEE) were performed with a binomial distribution, logit link function, and exchangeable correlation structure. GEEs were chosen because they correct for the non-independence associated with repeat measures and allow for a binary outcome variable. The following variables were included as explanatory variables in the preliminary GEE models: log-transformed concentrations of lactoferrin and sIgA, change in concentrations of both compounds from the previous month expressed as a percentage of the previous month's concentration, infant age (continuous), infant sex, maternal parity (continuous), and floor type (dirt or cement) of the infant's home. Infant age, maternal parity, and floor type were never significantly related to infant illness, and were removed from the final models. This resulted in an improvement in the QICc values of our models and these

variables were permanently excluded. The outcome variables were symptoms of infant illness (binary) at different times of interest.

Analyses were conducted for three time periods: symptoms in the month preceding the study visit, symptoms at the time of the study visit, and symptoms in the month following the study visit. (The data about future symptoms of illness was obtained at the following month's interview). Symptoms were further separated into gastrointestinal, respiratory, and total. This resulted in a total of 9 analyses. Significance was set at $p<0.05$. Results presented (Table 5.3) have been corrected for multiple models using the Benjamini-Hochberg false discovery method. Because some of the explanatory variables (monthly changes in immune compounds) relied on having information from previous or subsequent visits, the 110 study visits resulted in n=79 full data points for past and current symptoms, and n=52 data points for subsequent month's symptoms.

Plots of Pearson residuals vs. predicted responses were examined for outliers and heterogeneity. Four of the nine models had one outlier each. Outlying points (those with a residual greater than 3 standard deviations) were removed and analyses rerun. The direction and absolute significance of results at $p=0.05$ did not change when outliers were removed; however, the odds ratios were made more extreme. Because the outliers may represent meaningful variation in the population and do not change the interpretation of results, results presented include all data points. Plots did not reveal a clear heterogeneity of data and histograms of residuals were relatively normally distributed.

Results

All infants enrolled in the study experienced illness during the study period; N=30/30 (100%) infants were reported as having illness at any of their home visits. Of infants with more than 1 study visit, 27/28 (96%) experienced repeated bouts of illness. The incidence of symptoms varied by subtype (respiratory and gastrointestinal) and period of inquiry (symptoms concurrent with study visit or in previous month) (Table 5.1). Both lactoferrin and sIgA showed considerable variation among mothers and across time (Table 5.2). The concentration of both compounds was positively associated with age of the infants in our sample, but age was never a significant predictor of the concentration of either immune factor when included in the full model.

Table 5.1: Reported incidence of symptoms of illness experienced by 30 infants at 110 study visits, categorized by subtype

Infant symptoms	Incidence	Percent of total visits
Respiratory symptoms in past month	43	39.1
Gastrointestinal symptoms in past month	45	40.9
Any symptoms in past month	74	67.3
Current respiratory symptoms	47	42.7
Current gastrointestinal symptoms	12	10.9
Any current symptoms	53	48.2

Table 5.2: Descriptive statistics of milk compounds (n=109 samples)

Compound	Mean concentration	Median concentration	SD concentration	Range of all samples	Range of individual maternal means
Lactoferrin (ug/mL)	2621	2378	1199	864-6581	2240-3948
sIgA (ug/mL)	648	556	379	183-2724	308-855

Concentrations of lactoferrin and sIgA in milk were significantly related to the experience of symptoms of illness in infants both in the month prior to the study visit (Table 5.3) and the subsequent month (Table 5.4). At higher concentrations of lactoferrin, infants were more likely to have experienced illness in the preceding month and more likely to experience illness in the subsequent month (odds ratios >1). This was true when all symptoms were analyzed together, as well as when gastrointestinal and respiratory symptoms were analyzed separately. At higher concentrations of sIgA, infants were less likely to have experienced illness in the preceding month and less likely to experience illness in the subsequent month (odds ratios <1). This was true for gastrointestinal and total symptoms, as well as respiratory symptoms in the preceding, but not subsequent, month. No relationships between the immune composition of milk and symptoms of illness concurrent with the study visit were significant.

Table 5.3: Relationships between milk immunofactors and prior month symptoms of illness

Compound	Symptoms	Odds ratio	95% CI	p
Lactoferrin	Any	13.72	[2.71, 69.27]	0.0060
	Gastrointestinal	61.37	[12.27, 307.05]	0.0045
	Respiratory	9.46	[1.46, 61.44]	0.0342
sIgA	Any	0.03	[0.008, 0.135]	0.0045
	Gastrointestinal	0.02	[0.004, 0.120]	0.0342
	Respiratory	0.15	[0.032, 0.731]	0.0045

Table 5.4: Relationships between milk immunofactors and subsequent month symptoms of illness

Compound	Symptoms	Odds ratio	95% CI	p
Lactoferrin	Any	35.37	[1.65, 759.76]	0.0345
	Gastrointestinal	22.76	[3.75, 137.96]	0.0045
	Respiratory	15.12	[2.19, 104.48]	0.0135
sIgA	Any	0.04	[0.003, 0.515]	0.0293
	Gastrointestinal	0.04	[0.003, 0.478]	0.0293
	Respiratory	0.19	[0.946, 1.035]	NS

Tables 5.3 and 5.4: *Results from generalized estimating equations: relationships between milk immune compounds and symptoms of illness presented as odds ratios for the month preceding the study visit (Table 5.3) and the subsequent month (Table 5.4). The odds ratio represents the multiplicative change in odds of "success" (illness) for a 1-unit increase in the explanatory variable (with everything else in the model held constant). In this case, a 1-unit increase in the explanatory variable is a 1-unit increase in the log-transformed values of the concentrations. All odds ratios associated with lactoferrin are greater than 1, indicating a positive relationship with symptoms of illness in both time periods. All odds ratios associated with sIgA are less than 1, indicating a negative relationship with symptoms of illness in both time periods.*

We calculated intraclass correlation (ICC) and 95% confidence interval for several variables to assess how much variance was attributable to individuals. The ICC [95% CI] for individual mothers' lactoferrin and sIgA values (calculated using a generalized linear model with only maternal identity as a random factor) were 0.68 [0.51, 0.80] and 0.64 [0.48, 0.78], respectively. This indicates that a large portion of the variance in milk composition was attributable to individual mothers. Total monthly infant illness

(calculated using a generalized linear mixed model approach with only infant identity as a random factor) had an ICC of 0.071 [0.002, 0.779], monthly respiratory illness had an ICC of 0.075 [0.002, 0.720], and monthly gastrointestinal illness an ICC of 0.229 [0.056, 0.598]. Less of the variation in infant illness month to month was attributable to individual factors, but these estimates are less precise.

Conclusions and discussion

Consistent patterns emerged for the relationships between sIgA, lactoferrin, and symptoms of illness. Almost all relationships between symptoms during the month prior to the interview and the month following the interview were significant. When significant, the odds ratios for sIgA were always less than 1 and the odds ratios for lactoferrin were always greater than 1. Therefore, with everything else in the model held constant, as sIgA increases, it is associated with lower rates of illness. As lactoferrin increases, it is associated with higher rates of illness. These relationships indicate that there is some predictive power of milk immunofactors as biomarkers, predicting whether an infant is likely to have been healthy or sick in the two months surrounding the sample. Relatively higher sIgA can be thought of as a biomarker for a healthy infant, and higher lactoferrin as a biomarker for a sick infant.

The immune bioactives in breastmilk exhibit contrasting associations with infant illness when considered within the unified protective-responsive model presented in Figure 5.1. The sIgA pattern illustrates the "protective" paradigm, that higher levels of immune compounds in milk will protect the infant from illness. This is consistent with other

studies that found a protective effect of sIgA against diarrheal illness (Walterspiel et al. 1994; Ruiz-Palacios et al. 1990). However, lactoferrin better fits the alternative "responsive" pattern, that illness is associated with an increase in the production or transport of immune molecules to the mammary. While causality cannot be firmly established with the current data, these associations do lend support to the paradigms. These categorizations are not based on the temporal sequence of our own data per se, but from an understanding of the biology of the milk immunofactors and their function. When a positive association exists between an immunofactor and illness, as we see with lactoferrin, it is assumed that illness has induced a rise in the compound, rather than the less intuitive interpretation that lactoferrin is causing symptoms of illness. Similarly, when we see a negative association between a compound and illness, as we do with sIgA, we infer a protective role, because the other interpretation (that infant illness suppresses maternal production of sIgA) makes less biological sense. These models are presented as logical frameworks rather than strictly temporal explanations, because the timeframe of milk sampling and illness may obscure the temporal dimension of the relationship. Logically, then, a preventive compound is one that is upstream of infant health status, and a responsive compound is downstream.

Figure 5.1: The Protective-Responsive Model of Milk Immunity

Figure 5.1: *A schematic diagram of the Protective-Responsive Model of milk immune factors and infant illness. Milk is a complex bioactive fluid with the potential to protect against illness in the infant as well as to respond to illness with an adjustment of immune composition. This conceptual figure integrates the findings of the present study; sIgA follows the protective pattern, while lactoferrin exhibits a responsive role.*

Some findings were unexpected. For instance, it is not immediately clear why symptoms of illness experienced at the time of the study visit were never significantly associated with milk sIgA or lactoferrin. It may be due to the small sample size (110 study visits from 30 infants), with a relatively small proportion of infants exhibiting symptoms of illness at any given time. There may also be a delay between the onset of symptoms and the adjustment of milk composition. In addition, the recent findings by the Hassiotou group—that maternal or infant illness is associated with a rise in sIgA and no change in lactoferrin concentration (Hassiotou et al. 2013)—are seemingly in contrast to the results reported here. However, in their study maternal infections were analyzed together with infant infections, and maternal infection was more strongly related to increases in milk leukocytes and humoral immunity compounds, including sIgA. Because of this and because of the different time scales of inquiry, it is not clear that these results are necessarily in opposition. Additionally, as their study followed urban Australian women

(who are assumed to have access to more hygienic conveniences), the different patterns that emerge may be related to the very different pathogenic environments experienced by our study populations.

It will be crucial to integrate maternal illness data into future studies to further our understanding of these relationships. Because maternal and infant exposure is expected to be strongly positively correlated and concurrence of infection and symptoms high, functional explanations about the adjustments of milk composition to suit the needs of the infant are incomplete without also addressing the needs of the mother. If it is true that maternal and infant exposure to illness are positively correlated, it would be difficult to figure out *why* milk composition is altered; that is, to separate whether changes in milk immune composition were passively reflecting maternal circulating values, whether they were elevated to protect the mammary from infection, or whether the adjustments were targeted directly toward supporting infant health. This could be addressed by collecting maternal blood samples to investigate the correlation between maternal circulating values and milk values, the relationship between elevated circulating immunofactors and length of maternal and infant illness, and testing whether elevated milk immunofactors predict a reduced risk of mastitis.

The first evolutionary explanation that was investigated in an attempt to explain the opposite relationships that sIgA and lactoferrin exhibit with symptoms of illness is the idea that there may be a cost to the infant if lactoferrin is prophylactically elevated, perhaps related to its role in iron regulation. Whereas elevated sIgA may always provide

a net benefit to the infant, elevated lactoferrin may result in a cost to the infant, such that mothers modulate lactoferrin secretion into milk to reflect current immunological needs of the infant. However, this explanation does not seem to be supported by the existing literature on this topic. Iron-saturated lactoferrin enhances the proliferation and differentiation of intestinal epithelial cells in vitro (Oguchi et al. 1995). In addition, formula-fed infants who receive supplemental bovine lactoferrin were found to experience fewer lower respiratory tract infections and had higher hematocrits (indicating better iron status) at 9 months than those fed unsupplemented formula (King et al. 2007). This study also reported a near-significant trend toward greater weight gain during the first 6 months of life for the lactoferrin-supplemented infants, similar to the findings of an earlier study in which lactoferrin supplementation was associated with significantly greater gains in height and weight in formula-fed infants (Hernell and Lönnerdal 2002). Importantly, the infants in these studies were supplemented with bovine lactoferrin, and the infants were fed a dairy-based formula instead of human milk. Despite these caveats, it seems reasonably likely that higher lactoferrin is associated with many beneficial outcomes for the infant, weakening this particular argument as a potential explanation for the diverging patterns of sIgA and lactoferrin.

We cannot definitively explain the significance of the different patterns of sIgA and lactoferrin with the data from the present study, but we offer two potential explanations to guide future work. One compelling explanation for the divergence is that there are different costs to the mother, the infant, or both associated with the production and ingestion of these two compounds. For this to be the case, given our findings, we would

expect that lactoferrin would be more costly to produce, or have more costly consequences associated with elevation, than does sIgA, and would therefore only be elevated when an infant experienced an illness and required additional lactoferrin to help fight the infection. Another possibility is that the lactoferrin and sIgA proteins have different functional lifespans, or control of production is regulated on different time spans. If lactoferrin is degraded or excreted more quickly, raising lactoferrin in an effort to prevent illness would be an ineffective or inefficient strategy, whereas responding to an existing infection with a rise in lactoferrin might be more effective. One piece of evidence to support the idea of differently-timed regulation is that sIgA in milk is produced by maternal B-cells, which migrate to the mammary gland and release IgA into the secretory epithelium to be converted to sIgA and released into milk (Brandtzaeg 2003). This process of migration and release is able to proceed more or less continuously, whereas lactoferrin has to be transcribed in mammary epithelial cells and may be more susceptible to the presence of pathogens, cytokines produced by other immune compounds, or transcription factors.

Our data have some limitations that prevent us from extending the protective-responsive model beyond its current logical framework into a more causal temporal understanding of the dynamics of milk immune composition. This is highlighted by the fact that the relationships between illness and milk composition that we uncovered are the same going forward and backward—i.e. milk immunofactors have similar associations with past and future illnesses. This would not be expected from a strictly temporal interpretation of these relationships and can probably be attributed to a number of factors relating to our

methodology. The biggest limitation is the one-month sampling regimen. Mothers were asked to recall whether their infants had experienced any of a number of symptoms in the previous month, or since the previous study visit. In addition to problems of self-report and recall, this led to symptoms experienced up to four weeks prior receiving the same statistical weight as more recent or ongoing symptoms. There is also the concern that certain infants may simply be sick more often than others, or may take longer to clear infections. A related concern is that certain mothers may produce consistently high or low concentrations of milk immunofactors (this appears to be true given the fairly high ICC values for sIgA and lactoferrin for individual mothers). Combined with the long sampling interval, these chronically ill infants or their chronically high-producing mothers may be obscuring the nuances of the temporal dynamics of the adjustment of milk composition with respect to an individual bout of illness. As an example, if an infant is ill both in the month preceding and the month following a given study visit, then it may be more likely that an immune factor will have a similar relationship to illness in both time periods than if the infant is only falls ill during one of the months. Similarly, many study visits may have fallen in the middle of a long bout of illness, which could inappropriately be classified as two separate bouts.

The protective-responsive model advanced in this article lends itself to the construction of explicit temporal predictions about the role and timing of lactoferrin and sIgA in symptoms of illness in infants. A causal temporal understanding of the appearance and duration of these compounds in milk would be an interesting avenue for future work. A more fine-grained measure of infant illness is necessary, including daily experience of

symptoms and perhaps integrating circulating immune factors such as CRP. Combined with daily milk samples, these data would reveal the temporal dynamics of the adjustment of milk composition with respect to an individual bout of illness. This should result in a clearer picture of whether lactoferrin and sIgA are correctly classified as primarily responsive and protective, respectively.

If lactoferrin is indeed responsive to illness, rather than protective, another interesting area for future research would be to determine whether this is related to a cost to the mother or infant of persistently elevated lactoferrin, or due to some sort of biological constraint, such as a short half-life in the infant's digestive tract. An animal study in which infants are given milk enriched with lactoferrin, sIgA, or both and then exposed to a gastrointestinal pathogen would be an interesting first step to determine whether lactoferrin is even capable of preventing infection, and on what timescale.

Finally, the infants recruited to the present study had a mean age of 244 days at the first study visit, all received supplemental foods, and nearly all received dairy products over the course of the study. Future studies should investigate the strength and direction of the relationships presented here among younger, exclusively breastfeeding infants, and the way these relationships change with the introduction of supplementary food, crawling, and mouthing behaviors, all of which might increase pathogen exposure.

Chapter 6: Bioactive components in milk and infant growth

Background and hypotheses

In infancy, the major competing demands for energy are growth and maintenance. The previous chapter discusses one of the ways in which human milk may attenuate maintenance costs incurred by infants—through the provision of bioactive compounds that reduce the likelihood or severity of infection or symptoms of illness. One important consequence of a reduction in energy spent on maintenance may be an increase in the relative energy budget available to support growth. This chapter explores a variety of relationships between maternal, infant, and milk variables as they relate to infant rate of growth.

Milk energy and infant growth

Clearly the most important role of milk in supporting growth is purely nutritional; breast milk provides all of the calories necessary to support infant growth for at least the first six months of life. Available milk energy is a product of two variable qualities in milk: milk yield volume and milk energy density (Hinde 2009). Infants who consume a higher volume of milk consume more calories and therefore have a larger energy budget to allocate to growth and maintenance. Milk can also vary in its nutrient density. Of the three macronutrients (fats, proteins, and carbohydrates), fat is most likely to vary significantly between mothers and across time (Mitoulas et al. 2002). As fat contains the

highest number of calories per gram of the macronutrients, this variation can result in substantial differences in milk energy density.

Milk volume exerts a strong effect on infant growth in humans and other primates. In an experimental study of energy restriction in baboon mothers, infants whose mothers were fed at 60% of *ad libitum* intake were receiving 37% less milk (of similar quality) by the end of the study than infants of mothers fed *ad lib.*, and their growth rate was reduced by 10% (Roberts et al. 1985). Though data are not presented, Prentice's group reports that human infants born during the dry season in Gambia, when maternal energetic conditions are more favorable, experience faster growth relative to standard weight-for-age growth curves in the immediate prenatal period than infants born during the wet season (Prentice et al. 1981a). In this study, milk volume was reduced by 36% during the wet season while energy density of the milk was essentially unaffected, suggesting that volume of milk consumed is a significant contributor to growth of human infants. A study of American infants likewise found growth rate to be positively associated to volume, but not energy density, of milk consumed (Butte et al. 1984). This study found that milk volume accounted for up to 30% of the variability in weight gain of infants.

There is mixed evidence about the role of milk energy density on infant growth in humans and other primates, but most seems to suggest that it does not contribute as powerfully to variation in infant growth as milk volume. For instance, a study of Australian infants found no relationship between infant growth rate and the amount or concentration of fat, protein, or lactose in milk (Mitoulas et al. 2002). In one study of

rhesus macaques, gross energy of milk was not associated with infant weight (Hinde et al. 2009), though in another study, available milk energy (the product of energy density and milk yield volume) was a better predictor of infant mass than yield alone, suggesting some contribution of energy density to variability in infant mass (Hinde 2009). Human infants receiving milk that was relatively high in carbohydrates and low in lipids and protein grew less during the first 6 months of life than those receiving milk of higher quality and higher energy density (Qian et al. 2010).

Unfortunately, it was not possible to measure volume and milk energy density in the present study (see Chapter 3 for discussion). While I will therefore be unable to compare Toba infant growth to other populations in these respects, it is important to understand how these factors may influence growth to aid in cautious interpretation of the results presented below.

Milk bioactive compounds and infant growth

While nutritional factors in milk drive the bulk of infant growth, the effect may be modified directly or indirectly by milk bioactives. For instance, infants who consumed milk with higher concentrations of leptin were leaner (Fields and Demerath 2012).

With respect to the compounds under investigation in the present study, there has been very little research into their relationships with infant growth. One study of rhesus macaque infants found that higher milk cortisol at peak lactation was positively associated with infant growth (Hinde et al. 2015) and higher maternal cortisol was also

positively related to infant growth rate in red squirrels (Dantzer et al. 2013). In this chapter, I test the hypothesis that cortisol and monthly changes in cortisol will positively associate with growth rate in human infants as well. Alternative hypotheses are presented in Chapter 2.

Circulating insulin-like growth factor 1 (IGF-1) is an important promoter of growth, particularly growth in height. IGF-1 promotes growth both independently and synergistically with growth hormone (Laron 2001). Circulating IGF-1 in very low birth weight infants positively correlated with lower leg growth velocity in the immediate postnatal period (Kajantie et al. 2002). IGF-1 also has a strong trophic effect on human infant intestinal cells (Hirai et al. 2002). Premature infants fed formula supplemented with IGF-1 displayed reduced gut permeability as compared to unsupplemented infants (Corpeleijn et al. 2008). Milk IGF-1 may therefore support infant growth indirectly by promoting intestinal integrity and nutrient absorption. For this reason, I hypothesize that the concentration of IGF-1 will positively associate with infant growth rate. Alternative hypotheses are presented in Chapter 2.

There are few studies investigating the relationship between immune factors in human milk and infant growth. One study found that milk IgA was positively related to upper arm fat area of Ariaal infants (Miller 2011). When this regression was controlled for the amount of fat in the milk sample, this effect disappeared and new associations surfaced. Fat-corrected milk IgA concentration correlated positively with height- and weight-for-age, meaning that larger infants received more sIgA per gram of fat from their mothers.

However, these analyses did not control for volume. As discussed above, one way in which immune compounds have the potential to influence infant growth is through the attenuation of symptoms of illness. I therefore hypothesized that the concentration of sIgA and lactoferrin would positively associate with infant growth rate. Alternative hypotheses are presented in Chapter 2.

Infant illness and growth

The relationship between illness and growth in infants has been studied in several populations where children experience high pathogen exposure. Many of these studies have found that illness is associated with reduced growth. Early work in Guatemala found infants experiencing infectious disease were more likely to lose weight and stop growing in height (Mata et al. 1977). A recent study by Panter-Brick's group found elevated immunoglobulin G (IgG) in Nepalese children to be associated with a reduced weight-for-age Z score (Panter-Brick et al. 2009). A similar cost to immune activation was found among 2-4 year olds in lowland Bolivia; those children who had elevated C-reactive protein (an acute phase immune protein) at baseline grew less over the subsequent three months (McDade et al. 2008). I hypothesized that experiencing symptoms of illness would be associated with a reduction in rate of growth.

Maternal condition and infant growth

A mother's own energetic condition may also be expected to influence the growth of her infant. The variables chosen to represent maternal energetic condition are body mass index, monthly change in body mass index, C-peptide of insulin, and parity. I made

several predictions about maternal condition and infant growth that all start with the assumption that mothers in better energetic condition will support a faster rate of growth in their infants.

Body mass index is an estimate of energetic status at a single point in time. Higher BMI generally indicates more stored energy. Monthly *change* in BMI was chosen as an estimate of energy balance. A positive change in BMI indicates that energy is being stored, whereas a negative change in BMI indicates that energy is being mobilized. I therefore hypothesized that BMI and monthly change in BMI would positively associate with infant growth rate.

C-peptide is a protein that is produced in a 1:1 ratio with insulin. In general, higher insulin production indicates an energy storage state. However, individuals differ in their sensitivity to insulin. For this reason, a maternal average C-peptide value across the study period was calculated and individual values were adjusted to reflect their position relative to this average. Furthermore, there is evidence that insulin dynamics provide a stronger physiological signal than do static insulin values (Valeggia and Ellison 2004). C-peptide concentration was also expressed as a percentage change from the previous month's value to account for this. I hypothesized that monthly change in C-peptide and C-peptide relative to maternal average would positively associate with infant growth rate.

Lactation is very energetically costly and is associated with depletion of maternal fat stores (Prentice 1980; Miller et al. 1994). Increasing parity is associated with long-term

depletion of maternal energy reserves in some energy-limited populations (Shell-Duncan and Yung 2004; Tracer 1991; Little et al. 1992). In adequately-nourished populations, BMI tends to increase with increasing parity. Importantly, though, fat is redistributed away from the gluteofemoral region (where it is most accessible to support infant growth during late pregnancy and lactation) and increases in central fat depots—a phenomenon termed "covert maternal depletion" (Lassek and Gaulin 2006). However, according to the terminal investment hypothesis, investment in individual offspring is predicted to increase toward the end of a woman's reproductive career, as the potential for future reproduction diminishes. I hypothesized that parity would negatively associate with infant growth rate as a result of maternal depletion, but suspected that this relationship might be tempered or eliminated as a result of increasing investment in later offspring.

Variables and statistical methods

Milk variables

Concentrations of compounds in milk were log-transformed before analysis. Monthly changes in these compounds are expressed as the percent change incurred in the non-log transformed concentration of the initial value. Full laboratory methods are described in Chapter 3.

Maternal condition variables

Monthly change in C-peptide was expressed as the percent change incurred in the non-log transformed concentration of the initial value. Difference from maternal average C-peptide value was calculated by adding each month's log-transformed C-peptide value,

dividing by the number of study visits, and subtracting each month's value. Full laboratory methods are described in Chapter 3. BMI was calculated at each study visit and monthly change in BMI was calculated by subtracting each month's BMI from the subsequent month's BMI. Parity was self-reported by mothers and is assumed to represent the number of live-born offspring.

Infant illness

Infant illness was coded as a binary variable. Any experience of symptoms by the infant in the month prior to each study visit (as reported by the infant's caretakers) resulted in a coding of 1; infants who were symptom-free received a code of 0. A full description of interview methods, including prompts given about infant illness, can be found in Chapter 3.

Calculation of infant growth rates

Infant growth rates were calculated from raw measures of length (in cm.), mass (in grams), head circumference (in cm.), and arm circumference (in cm.) at each study visit. The change that occurred in each of these measures between study visits was the "growth" experienced, and the growth was divided by the number of days between measurements for the growth rates in centimeters per day and grams per day. The values in cm./day were multiplied by 100 to obtain results in mm./day, for ease of interpretation.

Measurement error resulted in 6 instances where monthly linear growth appeared to be negative. Some of these measurements occurred within a week of another measurement

event of the same infant by other researchers; in 2 of these cases, the value obtained by the other researchers resulted in a positive linear growth and that value was substituted for the previous value (with the number of days between measurements adjusted accordingly). The other 4 values had resulted in a total linear growth loss of 0.5cm, so 0 was substituted.

There were 9 instances where similar measurement error occurred with head circumference measures. Three of these values resulted in a circumference loss of 0.5 cm, and were situated between 2 identical values 0.5 cm higher. The problem value was therefore adjusted to be the same as the months surrounding it, resulting in 2 months of 0 cm. growth. Two of these measurements occurred within a week of another measurement event of the same infant by other researchers; in both of these cases, the value obtained by the other researchers resulted in a positive change in head circumference and that value was substituted for my previous value (with the number of days between measurements adjusted accordingly). Two values had a loss of 0.5 cm. and were replaced by 0. Two values had a loss of greater than 0.5 cm. and were removed entirely.

Arm circumference and mass were allowed to experience a negative monthly change.

Creation of time-standardized growth for length and mass

Some analyses used length and mass attained over a period of several months. These variables were constructed differently.

Length

Total growth in centimeters was calculated for each infant by subtracting length at his first study visit from length at his final study visit. This value was divided by the number of days between his first and final study visits, and multiplied by 115 days, the median number of days enrolled for all infants who had at least 2 study visits (n=28).

A subset of these data was created for infants who had 4 or 5 study visits (n=22). For consistency, the 5th visit was ignored for applicable infants and only the first 4 months of data were used. These values were standardized to 115 days, the median number of days in these approximately 4-month intervals.

Mass

Mass in grams was calculated using the same method described above for length.

Statistical analyses

Generalized estimating equations (GEEs) were chosen because they correct for the non-independence associated with the repeat measures found in this data set. Histograms of residuals were examined after analysis to check for outliers. In addition, scatterplots of these residuals versus the mean predicted value from each model were examined for outliers and heterogeneity. Three individuals had much lower growth than expected in the analyses investigating milk composition and linear growth and were withheld from those analyses. The Benjamini-Hochberg false discovery method was used to adjust p-

values for multiple comparisons within families. Significance was set at $p<0.05$, but some marginally significant results ($0.05<p<0.10$) are presented below.

Results

Basic correlates of growth

Average rates of growth and other descriptive statistics of the growth rates are shown in Table 6.1.

Table 6.1: Descriptive statistics for rates of growth in length, mass, head circumference and arm circumference for all infants

	Linear (mm/month)	Mass (g/month)	Head circumference (mm/month)	Arm circumference (mm/month)
Mean	156	244	56	13
Median	140	171	39	0
5th Percentile	0	-333	0	-123
95th Percentile	374	903	205	128

Table 6.1: *Values are not corrected for repeat measures of the same infants.*

Before testing the specific hypotheses related to infant growth, simple analyses were performed with the goal of better characterizing the patterns of growth of the infants in this study. First, I used GEEs to test whether there were relationships between the age and sex of the infants and their rates of growth. GEEs were chosen over simple linear regression because they correct for repeated measurements from the same individuals.

Infant age is significantly negatively associated with rate of growth in length, mass, head circumference, and arm circumference (Table 6.2). Younger infants grow at a faster rate

than older infants. Because infant age is significantly related to all four categories of growth, age was included as a covariate in every growth model.

Table 6.2: Relationships between infant age (in days) and rates of growth

	B	p
Linear growth rate (mm/day)	-.006	.022
Mass growth rate (g/day)	-.043	.001
Head circumference growth rate (mm/day)	-.007	<.001
Arm circumference growth rate (mm/day)	-.005	<.001

Table 6.2: *All relationships between infant age and rates of growth were significant and negative. B can be interpreted as the reduction in growth rate (in mm/day or g/day) as age increases 1 day.*

Sex of the infant is not related to rate of growth in length, mass, head or arm circumference (Table 6.3). However, before p-values were adjusted to account for multiple comparisons, sex was marginally related (at p=.074, with infant age in the model) to the rate of arm circumference growth, with females exhibiting a faster rate of growth. Alternatively, since arm circumference may experience a negative change in a given month, this might mean that male infants were more prone to *losing* arm fat than were females. Although this finding did not retain significance with the p-value adjustment, sex was included as a factor in models involving arm circumference, and additional tests were performed for some analyses involving arm circumference to see whether male and female infants differed.

Table 6.3: Relationships between infant sex and rates of growth

	B	p
Linear growth rate (mm/day)	-.772	.322
Mass growth rate (g/day)	-1.812	.788
Head circumference growth rate (mm/day)	.029	.930
Arm circumference growth rate (mm/day)	.535	.296

Table 6.3: *All models include infant age as a covariate. Reference group is male infants; a significant p-value indicates that B is significantly different between males (for whom B is set to 0) and females. B can be interpreted as the average difference in growth rate (in mm/day or g/day) for female infants compared to males.*

Intra-Infant Patterns of Growth

To better understand the patterns of growth in these infants, some simple analyses were performed to assess how the different types of growth relate to each other. The simple R^2 values of the linear relationships between linear, head circumference, mass, and arm circumference growth rates are shown in Table 6.4.

Table 6.4: R^2 values of the linear relationships between different rates of growth across infants

	Linear	Head	Mass	Arm
Linear	-	0.126	0.050	0.000
Head	-	-	0.076	0.082
Mass	-	-	-	0.136
Arm	-	-	-	-

Table 6.4: *These relationships do not account for repeat measures. Age of the infant is somewhat inherently controlled for by the nature of the data; in each comparison, both measures of growth rate that form one data point come from the same infant at the same age.*

In addition, GEEs were used to assess the predictive value of linear and mass growth rates on all others. All models included age of the infant as a covariate, and those involving arm circumference also included infant sex as a factor. Growth rate in mass

was found to be a significant predictor of linear growth rate [B=.079, p=.0045] and arm circumference growth rate [B=.053, p=.003], but not head circumference growth rates. Linear growth rate predicts head circumference growth rate [B=.159, p=.03] but not mass or arm circumference growth rate.

In general, those measures of growth that reflect skeletal development (linear and head circumference) are related, and mass and arm circumference, which reflect adiposity (among other things) are related. Growth in mass predicts growth in length, but not vice versa. The infants who gained weight were likely to grow in height as well, but those who grew in height did not necessarily gain weight.

Considering male and female infants together, arm circumference growth rate does not predict concurrent linear growth rate [B= -.154, p=.319] or subsequent linear growth rate [B=-.105, p=.529] in these infants. However, adding an interaction term to the model (sex*arm circumference growth rate) reveals that there is a different relationship for males and females between arm circumference growth rate and linear growth rate in the subsequent month. The relationship for male infants is negative and significant (B=-.491, p=.012); for female infants, the relationship is positive but not significant (B=.209, p=.283). This suggests that males may be mobilizing stored fat to fuel linear growth, although it is unclear why this occurs in the month prior to linear growth instead of concurrently. Arm circumference is an imperfect measure of adiposity. Furthermore, measurements were taken a month apart, so if the effect were happening on a shorter time scale, it may appear concurrent as growth is aggregated over the month.

Milk composition and infant growth

In this section, I examine the relationships between the four milk compounds of interest (cortisol, IGF-1, lactoferrin, and sIgA) and rate of infant growth in length, mass, head circumference, and arm circumference.

I tested the relationship between the concentration of each milk compound at each visit and growth rate in each category during the month following the study visit. I also tested whether the percent change exhibited in the concentration of each compound every month related to growth rates over the same month and the subsequent month. P-values were adjusted for multiple comparisons among all static concentrations for all growth rates (12 models), all models where growth was concurrent with changes in milk compounds (12 models), and for all models where the changes in milk compounds preceded growth (12 models).

Cortisol

Neither cortisol concentration nor monthly changes in cortisol were related to any measure of growth. The relationship between cortisol and arm circumference growth rate did not differ by sex.

IGF-1

Log-transformed concentration of IGF-1 was significantly positively related to linear growth rate (B=1.492, p=.016) in the month following the visit. Each 10% increase in IGF-1 is associated with a 0.1492 mm/day increase in linear growth rate.

Percent change in IGF-1 concentration was significantly negatively related to head circumference growth rate (B= -.003, p=.051) and arm circumference growth rate (B= -.006, p=.004) over the same month but not to linear, mass, or arm circumference growth rates. Effect sizes are small; they are presented in Table 6.5 (in mm/month for ease of interpretation).

Lactoferrin

Neither lactoferrin concentration nor monthly changes in lactoferrin were related to any measure of growth. The relationship between lactoferrin and arm circumference growth rate did not differ by sex.

sIgA

Concentration of sIgA was never significantly related to any measure of growth rate. The relationship between sIgA and arm circumference growth rate did not differ by sex.

Percent change in sIgA was negatively related to linear growth rate (B= -.034, p =.024) and head circumference growth rate over the same month (B= -.004, p=.051) but not to arm circumference or mass growth rates. Effect sizes are presented in Table 6.5 (in mm/month for ease of interpretation).

Table 6.5: Effect sizes of monthly change in milk compounds on linear and head circumference growth rates

	Percent increase (magnitude of change in concentration)			
	1%	**10%**	**25%**	**50%**
Linear (mm/month)				
ΔsIgA	-1.02	-10.2	-25.5	-51
Head circumference (mm/month)				
ΔIGF-1	-0.09	-0.9	-2.25	-4.5
ΔsIgA	-0.12	-1.2	-3	-6
Arm circumference (mm/month)				
ΔIGF-1	-.18	-1.8	-4.5	-9

Table 6.5: *The values in the table can be interpreted as the average reduction in growth rate associated with the corresponding increase in concentration of the milk compounds. Only statistically significant relationships ($p<0.1$) are shown.*

Because all of the significant relationships between monthly change in milk compounds and growth rate were so similar, I wanted to make sure that they were not an artifact of the data structure and statistical test. Specifically, I tested whether there was an interaction between starting concentration of the milk compound and the change exhibited in the concentration of the compound over the following month. A significant negative interaction would mean that a high starting concentration is more likely to predict a decrease over the following month (because "high" is a relative term within a limited data set, this is a reasonable assumption). If this were the case, it might be that the high starting concentration was responsible for the effect and the negative relationship between monthly change and growth would be a statistical artifact. However, none of the interactions between milk compounds and subsequent changes were significant.

Another more likely explanation is that an increase in milk volume is responsible for driving a decrease in the concentration of milk compounds from month to month. If this

is the case, the increasing milk volume, and not change in production or transfer of these compounds into milk is driving the increase in infant growth and the negative correlation of these compounds with indices of growth is a side effect. While this may be true for sIgA, IGF-1 typically displays a positive association with milk volume (Breier et al. 1993; Prosser et al. 1989).

Infant illness and growth

In this section, I examine the relationships between symptoms of illness experienced by the infant in a given month and growth rate (length, mass, head, and arm circumference) over the same month and over the subsequent month.

There were no significant relationships between illness and any measure of infant growth rate, either concurrently with growth or with illness preceding growth.

While there was no significant effect overall of illness on infant growth, I hypothesized that those infants who were ill but mobilized stored energy (fat) would grow more in length than those infants who were ill but did not mobilize fat. While it cannot be confirmed that negative changes in mass and arm circumference represent fat mobilization, they are the best measures available in the current data set to test these hypotheses. I tested the effect of the interactions between illness and mass and arm circumference growth rates on linear growth rate. After adjusting for multiple comparisons (8 models), there were no significant interaction effects between any measure of infant adiposity and illness on linear growth rate.

Another way to test this effect is to simply look at the interaction between arm circumference (controlling for age) and illness on linear growth rate. Using this method actually does show an important difference in infants who have more upper arm fat. At the mean infant age, those infants who were sick had a significant positive association between arm circumference and linear growth rate in the month following illness (B=.584, p=.014) whereas healthy infants had a (marginally significant) negative association (B= -.869, p=.070). This finding suggests that body fat may play an important role in buffering infant linear growth in the event of illness. This finding echoes the work of McDade and colleagues in older children, who found that elevated CRP impaired the growth of children with lower body fat (McDade et al. 2008).

Maternal energetic condition and infant growth

In this section, I examine the relationships between several maternal variables and infant growth rate in length, mass, head circumference and arm circumference. The maternal variables tested in this section are body mass index (BMI), monthly change in BMI, monthly change in C-peptide of insulin, difference from an individual mother's own average value of C-peptide, and parity.

Body mass index

No infant growth rate variables were related to maternal BMI. BMI at the first study visit and infant growth in length and mass over the entire study period were also not

significantly related. The relationship between maternal BMI and infant arm circumference growth rate did not differ by sex.

Monthly change in body mass index

As mothers gained weight, the infants were also gaining weight. Monthly change in maternal BMI was strongly positively related to mass growth rate of the infant (B=3.182, p=.008). For each unit that maternal BMI increased from the previous month, infant mass growth rate increased 3.2 g/day (or about a 96 g/month increase) over the same time period. For example, a 160-cm. (5'3") woman weighing about 60 kg. has a BMI of 23.5. If she gained 2.7 kg in a month, her BMI would increase 1 unit, to 24.5. Her infant, on average, would gain an additional 96 grams that month.

The relationship between change in maternal BMI and infant arm circumference growth was tested separately for male and female infants through the inclusion of an interaction term. There was a significant interaction between infant sex and maternal ΔBMI ($p<.001$). Males had a significant positive relationship between maternal ΔBMI and rate of arm circumference growth (B=.963, p=.001) and females had a significant negative relationship (B= -.796, p=.019).

There was no relationship between change in maternal BMI and head circumference or linear growth rates.

Difference from maternal average urinary C-peptide of insulin

Urinary C-peptide concentrations that were higher or lower than a mother's own personal average were not associated with any infant growth rate variables. The relationship between maternal relative C-peptide concentration and infant arm circumference growth rate did not differ by sex.

Monthly change in urinary C-peptide of insulin

There was no relationship between maternal monthly change in urinary C-peptide of insulin and any measure of infant growth. The relationship between maternal monthly change in C-peptide and infant arm circumference growth rate did not differ by sex.

Parity

Parity is negatively related to infant linear growth rate. Considering the first 4 months of growth for all infants who were studied for 4 or 5 months (n=22), maternal parity was significantly negatively associated with the rate of growth in length that occurred during the study period. Children with more older siblings grew more slowly (B= -.236 [-.463, -.008], p=.042). For each additional older sibling, the infant attained .236 cm. less linear growth over a 4-month period.

When modeled as a continuous variable, there was no significant relationship between parity and growth rate in mass. However, infants of primiparous mothers grew more over the 4 month study period than the infants of multiparous mothers. Controlling for infant age and mass at the initial study visit, first-born status was associated with an average of

820 additional grams of mass (95% CI [148, 1492], p=.033) and 2.1 additional centimeters of height (95% CI [0.147, 4.035], p=.035) gained in 4 months.

Conclusions and discussion

This chapter explores factors relating to infant growth (rates of linear growth, growth in mass, head circumference, and arm circumference). Explanatory variables tested include concentrations of the four milk compounds, whether the infant had experienced symptoms of illness, and maternal condition variables.

Milk composition

It is important to remember that hormone levels are only part of what determines hormonal action. For a complete understanding of the pathways discussed, it would also be necessary to measure receptor density on the infant tissues of interest. In addition, almost all of the infants in the study received supplemental dairy foods. It is unknown how this may have affected their growth or endogenous hormone production, particularly with regard to IGF-1. Finally, the ages of these infants span a very large range. There may be a critical window for any of these compounds to affect growth that is missed by this diverse sample.

If milk volume is a major factor in determining growth rate, and these compounds are diluted by high milk volume, we might naively expect to see a negative association between all milk compounds and growth. As previously described, though, this dilution effect is unlikely for IGF-1 and cortisol. In fact, the only significant relationship between

concentration of a milk bioactive and infant growth rate was a positive association between IGF-1 and linear growth. Given that IGF-1 is predicted to positively associate with milk volume (Breier et al. 1993; Prosser et al. 1989), this finding is consistent with the possibility that milk volume is a main source of variation in infant growth rate.

Cortisol

Cortisol concentration was not associated with any measure of infant growth rate. It was predicted that cortisol would be positively associated with growth rate (particularly mass) following the findings of Hinde's and Dantzer's groups (Hinde et al. 2015; Dantzer et al. 2013). It could be that cortisol is not a salient growth-promoting signal in human infants the way it is in rhesus macaque and red squirrel infants. It is also possible that this relationship exists in humans but is somehow being obscured by an interaction with milk energy. This putative relationship is explored in more detail in Chapter 7.

It was also hypothesized that cortisol may influence growth through a change in behavior. Because no overarching relationship between cortisol and growth was found, these hypotheses were not supported. However, milk cortisol may well have had interesting relationships with the behavior and temperament of these infants that were not measured.

IGF-1

IGF-1 was predicted to positively associate with infant growth rate, and it did display a positive relationship with linear growth rate. This effect may be driven by milk volume. Because IGF-1 and milk volume are positively correlated (Molento et al. 2002; Breier et

al. 1993; Prosser et al. 1989), it is possible that IGF-1 is driving an increase in milk yield which is responsible for the accelerated linear growth rate. Following this explanation, IGF-1 concentration would have no independent effect on growth at all. This hypothesis could easily be tested in a future study integrating 24-hour milk volume.

Alternatively, IGF-1 may have improved intestinal integrity and nutrient absorption, resulting in more efficient digestion and a relatively larger energy budget in infants receiving higher concentrations. These hypotheses are still interesting and viable, and certainly worthy of future study. A dual-sugar challenge (Nathavitharana et al. 1988) would be a good way to determine whether milk IGF-1 is associated with gut permeability. However, these explanations do not account for the finding that only linear growth was significantly related to IGF-1. If the mechanism leading to improved growth were simply milk volume or nutrient absorption, it seems likely that other measures of growth, particularly mass, would show a similar association.

It is tempting to interpret this finding as a direct contribution to linear growth by IGF-1 in milk. However, animal studies of radiolabeled IGF-1 have shown that less than 1% of orally consumed IGF-1 enter circulation (Vacher et al. 1995; Hammon and Blum 1997**;** Philipps et al. 1995; Burrin 1997; Donovan et al. 1997; Burrin et al. 1996). Another possibility is that IGF-1 in milk alters the sensitivity of the growth hormone-IGF-1 axis of the infant. Breastfed infants have lower circulating IGF-1 and a slower growth rate than formula-fed infants (reviewed by Schack-Nielsen and Michaelsen 2007). IGF-1 is not a common supplement in infant formula, so it is interesting that higher consumption

of IGF-1 by breastfeeding infants is related to lower circulating levels. The relationship between IGF-1 and breastfeeding status reverses later in childhood; by the age of 7, children who were breastfed as infants have higher IGF-1 than formula-fed children. It has been suggested that this is due to a "programming" effect on the IGF-1 pathway by recalibrating the pituitary sensitivity to IGF-1; if this is true, then high levels of IGF-1 in infancy reduce pituitary sensitivity, resulting in a blunting of negative feedback and lower production of growth hormone and IGF-1 later in life (Martin et al. 2012). Oral IGF-1 could also affect systemic receptor production (it is known to affect intestinal receptor expression in calves: Baumrucker et al. 1994) or systemic binding protein expression. This does not explain the current finding, but given that the process of IGF-1 programming is incompletely understood, it introduces a potential mechanism to investigate. Future studies could measure circulating growth hormone, binding proteins, and IGF-1 levels to compare to milk concentration, or test for variation in IGF-1 mRNA or IGF-1 receptor density in different tissues, such as the plates of long bones.

There was a very small but significant negative effect of monthly change in IGF-1 on head and arm circumference growth rates. An increase in milk volume may have driven this effect; milk volume may be responsible for the increased growth rate and decrease in IGF-1 concentration.

Immune factors (lactoferrin and sIgA):

Lactoferrin and sIgA did not relate to infant growth rate. The predictions that they would were predicated on their attenuation of symptoms of illness, so discussion of these hypotheses is combined in the following section.

There was a very small but significant negative effect of monthly change in sIgA on linear and head circumference growth rates. An increase in milk volume may have driven this effect; milk volume may be responsible for the increased growth rate and decrease in sIgA concentration.

Infant illness and growth

Infant illness was not associated with any measure of infant growth rate, either in the same month that illness was incurred or in the subsequent month. This finding was not expected; it may be that the interval between interviews and measurements was too long to detect a subtle effect of illness on growth rate or that the index of infant illness was too coarse. However, there was a significant interaction between arm circumference and illness on subsequent linear growth rate. Controlling for age, those infants with more upper arm fat who were sick experienced more linear growth in the month following illness. There was a negative relationship of marginal significance between arm circumference and linear growth among infants who were healthy. Thus, arm fat seems to buffer linear growth in the event of illness.

Because lactoferrin was positively related to symptoms of illness in these infants, it was predicted that lactoferrin would be negatively related to growth. sIgA, with its negative relationship to infant illness, was predicted to relate positively to infant growth rate. Neither lactoferrin nor sIgA were significantly related to any measure of infant growth rate. This is perhaps not surprising, given that illness itself was not associated with growth rate. Even if the interpretation that sIgA prevents illness is correct, if illness is not impairing growth rate, preventing illness should not be expected to promote growth.

An intriguing possibility is that lactoferrin was able to "rescue" growth in those infants who were ill; that is, illness *would have* negatively impacted growth were it not for lactoferrin's beneficial effects, and elevated lactoferrin is the *reason* illness does not affect growth. Perhaps lactoferrin is able to defray the cost of fighting infection enough to provide a substantial boost in the amount of energy available for growth. This hypothesis could be tested through an analysis of the relationship between lactoferrin and growth in the subset of infants who experienced illness. If the sick infants who received the most lactoferrin grew more, this hypothesis would be supported. This effect was not strongly present in the current data set. Only growth in head circumference concurrent with symptoms of illness was significantly related to lactoferrin (B=.859, p=.032). It does not seem likely that only head circumference growth would be rescued, and furthermore, after correcting for 8 additional analyses, the p-value would likely not retain significance. However, the effect of lactoferrin rescuing growth may be detectable with a larger sample size.

Maternal condition and growth

Monthly change in maternal BMI positively associated with growth rate in mass over the same period. This finding supports the hypothesis that improved maternal energetic condition contributes to improved infant growth. It seems unlikely that this is a spurious correlation. The youngest infants gain mass at a higher rate than older infants (Table 6.1), but in order for this to explain the finding, infant age would have to be negatively associated with ΔBMI, so that the mothers of the youngest infants would be gaining the most weight. In fact, the opposite is true. There was a significant *positive* relationship between age of the infant in months and ΔBMI (B=0.037, p=.012). The mechanism by which infant growth might be signaled is unknown. None of the milk components measured in this study associated with ΔBMI or infant growth in mass, but it is possible that there is another milk-borne signal that is mediating this relationship. There may have also been an increase in available milk energy as maternal BMI increased in this population. Moderate weight loss is not associated with changes in energy density or milk volume (Dewey et al. 1994), but weight *gain* might show a different pattern. This could be easily addressed by future studies. Milk composition changes through experimentally-induced weight gain would be particularly convincing.

Change in maternal BMI was positively associated with arm circumference growth rate for male infants, but the opposite relationship was found in female infants. Controlling for age, there is a significant difference in arm circumference between male and female infants (with females as reference, male B=1.226, p=.035), meaning that at any given age, a male infant is likely to have a greater upper arm circumference than a female

infant. Infant sex is not related to ΔBMI (p=.859), so sex of the infant does not predict whether a mother is more likely to gain or lose weight. It is possible that there is a signal in milk associated with changes in maternal weight that is read differently by male and female infants. For instance, male infants might store more fat when they receive a signal that maternal energetic status is improving, whereas females may mobilize stored energy (perhaps to fuel linear growth). However, there is no significant interaction between maternal change in BMI and sex on predicting linear growth rate (p=.175). Importantly (because change in BMI was modeled as a continuous variable), the salient signal could just as easily stem from weight loss or weight gain.

Modeled as a continuous variable, maternal parity was negatively associated with linear growth rate. Furthermore, primiparous mothers produced infants who grew significantly more in mass and height over the 4-month study period. These findings lend some support to the hypothesis that parity may lead to depletion of maternal energetic resources and reduced ability to support infant growth. They also echo the findings of Hinde's recent study of rhesus macaque growth (Hinde et al. 2015). Primiparous mothers in that study were found to produce milk with higher concentrations of milk cortisol, which was associated with greater infant gains in mass between early and peak lactation. The replication of the finding that first-born status is associated with faster growth in human infants is very exciting and discussed in greater detail in Chapter 7.

There are some alternative explanations for this finding. Infants with more older siblings are more likely to have other children competing for resources within their household.

Older children may also increase both the mother's and infant's exposure to pathogens and this may have consequences for growth and development. A theoretically interesting, but logistically difficult, way to approach this problem would be to study differences in the growth of infants of high-parity mothers where the older siblings lived together or apart from the mother and infant. That way, competition over household resources could be somewhat separated from maternal energetic reserves. Perhaps more feasibly, a supplementation study focusing on mothers, infants, or the household might resolve these explanations. If maternal supplementation eliminated the effect of parity on linear growth, it would provide support for the maternal depletion hypothesis. If household or infant supplementation eliminated the effect, it would provide more support for the idea that competition over household food resources is driving this pattern.

Chapter 7: Conclusions and future directions

This study represents an early attempt, and in many cases, a first attempt at describing the relationships between maternal condition, concentrations of cortisol, IGF-1, lactoferrin, and sIgA in milk, and infant growth. As a result, many of the investigations presented here were exploratory in nature and a large number of speculative hypotheses were advanced. Many significant relationships were uncovered, supporting the notion that lactation biology is an interesting and important area of research in public health and evolutionary biology.

While it has been addressed many times throughout this dissertation, it bears repeating that the ability to fully interpret the results of this study suffers from lack of inclusion of milk volume as a variable. Concentration is one important aspect of milk bioactive factors, but total amount transferred to the infant would also be useful to know. Given the crucial predictive role of milk volume on infant growth, the analyses relating milk bioactives to growth are particularly tenuous without the ability to properly control for volume. However, especially given the extreme difficulty of obtaining 24-hour milk volume from a large sample of women in field conditions, there is still a great deal of value in learning what we can about milk composition.

Main findings

A variety of maternal energetic condition variables were negatively associated milk bioactive compounds. Specifically, BMI was negatively associated with cortisol and IGF-1. Relatively high insulin production (compared to the individual's own average) was associated with lower cortisol. Primiparity was associated with significantly higher milk cortisol. Parity was positively associated with lactoferrin in male, but not female infants.

Milk sIgA was negatively associated with symptoms of illness in the month prior to milk sample collection and in the subsequent month. Lactoferrin, on the other hand, was positively associated with symptoms of illness.

IGF-1 was positively related to linear growth rate. Illness was not related to growth, but among the infants who were ill, those with larger arm circumferences had greater linear growth in the month following illness. Monthly change in maternal BMI was positively associated with infant growth rate in mass over the same month. Monthly change in maternal BMI also positively associated with arm circumference growth rate in male infants, but the opposite was true for females. Parity was negatively associated with linear growth rate. Primiparous mothers infants that gained significantly more mass and height over the four month study period.

Impact

One goal of this dissertation was to attempt to illuminate some of the first full pathways from maternal characteristics, through bioactive composition of milk, to infant growth and development. In essence, I was interested to determine whether any of the bioactive components in milk serve to communicate the energetic state of the mother in a way that affects infant growth. I initially predicted that such a pathway would operate so that mothers in better condition would signal the availability of energy to their infants who would interpret this signal as an indication that they should increase their rate of growth.

IGF-1 associated negatively with maternal condition and positively with infant linear growth. It is immediately clear that this relationship does not support the *a priori* prediction. If anything, the opposite appears to be true; mothers in poorer nutritional condition have higher IGF-1, and high IGF-1 is associated with faster linear growth. It is possible that what we're seeing is the effect of dilution—that mothers in better condition produce a higher volume of milk, leading to increased infant growth rate, but the increased volume of milk produced dilutes the IGF-1 concentration. This possibility cannot be eliminated without a reliable estimate of milk volume, but there is good reason to actually expect a positive relationship between IGF-1 and milk volume (Breier et al. 1993).

There is another interesting possibility to unify the mother-infant IGF-1 pathway, a framework I am calling the "Calorie Push" hypothesis (illustrated in Figure 7.1). Assuming again that mothers in better energetic condition produce milk that is better able

to meet the all the caloric needs of their infants, IGF-1 may represent a signal from mothers in poor condition that the infant should divert a larger portion of his relatively small milk energy budget to growth. This is analogous to the relationship between energy availability and circulating prolactin during lactation. Mothers in marginal energetic condition require more prolactin to produce the same amount and quality of milk (Lunn et al. 1984, Lunn et al. 1980). These women have lower plasma estradiol and progesterone (Lunn et al. 1984), suggesting that elevated prolactin in the case of calorie restriction may act to shunt calories toward milk production and away from the kind of somatic investment necessary for menstrual resumption. Similarly, a mother providing enough calories to support all of her infant's growth and maintenance functions would have no need to influence a tradeoff between growth and maintenance in her infant, because no tradeoff is occurring. However, if the infant is experiencing an energetic tradeoff, signals in the milk may importantly influence the allocation of calories.

I attempted to test for this effect statistically by including maternal BMI, IGF-1, and an interaction term in a GEE to predict infant linear growth rate. A negative interaction would lend support to this hypothesis (as BMI increases, the effect of IGF-1 on growth decreases). However, the interaction was not significant, meaning that the relationship between IGF-1 and linear growth is the same at all levels of BMI. While this particular analysis did not lend support the calorie push hypothesis, it is a promising idea that could be probed more systematically by future studies. Although there was no relationship between cortisol and infant growth in the current study, its positive relationship with growth in mass in infants of other species as well as the negative relationship between

maternal BMI and cortisol in the present study means that the potential for cortisol to show the calorie push pattern should be considered.

Figure 7.1: Conceptual model of the "Calorie Push" Hypothesis

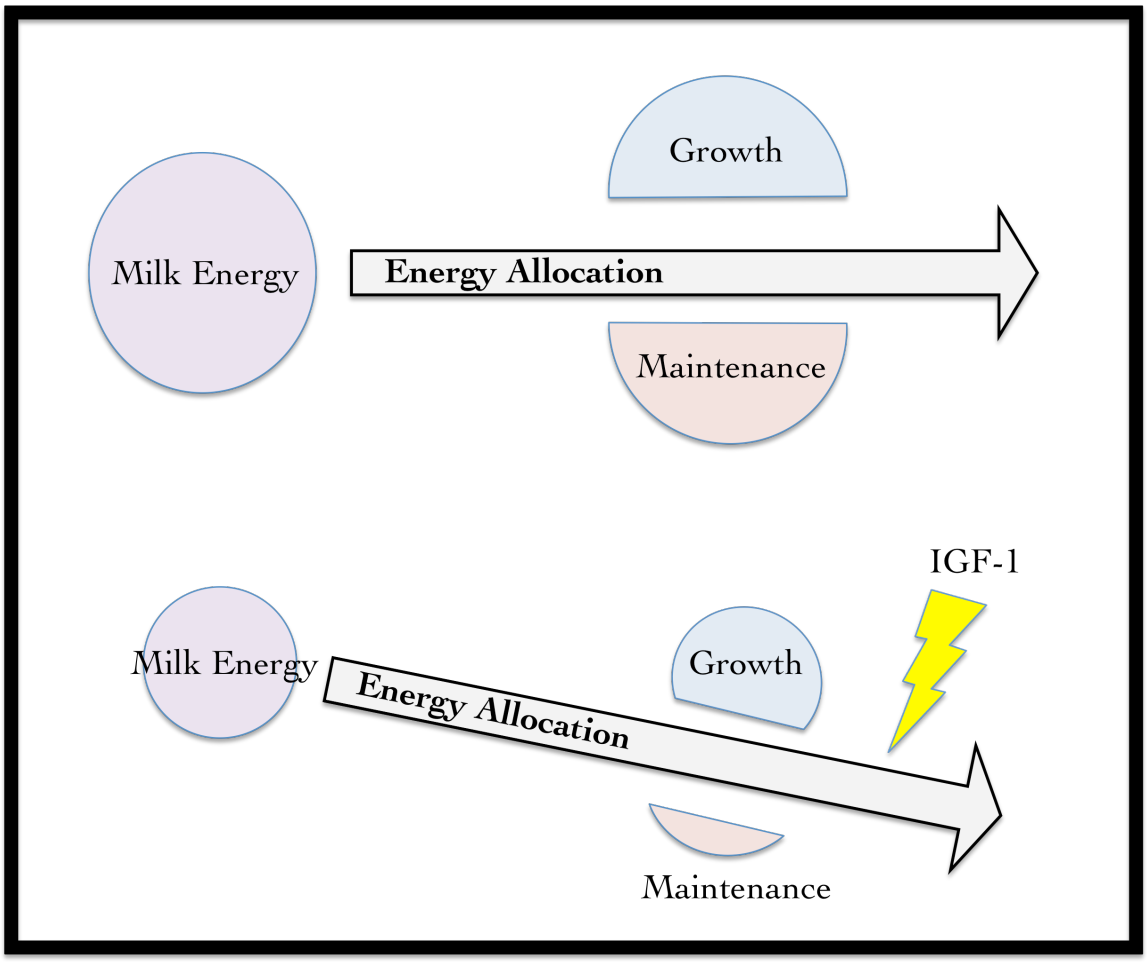


Figure 7.1: *A larger milk energy budget allows for ample energy to be allocated to both growth and maintenance processes as needed (top). To attain the same amount of growth, an infant with a smaller milk energy budget may be sensitive to milk-borne cues such as IGF-1 that influence the proportion of calories to invest in various functions (bottom).*

The "calorie push" framework could be a useful theoretical framework to guide future studies of the role of milk bioactives in the pathway from mother to milk to infant. These studies would be most informative if they focused on exclusively breastfeeding infants,

perhaps in a population that experiences seasonal availability of energy. Unlike the infants in the present study, some of whom received a substantial portion of their total calories from supplemental foods, exclusively breastfeeding infants will be receiving a pure signal from their mothers tailored to the true caloric consumption of the infant. A nutritionally seasonal environment would allow this hypothesis to be tested among mothers in poor energetic condition in whom the signal is expected to be the strongest, with women nursing in the "good" energetic season serving as a sort of control group. If milk volume is reliably measured and controlled for in such a future study, a negative relationship between IGF-1 or cortisol and infant growth rate would be very strong support for the calorie push hypothesis. This could be a very useful framework for understanding the role of milk bioactives in mediating the life history tradeoffs of infants.

An additional pathway from mothers to infants through milk builds from the findings that primiparous mothers have significantly higher concentrations of cortisol in their milk, and that infants of primiparous mothers attained significantly greater gains in height and mass over the study period. These results strongly parallel recent findings by Hinde's group in one of the only other studies linking maternal factors with milk bioactives and infant growth in primates (Hinde et al. 2015). Their study also found a negative relationship between maternal parity and milk cortisol concentration in rhesus macaques, and infants who received higher-cortisol milk gained more mass after correcting for available milk energy. In addition to the incredible benefit of dissociating the independent effect of cortisol on growth from milk energy, this study included behavioral data that led to the proposal of an interesting behavioral mechanism that unifies the

findings. After controlling for milk energy, milk cortisol concentration predicted a more nervous temperament in the infants. The authors propose that the high concentration of cortisol in the milk of low-parity mothers serves as a signal to their infants to conserve energy by restricting exploratory behaviors. Essentially, young mothers might program a "cheaper" infant; these infants start smaller but are able to grow faster by reducing behavioral expenditures.

The significant results from the present study are entirely consistent with this framework. On average, primiparous mothers produced milk with almost 40% higher cortisol concentration, and their infants gained an additional 820 grams and 2 centimeters of height over the four month study period. However, no direct link between milk cortisol concentration and infant growth rate was uncovered in the present study. This could be due to a confounding effect of milk energy or volume, which were not controlled, or to limitations of a small sample size. The conceptual pathway from maternal parity to infant growth through milk cortisol is presented in Figure 7.2. Cortisol is a very promising link between primiparity and increased infant growth rate, and should be more systematically investigated in future studies integrating milk energy and milk yield.

Figure 7.2: Conceptual model of relationships between primiparity, milk cortisol, and increased infant growth rate

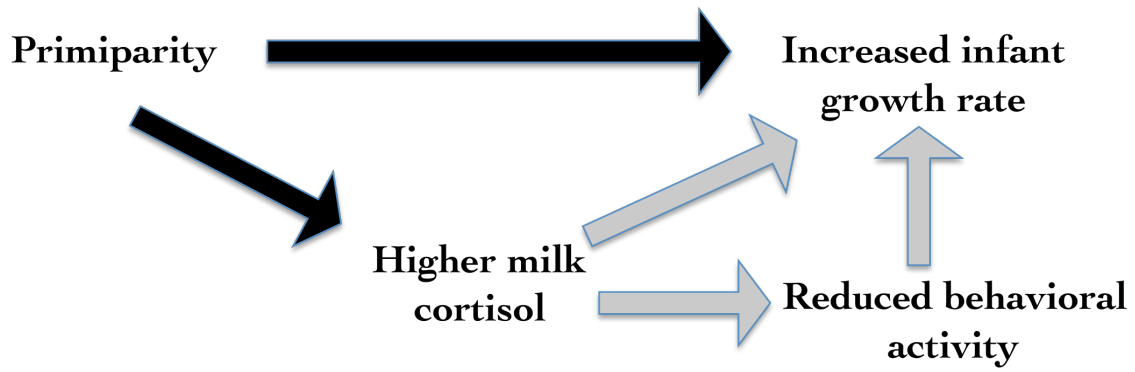


Figure 7.2: *Dark arrows represent significant relationships in the present study. Grey arrows represent proposed relationships mediating the relationship between primparity and increased infant growth rate, following the pattern shown in rhesus macaques (Hinde et al. 2015).*

Arguably the most interesting results to emerge from this study are the relationships between lactoferrin, sIgA, and infant illness. These results add to the body of literature about the protective effects of sIgA against infant illness, particularly diarrheal illness (Walterspiel et al. 1994; Ruiz-Palacios et al. 1990). This study is also among the first to study natural variation of lactoferrin in human milk and its relationship to symptoms of infant illness. These findings are important as first steps toward a source of potential intervention against infant illness, particularly diarrhea, which is a leading cause of infant mortality worldwide. In addition to the public health implications of these findings, this study contributes to the dialogue in evolutionary biology surrounding breast milk composition. The data presented here demonstrate the importance of breast milk as a determinant of infant health, and suggest that mothers may be able to modulate milk composition to address the needs of their infants. The role of breast milk as a mediator

between maternal and infant biology is worthy of further investigation. In particular, data that inform our understanding of the timeline of milk immune adjustment relative to infection and symptoms of illness, and about the relative efficacy of immune compounds in their protective and responsive roles would be useful in expanding the conceptual "protective-responsive" model presented in this paper into a comprehensive description of milk's dynamic role in infant immunity.

Future directions

The specific avenues for future research proposed above arise directly from results of the present study. Many other future directions relate more broadly to ideas presented within this dissertation, presenting exciting possibilities for research at the intersection of evolutionary biology and public health. A great example of this type of research avenue is investigating the role that maternal overweight and obesity have on milk composition. This is clearly an area worthy of study based on the increasing prevalence of overweight and obesity in the global West, but also in transitioning populations around the world. Typically, reproductive ecologists have been far more concerned with lean populations and the effects of caloric restriction on physiology, but this restriction limits our contribution to relevant dialogues in public health and neglects important human variation. We can reframe these dialogues in interesting ways. For instance, are there different "types" of obese mothers? Do disorders of the metabolic syndrome such as diabetes affect milk composition more strongly than adiposity *per se*? In the same range of body fat values, do historically fatter populations have different milk composition than historically thinner populations undergoing a metabolic transition?

Furthermore, a nuanced evolutionary perspective could be just what is needed to solve some of the more complicated puzzles in lactation biology. For instance, maternal BMI positively associates with milk leptin (Andreas et al. 2014), but infants who ingest high-leptin milk are leaner (Fields and Demerath 2012). These findings seemingly contrast with the very high correlations between maternal and childhood obesity that are known to exist (Gibson et al. 2007; Maffeis et al. 1998). Carefully investigating the sources and consequences of bioactive signals in milk through an evolutionary lens might uncover whether this is an adaptive functional system intended to prevent the transmission of obesity, or perhaps a system that evolved for a lower range of body fat and becomes dysfunctional at the extremes.

Another area that presents an interesting avenue for future research is the difference in milk composition between low- and high-parity mothers. While there may be good adaptive reasons for directly targeting infants with bioactive signals specific to their birth order, it is important to first understand how much of the variation in these compounds is simply due to differences in mammary architecture that are attributable to parity. For instance, the idea advanced by Hinde's group that low-parity mothers elevate cortisol as a way to extend the life of mammary epithelial cells and compensate for a lower volume of milk-producing tissue (Hinde et al. 2015) provides a functional explanation for a difference in milk composition that is ultimately targeted at *maternal*, not infant, physiology. However, infants may still identify this type of signal as a reliable indicator

of maternal environment or energetic condition and their behavior and physiology may be altered.

Finally, achieving a concrete understanding of the pathways from mother to milk to infant is not a simple undertaking precisely *because* the biologies of mothers and infants interface so closely. The finding that lactoferrin and sIgA, despite their broadly similar roles, have different relationships with infant illness provides a good example of this concept. Future studies should be constructed keeping in mind that infant biology may be able to influence milk composition as much as maternal biology. Where ethical, experimental work will help to resolve the ambiguities inherent to this complicated pathway.

This dissertation serves an important role as an exploratory study of the relationships between maternal condition, several milk bioactives, and infant health and growth outcomes. It also proposes some theoretical models, including the protective-responsive model of milk immunity and the calorie push hypothesis of maternal signaling via milk. These results will hopefully inspire more systematic studies of these and other milk bioactive factors to advance our understanding of a critical area of human biology.

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