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More than just birth weight: A longitudinal study of the reproductive ecology of infant growth and development

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An Abstract of
A dissertation submitted to the Faculty of the Graduate
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of the requirements for the degree of
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Department of Anthropology

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Abstract

Anthropological investigations of reproductive function have theorized that childhood development serves as a "bioassay" of environmental conditions, adaptively tuning the timing of maturation and adult reproductive function to energy availability. The relationship between somatic growth and hormonal development is not well understood during the critical period of infancy when growth is shaped in response to salient environmental variables. This project seeks to fill this gap by extending reproductive ecological models to infant growth and development.

The present research developed novel, non-invasive methods for measuring fecal sex steroid levels in infants and investigated: (1) sex-specific developmental trends in steroid production, (2) the relationship between hormonal levels and growth and body composition and (3) the effects of environmental factors, such as feeding and maternal characteristics, on sex steroid levels. The data used to address these aims came from a longitudinal sample of 32 infants followed weekly from age 1 week to 15 months. Growth and body composition were measured using standard procedures. Testosterone and estradiol were assessed from diaper samples using methanol extraction and microradioimmunoassay techniques. Feeding style, behavior and illness were measured through parental diaries and questionnaires.

The results identified novel patterns of endocrine activity during infancy. Sex steroid levels followed distinctive patterns in males and females and contributed to inter-individual variance in body size and composition. Relationships between sex steroids, body composition and feeding measures indicated that energy availability may also influence hormone levels.

These data provide the first frequent longitudinal documentation of infant sex steroid levels. The intensive nature of the study permitted investigation of sex steroid activation and growth, providing a potential mechanism for epidemiological observations that infant growth rate is linked to adult reproductive function and health. The multiple methodologies used allowed for discussion of the linkages between growth, energy availability and endocrine development, an issue central to anthropological debates concerning the determinants of growth and maturation. This mechanistic focus is critical amidst the rising incidence of infant and childhood obesity and the growing concern over whether childhood obesity contributes to early maturation, increased morbidity, and the risk of developing reproductive cancers.

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Table of Contents

List of Tables

List of Figures

Introduction and Chapter Summaries

The Myth of "Female Buffering"?

In her often cited 1985 paper, Sarah Stinson reviewed the evidence for what she described as the "pervasive idea" in physical anthropology that males are more sensitive than females to the effects of the environment during growth and development (Stinson 1985). Originally proposed by Greulich in the 1950's, the idea that female growth is better buffered from environmental perturbations, was derived from empirical evidence of sex differences in height, weight and body dimensions among Guamanian and Japanese schoolchildren who had survived nutritional deprivation and atomic bombing during World War II (Greulich 1951; Greulich et al 1953). In these samples, the height and weight of girls were more similar to those of the American reference than were the height and weight of boys. These data were interpreted as "consistent with view that the human male is less successful than the female in withstanding the rigors of an unfavorable environment" (Greulich 1951:69). Subsequent work by Stini (1969; 1972), Tobias (Tobias 1975) and others (reviewed in: Tanner 1962) extended these observations to populations undergoing chronic under-nutrition, where it was proposed that male stature was more affected than female stature by environmental conditions when compared to an external reference standard.

How these sex differences in size are achieved has been a focus of human growth studies, where it is widely assumed that sex differences in size are at least partially determined by these sex differences in the sensitivity of growth and development to environmental factors (Kuh et al 1991; Stini 1969; Stinson 1985), mediated through the differential energetic requirements of males and females during growth (reviewed in: Sellen 2006).

Secular increases in male height, for example, have been proposed to reflect environmental improvement and better nutrition during the period of juvenile growth, since male growth is presumed to be more plastic to improved conditions than female growth (Cole 2000; Wolanski and Kasprzak 1976). Similarly, females are thought to show greater canalization both over the course of the secular trend in height and also within individual children, being less easily thrown off their growth curves by adverse environmental circumstances (Tanner 1962). However, the interpretation of what greater male sensitivity should predict in terms of catch-up growth has been the subject of debate (reviewed in: Stinson 1985). Some auxologists believe that females should show greater catch-up growth, because they should return more quickly to their "normal growth path" when the environment ameliorates (Prader et al 1963; Tanner 1962), while others think that males should show greater catch-up growth, since their growth is more labile and should respond more quickly when environmental stress is removed (Bielicki and Charzewski 1977; Tobias 1975).

This difference in the interpretation of catch-up growth highlights an important issue complicating evolutionary and life history explanations of sexual size dimorphism. The assumption that sex differences in physiology underlie observed sex differences in size may be too simplistic, since it implies that the sexes share similar environmental and cultural exposures. However, the relative growth of males and female is also strongly influenced by socio-culturally mediated differences in access to resources and exposure to energetic stressors such as nutritional inadequacy or increased workload (Sellen 2000; Worthman 1993). For example, Sellen (2000) documents an age-specific reversal of sex differences in growth indicators that is associated with changing work roles of male and

female children. Similarly, Shell-Duncan and Obiero (2000) observe better female anthropometric status, despite a parental bias towards first-born male children, and suggest that this may be the result of social factors, such as access to food during meal preparation. Such observations suggest that underlying sex-specific physiological differences in environmental responsivity may be either accentuated or masked by variation produced by the local contexts where developmental niches may differ by gender.

Despite these difficulties, the idea of greater female buffering and Stinson's review article (1985) have been widely cited[1] in both biological anthropology and evolutionary biology. The idea of greater female buffering has been incorporated into human biological examinations of sex differences in infant growth (Tracer et al 1998), cardiovascular health and lipid metabolism in adolescents (Kuzawa and Adair 2004), and nutritional status of children and adults living in poor conditions in the US and worldwide (Crooks 1994; Crooks 1999; Dettwyler 1992).This idea of greater female buffering has further been applied as an explanatory model for observed differences in the sexual dimorphism of skeletal populations (e.g.Goodman and Armelagos 1989; King et al 2005; Tague 1989) and incorporated into evolutionary explanations for sex differences in survivorship that lead to skewed sex ratios (e.g.Badyaev 2002; Grant 1994; Wells 2000). However, despite the widespread appeal of this idea, very little evidence exists to support it. As Stinson states in the conclusions to her review, "the available evidence thus provides, at best, weak support for the hypothesis that males are less buffered than females against the environment during growth and development"(Stinson 1985:141).

[1] As of 4/6/2007, Stinson's review was cited 55 times in the Web of Science and 64 times in Google Scholar.

Although her review provides only weak support for a male bias in postnatal morbidity and mortality and for sex differences in growth in response to environmental conditions, her review and the idea of greater male sensitivity continue to be widely cited.

Perhaps the reason this idea has been so pervasive, despite the paucity of supporting evidence, is because it has been argued to make sense evolutionarily. From an evolutionary perspective, females are expected to be better buffered against environmental conditions, because of the greater energetic costs entailed in supporting pregnancy and lactation (Stini 1969; Stini 1972; Stinson 1985). This evolutionary argument is drawn from an evolutionary paradigm about sex differences, parental investment theory (PI). Derived from the Trivers-Willard hypothesis (Trivers and Willard 1973), PI models suggest that the development of sex differences in response to environmental conditions is linked to the differential possible reproductive success of male offspring versus female offspring (Cameron 2004; Rosenfeld and Roberts 2004; Wade et al 2003). PI models argue that, since males are limited in their reproductive success only by access to females, mothers, whose own time and energy to allocate to reproduction is limited, should invest preferentially in their sons when environmental conditions are good. Daughters, on the other hand, have less variable reproductive success and should be invested in when conditions are not as favorable, since they can not provide the same potential pay-off. Human biology applications of this model have suggested that genes that favor male health when the environment is good and male vulnerability when the environment is poor, should both be selected over time and that this selection can be tested by examining sex ratios fetally, at birth and postnatally (Crognier et al 2006; Elsmén et al 2004; Gaulin and Robbins 1991; Koziel and Ulijaszek

2001; Wells 2000). While the hypothesis that females are better buffered against the environment during growth because they have a higher reproductive cost in adulthood is "attractive on theoretical grounds" (Stinson 1985), how this greater buffering could be achieved physiologically during growth remains unclear.

An Alternative Viewpoint: Sex Differences in Reproductive Ecology

The idea of greater male sensitivity to the environment stands in sharp contrast to another field of study in biological anthropology, namely reproductive ecology. Reproductive ecological studies have documented greater *female* sensitivity to environmental conditions, particularly energetic stressors, both during maturation and the reproductive period (Ellison 1996; Ellison 2001; Ellison et al 1993b). Male fecundity and reproductive function, on the other hand, seem to be relatively unresponsive to all but the most extreme energetic deprivation (Bribiescas 2001; Campbell and Leslie 1995).

As many researchers in the field of reproductive ecology have noted reproduction is an energetically costly and risky undertaking for the human female (Dufour and Sauther 2002; Frisch 1990; Pike 2001; Wood 1994). Given the high energetic costs and mortality risks associated with pregnancy, parturition and lactation, mechanisms delaying reproduction until the probability of a successful pregnancy is high are likely to have been selected for in the course of human, and mammalian, evolution (Dufour 2003). To the extent that the probability of reproduction depends on energy availability, female reproductive physiology should show sensitivity to salient ecological variables, such as caloric intake, energy expenditure, and disease load. This sensitivity of adult ovarian function to ecological conditions has been well documented (Ellison 2001; Ellison et al 1993a; Jasienska and Ellison 1998; Panter-Brick 1993; Vitzthum 2001; Worthman and

Konner 1980). Among women in both Western and non-Western populations, fecundity and sex steroid production have been shown to be responsive to energy availability, workload, and nutrition (Ellison et al 1993b; Jasienska and Ellison 1998; Panter-Brick 1993; Valleggia and Ellison 2001). Ovarian responsivity is associated with not only acute changes in energy balance, such as weight loss during the hunger season (Bentley et al 1998), but also longer-term developmental influences, such as the tempo of growth in childhood and adolescence (Ellison 1996; Lipson 2001).

Male reproductive function, on the other hand, is less responsive to environmental conditions. Unlike female reproductive physiology which has been selected to be responsive to energetic conditions altering hormonal levels and consequently the probability of conception, male reproductive physiology is relatively unresponsive to energetic availability in modulating spermatogenesis (Bribiescas 2001). Testosterone levels do vary between populations (Beall et al 1992; Ellison et al 2002), however, and this variation, while not impairing spermatogenesis, is thought to be related to somatic investment in muscle mass (Bribiescas 2001; Bribiescus 2001; Campbell et al 2003). Thus, it has been theorized that population level differences in testosterone levels represent developmental responses to chronically poor conditions and reduced investment in somatic, particularly muscle, tissues. Rather than suggesting that one sex or the other is more buffered from the effects of the environment, such facultative adjustments to acute and chronic ecological conditions link local ecologies to individual and population variation reproductive physiology in both males and females and, ultimately, provide the variation on which selection can act.

Life History Theory

How this selection can actually work physiologically is elaborated by life history theory which views growth and reproduction as distinct and exclusive categories within an evolutionary framework. Life history theory posits that individual organisms must distribute limited energetic resources between the competing demands of growth, maintenance, and reproduction (Charnov 1991; Stearns 1992). From the life history perspective, phenotypic plasticity in growth rates and hormone levels may be best understood as an adaptation allowing the developing individual to track its environment and to be affected by it in ways that promote fitness (Chisholm 1993; Little 1997; Stearns and Koella 1986). On an evolutionary scale, therefore, growth rates represent adaptations to mortality risk due to environmental constraints in energy availability. This trade-off model further implies that growth rates, as well as age at maturation and adult body size, will vary between populations with different environmental risks of inadequate energy availability or mortality (Hill and Kaplan 1999). As the energetic costs of growth and reproduction differ between the sexes, the optimal timing of such trade-offs can differ by sex as well. On a broad, inter-specific level, the timing of life history trade-offs, such as continued growth versus sexual maturation, reflect stable equilibrium points along a reaction norm (Hill 1993; Hill and Hurtado 1996; Stearns and Koella 1986). Life history theory further suggests that the reaction norms underlying these energetic trade-offs are shaped during fetal and infant development when physical ontogeny is 'designed' to unfold in a dynamic relationship with the environmental milieu (Worthman 1993). However, little empirical evidence exists to test how these theoretical predictions correspond to individual growth.

While Hill (1996) may claim that life history decisions regarding energy allocation are made on the molecular, physiological, and behavioral levels, little research has been done describing exactly how life history parameters become translated into the physiology of the individual. Work by human biologists and endocrinologists, however, suggests that hormones may play this central role in allocating metabolic resources. Hormones both mediate energy distribution in the short-term and modulate life history priorities in the long-term, by monitoring the exogenous and endogenous environment and transducing this information to modify cellular function in target organs (Finch and Rose 1995). Acting as a signal system of energy availability, then, hormones orchestrate the tempo of growth and maturation through organizational or regulatory adjustments to the environment during ontogeny (Worthman 2000).The primarily energetic nature of hormonal signals is seen in the extensive diversification of hormone receptor isoforms that allow cell-type specific responses to the same hormones (Rose and Finch 1995). Such diversification allows a hormone, insulin for example, to send a general signal, energy availability, and, yet, elicit multiple, specific physiological responses, such as osteoblastic cell division, folliculogenesis, or lipogenesis (Poretsky et al. 1999). Further, the multiplicity of responses to metabolic hormones suggests that the classically formulated growth and reproductive axes are intricately intertwined. A closer focus on the physiology of the developing individual not only allows such interactions to be explored, but also allows growth and maturation to be envisioned as a dynamic, responsive process.

Why Infancy?

Infancy may represent a particularly important period from both a developmental and evolutionary perspective, since it is during infancy that rates of growth and development are shaped in response to salient environmental variables and hormonal axes are active at levels not seen again until puberty (Forest and Ducharme 1993). Growth during infancy may represent a sensitive marker of environmental conditions, such that poor early conditions result in reduced stature and delayed maturation (Ellison 1996). The importance of this period for later size and stature is illustrated by the incidence of stunting in the developing world where the majority of the deficit in adult height is due to nutritional deprivation in the first 3 years of life (Martorell et al, 1995; Neumann and Harrison, 1994), although some catch-up growth may be possible (Adair, 1999). On the other end of the spectrum, weight gain during infancy has been linked to the risk of obesity in later life. As a recent review of the many epidemiological studies of the link between infant weight gain and later obesity states, infants at the highest end of the distribution for weight and/or body mass index (BMI) or those who gain weight rapidly are at an increased risk of obesity in childhood and adulthood (Baird et al, 2007). Like the studies linking stunting to adult stature, these obesity studies similarly link environmental conditions in infancy, in this case excess nutritional intake, with adult body morphology.

Epidemiological research has increasingly documented that these early pre- and postnatal growth rates are shaped by the nutritional environment and, in turn, can shape later physiology, having long lasting effects on adult cardiovascular, metabolic and reproductive health (Barker 1995; Desai and Hales 1997; McDade et al 2001). Within this epidemiological framework, the period of infant development represents a critical

window during which organ-level growth is determined and metabolic set points are programmed (reviewed in: Cameron and Demerath 2002; Greenwood and Bell 2003).

Given the potential for lifelong reproductive effects, the interactions between energy availability, the tempo of growth and maturation, and reproductive function need to be explored from the beginning of the growth process, *in utero* and early postnatal life. Indeed, infancy may be the best time to explore not only the functional, physiological outcomes of fetal adjustments to the environment on reproductive function but also the relationship between the somatic and reproductive axes, since hormonal levels and rates of growth seen in infancy are not seen again until puberty. The postnatal period may be particularly important in shaping the future tempo of growth, since it represents a rapid and dramatic environmental transition from the maternal environment to the external world (Gluckman et al 2005) and is a time of high mortality risk (Kramer 1990; Rajanikumari and Rao 1987).

Sex Differences in Infant Growth and Development

Although less attention has been paid to sexual dimorphism in growth during this period, early life influences may be particularly important, because postnatal growth is rapid and susceptible to nutritional influences (Gluckman and Pinal 2003; Liu et al 1998). In humans, infant growth in size in length, weight and body tissues is dramatic. During the first year body weight triples and adipose tissue is deposited rapidly, reaching a peak around 6 to 9 months (Tanner 1989). Sex differences in size and growth velocity, however, are seen from fetal development, when the male fetus grows longer and puts on more weight than the female fetus (de Zegher et al 1998). The growth rates of male and

female fetuses also differ, though the direction of this difference is under debate. Some authors have found that male fetuses show early growth acceleration in comparison to female fetuses (de Zegher et al 1998).The more common view is that girls show greater early gains in growth and a generally faster maturational pattern (Lampl and Jeanty 2003; Tanner 1989). The result of these fetal sex differences in growth is that girls are typically born shorter and weighing less than boys (Hindmarsh et al 2002). A recent multinational survey of infant growth (WHO 2006) documents only a slight male advantage in length and weight at birth of 100 gm and 0.8cm, respectively. Greater sex differences have been documented in studies where mothers were supplemented during pregnancy. In the Bacon Chow nutritional intervention study, for example, McDonald et al (1981) found that male infants born to mothers supplemented with 800 kcal and 40g of protein daily before and during pregnancy were 200 gm heavier (3215.9gm for males versus 3071.3gm for females) at birth and 1.4cm longer (49.88 cm for males and 48.54 cm for females), suggesting that male fetal size was more responsive to maternal energy availability.

Postnatally, the length and weight differences between male and female infants are more pronounced. In the multinational WHO sample of exclusively breastfed infants, male infants were longer at all ages than female infants by approximately 1.6-1.7 cm and heavier by over half a kilogram at all ages measured after birth (WHO 2006). Male infants also gain length and weight more rapidly than do female infants (Nelson et al 1989), though the nature of the sex difference in weight tends to be more variable (Preece et al 1996).While male infants tend to be both heavier and longer than females at birth, female infants have both a greater fat mass for body weight at birth (Guihard-Costa et al 2002) and a greater amount of subcutaneous fat per kilogram body weight (Copper et al

1993; Rodriguez et al 2004). Female body fat percentage has been estimated to be as much as 22% greater than males' in the weeks following birth (Fomon et al 1982). The pattern of adipose distribution also varies by sex (Guihard-Costa et al 1997) with female newborns have higher absolute and relative subscapular and tricipital skinfold thickness than male infants, a sexually dimorphic pattern also seen in children, adolescents, and adults (Malina 1996).

Sex differences in growth rates, stature and body fat are documented during fetal development (Catalano et al 1995; de Zegher et al 1998), adolescence (Rogol 2003) and adulthood (Veldhuis et al 2005) when they are associated with sex steroid production. The presence of these sex differences in growth in infancy raises an interesting question: could sex steroid differences also underlie sexual dimorphism in infancy as well?

Hypothesis: Sex differences in hormonal production during infant development prime maturation and life-long reproductive function

These sex differences in growth, which are similar to those seen at times when the HPG axis is active, along with the postnatal rise in sex steroids documented in human infants (Forest et al 1974) suggest that, indeed, sex hormones may also play an organizing role in the ontogeny of sex differences in growth and serve as an interface between environmental conditions and growth rate. A role for HPG activation in shaping somatic growth during infancy makes mechanistic and theoretical sense. Growth hormone and IGF-I secretion are a fundamental determinant of body size in both sexes of vertebrates (Badyaev 2002) and this secretion is at least partially under gonadal hormone control (Veldhuis 1998). Both androgens and estrogens stimulate the secretion of GH, but their relative importance and biochemical effects differ in sex-specific ways (Berensztein et al

1995; Veldhuis 1996). While role of GH in infant growth continues to be debated with many researchers believing that GH is only active in the later half of infancy (Karlberg et al 1994; Tse et al 1989), recent research using newly-developed, ultrasensitive assays for GH, IGF-I and their binding proteins suggest that, not only is GH produced from birth, it also shows characteristic pulsatility and sex differences (Geary et al 2003; Ghigo et al 2000; Pirazzoli et al 1997), indicating functionality of the axis. In animal models, sex steroids have been shown to produce sex-specific density and distribution of GH receptors and hormone secreting cells, which persist into adult life (reviewed in: Badyaev 2002; Brandstetter et al 2000; Lopez et al 1995). Such evidence suggests that sex steroid production in human infants may prime future growth. Further, such production, if sensitive to the energetic availability, could prime growth rate and maturation in environmentally adaptive ways.

Although the interaction between the gonadal and hypothalamic axes has received little attention in infancy, maturation of the gonadal axis in adolescence has been closely tied to the final phases of physical growth (Tanner 1962; Worthman 1993). The synchronization between physical growth and sexual maturation suggests that the trajectory of ovarian maturation and subsequent adult levels of ovarian function may be determined by growth rate during childhood. Certainly, age at menarche varies significantly among populations (Eveleth and Tanner 1990) and this variation has long been theorized to represent a developmental response to ecological (Benefice et al 2001; Khan et al 1996; Pasquet et al 1999), cultural (Worthman 1993), or psychosocial (Chisholm 1993; Kim and Smith 1998) conditions experienced during childhood. Further,

within a single population, the timing of menarche predicts adult ovarian function, with girls who go through menarche relatively early having higher serum estradiol concentrations in adulthood than those who mature later (Apter 1996; Apter and Vihko 1985). Similarly, the effect of developmental conditions on the timing of maturation in males may be similar to that seen in females, as males chronically exposed to undernutrition have delayed maturation and diminished adolescent growth spurts (Sellen 1999). Less is known about the effects of childhood conditions on subsequent reproductive function, but recent research suggests that low birthweight is associated with smaller testicular volume, sub-fertility and lower adult height in males (Cicognani et al 2002; Francois et al 1997), linking energetic conditions experienced *in utero* by males, to reproductive function and adult size as well.

According to Ellison (1996), the timing of menarche, and the female reproductive system more generally, may have been molded by natural selection to link adult ovarian function to the tempo of childhood growth and maturation. Increasing evidence suggests that this may be the case with male reproduction as well (Bribiescas 2001; Campbell and Leslie 1995). Thus, childhood growth serves in both sexes as a "bioassay" of environmental conditions. In this model, poor childhood conditions significantly delaying maturation would also lead to the establishment of reproductive set-points that result in lower hormone levels, potentially increasing the likelihood of anovulatory cycles, extending the waiting time to conception and reducing the overall, lifetime energetic costs of reproduction in females and decreasing the metabolic costs of somatic investment in males.

Given the developmental and regulatory complexities of the HPG system, it is not surprising that a wide range of variation exists in steroid hormone production and reproductive function within subjects, between individuals, and among populations. Indeed, variation in endocrine function associated with age, developmental context, and energetics reflects a functional modulation in response to the probability of successful reproduction outcome and future reproductive success (Ellison et al 1993a). Rather than represent a failure of homeostatic mechanisms, such modulation represents an adaptive response designed by natural selection to enhance lifetime reproductive success. Occurring along a continuum of response based upon the duration and severity of local ecological conditions, steroidal responsivity reflects a norm of reaction through which populations adapt to their local ecologies. Such reaction norms can be considered, from an evolutionary perspective, adaptive mechanisms that receive the distribution of environmental variation and transform it into the distribution of phenotypic variation, thus mapping genotype onto phenotype as a function of the environment (Stearns 1989).

Recent work in developmental biology further suggests that the maternal environment actually modifies the unfolding of fetal development in expectation of the external conditions, creating an epigenetic model through which developmental conditions lead to intergenerational adaptations to the expected environment (Kuzawa 2004; Reik et al 2001). That variation in reproductive function is an adaptive strategy should not be surprising since the HPG axis lies at the heart of human life history strategies. The hormones produced by the HPG help to mediate resource partitioning and modulate the timing of life history events, a key element in differential reproductive success and long-term evolutionary fitness (Worthman 1999).

Reproductive Development during Infancy: Possible Mechanisms

While this model is valuable in linking conditions of growth to reproductive development, it overlooks the fact that, by puberty, the gonads have already undergone significant growth and differentiation. Consequently, the adult ovary or testes is not "checking" what happened in childhood, but rather is developing from a genetic plan that has already been physiologically altered by ecological and developmental experiences from conception onwards. These experiences may constrain the range of variability that can be expressed in adulthood if, for example, fewer thecal or Leydig cells exist to produce estrogen or testosterone or if sensitivity to feedback is reduced as a result of fewer hypothalamic receptors. The idea that a phenotype can vary as a continuous function of the environmental signal, known as a norm of reaction (Stearns 1989), is important in reproductive physiology, since the gonads may be especially sensitive to conditions limiting phenotypic plasticity. Ova , for example, reach their maximum number of 6 to 7 million at 20 weeks of gestation and no mechanism exists to create new eggs after that period (Speroff and Fritz 2005). Similarly, the testes are organized pre- and postnatally and evidence suggest that the number of Sertoli cells produced during these late pre-natal and early post-natal period determines testicular size and sperm characteristics in adulthood (Chemes 2001). Conditions compromising early ovarian and testicular development, then, could set limits on the adaptability of reproductive function that could be expressed in later life.

The effects of endocrine disruptors on sexual development and future reproductive health of human and nonhuman primates have been well described- for example, fetal DES exposure leading to infertility and reproductive cancers in women (Bamigboye and

Morris 2003) and androgen exposure altering primate gonadal anatomy and sexual behavior (Mann et al 1997). More subtle alterations in maternal environmental milieu have also been shown to affect reproductive development and function. Animal models have demonstrated that the developing gonads are sensitive to the maternal environment. Experimental manipulations of maternal diet in pregnant sheep, for example, was found to delay the onset of meiosis in female fetuses (Rhind et al 2001) and reduce the number of Sertoli cells in newborn lambs (Bielle et al 2002). Studies of human gonadal development have found more equivocal evidence of an effect of maternal under-nutrition and IUGR on reproductive development. Some researchers have found smaller ovarian volumes and retarded follicular development in growth restricted female infants (de Bruin et al 1998; Ibanez et al 2000) and impaired gonadotropin secretion in growth restricted male and female infants (Ibanez et al 2002). However, other studies have not been able to demonstrate an effect (Lumey and Stein 1997), likely because fetal effects depend on the timing and severity of the ecological insult. Although the extent to which maternal nutrition can affect human reproductive development is unclear, factors affecting tissue differentiation, gonad formation and establishment of the endocrine system are likely to prime subsequent reproduction function and contribute to inter-individual variability.

Inherent in the argument that birthweight is related to the timing of maturation and future adult function is the idea that the hormonal axis of smaller infants has been altered during fetal development. However, very little is known about the effects that fetal growth may have on hormonal levels during infancy, an important time for the organization of the hypothalamic-gonadal and growth axes. Previous cross-sectional studies of neonatal

IGF-I (Christou et al 2001), FSH (Ibanez et al 2002) and estrogen (Ibanez et al 2002) levels indeed suggest that the hormonal milieu of neonates may differ significantly by birthweight, but there are no published results from longitudinal studies documenting the nature of these differences. While potentially constraining the reaction norm of reproductive function in adulthood, the sensitivity of the gonads to conditions during development also inherently links environmental conditions to the tempo of physical growth and reproductive development. Further, differences in hormonal levels may have important functional consequences on growth, since the synergistic interaction between sex steroids and growth hormones, at least in adolescent growth, is well documented (Toublanc 1997). Androgens and estrogens have been shown to affect growth of the skeletal system and alter body composition through both direct action and also amplification of growth hormones (MacGillivray et al. 1998, Spelsberg et al. 1999), linking early conditions to later health and fitness.

Epidemiological Evidence

Understanding then that developmental conditions affecting reproductive maturation are the very same as those affecting what is more traditionally considered growth requires an investigation even further back in developmental time of the ways fetal and infant experience shape the tempo of growth and maturation. Epidemiological studies suggest that developmental influences on ovarian function extend into infancy and fetal life. Adair (2001) found that, among a cohort of teenage girls in the Philippines, those who were long and light at birth had the earliest age at menarche, particularly if they were fast growers in infancy. Similar correlations between fetal growth and the timing of menarche

have been seen among Swedish adolescents, with girls born small for gestational age experiencing menarche four months earlier than girls with a normal size at birth, though these results were no longer significant when childhood growth patterns were taken into account (Persson et al 1999). Conversely, in a large cohort of British women, Cooper et al. (1996) found that girls who were heavier at birth had later ages at menarche. Again, childhood growth patterns were important with those girls who were heavy at age seven having the earliest menarche.

Ecological conditions affecting the growth of the ovary during fetal and postnatal life and limiting the potential reaction norms would then have life long effects on subsequent reproductive function extending beyond the timing of menarche. Evidence for such effects comes from studies of infertility, polycystic ovarian syndrome (PCOS), and menopause. Ibanez et al. (2002) found that the prevalence of anovulation is higher among post-menarcheal girls born small for gestation age than those born at a normal weight despite similarities in current height and BMI. Not only did birthweight correspond with absolute measures of ovulatory sufficiency, but ovulation rate was also related to birthweight, with SGA girls who were ovulating doing so less frequently than AGA girls. If continuing into adulthood, these differences in ovarian function could result in fertility differences since fecundability is inherently related to ovulatory frequency and sufficient hormonal production. Birthweight has also been implicated as a risk factor in the development of polycystic ovarian syndrome (PCOS), an as yet poorly understood disorder associated with excessive androgen production, obesity, and menstrual irregularities that can result in lowered fertility and compromised ovarian function. Given the heterogeneous nature of the syndrome, the direction of the relationship between

birthweight and PCOS remains unclear, with some studies finding a relationship between large size at birth and increased incidence of the syndrome (Cresswell et al 1997) and others (Ibanez et al 1998) finding an increased incidence in those small at birth. Although not well understood, the relationship between birthweight and PCOS highlights the intertwined nature of the reproductive and energetic axes as insulin is implicated in both the obesity and increased diabetes risk associated with PCOS as well as the hyper-production of androgens and ovarian enlargement (Michelmore et al 2001). Finally, epidemiological evidence suggests that age at menopause, the endpoint of reproductive function in women, may also be related to birthweight with earlier age at menopause associated with both higher birthweight and shorter body length at birth (Cresswell 1997). A correlation between menopause and birthweight makes functional sense, since, as the endpoint of reproductive function, the timing of menopause should be related to the same developmental conditions affecting the onset and maintenance of reproductive function.

As discussed above, the effects on developmental conditions on male reproductive maturation and function have received less attention (Bribiescus 2001), but recent studies suggest that male subfertility may also be related to birthweight and infant growth rate (Cicognani et al 2002; Francois et al 1997). The importance of both birth weight, a marker of fetal development, and childhood growth rates on the timing of maturation suggests that the reproductive axis is sensitive to ecological conditions both *in utero* and postnatally. The tempo of growth during infancy is inherently linked to both genetic and environmental conditions and may establish the "dose response curve" relating

reproductive function to salient environmental variables such as diet, energy expenditure, and illness. However, the mechanisms underlying these associations remain unknown.

HPG Axis Development

These associations between infant growth and later reproductive maturation suggest that the activity of the reproductive axis in infancy is a valuable path of inquiry. The existing literature suggests that gonadotropin and sex steroid production peaks in male infants around 3 months of life, when LH and testosterone levels are similar to those in adolescence, and gradually decline after 6 months (Andersson et al 1998; Forest et al 1974). The pattern of gonadal steroid production in female infants, on the other hand, appears more complex and is less well understood (Lee 2003). Estradiol is measurable in infant girls through the end of the first year of life, with values falling within the adult mid-follicular range. This estradiol production is thought to be stimulated by high FSH production, which can exceed the normal upper range for adult women (Crofton et al 2002a).

The activation and function of this axis in infancy remains relatively unknown, but morphological studies of gonadal tissues and clinical administration of gonadotropins to hypogonadal males (Main et al 2000), suggest that the gonads are capable of producing steroid hormones in infancy. In studies to date, serum levels of sex steroids in infants are generally lower than adolescent and adult circulating concentrations (Bergada et al 1999; Crofton et al 2002b). However, clinical studies of neonatal boys suffering from hypogonadism indicate that even these low levels are physiologically important for normal genital development (Main et al 2000). Estradiol also appears to have a biological

function during infancy as ovarian size, follicular differentiation, and breast tissue mass are all correlated with estradiol levels in neonatal girls (Schmidt et al 2002).

Methodological Difficulties

Unfortunately, understanding of the potential functional consequences of the activation of the HPG axis for growth and development has been hampered by the ethical and methodological difficulties inherent in sampling serum from healthy infants. These ethical and technical considerations have meant that the majority of evidence to date comes from studies of the gonadal contents of autopsied infants (Bidlingmaier et al 1987), cross-sectional samples of infants undergoing clinical procedures (Burger et al 1991; Winter et al 1975; Winter et al 1976) and a single longitudinal study, with infrequent three-month intervals (Andersson et al 1998).

Even the relatively less-invasive salivary (Ellison 1988) and blood-spot (Worthman and Stallings 1997) measures employed in children and adults present practical and technical problems in young infants. Prior to teething, the provocation and collection of sufficient saliva quantities to assay is uncertain and requires the use of cotton swabs, known to interfere with quantifiable levels of testosterone and estradiol (Shirtcliff et al 2001). Blood spots are also impractical in longitudinal studies of infant hormonal development, since few parents or infants would tolerate repeated finger pricks. Given these difficulties, we decided to employ a novel approach and explore the possibilities of extracting sex steroids from infant fecal samples, a method previously employed in field endocrinology of non-human primates (Whitten et al 1998) and one that is particularly effective when non-invasive sampling collection is required.

Study Aims

With this new methodology for measuring sex steroids in infants, the goal for this dissertation was to examine the relationship between somatic growth and reproductive development, a question that has long been of interest to biological anthropologists, reproductive ecologists and human biologists. Previous research in adults has documented that, on the individual and population levels, body morphology, maturation and steroid levels are related, in sex-specific ways, to developmental and environmental conditions. Despite the documented importance of childhood development for adult reproductive function (Berkey et al 2000; Ellison 1996; Khan et al 1996; Worthman 1993), reproductive ecological studies of infancy to date are limited to birth seasonality (Bailey et al 1992; Leslie and Fry 1989), immune function (Collinson et al 2003), and breastfeeding (Lunn 1994). While comparatively less longitudinal research has been done on the early development of the hypothalamic-pituitary-gonadal (HPG) axis during postnatal development, it is well known that patterns of variation in adult reproductive function and health reflect differences in this ontogenic experience (Ellison 1996; Lipson 2001; Lummaa 2003). Hormones mediate the relationship between socioecological and developmental context and later function by setting regulatory set-points and mediating resource partitioning, adjustments that determine such life history parameters as adult body size, age at maturation, and longevity (Finch and Rose 1995; Worthman 1999). Thus, a careful description of HPG development and the pathways through which its development may be influenced by environmental and social conditions provides insight into the key determinants of individual and population reproductive function and adaptive strategies. This project addresses the anthropological question of whether infancy serves

as a bioassay, linking environmental conditions to the physiology of growth and reproductive development.

Specifically this study asks:

1. Are there sex differences in the developmental trajectory of hormones in infants?
2. Are size and body composition in infancy related to steroid levels in a sex-specific ways?
3. Do energetic markers have sex-specific effects on variability in sex steroid levels?

Chapter Summaries

II. **Methods**

Chapter 2: Estradiol

This chapter details the methodology of fecal steroid extraction from the diaper samples and presents assay validation for an unmodified and modified assay for use in measuring estradiol from infant fecal samples. After a review of the methodological issues raised by the need for non-invasive measures in infants and findings of previous fecal analyses in human and non-human primates, this paper presents extraction methods, provides detailed assay procedures and validation and examines the effect of potential confounders such as the use of cotton diaper liners, time of day, storage conditions and heterogeneity of fecal samples. Finally, potential applications of this method are presented.

Chapter 3: Testosterone

As a follow-up to the extensive description of the development of the extraction procedures in the estradiol chapter, this shorter chapter presents the assay procedure and validations for the measurement of fecal testosterone in infant diaper samples. This

article addresses some of the issues raised in the measurement of testosterone in salivary studies and suggests that fecal measures may be useful for assessing hormone-behavior relationships in younger infants and children.

III. Results

Chapter 4: HPG Gender and Developmental Trends

This chapter presents a mostly descriptive summary of estradiol and testosterone levels. After descriptive statistics of testosterone and estradiol levels are presented, mixed-modeling approaches for correlated data are employed to assess sex differences in hormonal levels and developmental trends. This chapter presents the results that sex steroid levels: 1) can be measured throughout the first 15 months of infancy in both male and female infants, 2) show significant variation within- and between- individual infants, 3) vary between the sexes in age-specific ways, and 4) overlap adult values.

Chapter 5: HPG and Size

Given links between infant growth and later size and maturation, this chapter explores whether the patterns of sex steroid excretion in infancy documented in the previous chapter were associated with sex differences in growth and body composition. After a review of the evidence for sex differences in infant growth and hormonal contributions to sex differences in growth in adolescents, data are presented that sex steroid levels contribute to inter-individual variance in length, weight, and measures of body composition, including subcutaneous fat folds and trunk and limb circumferences. This chapter documents, using mixed-model regression analysis for correlated data, that differences in sex steroid levels are associated with sex-specific growth and body compositional changes. The

implications of these associations for epidemiological observations that growth rate is linked to maturation and adult health are discussed.

Chapter 6: HPG, Feeding and Energetics

As discussed above, a wide range of variation in adult ovarian function exists across individuals within the same population and across populations and this variation is related to differences in the environmental context of those individuals and populations (Ellison 1993).Given the assumption that this sensitivity to ecological and energetic constraints develops during infancy and childhood (Worthman 1999), this chapter investigates the environmental sensitivity of sex steroid production during infancy by assessing the association between hormonal levels and feeding style, maternal anthropometric characteristics, and other socio-behavioral factors. The results of this chapter indicate that hormonal levels have a strong association with feeding style, but that this relationship is biologically confounded by infant size. The results of this chapter suggest that infant feeding style and hormonal levels may interact to establish be important in priming metabolic development.

IV. Summary and Conclusions

In this concluding section, the results of the present study are reviewed and "best" statistical models linking infant sex steroids to infant growth to energetic availability are presented. The implications of these models for arguments about female buffering and evolutionary explanations for sex differences in infant growth are discussed. Finally, the practical significance of these results for cultural models of infant feeding and public health are proposed.

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Chapter 2: Noninvasive methods for sex steroid recovery in infants: Fecal estradiol

Introduction

Although the effects of early androgen exposure have been well documented in animal models due to an interest in the masculinizing effects of androgens on postnatal brain development (Herman et al 2000; Mann and Fraser 1996; Resko and Roselli 1997), the role of early estrogen exposure has received attention recently in response to growing concerns about soy formula use in human infants (Irvine et al 1998; Setchell et al 1998; Sharpe et al 2002) and the effects of environmental estrogens used in industry and agriculture (Aksglaede et al 2006; Kelce and Wilson 1997). These studies have highlighted the important organizing role estrogens may play in infancy on the brain and sex-specific behavior of both rodents and non-human primates (Arnold and Gorski 1984). Modification of the sex steroid milieu in neonatal rodents and nonhuman primates, through the blockage of endogenous sex hormones and/or the administration of exogenous ones, alters reproductive axis function, leads to structural changes in the brain and affects sexual behavior (Arnold and Gorski 1984; Mann and Fraser 1996). In humans, the potentially disrupting effects of estrogens on male sexual development and behavior has become an area of intense research given the rise in environmental estrogens and the increasing use of soy formula, two factors whose long-term developmental effects remain unknown (Irvine et al 1998), but have been implicated in declining sperm counts and the prevalence of reproductive disorders (Sharpe 2003).

Despite the importance of estrogens in the postnatal development of the body, brain and behavior, obstacles in the collection of samples suitable for the analysis of sex steroids

have limited our understanding of the patterns of estradiol production and utilization in during this critical period of neonatal development. The activation of the infant hypothalamic-pituitary-gonadal (HPG) axis, the existence of a postnatal gonadotropin surge and the postnatal production of estradiol were first documented in the mid-1970's (Forest et al, 1974; Winter et al 1975). According to these early reports, levels of estradiol were low after the first month of life, showed little sexual dimorphism and were predominantly below the level of detection of standard radioimmunoassay (Chellakooty et al 2003; Forest and Ducharme 1993; Juul 2001). Due to these lower levels of estradiol and the greater heterogeneity in observed values (Chellakooty et al 2003; Lee 2003; Quigley 2002), less has been generally known about estradiol production, giving the impression that estradiol production is less physiologically important than testosterone production in early infancy. However, the development and use of new, more sensitive assay techniques have documented that very low concentrations of estradiol are present in both prepubertal boys and girls (Klein et al 1994) and that even brief exposure to estrogens can induce a multitude of changes in the testes and their function (Sharpe et al 2002; Toppari et al 1996). More recent studies utilizing these ultrasensitive assay techniques, nonetheless portray a complex pattern of sex steroid production in infant girls with notable inter-individual variability in hormonal levels documented at every age measured (Andersson et al 1998; Chellakooty et al 2003). In spite of the potential disrupting effects of estradiol in male postnatal sexual development, the production of estradiol in infant boys using more sensitive assays has not been reported.

The paucity of data on the production of estradiol is due in large part to the ethical and methodological difficulties inherent in sampling the relatively low levels of sex steroids

serum from healthy infants. The evidence to date of postnatal sex steroid levels is derived predominantly from studies of the gonadal contents of autopsied infants (Bidlingmaier etl al 1987; Forest and Ducharme 1993), small clinical samples (Ibanez et al 2002, Main et al 2000), cross-sectional studies of infants undergoing operative procedures (Forest et al 1974; Winter et al 1976), infants within a limited age range (Chellakooty et al 2003, Schmidt and Schwartz 2000) and infrequent longitudinal serum assessment (Andersson et al 1998) due to the invasive nature of serum sampling in young, healthy infants. Consequently, existing data can only describe the range of variability in hormonal levels and cannot assess developmental trends or the meaning of this variability in individual development.

Previously, researchers have developed salivary assays (Ellison 1988; Ellison and Lipson 1999; Shirtcliff et al 2000) and blood-spot assays (Shirtcliff et al 2000; Worthman and Stallings 1997) considered minimally invasive for sample collection in children and adults. However, both of these methods present practical and technical problems in young infants. Prior to teething, the provocation and collection of sufficient saliva quantities to assay, normally a volume 0.4 to 5mL per assayed hormone (Lu et al 1999), is uncertain and requires the use of cotton swabs, known to interfere with quantifiable levels of estradiol and testosterone (Granger et al 1999; Shirtcliff et al 2001). Additionally, salivary levels of sex steroids are estimated to be between 0.2 and 7.90% of circulating serum levels (Lu et al 1999), levels that may be too low to be detected in infants. Finger-prick blood spots have been also validated as a viable alternative to salivary samples in a diverse range of settings (Worthman and Stallings 1997); however, frequent blood spots

are also impractical in longitudinal studies of infant hormonal development, since few parents or infants would tolerate repeated finger pricks.

Consequently, we chose to utilize non-invasive methods for extracting estradiol from infant fecal samples, a method previously employed in field endocrinology of non-human primates (Brockman and Whitten 1996; Stavisky 1995; Wasser et al 1988; Whitten et al 1998a) and one that is particularly effective when non-invasive sampling collection is required (Whitten et al 1998a). Steroid hormones, such as estradiol, are eliminated from the body via the kidney, where they are conjugated and excreted in urine, and the GI tract, where they are excreted in the feces (Adlercreutz and Martin 1980). Unlike urinary hormones, the majority of estradiol excreted in feces (98%) is unconjugated; consequently, fecal estradiol levels may provide an analytically useful estimate of free, bioactive concentrations in the body (Adlercreutz and Jarvenpaa 1982). Additionally fecal steroidal measures may be particularly useful indicators of hormone production, because unlike serum samples which can be influenced by the pulsatile nature of sex steroid production, they provide a time-integrated measure over several hours to several days (Whitten et al 1998). This summary, time-integrative effect may serve to make fecal estimation of estradiol both more representative of 24-hr estrogen secretion in infants and also increase the probability of capturing levels of estradiol above the detection limit of commercial assays.

Fecal hormonal concentrations have been only infrequently utilized in human studies to assess the effects of dietary content, such as fat, fiber and protein (Goldin et al 1982; Gorbach and Goldin 1987; Lewis et al 1997), or medicines, such as antibiotics (Martin et al 1975), on the intestinal metabolism and clearance of estradiol, or, in one example, to

assign sex to paleofecal samples (Sobolik et al 1996). Fecal analysis has been more extensively validated in the study of non-human primates. Initially utilized to detect menstrual cycling changes and pregnancy (Wasser et al 1988), fecal hormonal analysis has been used to study reproductive seasonality (Ziegler et al 2000), stress (Whitten et al 1998b), dominance hierarchies and rank (Beehner et al 2005), and parenting behavior (Ziegler and Sousa 2002) in a wide variety of primate and other mammalian species. Not only has the analysis of fecal steroids proven to yield values highly correlated to those of serum (Whitten and Russell 1996), the collection of fecal samples has been shown to be feasible in field studies because sample collection does not interfere with a subject's behavior or endocrine state and requires only small quantities of fecal samples (usually less than 1gm) (Whitten et al 1998a).

Since the study of hormonal measures in human infants presents some of the same methodological problems as the study of non-human primates, namely the necessity of using non-invasive measures that do not require subject compliance and do not interfere in daily life, we have adapted methods developed for non-human primate field studies to document steroid hormone concentrations in human infant feces. These methods are particularly well suited to the study of human infant feces, since microradioimmunoassay techniques used in fecal analysis (Stavisky 1995) have the advantages of being highly specific for the measured hormone, reducing the potential effects of cross-reactivity and having a high sensitivity to allow the quantification of even low steroid levels. Thus, we aimed to 1) develop a collection and extraction protocol to allow for the longitudinal examination of infant fecal sex steroids; 2) validate commercially-available radioimmunoassay kits for the measurement of estradiol in human infant feces and 3)

modify a commercially-available kit to create and validate a microassay for the assessment of infant fecal estradiol. Here we describe a method for the collection, extraction and radioimmunoassay of fecal estradiol from the feces of human infants aged 1 week to 15 months of age. This extraction method is a modification of the one developed by Stavisky (1995) and used in the Laboratory for Reproductive Ecology at Emory University. The microimmunoassay techniques are modified from those developed by Shirtcliff and colleagues for use in bloodspot samples (2000). Assay performance is presented for both the straight, commercially-available kit and the modified assay and is characterized in terms of specificity, sensitivity, reliability, precision, accuracy and linearity.

Sample and Methods

Subjects

Thirty-two infants (15 male, 17 female) born after uneventful pregnancies participated in a prospective, longitudinal hormonal and growth research study after parental informed, written consent of an Emory University IRB-approved protocol. Subjects were recruited from an Emory University affiliated daycare center and opportunistically from university academic departments, Atlanta-area mothers' groups and lactation support groups. Infants entered the study between the ages of 7 days and 11 months and were followed with weekly sample collection and growth measurements for a median of 29 weeks (range: 4 days-15 months).

A subsample of 10 (5 female, 5 male) of these infants participated on a daily basis in the methods development phase of the study. These infants were 5 months or younger at enrollment and were followed for a period of 4 days to 12 months, with the majority

participating for over 6 months. All participating infants had a full term birth (birthweight >2500g and gestational age>37 weeks), were singletons and were born following uncomplicated pregnancies. While the infants had common respiratory and intestinal illnesses during the course of the study, there were no infants with known endocrine, metabolic or other significant pathologies.

Sample Collection and Preparation

For the initial phase of methods development, three types of diapers were provided for the study collection, traditional gel-based disposable diapers, an all-natural cotton diaper, and rice paper liners used inside cloth diapers, according to parental preference. After the initial methods development phase, parents were provided with all-natural cotton diapers and asked to collect all urine in these diapers. Parents were asked to retain all feces during the study interval, including those samples in gel-based diapers. The majority of parents nonetheless used the provided all-natural cotton diapers for both samples. Only the fecal samples were used in the present analysis.

The ten sets of parents participating in the initial methods development portion of the study retained and recorded **all** daytime and nighttime diapers during the course of the study. The subsequently enrolled parents (n=22) retained all overnight samples on the day of measurement. In both cases, soiled diapers or liners were placed in plastic storage bags and labeled for date and time of collection. Bagged diapers were stored in portable coolers equipped with dry ice packs frozen at -80°C. Within 24 hours, the diapers were collected and transported to the Laboratory of Reproductive Ecology, Emory University for storage at -80° C. Samples were analyzed between 24 hours and 12 months after collection.

Extraction Methods

Diapers were thawed in preparation for analysis overnight at 2-8° C. As this was the first time infant diapers were used to collect fecal samples for RIA analysis, several sample sizes, portions of the diaper and extraction methods were tested to develop the most efficient and reliable method. Initially, samples were subjected to four treatments. First whole rice paper liners (n=10), containing between 2.4 and 4.0 gm of feces, were submerged in 125mL of methanol, covered with parafilm and stirred for 3.5 hours. Samples were evaporated under nitrogen and reconstituted with 3mL of working buffer (0.1% gelatin-phosphate-buffered saline (pH 7.4), made by adding 0.1 gm of gelatin to 100mL of Delbecco buffer (Gibco, Grand Island, NY)) in preparation for immunoassay.

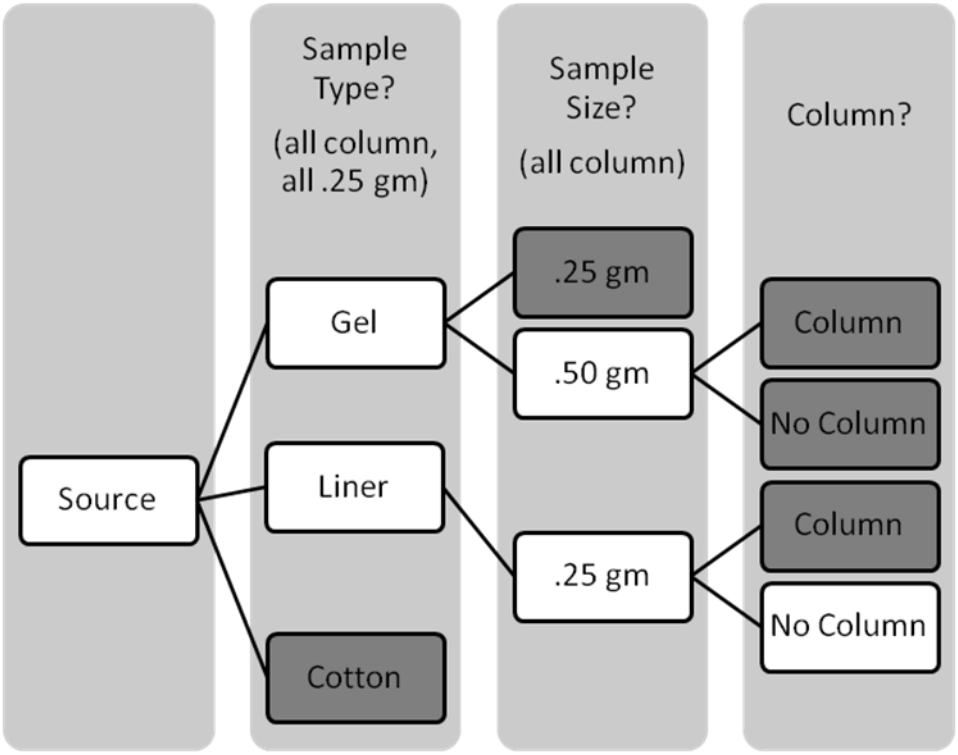


Figure 2.1: Extraction Method Development
Dark boxes represent methods that were not found less feasible or reliable

In the next 3 treatments (illustrated in figure 2.1, above), 5 samples were processed in each treatment group yielding 45 samples used in methods development. First, source of the sample was assessed; 0.25gm of fecal sample was gathered from the gel insert of the traditional diapers, the cotton insert of the all-natural diapers, or excised from the cotton liner of either type of diaper using disposable razor blades. All samples were placed in borosilicate glass tubes with 5mL of methanol and vortexed for 10 minutes.

For samples that would be column extracted, 3mL of the extract was then pipetted into 0.2 µm nylon microfilter centrifuge tubes (Centrex, Whatman Laboratory, Clifton, NJ) and centrifuged at 1500*g for 5 minutes. Samples were next extracted using small columns of reversed-phase octadecylsilane (C18) bonded to silica (Sep-Pack C18, Waters Associates, Inc. Milford, MA). The sample was diluted by an equal volume of distilled water and layered onto the column, primed according to manufacturer's instructions. The column was then washed with 5ml of distilled water and the steroid fraction was eluted with 3mL of methanol. This procedure was repeated for gel-based samples with initial mass of 0.50gm.

For samples not subjected to column extraction, 3ml of extract was pipetted into polypropylene tubes and centrifuged at 1500*g for 5 minutes. After spinning, the liquid phase of the samples were then decanted into 0.2 µm nylon microfilter centrifuge tubes (Centrex, Whatman Laboratory, Clifton, NJ) and centrifuged at 1500*g for an additional 5 minutes. Samples were transferred to labeled polypropylene tubes for storage and stored in -80 C until analysis.

The performance characteristics of each of these treatments are presented below in the results section below. Following the method development phase, all samples were excised, when soaked in to the cotton liner, or scraped from the cotton diaper liner if resting on top, spun once to remove solid particles and filtered through centrifuge tubes. A minimum of 2 samples was extracted from each diaper sample.

Estradiol Assay

Unmodified Assay

During the initial method development and validations, fecal estrogen was quantified using a commercial double antibody third-generation radioimmunoassay kit for the quantification of serum estradiol (DSL-39100, Diagnostic Systems Laboratories, Webster, Texas). Aliquots (200µl) of the methanol extracts of fecal samples were evaporated under nitrogen and reconstituted with 1mL of working buffer[2] before assay, yielding a dilution of 1:5. The estradiol standards provided with the DSL kit contain standard concentrations of 0, 1.5, 5, 15, 50, and 150pg/ml in human serum with sodium azide as a preservative. The antiserum is rabbit anti-estradiol in a protein based buffer (BSA) that cross-reacts 6.9% with estrone and <1% with equilinin, equilenin, 17β-estradiol-3-glucuronide and estriol. The second antibody is goat anti-rabbit gamma globulin in a protein-based buffer with polyethylene glycol. Controls are DSL internal controls and Lyphocheck immunoassay control sera Levels I and II (Bio-Rad/MP-Biomedicals, Anaheim CA) containing estradiol in human serum. The remainder of the assay followed the manufacturer's directions for an overnight incubation.

[2] 0.1% gelatin-phosphate-buffered saline (pH 7.4), made by adding 0.1 gm of gelatin to 100mL of Delbecco buffer (Gibco/Invitrogen, Grand Island, NY) and incubating for 4 hours at 45°C

Modified Assay

Once an appropriate extraction method had been established and the commercial assay had been validated for use in infant fecal samples, a microassay procedure was validated for use with the diaper extracts. This modified assay was based on the Shirtcliff et al.(2000; 2001) modifications of the DSL double antibody RIA kit for estradiol (DSL-39100, DSL, Webster, Texas) developed for use with salivary and blood spot estradiol. In this procedure, estradiol standards and controls provided with the DSL kit were diluted by 4 with working buffer to give concentrations of 0, 0.375, 1.25, 3.75, 12.5, and 37.5 pg/ml of estradiol. The first antiserum (rabbit-produced anti-E_2) was diluted 1:4, the DSL ^{125}I tracer was diluted 1:3 and, as in the unmodified assay, diaper extracts were diluted 1:5 with the working buffer. Duplicate aliquots (250µl) of the diluted standards, controls, and reconstituted samples were pipetted into labeled duplicate 12 x 75 polypropylene tubes. Diluted 1st antibody solution (100 µl) was pipetted into all tubes except the non-specific binding (NSB) and Total Count tubes and an equal amount of gel buffer was added to NSB tubes. All tubes were vortexed and incubated at room temperature for four hours. Following incubation, 100µl diluted ^{125}I E_2 tracer was added to all tubes, tubes were vortexed and incubated for 18-22 hours at 4° C. Following the overnight incubation, tubes were brought to room temperature and precipitating reagent (500 µl) was added to all tubes except the total count tubes. The tubes were vortexed, incubated at room temperature for 20 min, and then centrifuged at 1500*g* for 1 hour. Finally, the supernatant was decanted, blotted and counted for 5 min in a RIASTAR gamma counter (Packard, Downer's Grove, IL) using RIASMART and Expert QC software. Results are calculated in pg/ml using log-linear regression.

RIA Validation

Both RIA assay methods were validated for sensitivity, accuracy, precision, and parallelism. Assay sensitivity was determined by the quantity of unlabeled steroid required to inhibit binding of tracer by an amount equal to two standard deviations below the mean in the absence of unlabeled hormone. The accuracy of the method was assessed by measuring the recovery of incremental known quantities of steroid to low diaper extract samples. Within-assay precision was tested by the mean coefficient of variation of duplicate fecal extracts and replicate determinations of high and low serum controls samples within the same assay, while between-assay precision was measured by the coefficient of variation of serum controls run across multiple assays. Controls were included in each assay and included a pool of fecal extracts, 2 serum controls (low and high) provided with the kit and 2 Lyphocheck serum controls (low and high) purchased separately. Parallelism was assessed by serially diluting a high sample and comparing the slope of the expected dose versus the percent bound to the slope of the standard curve for the RIA.

Treatment Groups

Sample type

Given the novelty of the extraction of fecal steroids from infant diapers, several treatments were applied to fecal samples to test for potential interference of the diaper matrix and to assess sources of variability that could influence assay results. First, to test for potential interference by the diaper cotton matrix, samples of clean cotton liner were run through the extraction and assay procedures to test for the presence of any cross-reacting metabolite in the cotton liner. In a second test, clean samples of cotton liner

(n=25) were cut at sizes similar to those used in the sample collection process and weighed. Similarly sized liner samples (n=25) were then cut from soiled diapers and the weight difference between the clean and soiled liners were assessed to address the proportion of the fecal/cotton sample that was accounted for by the cotton liner. Additionally, in samples of sufficient size and proper consistency (n=205), a scraped sample and a liner sample were collected to test for any differences introduced by sample type. Differences between sample types were assessed with matched pair t-tests.

Within-sample variability

We then addressed other sources of variability that could influence the assay results. First, we measured the potential effects of within-sample variability. Steroid hormones have been documented to vary within an individual fecal sample (Adlercreutz and Jarvenpaa 1982; Millspaugh and Washburn 2003). To test the homogeneity of fecal steroid distribution across the individual diaper, we assessed duplicate samples (n=352) from the same diaper using the same sampling strategy (i.e. 2 liner samples or 2 scraped samples) but sampled from different portions of the diaper. Additionally, in a subsample of diapers (n=4), multiple samples were extracted from the same diaper, with 2 adjacent samples being collected and 2 samples collected from different parts of the diaper. Differences in CV's between were assessed between the sampling strategies.

Between sample variability

Circadian variability

To determine the proper sample collection for the expansion of the weekly component of the study, we tested whether fecal estradiol in infants showed diurnal variability in the

methods development phase. Serum studies have documented a diurnal pattern of estradiol secretion in adolescent girls and adult women (Licinio et al 1998; Norjavaara et al 1996), but fecal samples have less consistently shown a diurnal pattern (Beehner and Whitten 2004) likely due to the time-integrated nature of the fecal sample. To test for a diurnal effect in infant diaper samples, morning estradiol values (defined 3 ways: 0:00-11:59am, 0:00-10:00am, or 5:00-9:00am; n=625, 393, and 259, respectively) were compared to non-morning estradiol values (the samples not included in above definitions; n=591, 769, and 907, respectively) across the sample, using a two-sample t-test with unequal variances. Mixed model multiple regression analysis was then used to test for a predictive relationship between estradiol dose and time of day controlling for individual effects on estradiol levels.

Time between samples

The number of fecal samples varies between individual infants and changes with age in this sample. Under 3 months of age the number of daily fecal samples ranged from 0-5, while at 6 months this range was 0-3 diapers per day. We tested whether estradiol levels were significantly associated with the duration between samples among the subset of infants (n=8) for whom we had a complete multiday series of diapers (n=557).

Time to assay

Finally, to determine whether steroid concentrations differed in relation to the length of time between collection and assay, we tested whether this length of time was significantly associated to hormone concentration by using multiple regression analysis. Additionally, we reran diaper extracts that were assayed early in this longitudinal study again towards the end of analysis. These samples, along with other repeated samples, were used to test

whether the change in time between assays was associated with the change in assayed values using Spearman's rank correlation.

Results

Sample collection

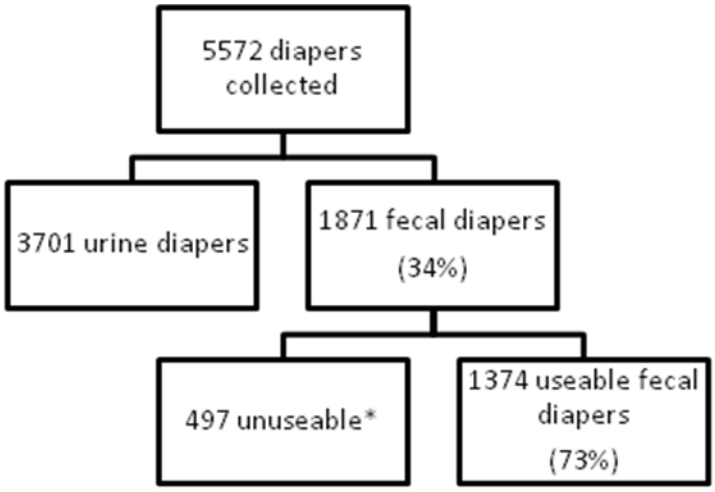


Figure 2.2: Sample collection
*unuseable diapers were contaminated with urine, left unchilled or of insufficient size to sample.

5572 diapers were collected in the development and weekly phases of the study. Of these 34% contained fecal samples, yielding 1871 fecal samples. Fecal samples that were of insufficient weight for extraction (<0.25gm), heavily contaminated with urine or not kept chilled were excluded from analysis, yielding a final sample size of 1374 useable fecal diapers.

Effects of sample type and extraction method

Table 2.1: Comparison of assay performance by diaper type

DIAPER TYPE	Mean pg/gm (SD)	%CV
Rice paper liners (n=10)		
no column	.637 (.175)	27.57
Cotton inner layer (n=5)		
Column	.021 (.005)	28.47
Gel inner layer (n=20)		
column	.056 (.014)	24.40
no column	.097 (.013)	12.85
Cotton liners (n=20)		
column	.381 (.099)	28.60
no column	.612 (.013)	16.20

Mean values and between sample coefficients of variation were calculated for the different extraction treatments (depicted in figure 2.1) and are presented in the **Table 2.1** above. While the highest mean estradiol values were measured using the rice paper liners and no column extraction method, this technique also yielded the highest %CV between tubes and required the most time and materials to process. The methanol volume of 125mL required to process the samples was prohibitively time-consuming for larger sample sizes and was more prone to error due to incomplete recovery of volume due to spillage during mixing. The cotton inner layer of the all-natural diapers was excluded due to low recovery of steroid hormones from the pulpy cotton matrix and the number of samples which were below the detection limit of the assay (3 out of 5). While both the gel inner layer component of the traditional diapers and the external cotton liners, from both the traditional and all-natural diapers, had comparable %CV, the recovery from the gel inner layer was lower even at sample sizes that were twice as large (0.50gm) as those used with the cotton liner (0.25gm). Finally, the effect of column extraction on recovery was assessed. Samples not run through the column, but spun an extra time before being

filtered through the centrifuge tubes, had both higher recovery in the gel inserts and cotton liners and lower %CV. Cotton liners and a column-free technique therefore provided the best conditions for recovery and reliability, with a relatively higher mean dose of 0.612ng/gm and a CV of 16.2% between the 5 assayed test samples.

Assay Performance

Table 2.2: Performance of DSL Estradiol RIA and Diluted Assay

Assay Performance	DSL-39100 kit	Diluted Assay
Range of standards pg/ml	1.5-150 pg/ml	0.375-37.5 pg/ml
Range, pg/tube	0.3-30 pg/tube	0.08-7.5 pg/tube
Sample volume	200 µl	250 µl
Sample dilution	1:5	1:5
Analytical sensitivity	0.95 pg/ml (N=4)	0.35 pg/ml (N=25)
R of standard curve	1.000 (N=4)	0.992 (N=25)
Analytical recovery %	105.8 +/- 2.2	88.8 +/- 5.9
Parallelism	Yes	Yes
Precision		
Intra-assay CV % (Mean)		
Low serum control	6.8 (5.9 pg/ml)	10.3 (3.7 pg/ml)
High serum control	5.8 (29.2 pg/ml)	7.2 (10.8 pg/ml)
Low sample	11.8 (3.4 pg/ml)	5.8 (3.4 pg/ml)
Medium sample	6.2 (13.0 pg/ml)	-
High sample	3.9 (123.1 pg/ml)	2.2 (8.7 pg/ml)
Inter-assay CV % (Mean)		
Low serum control	4.8 (8.5 pg/ml)	11.0 (3.5 pg/ml)
High serum control	5.8 (29.2 pg/ml)	12.9 (9.0 pg/ml)
Low sample	7.7 (12.4 pg/ml)	18.5 (3.3 pg/ml)
Medium sample	3.4 (37.1 pg/ml)	16.2 (10.8 pg/ml)
High sample	8.9 (62.9 pg/ml)	9.9 (34.3 pg/ml)

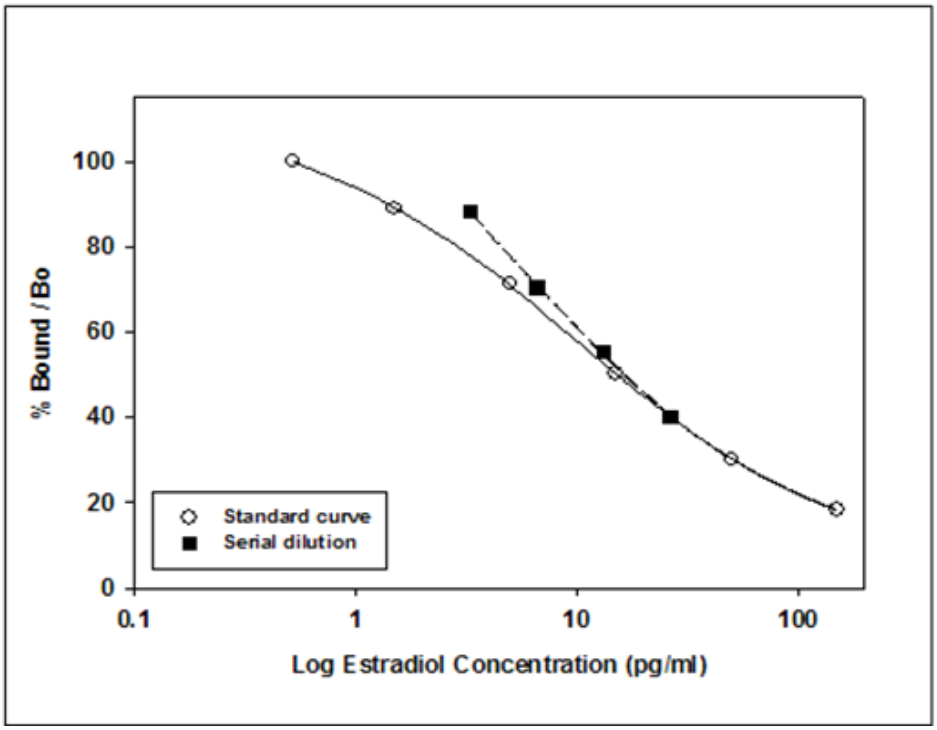
Unmodified DSL kit

Assay validation results for the standard, commercial estradiol assay (DSL-39100) are presented in **Table 2.2** above. Sensitivity, estimated by interpolation of the mean minus two standard deviations for 5 sets of duplicates at the zero standard, was 0.95pg/ml. The accuracy of the method was assessed by the quantitative recovery of estradiol standards added to aliquots of fecal extract. The percent recovery was high (106%) over a 1.5-150 pg/ml range of concentrations **(Table 2.3)**. Serial dilution of diaper extracts gave displacement curves paralleling the curve obtained for the estradiol standard over the range 5-37 pg/ml **(Figure 2.3)**. Within-assay precision was tested by replicate determinations of control, high, medium and low samples and ranged from 3.9-11.8% CV's. Inter-assay CV's, tested by replicate determinations of serum controls and high, medium, and low samples across assays ranged from 3.4-8.9%.

Table 2.3: Recovery of estradiol added to diaper extract with low endogenous estradiol

Endogenous pg/ml	Added pg/ml	Expected pg/ml	Observed pg/ml	Recovery %
DSL 39100				
15.00	0.00			
15.00	1.50	16.50	18.21	110.36
15.00	5.00	20.00	21.31	106.55
15.00	15.00	30.00	29.96	99.87
15.00	50.00	65.00	71.93	110.66
15.00	150.00	165.00	167.80	101.70
			Mean+/-SEM	105.8+/-2.2
			y=1.02 x + 16.47, r = 0.998	

Figure 2.3: Serial dilutions of diaper extract compared to standard curve from DSL Estradiol Double Antibody Kit.



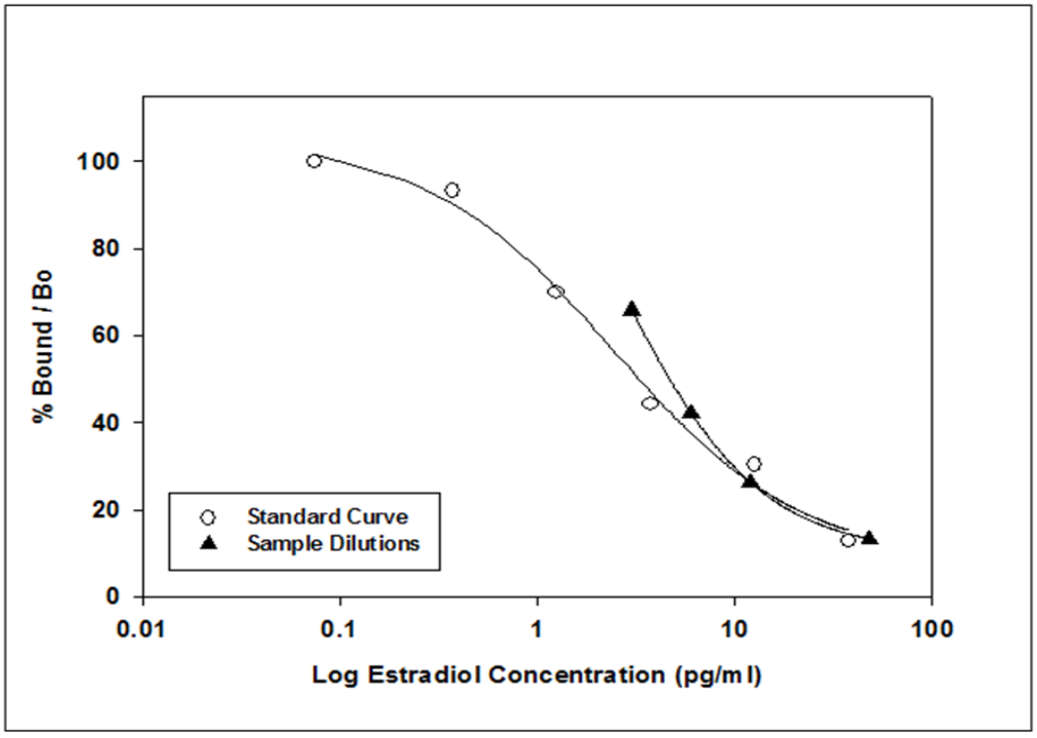
Diluted Assay

Assay validation results for the modified, microassay (DSL-3900) are also presented in **Table 2.2**. The analytical sensitivity of the diluted assay was 0.35pg/ml. In tests of accuracy, the percent recovery of estradiol was lower and averaged 89% over a 0.4-12 pg/ml range of concentrations **(Table 2.4)**. Serial dilution of extracts gave displacement curves that paralleled the standard curve over the range of 5-48 pg/ml, diluted 1:1 to 1:8 **(Figure 2.4)**. Therefore, samples were diluted 1:5 for assay. Intra-assay coefficients of variation ranged from 7.2-10.3% for high and low serum controls and 2.2-5.8% for high and low fecal samples. Inter-assay CVs were 11-13% for high and low serum controls and 9.9- 18.5% for low, medium and high samples.

Table 2.4: Recovery of estradiol added to diaper extract with low endogenous estradiol

Endogenous pg/ml	Added pg/ml	Expected pg/ml	Observed pg/ml	Recovery %
Diluted Assay				
1.96	0.00			
1.96	0.38	2.34	1.70	72.81
1.96	1.25	3.21	2.83	88.16
1.96	3.75	5.71	5.34	93.52
1.96	12.50	14.46	14.57	100.76
			Mean+/-SEM	88.8 +/-5.9
			y=1.05 x - 0.67, r = 1.000	

Figure 2.4: Serum dilution of a diaper extract to standard curve of diluted estradiol assay

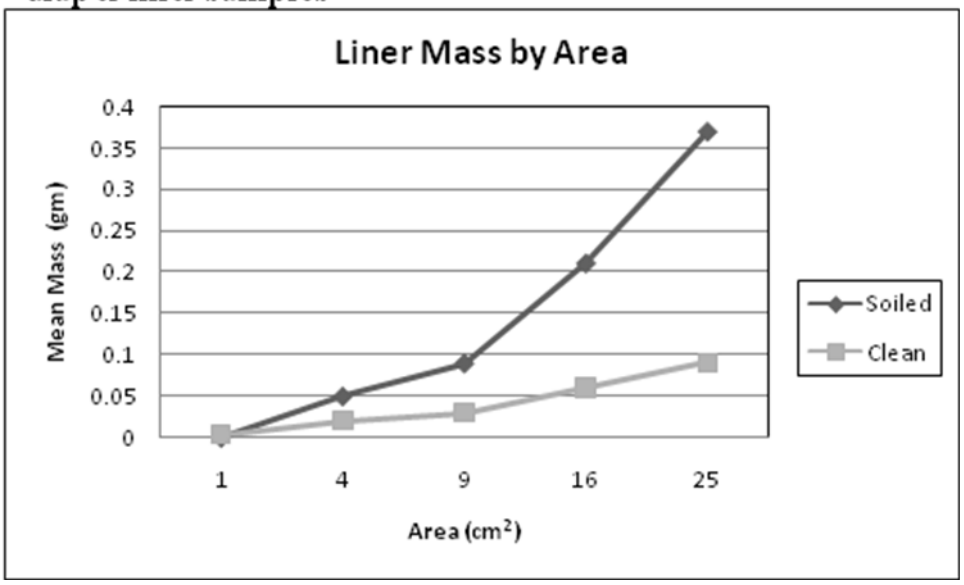


Effects of treatment group

Sample Type

There was no evidence of interference from the cotton liners in the measurement of fecal estradiol from the infant diaper samples. First, the cotton liners subjected to the extraction and assayed following the diluted procedure yielded no measureable estradiol levels; these results were below the lower limit of the curve. Second, the proportion of cotton liner:feces was calculated. These results are shown in **Figure 2.5** below. At the liner size used most commonly in the assay, between 16cm^2 and 25cm^2, the proportional difference between clean liner mass and soiled liner is greatest, indicating that the cotton liner contributes relatively little to sample weight.

Figure 2.5: Comparison of the mass of soiled and clean diaper liner samples



Differences in repeated samples using different sampling strategies, one liner sample and one scraped sample, were assessed by matched pair t-tests. The mean estradiol dose of liner samples was 0.51pg/gm (SD=0.58) while the mean dose of scraped samples was

0.49pg/gm (SD=0.47). These differences were not significant in the 205 paired samples (t=0.80, p=0.42).

Effect of within-sample variability

To assess the potential heterogeneity of estradiol levels across the fecal sample, different sampling strategies within the same fecal sample were assessed. Results from a subset of diapers (N=4) that had both discordant and adjacent areas sampled indicated that samples taken from different areas of the diaper had higher %CV than samples that were taken from more adjacent areas. %CV from duplicate samples taken from adjacent areas was 5.3%, while those taken from different areas were 16.2%. To control for these potential effects, 2 samples from different areas were collected from future samples. Those with high %CV between duplicate samples were pooled. Pooling the samples reduced the variability, yielding a %CV of 6.4%. However, differences in dose between the first and second samples (N=352 pairs) from the whole study were not significant in matched-pair t-test analysis (t=0.28, p=0.78).

Table 2.5: Effect of sample location on within-diaper variability

	Discordant-Area Sampling		Adjacent-Area Sampling		Pooled Aliquot	
	Mean pg/ml (SD)	**%CV**	**Mean pg/ml (SD)**	**%CV**	**Mean pg/ml (SD)**	**%CV**
1	10.76 (2.02)	18.72	11.57 (1.52)	13.17	11.66 (.81)	6.94
2	8.68 (.62)	7.10	8.86 (.94)	10.56	7.66 (.66)	8.60
3	4.87 (1.39)	28.60	6.96 (.74)	10.61	5.25 (.18)	3.44
4	4.18 (.44)	10.54	3.04 (.01)	0.29	3.62 (.23)	6.44
Summary		16.24%		5.32%		6.36%

Between sample variability

Effect of circadian variability

The relationships between time of day and estradiol dose were assessed using two sample t-tests and are presented in **Table 2.6.** Mean estradiol levels from the morning fecal samples did not differ from those at other times of the day when defined as midnight to noon or midnight to 10:00am. There was a trend towards lower mean estradiol levels in early morning samples, defined as 5:00-9:00 am[3], than those from later in the day.

Table 2.6: Effect of time of day on estradiol level

Time of day	N	Mean E_2 (SD) (1= in time period)	N	Mean E_2 (SD) (0=not in time period)	T value (p)
0:00-11:59am	625	.56 (1.26)	591	.50 (.86)	1.04 (.30)
0:00-10:00am	393	.49 (.77)	769	.52 (.97)	.55 (.58)
5:00-9:00am	259	.43 (.81)	907	.53 (.84)	1.71 (.09)

To assess the potentially confounding effects of individual variability on these relationships, if infants with generally higher estradiol levels were more likely to defecate later in the day, mixed model regression analysis, controlling for individual, was conducted. These models found no significant association between any of the time period and mean estradiol dose, except for the 5:00-9:00am time period, which showed a negative trend with estradiol level (β=-.10, p=.09).

[3] The 0:00 to 10:00 am time period was chosen based on previous research conducted on urinary cortisol in this sample of infants (Rikin, Thompson and Lampl, unpublished data). The 5:00-9:00 time period was chosen to correspond the normal time range of awakening times in this sample of infants.

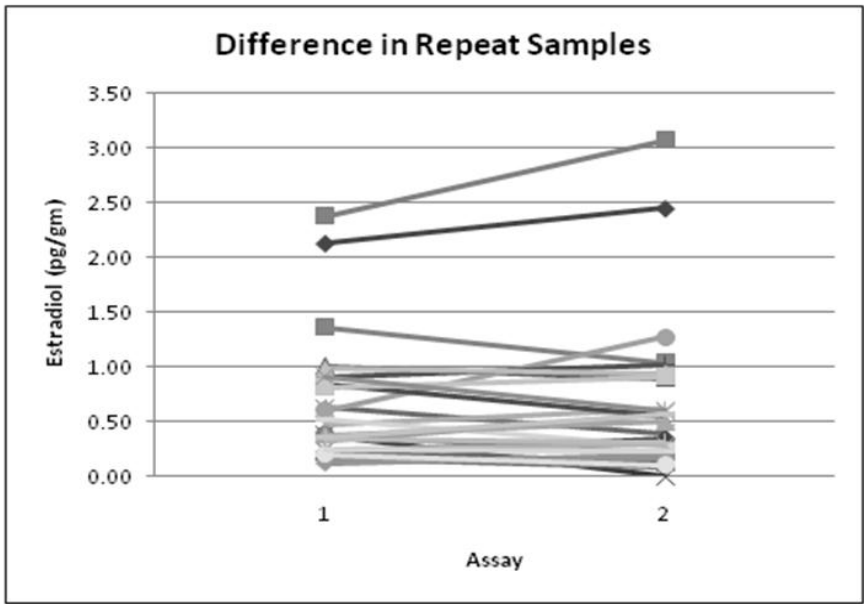
Effect of time between samples

Time between diaper samples was calculated in a sub-sample of infants for whom we had complete multiday fecal series (N=494). Mixed-model regression analysis, controlling for age, the interaction between age and hours between diapers and the random effect of individual, was used to test whether the time lapse between diaper samples influenced estradiol values. Results of the modeling indicated that the hours between diaper samples had a slight negative effect on estradiol level (β=-0.02, p=0.003).

Effect of time to assay

The mean length of time between sample collection and assay was 103 days, with a range of 1-489 days. Mixed model analysis was used to test whether length of time between sample collection and assay had an effect on estradiol values. There was no significant association between time to assay and estradiol value, controlling for individual subject as a random effect, over the whole sample (β=0.0003, p=0.23). To further test this relationship, we reran samples at various time spans during the course of the longitudinal study for quality control. The estradiol doses in the first and second assay runs are plotted in **Figure 2.6.**

Figure 2.6: Effect of time between collection and assay on estradiol values in repeat samples



Spearman's correlation was used to whether time between assays was associated with the difference in dose between the 2 assay runs in the samples shown graphically above (N=31 paired observations). The median length of time between assay runs was 15 days, with a range of 0 to 554 days. Changes in estradiol dose between samples was not correlated with the length of time between assay runs (r=-0.17, p=0.35).

Discussion

Previous investigations indicated that fecal sampling provides accurate and reliable results in the study of primate sex steroid levels (Whitten et al 1998a); the current study demonstrates the utility of fecal analysis in the measurement of sex steroids in human infants as well. The developed extraction and assay procedures have the advantages of (1) requiring a low sample volume (≤0.25gm/sample), (2) having adequate sensitivity to detect the full range of individual differences in both male and female infants

(sensitivity=0.35pg/ml), and (3) being well-suited to use in a longitudinal protocol (easy to collect and well-tolerated by participants).

Based on the assay results from the different extraction techniques, we choose to employ a method of sampling feces from cotton diaper liners, using a double centrifuge method and no solid-phase column extraction step. This method had the advantage of requiring smaller sample size (0.25gm) and producing more reliable %CV than the other methods tested. Interestingly, the use of column extraction, which has been shown to be important in processing primate fecal samples (Stavisky 1995), yield both lower and less reliable results than samples not run through the column. This difference may be explained by a reduced efficiency of the column due to diaper residue remaining in the sample or, conversely, to the benefit of the extra spin in the no-column method. This latter explanation seems more likely as the first spin in the centrifuge has the effect of removing a measureable amount of solid particles from the fecal sample. An additional advantage of the double-spin procedure implemented here is the reduction in the time and cost associated with the solid-phase, column extraction, an issue which is not insignificant in a sample of this magnitude.

While both the straight commercial assay and the modified assay were accurate and precise in assessing fecal estradiol levels, with inter- and intra-assay coefficients of variation within the accepted range for radioimmunoassays, the modified assay had the additional advantage of increased sensitivity (0.35 pg/ml for the modified assay vs. 0.95pg/ml for the commercial assay). The modified assay also had a lower recovery, 88.8% (±5.9), than the non-modified assay, 105.8% (± 2.2); this result is likely due to the dilution of the modified assay, which may have enhanced specificity by reducing the

potential for cross-reactivity with other estrogen metabolites. Recovery was higher for the range of 5-48 pg/ml over which parallelism was observed. The modified assay then was preferable for the assessment of estradiol in infant samples because the greater sensitivity of the assay facilitated the measurement of declining estradiol levels with age (Andersson et al 1998), allowed the dilution of samples to reduce matrix effects and potential cross-reactivity of other metabolites and reduced the cost of the assay for use in frequent longitudinal sampling by reducing the amount of reagents used per assay.

These chosen extraction and assay procedures were able to quantify estradiol in 97% of samples from both male and female infants, levels that are higher than those seen in infant serum studies (Andersson et al 1998; Chellakooty et al 2003). This greater level of detection highlights one of the main advantages of fecal sampling in infants; the time-integrative nature of the samples, while nonetheless showing intra-daily variation, provides a composite, summary measure of estradiol production over several hours to a whole day. Unlike serum samples, fecal steroids document a time-lag from production to excretion of several hours to several days in primates (Whitten et al 1998a). The faster gut transit time of human infants however may reduce the magnitude of this delay. A wide range of gut transit times have been reported from infants, with a low of 90 minutes in infants irrespective of feeding type, age or sex (Khin et al 1999) to 17.42 hours in 4-month old formula fed infants (Sievers et al 1993). Observations from tracking the appearance of undigested solid foods in the stool of infants in this sample suggests that transit of solid foods may be as little as several hours.

Fecal levels of estradiol and other sex steroids have been documented to closely parallel serum values in primates (Whitten and Russell 1996), allowing assessment of endocrine

function without invasive blood sampling. In their study of sooty mangabeys, Whitten and Russell (1996) documented that fecal steroids parallel the peaks and troughs seen in the longitudinal profile of serum hormone measurement in individual animals. Such results indicate that fecal analysis can be sensitive enough to fluctuations in circulating serum concentrations to detect individual differences as well as response to external constraints, such as seasonality. This evidence, along with the high rates of recovery and sensitivity of the assays detailed here, indicates that the longitudinal assessment of fecal steroids in human infants should elucidate both individual developmental changes and the potential associations between fecal steroid levels and measures of growth, nutrition and behavior.

While the relatively elevated levels of sex steroids in human feces (Adlercreutz and Jarvenpaa 1982) provide the methodological advantage of permitting analysis of infant estradiol levels throughout the ages measured here, one week to 15 months of life, the analysis of estradiol from infant diaper samples introduces challenges not seen in serum studies. First, there is the potential issue of cotton interference from the diapers used to collect the fecal sample. Any interference of cotton would be more likely at earlier ages in breastfed infants whose stool is looser and more highly incorporated into the cotton liner. Previous examination of the effects of a cotton collection matrix on salivary levels of estradiol documented that cotton dental swabs used to collect saliva in infants and children can bias results (Shirtcliff et al 2001). However, the use of cotton diaper liners did not appear to affect the results of the current analysis. Estradiol values did not significantly differ between samples scraped from the feces on top of the liners and those excised from the cotton liner within the same diaper, suggesting that the cotton matrix did

not interfere with steroid recovery. Further, unsoiled cotton samples run through the extraction procedure and assayed documented no measurable estradiol levels, indicating that the cotton itself does not cross-react with the assay. These differences from past assessment may be due to the relatively higher levels of steroids present in feces when compared to saliva or to the use of methanol extraction to draw the steroids out of the cotton matrix, a step that was not taken in the salivary analysis.

Another issue in the analysis of diaper samples is the possibility for urine contamination of feces in diapers that contain both fluids. To minimize this potential for cross-contamination, diapers with an obvious mixture of urine and feces were not used in analysis. In diapers that contained both substances, care was taken when extracting samples to avoid fecal samples that were obviously mixed with urine. However, it is unlikely that inadvertent urine contamination plays a large role in the variability evidenced between samples. Urine and feces differ in their main metabolite of estrogen, estrone and estradiol respectively, and only a minimal fraction of urinary estradiol (Adlercreutz and Martin 1980) is present in the unconjugated form measured by this assay. In testing the effects of urinary contamination in primate samples, Wasser and colleagues (1988) found that the addition of 1 ml of urine per gram of feces in ethanol-extracted fecal samples had no effect on calculated doses of estradiol. The amount of urine added in this experiment is greater than that expected to occur physiologically.

Another potential confounder in the analysis of fecal steroids is that the amount of steroid hormone may be heterogeneous within the same fecal sample (Adlercreutz and Jarvenpaa 1982; Millspaugh and Washburn 2003). Comparison of the coefficients of variation between adjacent sampled areas and more divergent areas, ranging between 4.1-19.2%,

suggested in this may introduce variation in this study. We addressed this variability by taking duplicate samples from different areas of the same diaper and using a pooled aliquot with the values varied. Overall analysis of the differences in estradiol dose between these duplicate samples showed no significant differences, indicating that sample heterogeneity is unlikely to have a large effect.

While comparative assessments suggested that variability in estradiol concentrations may result from the effects of sample dispersion within and between diapers and the unequal inclusion of liner in the recovery from diaper to test tube, this effect was less important for large samples than for smaller samples, where the liner content contributed a greater proportion to the sample. These assessments highlight the need to standardize by weight and/or size when extracting diaper samples. Time of day and time to assay, however, had no significant effects on heterogeneity between samples indicating that this method is robust to different sampling strategies and can be employed without bias in long-term studies.

The direct correlation between serum and fecal steroid levels was not measured. Such a comparison, which has been conducted in primate models (Whitten and Russell 1996) would require repeated serum blood draws, something that is unethical in healthy infants. However, the levels of fecal steroids in these infants showed overlap with documented levels of fecal steroids in human adults (Goldin et al 1982; Sobolik et al 1996). This is a pattern that is also seen in infant serum when compared to adult serum, indicating that fecal hormonal levels are reflecting serum production. Unlike serum analysis, fecal samples can be collected both frequently and over a long period of time; longitudinal analysis, therefore, would allow the association between fecal levels of

steroids and physiological measures to be assessed and provide physiological validation independent of serum measures. These advantages of frequent, repeated sampling are unique to fecal analysis and, we believe, outweigh the potential disadvantages introduced by intra- and inter-sample variability.

Conclusion and Future Directions

The validity of this novel, non-invasive method for assessing estradiol in infants was supported by a steroid recovery rate of over 89%, a sensitivity of 0.35 pg/ml, and intra- and inter-assay coefficients of variations of less than 10 and 20%, respectively. The use of fecal steroids assays –as in primates- may be a powerful tool in monitoring the development and activation of the HPG axis in early infancy in humans, yielding a method suitable for examining both inter- and intra-individual differences in hormonal concentrations. The ability to frequently sample hormonal levels presents the unique opportunity to explore the longitudinal associations between steroid hormone levels and measures of infant growth and development.

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Chapter 3: Measurement of Testosterone in Infant Fecal Samples

Introduction

The relationship between salivary testosterone and a variety of physiological, behavioral and biosocial variables in children has been the focus of much recent research (Granger et al 1999). Studies have documented a possible link between testosterone and social dominance (Booth et al 2006), aggression (Scerbo and Kolko 1994) and psychological/behavioral disorders (Constantino et al 1993; Granger et al 2003) in prepubertal and adolescent children. In adults, salivary testosterone has been used to measure moment to moment changes in testosterone in response to challenges such as sports competitions (Passelergue and Lac 1999), chess games (Mazur et al 1992) and aggressive encounters (Mazur and Booth 1998). It has been hoped that salivary measures of testosterone in children could be used to measure response to external stimuli as well (Granger et al 1999). Salivary measures are advantageous in this context because they do not require invasive blood sampling, can be repeated at short time intervals and can be collected without causing stress, thus reflecting physiological state (Vining and McGinley 1987). Additionally, salivary measures are thought to quantify the serum proportion of free testosterone, acting as a valuable marker of bioactive testosterone (Rilling et al 1996; Vining and McGinley 1987).

However, the results of socioendocrinological studies in children are not consistent, with only some finding an association between the behavioral outcome of interest and testosterone level (Rowe et al 2004; Strong and Dabbs 2000). These disparate findings may be due to the ages of the children measured, with more consistent associations being seen through the pubertal transition and later adolescence than from studies of younger

children (Ostatnikova et al 2002; Strong and Dabbs 2000). The confounding effect of age may reflect real, physiological differences in the secretion of testosterone, or may, on the other hand, indicate a lack of analytical power to reliably measure testosterone in the saliva of younger children (Granger et al 1999).

While the use of salivary sampling is convenient in studies involving children or those requiring repeat sampling, its utility may be limited by the low levels of testosterone in saliva, generally considered to be .01-.001% of serum levels (Vining and McGinley 1987). Salivary testosterone studies are further complicated by the substances used to stimulate salivary flow (Granger et al 2004), gender differences in reliability (Shirtcliff et al 2002), and questions about the power of salivary measures to detect behavioral associations (Shirtcliff et al 2002). These problems are further exacerbated in young infants where, prior to teething, the provocation and collection of sufficient saliva to assay is uncertain and requires the use of cotton swabs, known to interfere with quantifiable levels of testosterone (Shirtcliff et al 2001).

For these reasons, a need for an accurate and sensitive non-invasive method for measuring testosterone in infants and young children exists. Having established an efficient extraction procedure and a reliable, sensitive assay procedure for the measurement of estradiol from infant diaper samples (presented in chapter 2), we tested whether these protocols would yield similar success in the measurement of testosterone in fecal samples of male and female infants during the first 15 months of life. Here we detail our assay protocol, modified from Granger et al (1999), and validate this modified micro-radioimmunoassay for the use in human infant diaper samples.

Subjects and Methods

Subjects

Thirty-two infants (15 male, 17 female) born after uneventful pregnancies participated in a prospective, longitudinal hormonal and growth research study after parental informed, written consent of an Emory University IRB-approved protocol. Subjects were recruited from an Emory University affiliated daycare center and opportunistically from university academic departments, Atlanta-area mothers' groups and lactation support groups. Infants entered the study between the ages of 7 days and 11 months and were followed with weekly sample collection and growth measurements for a median of 29 weeks (range: 4 days-15 months).

A subsample of 10 (5 female, 5 male) of these infants participated on a daily basis in the methods development phase of the study. These infants were 5 months or younger at enrollment and were followed for a period of 4 days to 12 months, with the majority participating for over 6 months. All participating infants had a full term birth (birthweight >2500g and gestational age>37 weeks), were singletons and were born following uncomplicated pregnancies. While the infants had common respiratory and intestinal illnesses during the course of the study, there were no infants with known endocrine, metabolic or other significant pathologies.

Sample Collection and Preparation

Parents were provided with all-natural cotton diapers and asked to retain all feces during the study interval, in the case of infants in the daily, methods development phase of the study, or in the day preceding measurement in the weekly study. Parents were instructed to save any fecal samples during the period, including those samples that may be in

traditional, gel-based diapers. The majority of parents nonetheless used the provided all-natural cotton diapers for the fecal sample.

Soiled diapers were placed in plastic storage bags and labeled for date and time of collection. Bagged diapers were stored in portable coolers equipped with dry ice packs frozen at -80°C. Within 24 hours, the diapers were collected and transported to the Laboratory of Reproductive Ecology, Emory University for storage at -80° C. Samples were analyzed between 24 hours and 12 months after collection.

Diapers were thawed in preparation for analysis. Samples that were contaminated with urine, of insufficient size to assay (<.25gm) or left un-chilled were not subjected to analysis. This protocol yielded 1374 diaper samples. Fecal samples were excised from the cotton liner of the diaper if soaked in or scraped from the liner if resting atop the liner and weighed. At least two samples were collected from the same diaper when possible and were subjected to methanol extraction (described in greater detail in chapter 2).

Assay Procedure

Fecal testosterone was quantified using a commercial double antibody third-generation radioimmunoassay kit for the quantification of serum testosterone (DSL-4900, Diagnostic Systems Laboratories, Webster, Texas), following Granger et al (1999). The antiserum is rabbit-produced anti-testosterone in a protein based buffer (BSA) that cross-reacts 6.6% with 5α-dihydrotestosterone, 2.2% 5-andristane-3β, 1.8% 11-oxotestosterone, <1.0% androstenedione, 5β- dihydrotestosterone, and estradiol. Aliquots of 50 µl (for male samples) or 100 µl (for female samples) of the methanol extract were evaporated under

nitrogen and reconstituted with 1mL of working buffer[4] before being assayed, yielding a dilution of 1:20 for male samples and 1:10 for female samples. Testosterone standards and controls provided with the DSL kit were diluted by 100 with working buffer to give concentrations of 1, 5, 10, 25, 50, 100 and 250pg/ml. The low and high internal kit controls were diluted by 100 and 30, respectively, to obtain values of 5pg/ml and 100pg/ml. Controls are DSL internal controls and Lyphocheck immunoassay control sera Levels I and II (Bio-Rad/MP-Biomedicals, Anaheim CA) containing testosterone in human serum. The first antiserum, DSL ^{125}I tracer and precipitating reagent were used without modification.

Diluted standards, controls, and reconstituted samples were pipetted into labeled 12 x 75mm polypropylene tubes in duplicate. 20µl of diluted 1st antibody solution was pipetted into all tubes except the non-specific binding (NSB) and Total Count tubes. An equal amount of working buffer (20µl) was added to NSB tubes. All tubes were vortexed and incubated in a 37° C warm bath for 30 minutes. Following incubation, 50µl ^{125}I T tracer was added to all tubes, and tubes were vortexed and incubated for 3 hours in a 37° C warm water bath. Precipitating reagent (500µl) was added to all tubes except the total count tubes. The tubes were vortexed and incubated at room temperature for 20 min. All tubes except the total count tubes were centrifuged at 1500*g* for 20 minutes. Finally, the supernatant was decanted, blotted and counted for 5 min in a RIASTAR gamma counter (Packard, Downer's Grove, IL) using RIASMART and Expert QC software. Results were calculated in pg/ml using log-linear regression.

[4] 0.1% gelatin-phosphate-buffered saline (pH 7.4), made by adding .1 gm of gelatin to 100mL of Delbecco buffer (Gibco/Invitrogen, Grand Island, NY) and incubating for 4 hours at 45°C

Statistical Analysis

After assay validation, we next assessed other sources of variability that could influence the assay results. To test the ability of the method to distinguish inter-individual differences in testosterone, Kruskal-Wallis non-parametric tests were used to determine whether median testosterone levels differed between individuals. Mixed-model multiple regression analysis, with individual entered as a random effect, was used to test for a predictive relationship between testosterone dose and time of day.

Results

Assay Performance

Table 3.1: Modified Assay Performance Characteristics

Assay Performance	DSL-4100 kit
Range of standards pg/ml	1.0-250 pg/ml
Range, pg/tube	0.4-100 pg/tube
Sample volume	400 µl
Sample dilution	1:10 (female) 1:20 (male)
Analytical sensitivity	.35 pg/ml (N=24)
R of standard curve	.99 (N=22)
Analytical recovery %	112.2±3.98
Parallelism	Yes

Assay validation results for the modified testosterone radioimmunoassay (DSL-4100) are presented in **Table 3.1** above. Sensitivity, estimated by interpolation of the mean minus two standard deviations for 24 sets of duplicates at the zero standard, was 0.35 pg/ml.

Table 3.2: Recovery of Steroid in Diluted Samples

Recovery		Expected	Observed	%Recovery		Expected	Observed	%Recovery
Dilution	1:10				1:20			
STD	0		39.47		0		17.81	
	1	40.4	36.17	89.4	1	18.81	14.71	78.2
	5	44.47	46.02	103.5	5	22.81	22.88	100.3
	25	64.47	94.26	146.2	25	86.81	55.22	63.6
	100	139.47	250.21	179.4	100	117.81	167.51	142.2

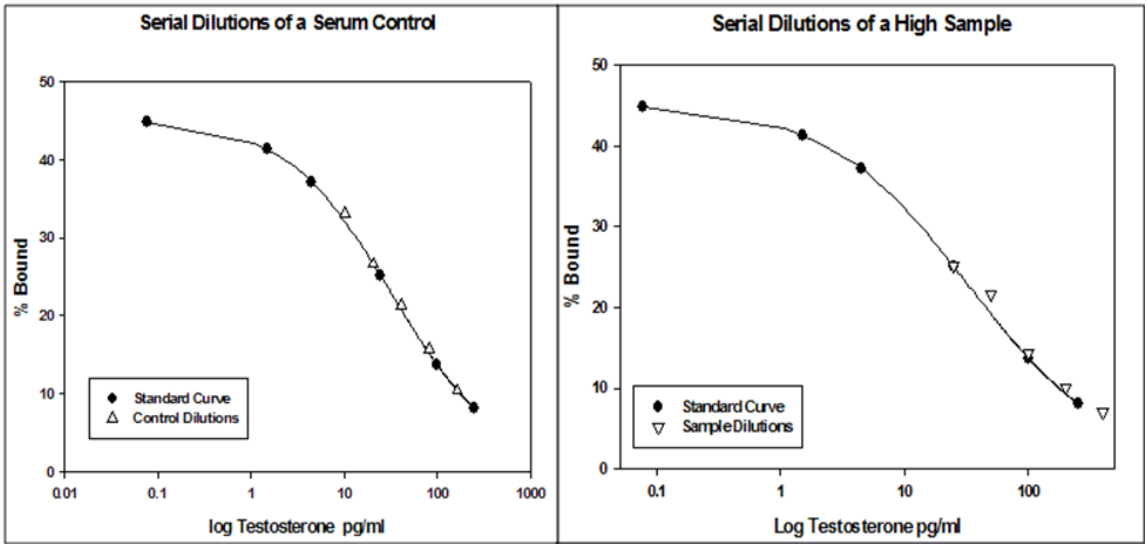
The accuracy of the method was assessed by the quantitative recovery of testosterone standards added to aliquots of fecal extract. The percent recovery was high (112.2±3.98) over a 14.7-250.2 pg/ml range of concentration. Consequently, samples were diluted to achieve a dose of ≤ 50 pg/ml, 1:10 for females and 1:20 for males. This dilution yielded a recovery closer to 100% and may have reduced the competitive binding of testosterone metabolites.

Table 3.3: Precision of Modified Assay

Intra-assay Variability	**N**	**%CV (Mean pg/ml)**
Low Serum Control	10	7.06 (5.35)
High Serum Control	10	5.59 (111.72)
High Sample	10	8.09 (39.34)
Inter-assay Variability		**% CV (Mean pg/ml)**
Serum Control	30	11.6 (78.70)
Male Pool (1:20)	30	10.8 (21.86)
Female Pool (1:10)	30	15.39 (21.3)

Within-assay precision was tested by replicate determinations (n=10) of the control and male and female pooled samples. Intra-assay coefficients of variation ranged from 5.6-8.1%. Inter-assay CV's, tested by replicate determinations (n=30) of an external serum control and pooled male (diluted 1:20) and female (diluted 1:10) samples across assays, were10.8-15.4%.

Figure 3.1: Serial Dilutions for the Modified Testosterone Assay

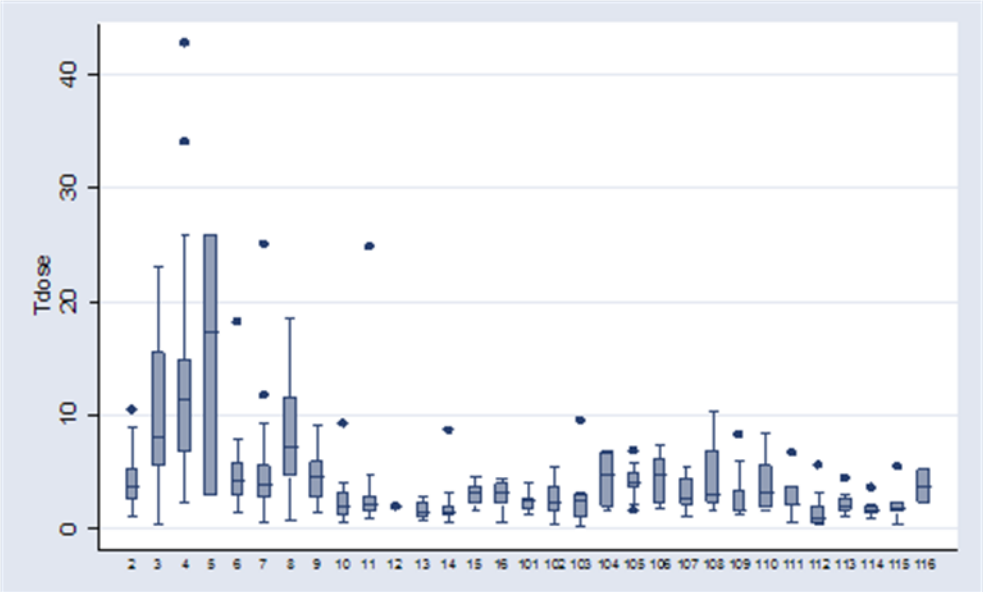


Serial dilutions of serum control and infant fecal extracts (**Figure 3.1**) gave displacement curves paralleling the curve obtained for the testosterone standards.

Sources of Variability in Testosterone Levels

Testosterone levels varied between individual infants (K-Wallis, p=0.0001). Diurnality was explored and testosterone levels showed a slight negative association with time of day, such that samples from later in the day tend to be higher than samples collected upon arising (5-9am) (xtreg, β=-0.64, p=0.097). There were no significant differences between testosterone in am and pm samples or between the quartile measures of time (0-5:59am, 6:00-11:59am, 12:00-5:59pm and 6:00-11:59pm).

Figure 3.2: Intra- and Inter-Individual Variability in Testosterone



Discussion

The sensitivity of this modified assay (detection limit=0.35 pg/ml), permits intensive frequent assessment of testosterone production in both male and female infants. Using this extraction and assay method, the current study was able to document detectable testosterone levels from 1 week to 15 months of life in both male and female infants and to discern individual differences in testosterone levels. This protocol was able to quantify testosterone in 100% of samples from both male and female infants, a proportion that is higher than that seen in infant serum studies (Andersson et al 1998; Chellakooty et al 2003).

The modified assay was accurate and precise in assessing fecal testosterone levels, with inter- and intra-assay coefficients of variation within the accepted range for radioimmunoassays. The assay is sensitive (0.35 pg/ml) and demonstrates a high recovery of fecal steroids, 112.2±3.98. The higher than 100% recovery of testosterone

added to fecal samples suggests that, at high doses of testosterone, the assay may be cross-reactive to testosterone metabolites. The dilution of the fecal samples to a 50pg/ml range, with a dilution of x10 in females and x20 in males, yielded recovery levels between 75 and 90%. Thus, this dilution may have reduced any matrix effects and potential cross-reactivity of other testosterone metabolites. Use of this diluted assay has the additional benefit of reducing assay costs, increasing the feasibility for use in frequent longitudinal sampling.

Testosterone levels in this sample of infants were variable within and between individuals. Testosterone also varied over the course of the day in individual infants. There was a trend ($p<0.10$) for testosterone levels in the early morning period to be lower compared to those from later in the day. While preliminary, this data may evidence a diurnal pattern of testosterone production, documented in salivary and serum samples of adults (Dabbs 1990; Valero-Politi and Fuentes-Arderiu 1996), with early morning samples representing those processed by the gut in the afternoon to evening of the preceding day. However, even in this large sample (n=722 timed samples), the level of significance only reached 0.097.

Like salivary studies, fecal testosterone measures have the advantage of allowing frequent, non-invasive sampling. However, as the results of this assay validation show, fecal testosterone levels in infants may be higher than those seen in salvia, due to the time integrative nature of fecal sampling, allowing assessment of testosterone in young infants and children. While fecal samples may not be able to detect minute to minute changes in serum testosterone, they may be able to address behavioral questions that span hours to days. Studies of the gut-transit time in infants support this supposition that fecal

hormonal levels represent conditions occurring between within as little as 90 minutes (Khin et al 1999) or as long as 17 hours (Sievers et al 1993), depending on infant age and feeding style. Individual level examination of the data suggests that biobehavioral factors have the potential to contribute to the documented variation in testosterone levels. Although preliminary, such associations indicate that this method will allow the assessment of the relationship between biobehavioral factors and testosterone production in infants.

Conclusion

The validity of this novel, non-invasive method for assessing testosterone in infants was supported by a steroid recovery rate around 100%, a sensitivity of 0.35 pg/ml, and intra- and inter-assay coefficients of variations of less than 10 and 1-15% respectively. While preliminary, the validated results of this extraction procedure and microassay technique for the measurement of testosterone from infant diaper samples suggest that fecal measurement of testosterone may be a powerful non-invasive tool for exploring both the development and function of testosterone in human infancy and the relationship between testosterone levels and salient bio-behavioral variables, such as infant feeding, developmental milestones or illness markers.

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Chapter 4: HPG Activation in Infancy: Developmental and Gender Trends

Introduction

HPG activation in infancy

The activation of the infant hypothalamic-pituitary-gonadal (HPG) axis and the existence of a postnatal gonadotropin surge were first documented in the early 1970's with Faiman and Winter's observation that follicle stimulating hormone (FSH) and luteinizing hormone (LH) levels in female infants, aged 0-2 years, were higher and showed greater variability than those seen in similarly aged male infants (Faiman and Winter 1971). The functional effects of these sex differences in gonadotropins were then more fully described by Forest and colleagues who found that not only did gonadotropin levels differ at birth and during the first year of life, but that sex steroid levels differed as well. Forest and colleagues categorized this surge in the HPG axis as a "mini-puberty"(Forest et al 1974). However, despite the widespread acceptance of this "mini-puberty" by pediatric endocrinology texts and human biologists, the patterning and potential functions of this surge have only been described more recently (Andersson et al 1998; Chellakooty et al 2003; Schmidt and Schwarz 2000; Schmidt et al 2002).

These studies (summarized in Tables 4.1 and 4.2) document a peak in gonadotropin and sex steroid production in male infants around 3 months of life, when follicle stimulating hormone (FSH), luteinizing hormone (LH) and testosterone levels are all elevated, followed by a gradual decline to nearly undetectable levels after 6 postnatal months (Andersson et al 1998; Forest et al 1974; Winter et al 1975; Winter et al 1976). LH is much higher in male than female neonates and its elevation stimulates postnatal testosterone production (Waldhauser et al 1981). Support for the hypothesis that LH

stimulates testosterone production comes from studies of the pulsatile nature of LH in neonates. Repeated serum sampling at 20-minute intervals for 160-360 minutes in infants receiving therapeutic partial exchange transfusion on the day of birth documented that LH release differed by sex, with males having a pulsatile secretion of LH at a median interval of 78 minutes and females having no detectable LH (de Zegher et al 1992). This production has been hypothesized to be necessary for normal scrotal and testicular development. Studies of neonatal boys suffering from hypogonadism revealed lower than normal testosterone levels and suggested to researchers that normal phallic and scrotal development in humans is dependent on intact testosterone secretion during early infancy (Main et al 2000; Main et al 2006). Inhibin B, a hormone produced by the Sertoli cells, is similarly elevated at 3 months of age, with peak levels that fall into the supra-adult range and remain elevated longer than the other hormones (Andersson et al 1998; Bergada et al 1999; Crofton et al 2002b). As inhibin B is a marker of Sertoli cell function and the total number of Sertoli cells is important for spermatogenic potential later in life, this sustained elevation suggests that the neonatal period may be an important window for Sertoli cell proliferation and maturation (Crofton et al 2002b). These high neonatal levels of gonadotropins and gonadal hormones have been documented to subside within the first year of life and remain low throughout childhood (Crofton et al 2002a; Crofton et al 2002b; Demir et al 1995; Raivio and Dunkel 2002). A relatively constant amount of inhibin B, however, is secreted throughout childhood and this secretion is hypothesized to contribute to the quiescence of the HPG axis during childhood by exerting negative feedback on the hypothalamus (Crofton et al 2002b).

Although the hormonal profiles during the fetal and postnatal surges have been well characterized in male infants, analogous studies in female infants have been hampered by the greater complexity of hormonal patterning in females, the extensive inter-individual variability in hormonal concentrations, and the greater frequency of values falling below the threshold of detection (reviewed in: Chellakooty et al 2003; Lee 2003). Similar to males, an activation of the HPG axis occurs during early postnatal development in females. In contrast to males, LH levels remain at a comparably low range, whereas those of FSH are 5- to 10-fold higher in females than similarly-aged males (Burger et al 1991). A large cross-sectional study of neonatal hormonal levels demonstrated that within the first 28 days of life, not only were FSH levels in females much higher than in males, but the concentrations of FSH were frequently above the normal upper range for adult women (Schmidt and Schwarz 2000). FSH release showed peaks and troughs when measured at 30 minute intervals in female infants aged 6-12 weeks (Waldhauser et al 1981). These sex differences may reflect the different function of FSH in male and female infants; in girls, high FSH levels result from low inhibin levels and likely permit neonatal estradiol (E2) production and follicular development. Estradiol production is measurable in infant girls through the end of the first year of life, with values falling within the adult mid-follicular range of 280-550 pmol/L (Burger et al 1991). Although FSH and E2 levels show a large range of variation between individuals, estradiol nonetheless appears to have a biological function during infancy. Research among deceased infants has shown that estradiol content of the ovaries is correlated with ovarian size and follicular differentiation, suggesting a role for infant E2 in gonadal growth (Bidlingmaier et al 1987). More recent studies of living infants also suggest that E2 levels

are correlated with breast tissue size (Schmidt et al 2002) and body weight at 3 months postnatal age (Lee 2003).

The physiology of sex steroids in males and females

Given the documented mitogenic and growth-promoting character of sex steroid hormones in adults, it is perhaps not surprising that sex steroids may have a role in infant development as well. In males, testosterone plays a role in many physiological processes including: prenatal and postnatal sexual differentiation and development, muscle protein metabolism, pubertal masculinization, initiation of spermatogenesis and adult cognitive and sexual function (reviewed in: Allen and Key 2000; Beauchet 2006; Bhasin 2000; Herbst and Bhasin 2004; Resko and Roselli 1997; Zhang et al 2000).

In adult males, testosterone is synthesized from cholesterol in the Leydig cells of the testes under the stimulation of pituitary-derived luteinizing hormone (LH) at a rate of approximately 5 mg/day. Once produced, testosterone enters the general circulation. Approximately 50% of this circulating testosterone is tightly bound to sex-hormone binding globulin (SHBG) and an additional 48% is bound with lower affinity to albumin, rendering these portions biologically unavailable (Vermeulen 1988). The remaining 2% of testosterone produced is considered "free" or unbound. It is generally assumed that only this free component can stimulate the gonadal and extra-gonadal effects of testosterone, although this has not been firmly established (Vermeulen 1988). The amount of bioavailable testosterone present is largely controlled by circulating SHBG concentrations and an increase in total testosterone production is required to increase the bioavailable component (Vermeulen 1988). The production of total testosterone is controlled by a negative feedback loop between circulating testosterone and LH release,

with high levels of testosterone, directly or after aromatization to estradiol (reviewed in: Raven 2006), reducing the frequency of the pulsatile release of LH by the pituitary.

In adult females, testosterone is mainly derived from ovarian production (Davis and Tran 2001) and the peripheral conversion of androstenedione (reviewed in: Speroff and Fritz 2005). The production rate in adult women is approximately 0.3 mg/day, a production 20 times lower than in adult men (reviewed in: Speroff and Fritz 2005). The physiological function of these low levels of testosterone in females is not well understood, though the maintenance of circulating androgen levels is hypothesized to be necessary to ensure an adequate supply of precursor hormone for estrogen biosynthesis in extra-gonadal sites, such as the brain, bone and adipose tissues (Davis 1999). Additionally, testosterone appears to be necessary for promoting and maintaining hair growth and bone mineral density (Greendale et al 1997) and is associated with better performance on cognitive function tests (Barrett-Connor and Goodman-Gruen 1999) and improved libido in older women (Davis and Tran 2001).

Estrogens, on the other hand, have wide ranging physiological effects in both males and females. In females, estrogen stimulates ovarian growth and development, breast development, gynoid fat deposition (adipose distribution on the thighs, hips and buttocks), and bone growth and epiphyseal fusion (Gruber et al 2002). Studies of estrogen-deficient or insensitive males have indicated that estrogen also plays an important role in bone growth and homeostasis, the initiation and completion of the pubertal growth spurt, cardiovascular health, and pituitary-gonadal interactions (de Ronde et al 2003). As with testosterone, the majority of estradiol, the most biologically active of the estrogens, is bound to SHBG and albumin, leaving a “free,” bioactive

component of approximately 2-3% (reviewed in: Rosen and Cedars 2007; Speroff and Fritz 2005). Estradiol is produced at levels much lower than testosterone in both women and men, with non-pregnant adult women producing 100-300 µg/day and adult males producing 35-45 µg/day (deRonde et al, 2003).

In females, estradiol is synthesized in a biphasic manner in the ovaries from circulating cholesterol. The pre-ovulatory follicle produces estradiol in a paracrine interaction between the theca and granulosa cells under the stimulation of both FSH and LH. During this phase, the theca cells produce testosterone which is rapidly aromatized into estradiol by the granulosa cells (Strauss and Hsuesh 2001). During the early and mid-follicular phases in adult women, low levels of estradiol have a negative feedback effect on FSH production; this feedback becomes positive in the later follicular phase with higher estradiol levels promoting further follicular development and estradiol secretion (reviewed in: Rosen and Cedars 2007). During the luteal phase, estradiol levels decline and low levels of estradiol again promote FSH secretion.

In both males and females, estradiol also arises from the conversion of androstenedione via estrone in extra-gonadal sites such as skin cells and adipose tissue (Speroff et al, 1983). In males, this peripheral conversion accounts for nearly 80% of daily estradiol production with only 15-20% estradiol produced by the testes. Enough estradiol can be produced from the conversion of free androgens to produce menstrual bleeding in postmenopausal women (Sitteri and McDonald, 1973), indicating that this extra-gonadal conversion can have physiological effects. Much less is known about the physiology and metabolism of sex steroids during early development, but studies of males with hypogonadism (Main et al 2000) and a female infant with aromatase deficiency (Mullis et

al 1997) point toward a physiological role in sexual development for the relatively low levels of sex steroids found in infancy.

Study Aim

While the image of a neonatal gonadotropin and sex steroid production peak between 3 and 6 months and a decline to childhood levels after 6 months is commonly found in studies of developmental endocrinology, remarkably little is known about the physiology of sex steroid production and utilization during this period of infant development from a longitudinal perspective. Instead, the evidence to date is derived predominantly from convenience samples of infants receiving operative procedures (Burger et al 1991; Winter et al 1975; Winter et al 1976), studies of the gonadal contents of autopsied infants (Bidlingmaier et al 1987), small clinical samples (n=4 and n=10, respectively) (Main et al 2000; Waldhauser et al 1981), and, more recently, from two large cross-sectional studies of 1126 3-month old infants (Schmidt et al 2002) and 473 3-month old girls (Chellakooty et al 2003). These studies, along with a single longitudinal study of 30 infants measured every four months for a period of two years (Andersson et al 1998), do provide support for a trend of high neonatal gonadal hormone levels with a gradual decline across the first year of life (**Figure 4.1**). However, the cross-sectional or infrequent longitudinal nature of these data has not permitted assessment of developmental trends in individual infants and particularly notable in these studies is the large range of inter-individual variability in hormonal levels at every age measured.

Given the large variation in previously documented physiological levels of sex steroid hormones, this study aims to employ novel, non-invasive fecal sampling of testosterone and estradiol to describe the weekly pattern of sex steroid production and variability over

the first 12 months of postnatal life. More specifically, this chapter aims to (1) assess the effects of increasing age on sex steroid levels in infants, (2) test whether hormonal levels vary by sex and (3) explore individual developmental trends as a source of previously documented sample-level variability in sex steroids.

Sample and Methods

Sample

Thirty-two infants (15 male, 17 female) participated in a prospective, longitudinal hormonal and growth research study. Subjects were recruited from an Emory University affiliated daycare center and opportunistically from university academic departments, Atlanta-area mothers' groups and lactation support groups. Infants entered the study between the ages of 7 days and 11 months and were followed weekly for a median of 29 weeks (range: 4 days-15 months) (**Table 4.3**). A subsample of 8 of these infants participated on a daily basis. These infants were 5 months or younger at the beginning of the study and were followed for a period of 4 days to 12 months, with the majority participating for over 6 months. All participating infants had a full term birth (birthweight >2500g and gestational age>37 weeks), were singletons and were born following uncomplicated pregnancies. All infants were clinically healthy during the course of the study with no diagnosed endocrine problems.

The study site varied by sample sub-group. All 8 of the daily infants and 8 of the weekly infants were measured during home visits with their mother and/or caretaker present at the time of measurement. The remaining 16 infants were measured weekly at a daycare center. These study protocols were approved by the Emory University Social/Humanist and Behavioral Institutional Review Board.

Fecal Sample Collection and Hormone Analysis

Parents were asked to retain all diapers from the evening prior to through the morning of the day of measurement in the case of the weekly samples and all daytime and evening diapers in the case of the daily sample. Soiled diapers were stored in portable coolers and chilled with freezer blocks frozen to -80° C until collected. Upon collection, samples were stored at -80°C until processed. Fecal samples that were of insufficient weight for extraction (<0.25gm), heavily contaminated with urine or not kept chilled were excluded from analysis. This protocol yielded 487 useable, weekly fecal samples.

Fecal samples were excised from the diapers, extracted with methanol, spun down to remove any remaining solid particles and filtered through 0.2 µm nylon microfilter centrifuge tubes (Centrex, Whatman Laboratory; for a detailed description of this method, see chapter 2). Extracted samples were then assayed using validated modifications (Shirtcliff et al 2000) of commercially-available RIA kits for testosterone and estradiol (DSL labs, Weber, TX). All samples were assayed in duplicate and the mean value of these duplicate samples was used for analysis. Samples were standardized for weight and values are expressed as pg/gm. These modified microassays had a detection limit of 0.35 pg/ml. Intra-assay CV's were between 3.3% and 8.1% and inter-assay CV's were between 8.9 and 15.4%, values within the accepted range for RIA assays. Steroid recovery was high and samples were diluted to reduce potential competitive binding from other testosterone (1:10 for females, 1:20 for males) and estrogen (1:5 for both sexes) metabolites.

Statistical Analysis

SAS version 9.0 (SAS Institute, North Carolina) and STATA/SE version 8 (Stata Corporation, College Station) statistical software were used for all statistical analyses. Steroid fecal values were corrected for fecal weight and are expressed in pg/gm. Descriptive statistics of hormonal measures are given as median values and reference ranges (minimum to maximum). If a measured hormone was below the detection limit, the value was expressed as 0 pg/gm for subsequent analysis. Analyses were repeated with undetectable values set at the limit of detection, but this did not change the results. Sex differences in steroid levels were assessed using ANOVA, weighted for sample frequency, and Kruskal-Wallis non-parametric tests. Repeated-measures mixed models were used to assess significant effects of age and sex on testosterone and estradiol levels. Subject was included as a random effect in these models to control for subject-specific effects such as measurement dependability and to account for the within-subject correlation. Statistical significance was defined as $p < 0.05$.

Results

Sample descriptives

Table 4.3 shows the basic background and anthropometric characteristics of the study sample. This sample of mothers and infants were generally healthy with normal ranges of birthweight and maternal BMI. The samples differed in 3 main ways. First, parity differed between the samples with 14 of the 15 mothers in the home-based sample being primiparous, while only 9 of 16 mothers in the daycare sample had no other children ($p=.037$). Secondly, gestational age at birth was slightly lower in the daycare sample than

in the home-based sample (39.16 *vs*. 40.35 weeks, p=0.01). More importantly, the median infant age at enrollment in the study differed between the study samples. Home-based study infants had a mean age of 2.97 months at study entry (sd±2.39, range= 0.23-7.67 months) while the daycare sample had a mean age of 7.42 months (sd: ±2.64, range=2.91-11.97; p=<.001). However, the home based sample was followed for a longer median duration, 18 weeks (range: 1-63) *versus* 14 weeks (range: 7-15, p=.03) leading to substantial overlap in measured ages. There were no significant differences in other maternal or infant characteristics between the groups. No mothers in either group reported a history of gestational diabetes, pre-eclampsia or smoking during pregnancy and/or the study interval.

Table 4.3: Sample Characteristics by Study Site

	Home-based sample	**Daycare Sample**
Mothers	*n=15*[†]	*n=16*
Age (*years*)	32.90 (4.05)	34.06 (3.91)
Primiparity (%)	93	56[*]
Pre-pregnancy Weight *(kg)*	62.96 (8.74)	63.29 (5.48)
Height *(cm)*	166.65 (6.69)	165.16 (7.18)
Pre-pregnancy BMI	22.5 (2.37)	23.29 (2.34)
Pregnancy weight gain *(kg)*	16.19 (4.37)	15.21 (6.57)
Infants		
Gestational Age *(weeks)*	40.35 (1.32)	39.16 (1.29)**
Birth weight *(kg)*	3.45 (4.24)	3.41 (4.38)
Birth length *(cm)*	51.25 (2.74)	52.30 (2.74)
BMI at birth	13.18 (1.65)	12.45 (1.00)
Male sex (%)	46	54
Age at Study Entry *(months)*	2.97 (2.39)	7.42 (2.64)***
Length of participation *(weeks)*	18 (1-63)	14 (7-15)*

[†]One mother in the home-based sample did not fill out the background questionnaire
*p <.05, **p<.01, ***p<.001

Inter- and Intra-Individual Variation in Sex Steroids

The sensitivity of these modified assays (described in Chapters 2 and 3), 0.35 pg/ml, allowed us to detect both estradiol and testosterone levels from one week of age to 15 months in both male and female infants. Testosterone was measurable in all 487 samples. Estradiol was measurable above the detection limit in approximately 90% of our samples. Fecal testosterone levels were generally an order of magnitude higher than estradiol samples, although there was considerable overlap at lower values. Estradiol levels tended to be lower than testosterone levels in both males and females and ranged from below the detection limit to 3.51 pg/gm with a median of 0.26 pg/gm. Testosterone levels were both higher, with a median value of 3.2 pg/gm, and showed more variation, with a range of 0.38 pg/gm to 42.8 pg/gm. The sample-level steroid concentrations are shown in the Figures 4.2 and 4.3. These scatterplots document a great degree of variability in testosterone and estradiol levels, with seemingly little developmental pattern.

An examination of median sex steroid values by subject indicates that inter- and intra-individual variability is present in both testosterone and estradiol levels (Figure 4.3).To illustrate individual differences and trends in sex steroid secretion, **Figure 4.4** shows the percentiles of testosterone and estradiol for both male and female infants. Infants in the highest percentiles have 10 to 20-fold higher testosterone levels than infants in the lowest percentiles for age. Similarly, infants in the highest percentiles have estradiol levels that are 1-2 fold higher than those in the lowest percentiles.

Developmental Trends

Testosterone

Graph 4.5 shows the median sample level of testosterone over the first 15 months. The sample-level curves of fecal testosterone versus age in general showed the highest values closest to birth and then a gradual decline to 6 months of age in both male and female infants. Male infants, but not female infants, show a slight increase in testosterone between 2 and 3 months. Median testosterone level at 3 months, however, is not statistically significantly greater than at 2 months when assessed using K-Wallis non-parametric comparison ($p=0.31$). Median testosterone levels were significantly higher before 6 months than after 6 months in males (KWallis, $p=0.001$). The reverse was found in females where median testosterone levels were higher after 6 months than before 6 months (KWallis, $p=0.02$).

In mixed model analysis controlling for within-subject correlation, testosterone was negatively associated with increasing weekly age over the first 13 months ($\beta=-0.04$, $p=0.03$). When stratified by sex, this negative association between testosterone levels and age is evidenced only in male infants, where increasing weekly age is associated with a decline in testosterone levels ($\beta=-0.11$, $p=0.001$).

Estradiol

Figure 4.6 shows the median sample level of estradiol over the first 15 months. Like testosterone, the sample-level curves of fecal estradiol versus age showed the highest values closest to birth in both male and female infants. Unlike testosterone, however, median fecal estradiol levels declined within the first month and remained relatively constant until 6 months in both male and female infants. After 6 months, female infants had a slight increase in estradiol levels, while male levels remained relatively constant.

In mixed model analysis controlling for within-subject correlation, there was no significant relationship with age over the entire 15 months of age in both male and female infants (β=0.002, p=0.32).

Sex differences

Table 4.4: Sex Differences in Testosterone and Estradiol

	Male *Median (range)*	**Female** *Median (range)*
Testosterone (pg/gm)	3.29 (.19 - 42.9)	3.17 (.39-25.03)
Estradiol (pg/gm)	.22 (0-2.47)	.33 (0-4.30)*

*p=.001

Testosterone

Sex-specific median levels of testosterone are presented in **Table 4.4** Testosterone levels in boys ranged from 0.19 to 42.9, with a median of 3.29 pg/gm. Testosterone levels in girls were slightly lower, with a range of 0.39 to 25.03 pg/gm and a median value of 3.17 pg/gm. There were no significant sex differences in testosterone levels assessed over the first 14 months of life when assessed by mixed-model analysis controlling for age and within-subject correlation (xtreg: β=-0.99, p=0.30). When the data are analyzed by age, as is indicated by the sample-level graph of testosterone by sex (**Figure 4.5**), sex is a significant predictor of testosterone before 6 months of age when boys have significantly higher testosterone levels than girls (β=-3.73, p=0.02). There are no significant sex differences in testosterone levels after 6 months (β=-0.30, p=0.66).

Estradiol

Sex differences in the median and range of estradiol are also presented in **Table 4.4.** Median estradiol levels were 0.22 pg/gm in boys, with a range of below the detection limit to 2.47 pg/gm. Median estradiol levels in girls were 0.33 pg/gm with a range of below the detection limit to 4.30 pg/gm. These differences in estradiol levels showed a trend towards significance in mixed models controlling for age and within-subject correlation, with estradiol levels positively predicted by female sex over the first 14 months of life (xtreg: β=.13, p=.045; model p=.08).

Female infants were 1.8 times more likely to have estradiol values above the median in comparison to male infants over this time period (OR=1.8, p=0.002). Comparison of the graph of median estradiol values by sex **(Figure 4.6)** indicates that, while boys and girls have similar values and slopes before six months, the lines diverge after months. When the data are stratified by age greater than or less than 6 months, sex is a significant predictor of estradiol level after six months (*xtreg*, β=0.15, p=0.046) but not before (*xtreg*, β=0.03, p=0.71).

Discussion

In this frequent longitudinal study, testosterone was detectable in all samples and estradiol in approximately 90% of samples, a proportion similar to a previous serum studies of estradiol level in infant girls (Chellakooty et al 2003) and significantly higher than other serum studies of estradiol and testosterone in 3 month old male and female infants (Schmidt et al 2002). Consistent with previous serum studies (Chellakooty et al 2003; Forest et al 1974), a wide range of variation in testosterone and estradiol levels was

detected in fecal samples. Levels of testosterone over the first 14 months of life ranged from 0.79 pg/gm at the 5th percentile to 14.38 pg/gm at the 95th percentile in this sample. Estradiol ranged from 0.03 pg/gm at the 5th percentile to 1.33 pg/gm at the 95th percentile during this same time period. On the sample level, these results are quite similar to the sample-level estradiol variability documented in a cross-sectional study of 473 3-month old infant girls (Chellakooty et al 2003). However, unlike that study, the longitudinal nature of this study permits the exploration of the sources of this sample level variability. Statistical analysis indicates that three main sources of this variability are individual developmental trends, age and sex.

Sample level and Individual Developmental Trends

Unlike previous cross-sectional serum studies (Burger et al 1991; Forest et al 1974; Winter et al 1976) and similar to a single, longitudinal salivary study (Huhtaniemi et al 1986), we found no single peak of testosterone or estradiol between 1 and 3 months or a decline to undetectable levels by the end of the first year of life. Rather, testosterone was negatively associated with age while estradiol showed no statistically significant association with increasing age. On the sample level, fecal testosterone levels were highest closest to birth, declined to 6 months, and then remained detectable throughout the first 14 months of life. Fecal estradiol declined within the first month and remained variable over the first 14 months. These results, however, only properly characterize the sample level pattern of median testosterone and estradiol levels. When individual weekly hormonal levels were plotted, a much more variable peak and trough pattern was

evidenced **(Figures 4.7** and **4.8)**, indicating that longitudinal examination of the physiological correlates of these increasing and decreasing hormonal levels is critical.

Sex Differences

The data indicate that infant sex contributes to inter-individual differences in sex steroid levels. These results document sex-specific developmental patterns with male testosterone levels significantly higher than female values before 6 months and female estradiol levels significantly higher than male values from 6 months through 12 months. These results for testosterone are in line with previous serum studies that have documented significantly higher testosterone in male infants from birth through 7 to 9 months of age (depending on the ages measured), but not at later ages (Bolton et al 1989; Forest et al 1974; Schmidt et al 2002; Winter et al 1976). Unlike the testosterone results, the results for estradiol are not as easily comparable with previous research. Previously, only a single cross-sectional study found significant sex differences in estradiol and none have found sex differences in the later half of infancy. In the single study documenting sex differences, serum estradiol levels were significantly higher in infant girls than infant boys at 3 months of age (Schmidt et al 2002). These results were not duplicated in the present study where sex differences are only evidenced after 6 months of age. These disparate results are likely due to the wide variability seen in estradiol at any particular age, which necessitates a large sample size to detect sex differences at any single point in time.

While this unique pattern of sex differences may be an artifact of small sample size, the exactly opposite results for testosterone and estradiol may indicate a sex-specific

developmental trajectory in which the female ovary continues to be active in producing both testosterone and estradiol later in infancy while the male testes production of sex steroids slows after 6 months of age. This explanation, while requiring further exploration, is consistent with both the lack of sex differences in testosterone after 6 months of age and the onset of sex differences in estradiol after 6 months. This continued activity of the female ovary is supported by previous serum studies that found a negative association between testosterone and age in infant males but no negative association between estradiol and age in infant females (Burger et al 1991). Further, inhibin, a marker of ovarian activity, remains low but measurable in infant girls throughout the first 4 years of life and is positively correlated with FSH levels, indicating sporadic follicular development during infancy and early childhood (Andersson et al 1998; Crofton et al 2002a).

Source of Hormones

The present data document that testosterone and estradiol are measurable in the feces of both male and female infants over the first 15 months of life. While the current study is unable to discern whether the source of these sex steroids is gonadal or extra-gonadal, previous documentation of elevated LH and FSH in infancy indicates that a gonadal source is likely. Both LH and FSH have been shown to follow an adult-like and sexually-dimorphic pulsatile secretory pattern from birth through early infancy (de Zegher et al 1992; Waldhauser et al 1981). This activation of the gonadotropins is generally thought to be responsible for stimulating testosterone and estradiol production by the testes and ovary, respectively (Winter et al 1975). Experimental administration of human chorionic gonadotropin (hCG) to male infants aged 5-180 days led to increased testosterone levels

in both saliva and serum, suggesting to researchers that the postnatal increase in serum testosterone in male infants is both testicularly-derived and gonadotropin-mediated (Dunkel and Huhtaniemi 1990).

Additionally, clinical examination of the morphology and steroid contents of the gonads of deceased infants document that sex steroid levels also are associated with morphological changes in the gonads (Bidlingmaier et al 1987), further supporting a gonadal origin for infant sex steroids. The neonatal ovary is known to be an active site of follicular development (Burger et al 1991), with the most rapid increase in follicular maturation occurring during the first four months of neonatal life, concurrent with the activation of the HPG axis (Polhemus 1953). In female infants, ovarian estrogen concentrations were found to parallel changes in ovarian weight and follicular differentiation, providing support for the hypothesis that the infant ovary is the main source of estradiol production in infant girls (Bidlingmaier et al 1987). Similarly, testicular estradiol was closely correlated with both Leydig cell development and testicular testosterone concentrations, suggesting that the male testes are also producing estradiol in response to gonadotropin activation (Bidlingmaier et al 1987).

Comparison to fecal values in adults and the range of infant serum to adult serum

The use of fecal samples for the assessment of testosterone and estradiol, while not used commonly in human studies, allows frequent non-invasive measurement. Enterohepatic circulation of steroids plays an important part in sex steroid metabolism (Adlercreutz and Martin 1980). Circulating steroids are eliminated from the body via the kidney, where they are conjugated and excreted in urine, and the liver, where they are metabolized and secreted into bile and enter the small intestine. Approximately 80% of the steroids in the

intestine are hydrolysed by intestinal bacteria and are reabsorbed into the bloodstream (Adlercreutz and Martin 1980). The remaining steroids that escape hydrolysis are excreted unconjugated in feces. While this fecal component only represents a small fraction of the circulating steroid levels, fecal estradiol concentrations have been shown nonetheless to parallel serum concentrations in primates and to reflect physiological events like ovulation and pregnancy (Whitten and Russell 1996).

Figures 4.9 and **4.10** compare fecal steroids levels in infants to adult fecal values. These adult values come from a small sample of American adults that were also analyzed in the same laboratory using a similar methodology (Sobolik et al 1996). Infant testosterone levels overlap in testosterone with adult values, particularly with the adult female range. Estradiol levels at the highest end of the infant fecal range overlap with those at the lowest end of the adult female range, which corresponds to the early follicular phase (days 5 and 7).

These results are in line with previous serum studies which documented infant estradiol and testosterone levels overlapping the adult range, particularly the adult female range (Burger et al 1991). However, the differences between fecal infant levels and fecal adult levels seem to be greater and these may be attributed to small sample size. Alternatively, the differing gut transit times between infant and adult humans (discussed in chapter 2) may result in a greater time delay and, thus, concentration of fecal steroids in adults as compared to infants.

Conclusion and Implications of Varying Levels of Exposure

Clinical studies have documented that the symptoms of micropenis and maldescended testes are associated with the lack of an early postnatal peak in testosterone (Main et al 2000; Main et al 2006). This observation suggests that not only are the testes the likely site of testosterone production, but also that this production is necessary for normal scrotal and testicular development. This hypothesis is supported by experimental research in rhesus macaques which documented that penile and clitoral growth in neonates could be significantly altered by suppressing or augmenting testosterone levels (Brown et al 1999). Further, in neonatal primate studies of male rhesus macaques and marmosets, gonadotropin and androgen production appears to prime Sertoli cell proliferation and germ cell differentiation, having effects on gonadal development and sexual behavior that can be traced into adult life (Mann and Fraser 1996). As Sertoli cell number is a determinant of spermatogenic potential, the postnatal activation of the hypothalamic-pituitary-gonadal axis has the potential to shape Sertoli cell proliferation and subsequent spermatogenesis (Quigley 2002).

Although the potential functional and developmental importance of the postnatal surge in female infants has received less attention, the infant surge may be critical for female sexual development as well. *In utero* growth restraint has been associated with both a reduction in primordial follicles and the hypersecretion of FSH (Ibanez et al 2002). Researchers have hypothesized that these results may link early growth conditions to both abnormal pubertal timing and subfertility in adulthood (Ibanez et al 2000; Ibanez et al 2002). Further, palpable breast tissue in infancy was associated with estradiol levels in 3-month old infant girls, suggesting to researchers that breast tissue was sensitive to sex

steroids early in life and that this time period may represent a developmental window during which breast physiology is primed (Schmidt et al 2002).

Such studies, along with the variability in sex steroid levels between individuals in the present study, suggest that hormonal exposure in infancy may alter gonadal development, having potential lifelong effects on reproductive function and fertility. Certainly, the effects of endocrine disruptors on sexual development and future reproductive health of human and nonhuman primates have been well described- for example, fetal DES exposure leading to infertility and reproductive cancers in women (Bamigboye and Morris, 2003) and androgen exposure altering primate gonadal anatomy and sexual behavior (Mann et al., 1997). However, the current results suggest that more subtle alterations may also have the potential to affect reproductive development and function.

Table 4.1: Serum Studies of Infant Testosterone Levels

Ages Examined	Study Protocol‡	Male level *Median (range)*	Female level *Median (range)*	Sex Difference?	Citation
Testosterone					
Birth (cord)	CS	39 ng/dl (±11)*	30 ng/dl (±7)*	P<.05; boys higher	(Forest et al 1974)
1-15 days		68 ng/dl (±60)*	12 ng/dl (±6)*	P<.05; boys higher	
1-3mo		208 ng/dl (±68)*	9 ng/dl (±4)*	P<.05; boys higher	
3-5mo		95 ng/dl (±53)*	6.7 ng/dl (±2.7)*	P<.05; boys higher	
5-7mo		23 ng/dl (±18)*	6.8 ng/dl (±3.1)*	P<.05; boys higher	
7-12 mo		6.6 ng/dl (±2.5)*	5.5 ng/dl (±2.8)*	No	
Birth (cord)	CS	24 ng/dl (5-49)	28 ng/dl (5-49)	No	(Winter et al 1976)
2 mo		175 ng/dl(53-560)	20 ng/dL	P<.001; boys higher	
4-6mo		41 ng/dl (<5-90)	20 ng/dL	P<.001; boys higher	
6-12 mo		5 ng/dl (<5-42)	20 ng/dL	Before 9 mo; P<.001; boys higher	
Birth (cord)	CS	1.29 nmol/L (±.85)*	.26 nmol/L (±.13)*	P<.01; boys higher	(Bolton et al 1989)
Birth (serum)	CS	416 ng/dl	46 ng/dl	NA	(de Zegher et al 1992)
Birth (cord)	L	4.02nmol/L (1.83-6.54)	NA	NA	(Andersson et al 1998)
3-24†		<.23 nmol/L	NA	NA	
0-6 mo	CS	3.2 nmol/L (±.75)*	NA	NA	(Bergada et al 1999)
6-24mo		.4 nmol/L (±.06)*	NA	NA	
3mo	CS	3.28 nmol/L (<.23-12.1)	<.23nmol/L (NA)	P<.001; boys higher	(Schmidt et al 2002)
3 mo	CS	3.23 nmol/L (.60-7.79)**	NA	NA	Boas et al, 2006

‡ CS= cross-sectional sample; L= longitudinal sample

* values reported are mean (±SD); ** values reported are median(2.5 and 97.5 percentiles);

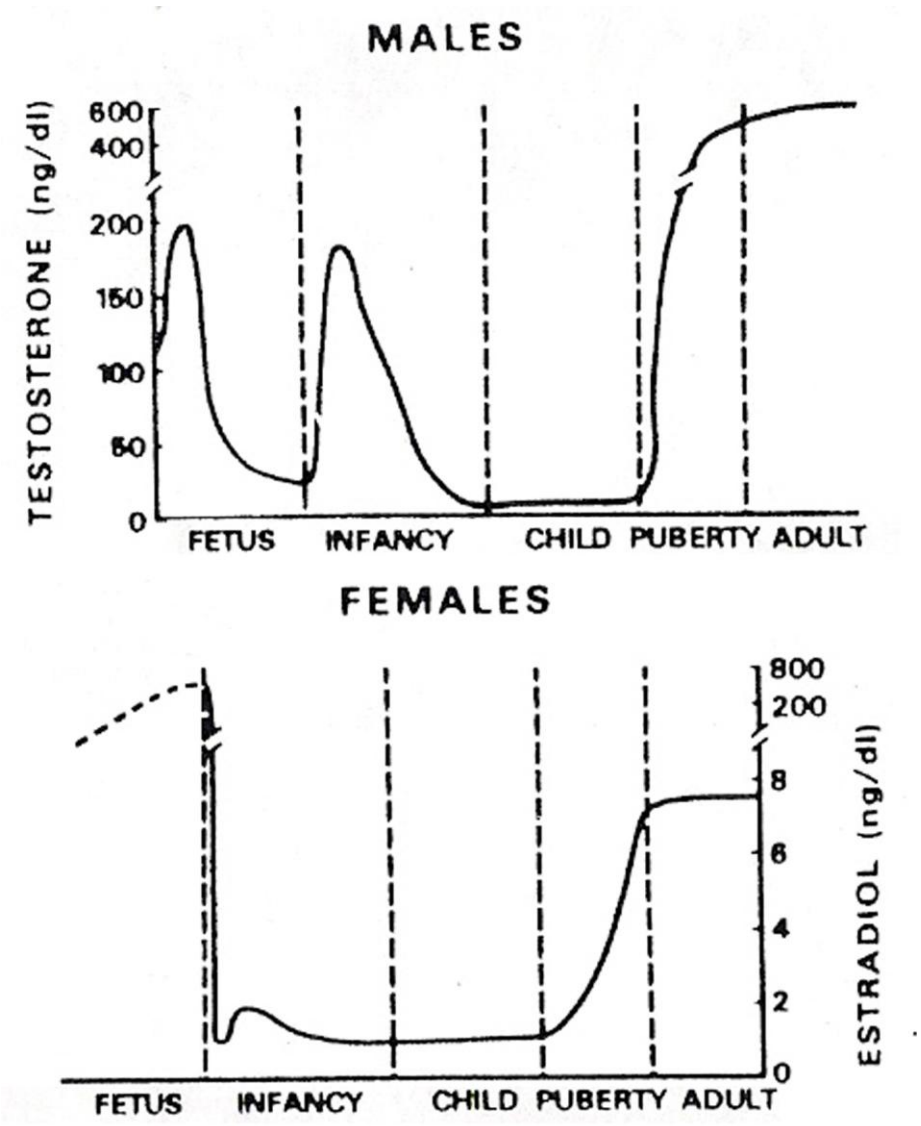
† measured at 3-month intervals

Table 4.2: Serum Studies of Infant Estradiol Levels

Ages Examined	Study Protocol‡	Male level *Median (range)*	Female level *Median (range)*	Sex Difference?	Citation
Estradiol					
Birth (cord)	CS	266-1569 ng/dl	266-1569 ng/dl	No	(Winter et al 1976)
Birth (serum)	CS	58 pg/ml	37 pg/ml	NA	(de Zegher et al 1992)
0-6mo	CS	NA	60.0 pmol/L(±16.95)*	NA	(Bergada et al 1999)
6-24mo	CS	NA	33.2 pmol/L(±1.24)*	NA	
4-6mo	CS	NA	91 pg/ml (±33)*	NA	(Ibanez et al 2002)
3 mo	CS	21.0 pmol/L (<18-58)	30.0 pmol/L (<18-100)	P<.001; girls higher	(Schmidt et al 2002)
3 mo	CS	NA	31 pM(18-83)	NA	(Chellakooty et al 2003)
3 mo	CS	18 pmol/L (.18-40)**	NA	NA	Boas et al, 2006

CS= cross-sectional sample; L= longitudinal sample
* values reported are mean (±SD); ** values reported are median(2.5 and 97.5 percentiles);
†measured at 3-month intervals

Figure 4.1: Previously Documented Pattern of Sex Steroid Production



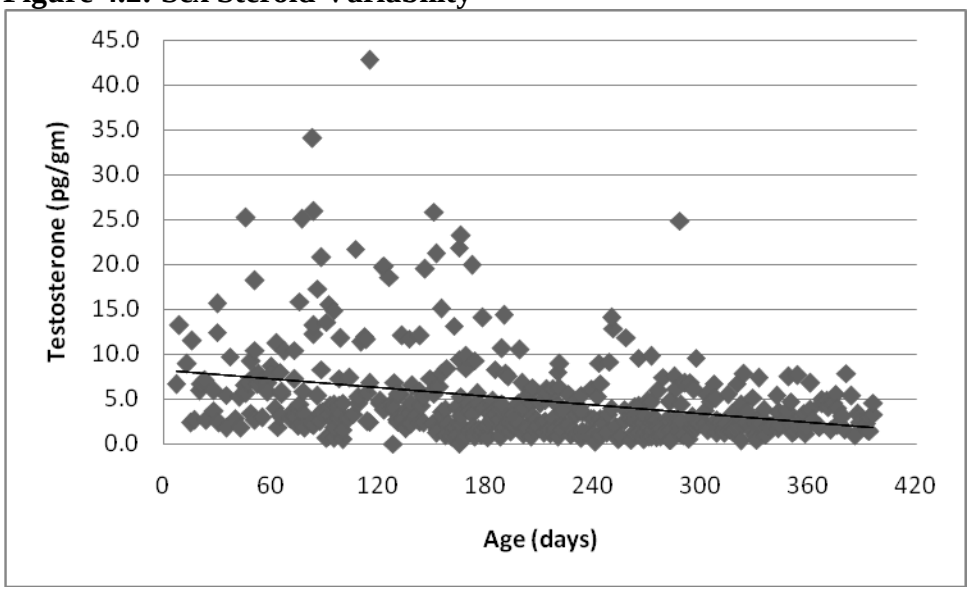
From Winter et al, 1976

Table 4.3: Study Measurement Protocol

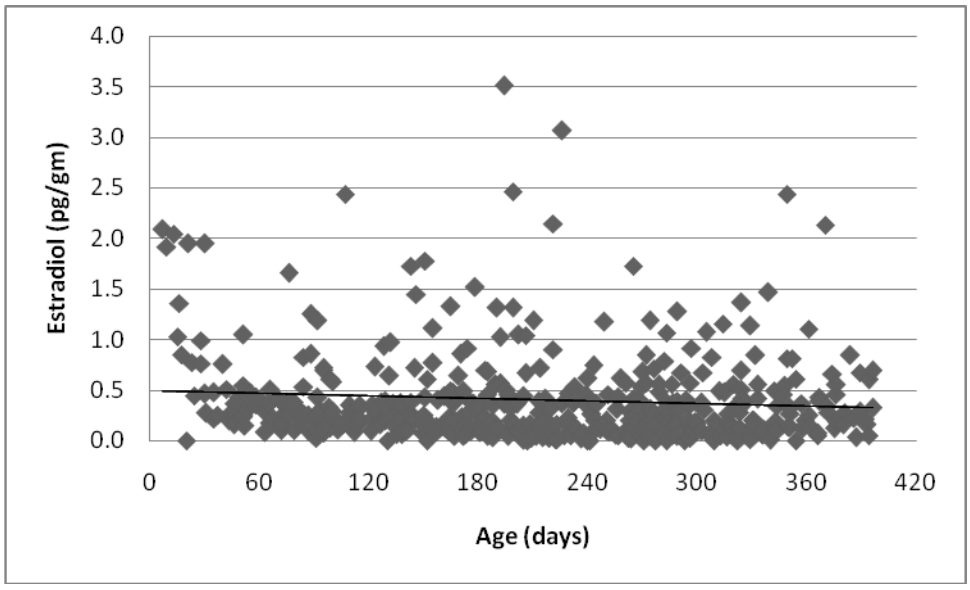
Subject Id num	Sex	Protocol	Ages of Study (days)	Diaper -weeks
1	F	D	14-22	1
2	F	D;W	7-456	45
3	F	D; W	13-245	53
4	M	D; W	9-355	36
5	M	D	83-87	1
6	M	W	40-109	14
7	F	D;W	17-436	54
8	M	D	77-291	29
9	F	W	137-465	23
10	F	W	89-293	18
11	M	W	232-407	19
12	M	D	219-223	1
13	F	W	135-227	14
14	F	W	139-230	12
15	F	W	60-176	12
16	M	W	79-194	11
101	M	W	187-299	13
102	F	W	287-399	12
103	M	W	185-297	14
104	M	W	266-378	7
105	M	W	115-227	13
106	F	W	272-384	11
107	F	W	177-289	12
108	F	W	341-453	8
109	M	W	282-394	10
110	F	W	74-186	7
111	M	W	312-424	7
112	M	W	219-331	12
113	F	W	117-229	14
114	M	W	253-365	10
115	F	W	227-339	10
116	F	W	68-180	3

D-Daily measurement protocol; W- Weekly measurement protocol

Figure 4.2: Sex Steroid Variability

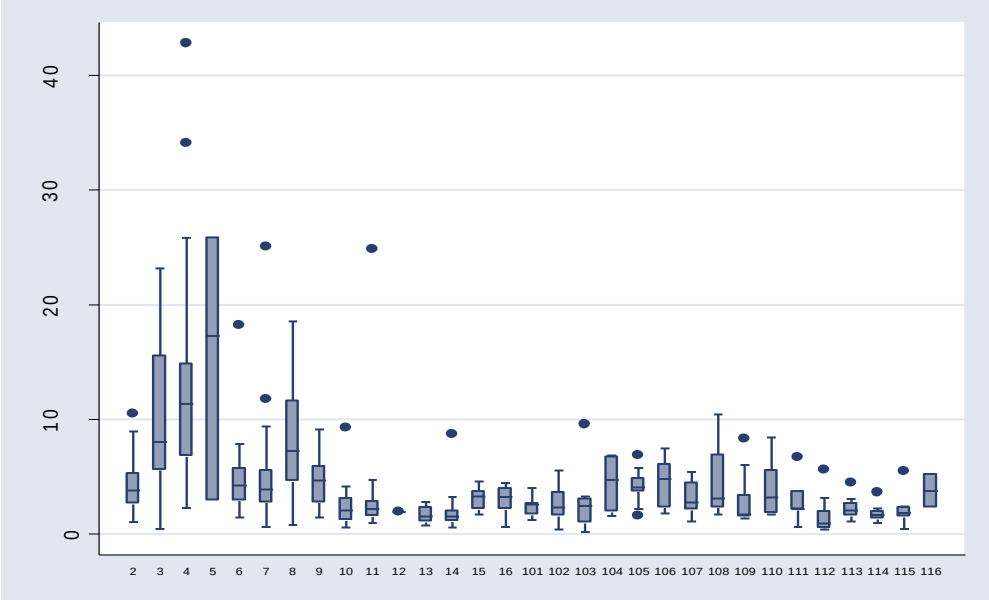


Measured testosterone in fecal samples by day of life

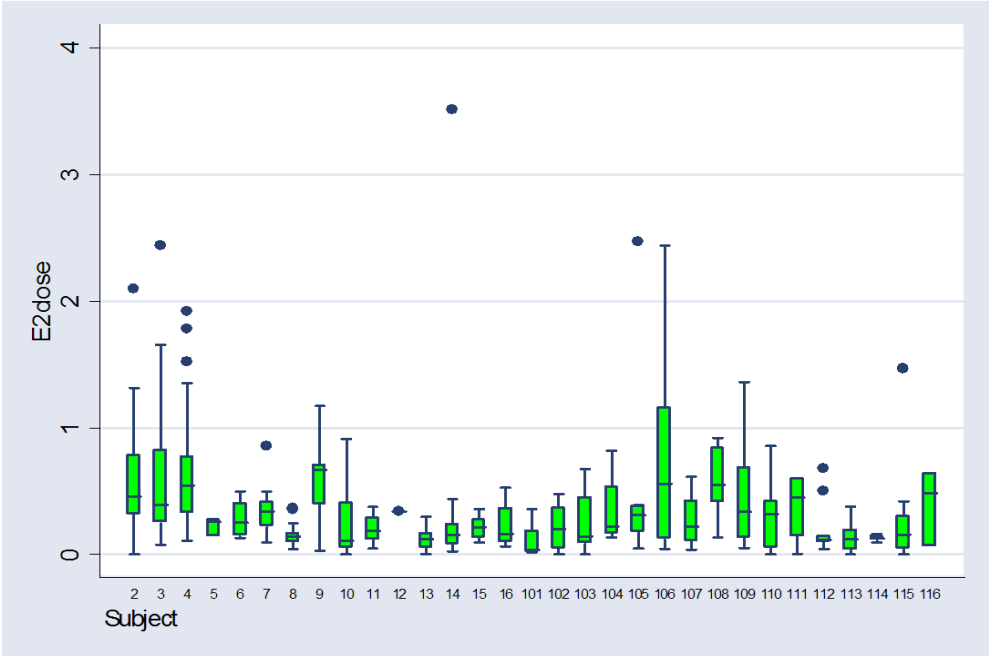


Measured estradiol in fecal samples by day of life

Figure 4.3: Inter- and Intra-Individual Variability

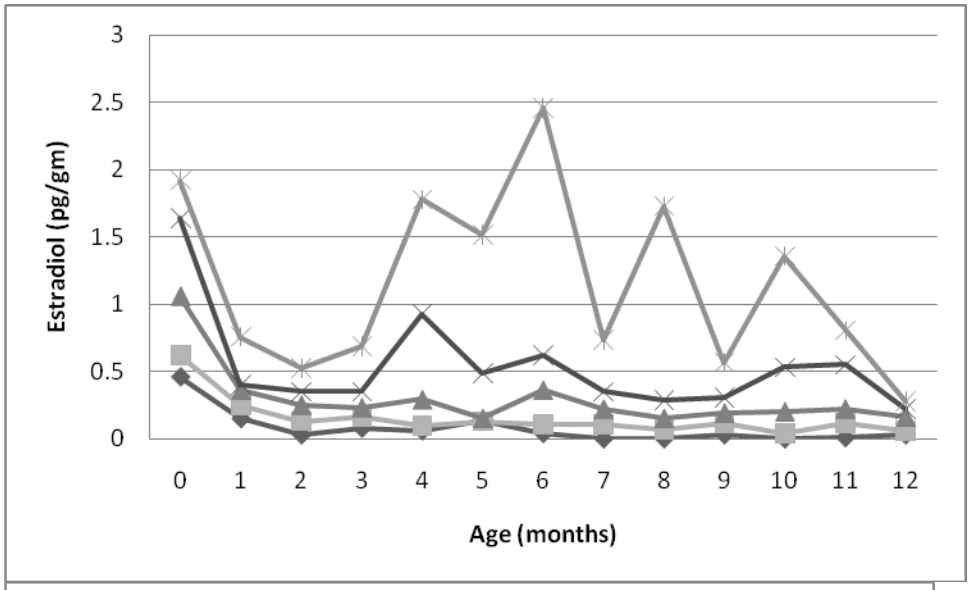


Inter- and intra-individual variability in testosterone levels (pg/gm).



Inter- and intra-individual variability in estradiol levels (pg/gm).

Figure 4.4a: Percentiles of Sex Steroids by Month of Age in Male Infants



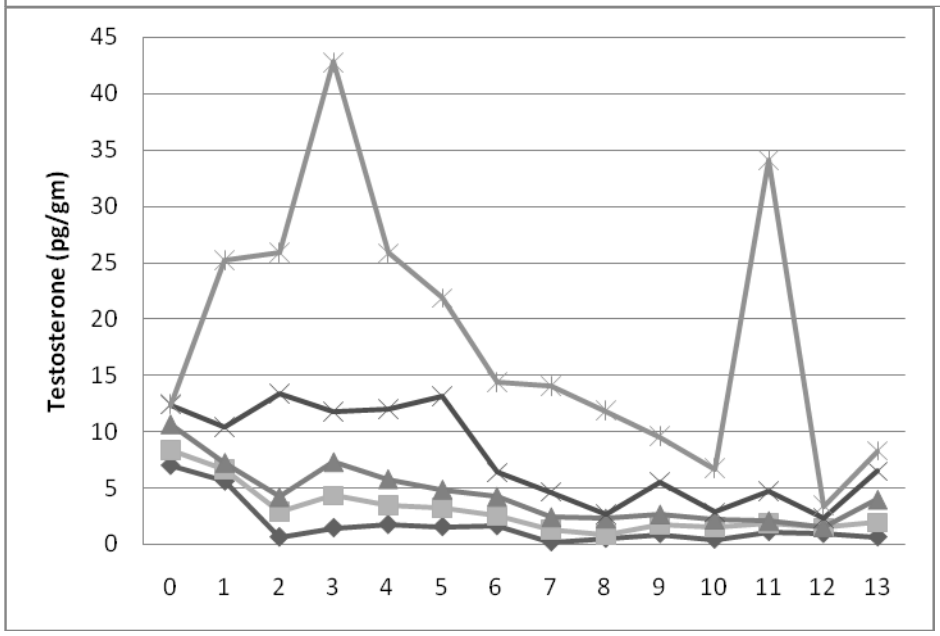


Figure 4.4b: Percentiles of Sex Steroids by Month of Age in Female Infants

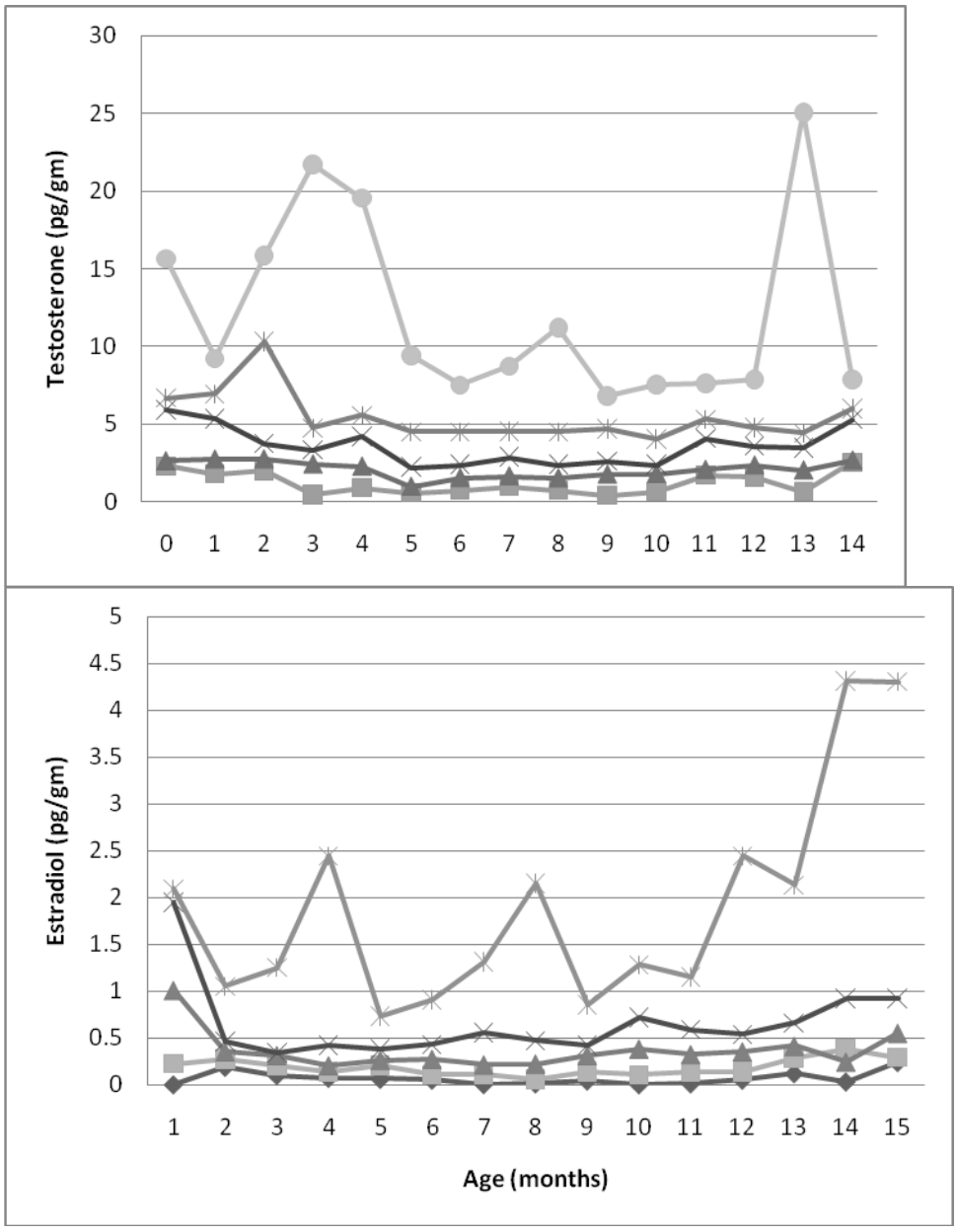
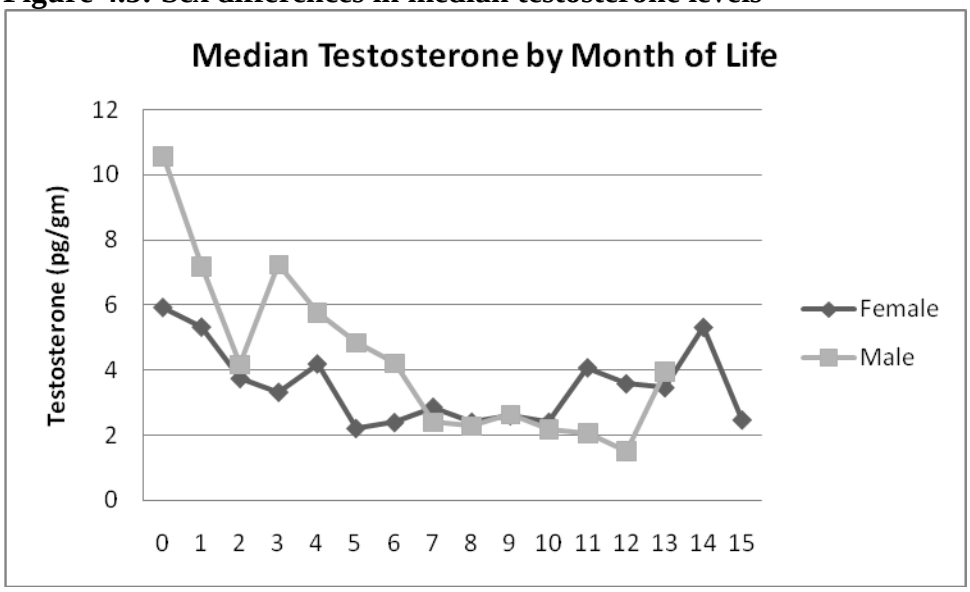
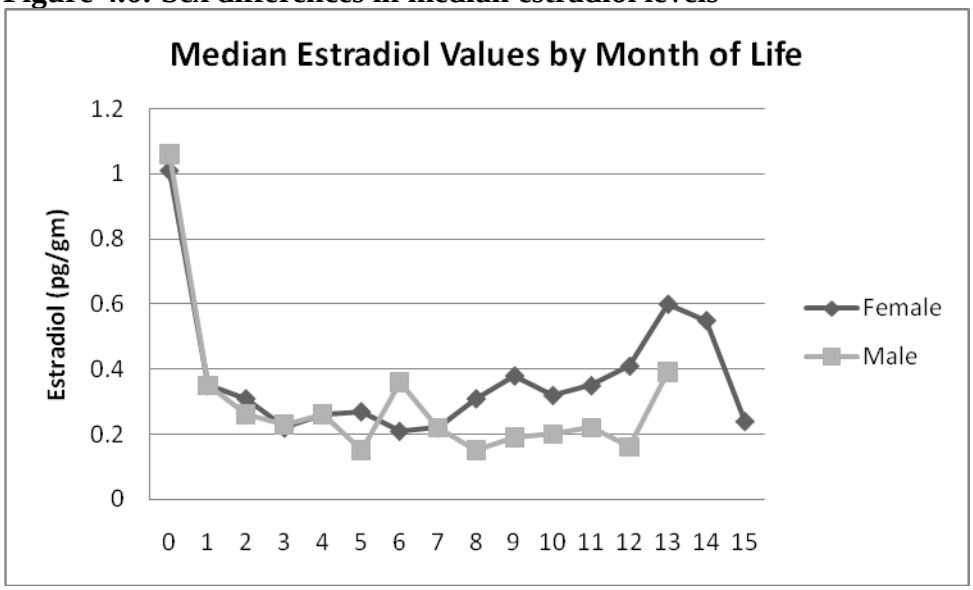


Figure 4.5: Sex differences in median testosterone levels



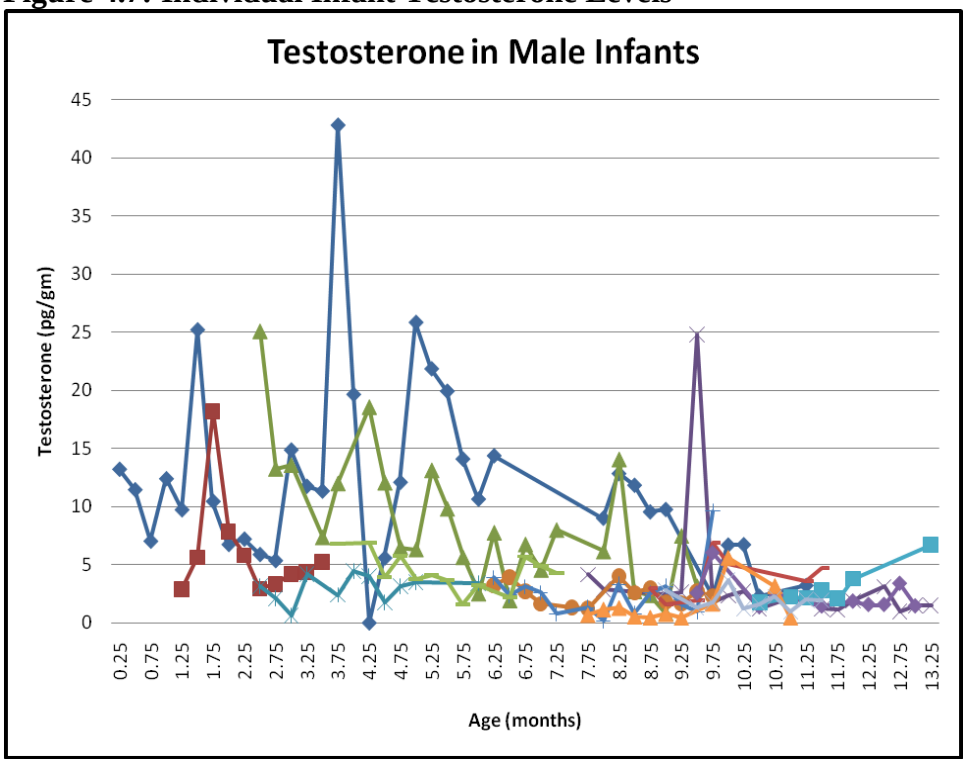
Sample median levels of testosterone (pg/gm) by sex and month of life

Figure 4.6: Sex differences in median estradiol levels

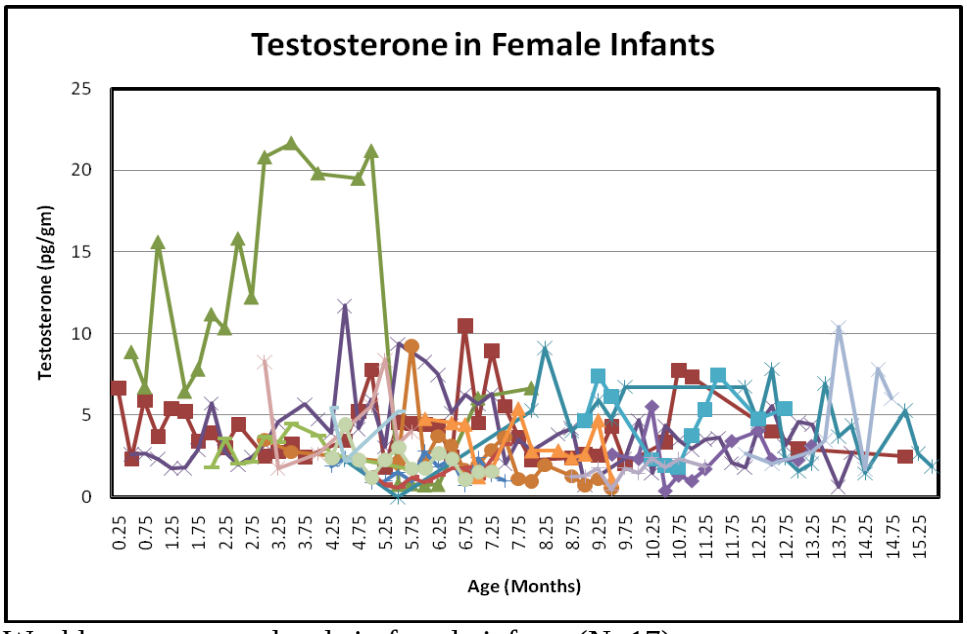


Sample median levels of estradiol (pg/gm) by sex and month of life

Figure 4.7: Individual Infant Testosterone Levels

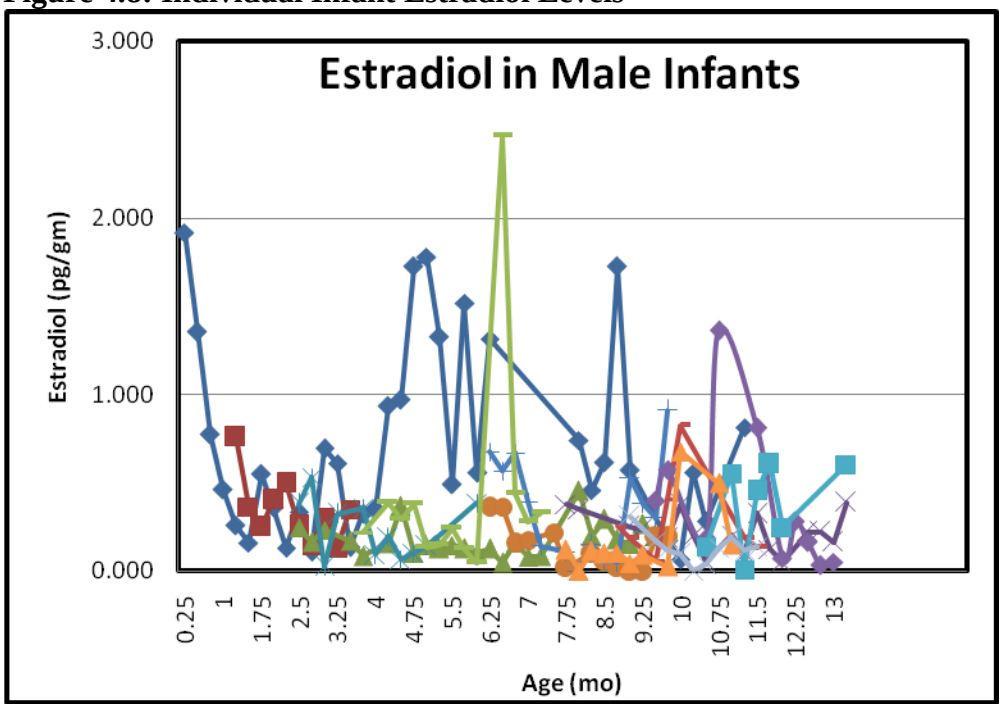


Weekly testosterone levels in male infants (N=15)

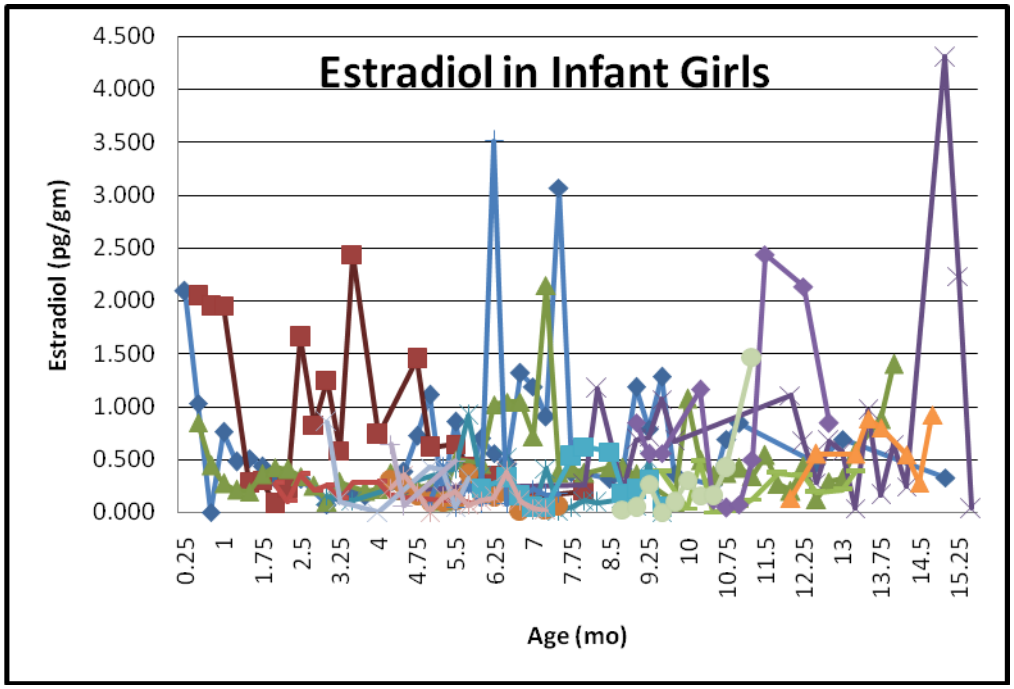


Weekly testosterone levels in female infants (N=17)

Figure 4.8: Individual Infant Estradiol Levels



Weekly estradiol levels in male infants (N=15)



Weekly estradiol levels in female infants (N=17)

Figure 4.9: Fecal Testosterone (pg/gm) Range in Infants versus Adults

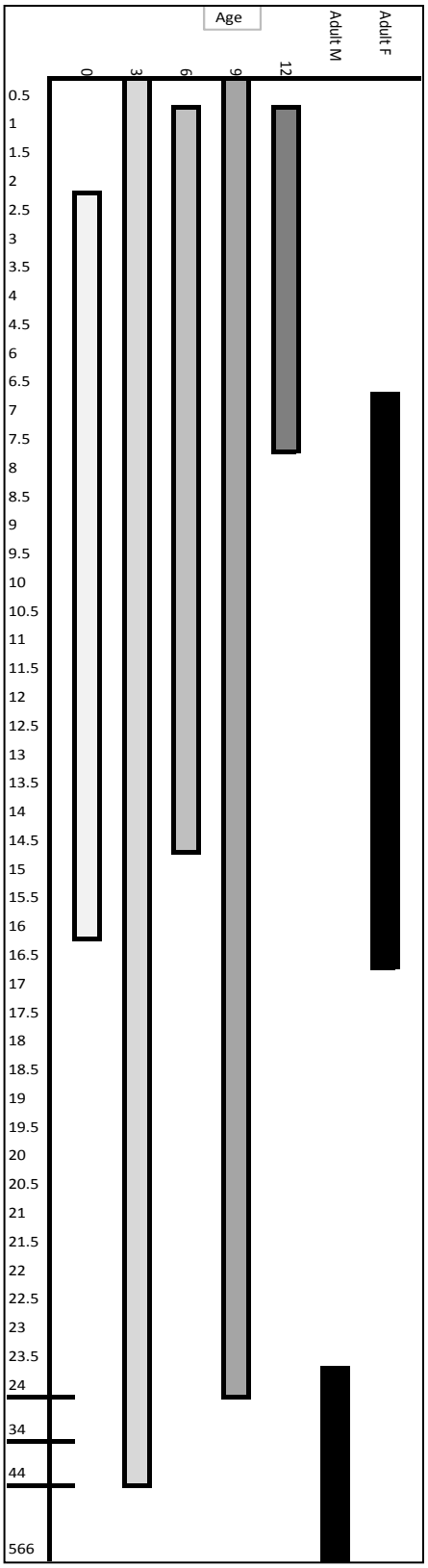
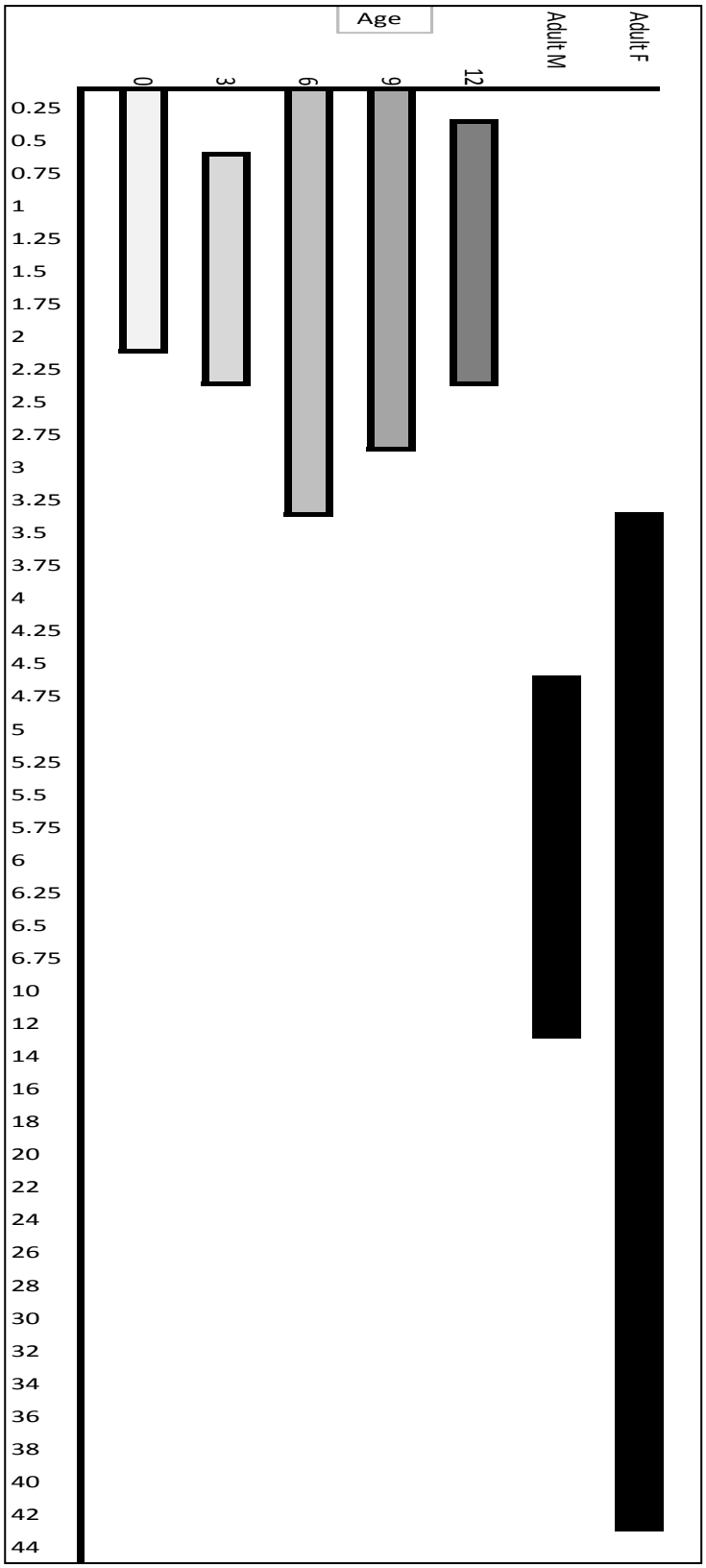


Table 4.10: Fecal Estradiol (pg/gm) Range in Infants versus Adults



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Chapter 5: HPG Activation and Infant Growth

Introduction

The period of infant development may be a particularly important time from both a developmental and evolutionary perspective, since, during infancy, rates of growth and development are shaped in response to salient environmental variables. Epidemiological research has increasingly documented that these early pre- and postnatal growth rates are shaped by the nutritional environment (Bispham et al 2003; Boulton et al 1999; Dewey 1998; Jackson 2000) and, in turn, can shape later physiology, having long lasting effects on adult cardiovascular, metabolic and reproductive health (Barker 1995; Fisher et al 2006; Godfrey and Barker 2000). Within this epidemiological framework, the period of infant development represents a critical window during which organ-level growth is determined and metabolic set points are programmed (reviewed by: Cameron and Demerath 2002; McCance 1976). These programming studies suggest that, along with cardiovascular and metabolic health, adult reproductive function also can be programmed early in development through structural alterations in the gonads or modifications in hormonal messaging systems (Hardy and Kuh 2002; Main et al 2006a; Michels et al 2006). Research into the postnatal surge in the hypothalamic-pituitary-gonadal axis (HPG) in humans and non-human primates indicates that infancy also may represent an important period for gonadal development (Boas et al 2006; Brown et al 1999; Main et al 2006b; Schmidt et al 2002).

Results from the previous chapter documented sex-specific developmental trends in steroid production in infancy and identified testosterone production falling within the adult range throughout the first year of life, with higher and sexually dimorphic levels

closer to birth and estradiol production at the lowest adult levels with pronounced sexual dimorphism only after 6 months of age. Whether such sex differences in testosterone and estradiol persist through childhood is debated (Garnett et al 2004; Juul 2001). However, similar sexual dimorphism is apparent in puberty, when increasing nocturnal gonadotropin secretion stimulates increased sex steroid production (Brook 1999; Plant and Shahab 2002). Testosterone and estradiol, in turn, promote gonadal growth, germ cell maturation and secondary sex characteristic development (reviewed in: Brook 1999). Accompanying pubertal sexual development are increases in weight accumulation, adipose tissue and muscle deposition, and bone growth and density, all of which are also linked in sex-specific ways to rising levels of sex steroids (Garnett et al 2004; Roemmich and Rogol 1999; Rogol 2003; Tanner 1989). During puberty, increased circulating testosterone preferentially promotes an increase in lean tissue mass and truncal adipose tissue, while circulating estrogens promote fat storage, particularly in peripheral tissues, and at low concentrations, stimulate skeletal growth in both males and females (Garnett et al 2004; Rosenbaum and Leibel 1999). These sexually dimorphic patterns of growth and body compositional changes result in the greater lean body mass in males and higher overall fat mass in females, differences that also may be seen from earliest fetal development onward (reviewed in: Malina 1996).

Many similarities exist between the periods of infant and adolescent growth. Both are characterized by rapid rates of linear growth and weight gain. During the first year of life, infants may gain up to 50% of their birth length and triple their birth weight (Tanner 1989). In healthy Western infants, this corresponds to a linear growth velocity of approximately 25 cm in the first year and a weight gain 9 kg/year, rates almost identical

to those seen during the adolescent growth spurt (Marshall and Tanner 1969; Marshall and Tanner 1970; Rogol et al 2002; Tanner 1989).

Along with a high rate of growth in length and weight, both periods are characterized by increases in fat mass and perhaps sexually dimorphic changes in the distribution of fat stores. Human infants are characterized by a high level of adiposity, with an estimated body fat percentage of 13.7% and 14.9% at birth for males and females, respectively (Butte et al 2000a; Fomon et al 1982; Fomon and Nelson 2002)2. Percent body fat has been documented to increase during early infancy, with infants reaching an adiposity peak of approximately 25% fat mass by 6 to 9 months of age (Butte et al 2000a; Fomon et al 1982). Similarly, skinfold thickness, the most common measure of subcutaneous fat mass and distribution in infancy, also increases rapidly after birth, reaching a maximum between 6 and 9 months, in all measured depots (Karlberg et al 1976; Prader et al 1976; Roche and Sun 2003b). This higher level of adiposity in females persists throughout infancy with females having skinfold thickness percentiles that are 0.5cm higher from 9 to 36 months (reviewed in: Roche and Sun 2003b).

The question of whether sex differences in the distribution of body fat stores exist during infancy or at any time prior to puberty remains unresolved (Arfai et al 2002). Researchers using skinfold calipers to assess subcutaneous fat have consistently documented greater skinfold in female infants (Arfai et al 2002; Guihard-Costa et al 1997), while results using dual-energy x-ray absorptiometry (DXA) are more conflicting (Harrington et al 2004; Koo et al 2000). The results of such studies range from no measurable sex differences in body composition (Harrington et al 2004), to greater fat mass, but similar muscle mass, in girls compared to boys to greater fat and reduced

muscle mass in girls (Koo et al 2000; Rodriguez et al 2004). These discrepancies in DXA results may be attributable to small sample sizes and differences in the age and maturational stage of study participants or to methodological limitations of using the DXA procedure. DXA results, particularly in infants, appear to be sensitive to type of energy platform used (pencil beam vs. fan beam), scan intervals, and subject movement during the scan (Koo 2000). Perhaps more importantly for comparison with the skinfold results, using DXA to measure regional body composition reduces the precision of the method (Koo 2000). Additionally, these regional measurements are traditionally limited to limb vs. trunk or upper vs. lower body and cannot distinguish more fine-grained depot level fat mass (Koo et al 2000). Anthropometric measures, while highly correlated with DXA estimates of whole body fat mass (Koo 2000; Schmelzle and Fusch 2002), also allow regional assessment of fat and lean body tissue and evaluation of sex differences in adipose tissue distribution. These differences may underlie the conflicting results seen in studies of sex differences in body composition in infancy and early childhood.

Despite these limitations in methodologies, the presence of sex differences at birth and even during the adiposity nadir of early childhood (Mast et al 1998) nonetheless suggests that female infants remain both fatter and more peripherally fat during the intervening period of infancy. Female infants have been shown to have greater fat mass for body weight at birth (Guihard-Costa et al 2002) as well as a different pattern of fat distribution (Guihard-Costa et al 1997). Female newborns have higher absolute and relative subscapular and tricipital skinfold thickness than male infants, a sexually dimorphic pattern also seen in children, adolescents, and adults (Antoszewska and Wolanski 1992; Guihard-Costa et al 1997; Malina 1996).

Similar sex differences in growth rates, stature and body fat are documented in fetal development (Bernstein 2005; Bernstein et al 1997; Lampl and Jeanty 2003), adolescence (Rogol 2003; Veldhuis et al 2005) and adulthood (Bjorntorp 1996; Guo et al 1997) when they are associated with sex steroid production. This raises an interesting question: could sex steroid differences also underlie sexual dimorphism in infancy as well?

Although the development and function of this complex interaction between somatic growth and sex steroids has been extensively studied in adolescence, less is generally known about the endocrinology of normal infant growth and about sex steroid production in infancy in particular. However, population-level epidemiological studies do provide support for the hypothesis that infant growth may also be linked to reproductive development. For example, Adair found that, in the Philippines, birth size predicted menarcheal age, with girls who were long and light at birth maturing earliest particularly if they were fast growers in infancy (Adair 2001). Similarly, growth in infancy and early childhood are also associated with age at menarche as in seen in rural Guatemala where girls who were severely stunted at age 3 matured later than girls not stunted (Khan et al 1986). The effects of developmental conditions on male reproductive maturation have received less attention. However, several recent studies suggest that male subfertility may also be related to birthweight and infant growth rate (Main et al 2006b). These associations between infant growth and maturational timing, along with the predictability of adolescent health outcomes from infant size (Adair and Cole 2003; Falkner et al 2004; Gillman et al 2003; Kim et al 2006; Kuzawa 2004; McDade et al 2001; Owen et al 2003; Singhal et al 2001), supports a functional linkage between infant growth and HPG activity.

Study Aims

The mechanisms underlying endocrine development and normal physical growth in infancy, and the relationship between these two developing axes, remain largely unexplored. Understanding this relationship is critical for understanding the maturation of the reproductive system and the tuning of neuroendocrine regulating mechanisms. As stated by Marshall (1976), such relationships will remain unclear unless conclusions can be based on longitudinal studies in which hormone production is related precisely to different parameters of sexual development and somatic growth.

Given the existence of hormonal differences already in infancy (documented in chapter 4) that are linked to sexual dimorphic patterns of growth and body compositional changes in adolescence and some evidence to support sex differences in infant growth and body composition, this study utilized a frequent, longitudinal study design to hypothesize that: 1) sex differences in infant growth and body composition, measured by body circumferences and skinfold thicknesses, will be present in this sample of healthy, American infants and 2) these sex differences in growth will be explained by sex steroids levels. Specifically, we asked: do levels of testosterone and estradiol predict linear growth, weight and body composition in sex-specific ways?

Sample and Methods

Sample

The data used to address these questions come from a sample of 32 mothers and infants enrolled in a weekly, longitudinal study of infant growth and hormonal development

following parental informed, written consent of an Emory University Human Subjects approved protocol.

Infants were followed longitudinally between the ages of 7 days and 15 months. The first 12 months of life are the focus of this analysis. Infants were enrolled and measured at two different sites; half of the sample was measured in their homes and the other half was measured in a university-affiliated daycare. The median length of weekly participation was 29 weeks.

Upon study entry, participating parents filled out a brief questionnaire detailing their height, weight, health and pregnancy background and infant characteristics such as gestational age, birth weight and length. These measurements were performed independently of the study as part of the standard hospital examination after birth and were provided by the parents.

Fecal Sample Collection and Hormonal Assays:

Parents were asked to retain all diapers from the evening prior to through the morning of the day of measurement. Soiled diapers were stored in portable coolers, chilled with freezer blocks frozen to -80° C and were collected during the measurement appointment. Samples were then stored at -80° C until further analysis. This protocol yielded 487 useable, weekly fecal samples.

Fecal samples were excised from the diapers, extracted using methanol and filtered. Extracted samples were then assayed using validated modifications (Shirtcliff et al 2000) of commercially-available RIA kits for estradiol and testosterone (Diagnostic Systems Laboratory, Weber, TX). All samples were assayed in duplicate and the mean value of

the duplicates was used for analysis. These modified microassays were able to detect steroid concentrations of 0.35 pg/ml and inter- and intra-assay CV's were within the accepted range for RIA assays. Steroid recovery was high and samples were diluted to reduce potential competitive binding from other estradiol or testosterone metabolites (for further details, see chapters 2 and 3).

Anthropometric Methods

Recumbent length, weight and head, body and limb circumferences, and trunk and limb skinfold thickness were measured following standard techniques (Frisancho 1990). Total recumbent length was measured to the nearest 0.1 cm by two observers using an infant measuring board equipped with fixed headboard and a mobile footboard (Precision Enterprises; Portage, MI). Length measurement protocol differed depending on the subsample of the study and the location of measurement. At the daily home and weekly daycare visits, both observers were trained in the maximal stretch technique (Lampl et al 2001). At the weekly home visits, mothers and/or caretakers acted as assistants, after training in holding the infant's head during the measurement, using the same technique. Length was measured in duplicate in the majority (~80%) of measurements, unless the state of the infant precluded additional measurement.

During the home visits, unclothed infant weight was measured using a portable, digital scale accurate to the nearest 10gm, calibrated before measurement (MedWeigh, MS-2410). At the daycare center site, infants were weighed clothed in a diaper. The average diaper weight for the infant was subtracted from the weight measurement in analysis.

Five limb and trunk circumferences (mid-upper arm (UAC), abdominal (ABDC), supra-iliac (SILC), mid-thigh (QC) and calf (CC)) were measured to the nearest 0.1 cm using a soft, non-stretchable fiberglass tape and following standard procedures for tape placement (Frisancho 1990). Six trunk and limb skinfolds were measured with Holtain skinfold calipers (subscapular (SSF), supra-iliac (SILSF), abdominal (ABDSF), triceps (TSF), quadriceps (QSF), and calf (CSF)) to the nearest 0.1mm, according to standard techniques (Frisancho 1990; Roche and Sun 2003a).

Data Analysis:

SAS version 9.0 (SAS Institute, North Carolina) and STATA/SE version 8 (Stata Corporation, College Station) statistical software were used for all statistical analyses. Steroid concentrations in fecal extracts were corrected for fecal weight and are expressed as pg/gm. The resultant fecal steroid concentrations were not normally distributed due to a right skew of the values. However, given the large sample size of hormonal values (n=487), results did not differ whether the actual values or the log-transformation of the values were included in analyses. Untransformed variables were used for ease of interpretation.

Descriptive statistics of hormonal measures are given as median values and reference ranges (minimum to maximum). If a measured hormone was below the detection limit, the value was expressed as 0 pg/gm for subsequent analysis. Analyses were repeated with undetectable values set at the limit of detection, but this did not change the results.

Sex differences in growth and body composition measures were assessed using Mann-Whitney two-sample rank sum non-parametric tests. Repeated-measures mixed models

were used to assess significant effects of testosterone and estradiol levels on growth and body composition measures. Repeated-measures logistic models were used to assess the effect of hormone levels on weekly incremental gains in length, weight and body composition measures. These models were stratified by sex to control for confounding by sex and age was included as a covariate. Additionally, in models assessing the effect of sex steroid predictors on velocity measures, size at the beginning of the weekly interval was included as control variable to control for any potential effect of size on velocity.

As no previous work has assessed the relationship between hormonal levels and size longitudinally in infancy, graphical methods were first employed for exploratory analysis. Based on the visual inspection of longitudinal graphs of hormonal values (Figures 4.5 and 4.6, in chapter 4), 2-monthly intervals were chosen for exploring the association between hormone levels and growth. Thus, models assessing the relationship between hormone values and growth measures were stratified by 2-monthly intervals to account for developmental trends evidenced in the data. This interval duration provided the smallest time frame with statistical power to assess differences. Additionally, 6-month intervals were also explored based on the sex difference results in the previous chapter.

Subject was included as a random effect in all repeated measures models to control for subject-specific effects such as measurement dependability and to account for the within-subject correlation. Statistical significance was defined as $p < 0.05$. Statistical significance of $p < 0.10$ was taken as evidence of a trend in association.

Derived Variables

Weight and length data were used to calculate ponderal index [PI=(weight in g/length in cm^3) *100]. Weekly incremental values for growth and body composition measures (length, weight, circumferences and skinfolds) were converted to velocities to account for any variation in the length between measurement intervals. Several composite measures and ratios were created to assess body fat distribution differences, based on previous analyses of sex differences in body fat distribution (Garnett et al 2004; Lampl et al 2005; Webster-Gandy et al 2003). These included:

Trunk2= abdominal+ suprailiac skinfolds
Trunk 3= subscapular +abdominal +suprailiac skinfolds
Leg= quadriceps + calf skinfolds
Trunk to leg= trunk2/leg
WHR= abdominal circumference/suprailiac circumference
TSF to SSF ratio= triceps skinfold/ subscapular skinfold
Skinfold to circumference ratio= skinfold (tsf, abdsf, silsf, qsf, csf)/ circumference (uac, abdc, silc, qc, cc)

Technical Error of Measurement

Reliability of length measurement was assessed through calculation of the standard deviation, coefficient of variation and technical error of measurement (TEM) between and within-observers. These results are presented in Table 5.1 below.

The TEM was calculated using the formula:

$$TEM = \sqrt{(\sum_{i=1}^{N}(M_{i1} - M_{i2})^2)/2N}$$

where M_{i1} and M_{i2} are the duplicate measures taken by a single observer or by 2 different observers on the same infant. 95% of the differences between replicate measures are expected to fall within ± 2*TEM (WHO, 2006). This 95% precision interval was used to determine whether significant length growth had occurred within the weekly interval.

Table 5.1: Reliability of Length Measurements

	N	SD (cm)	CV	TEM
Inter-Observer	371	0.15	0.23%	0.23
Intra-Observer (ALT)	191	0.11	0.16%	0.18

Significant weekly length gain was defined as an incremental gain of at least 0.5 cm during the weekly measurement interval based on the 95% precision interval for TEM. This value is more conservative than previous longitudinal studies of infant growth which have documented a technical error of measurement (TEM) of .124 to.145 cm (Harrison et al 1990; Lampl et al 1992). This more conservative figure was chosen to account for any additional variability associated with the use of different assistants in measurement or infant state.

Significant weekly weight gain was defined as an incremental gain of greater than 75 gm during the weekly measurement interval. This value has been shown in previous research (Lampl et al 2005) to correspond to the 95% reliability for weight change unaffected by intradaily processes such as hydration, defecation or variations due to eating.

Circumference/Skinfold Gain were coded dichotomously from the weekly incremental values for circumference and skinfold measures. A value of 1 indicates a gain in diameter or thickness during the interval between measurements, while a value of 0 indicates no change or loss over the interval.

Greater than the median testosterone/estradiol were defined as dichotomous variables, with a positive value when the testosterone or estradiol value is above the median value

for the individual infant. Thus, a value of 1 for the variable indicates a higher than usual hormonal level in the individual infant.

Results

Sample Characteristics

Table 5.2: Sample Characteristics by Infant Sex

	Male Infants	**Female Infants**
Maternal Characteristics	*n=15* Mean (SD)	*n=16** Mean (SD)
Age	33.7 (4.0)	35.6 (4.0)
Primiparity %	60%	87.5%
Pre-pregnancy Weight *(kg)*	62.0 (6.3)	64.1 (7.6)
Height *(cm)*	164.2 (6.3)	167.2 (7.2)
Pre-pregnancy BMI	23.1 (2.6)	22.9 (2.2)
Pregnancy weight gain *(kg)*	15.4 (4.5)	15.8 (6.5)
Infants		
Gestational Age *(weeks)*	39.1 (1.4)	40.1 (1.4)
Birth weight *(kg)*	3.69 (.37)**	3.38 (.47)
Birth length *(cm)*	53.2 (2.3)**	50.8 (2.7)
BMI	12.6 (1.2)	12.9 (1.5)
% Daycare	60%	50%
Age at Study Entry (months)	5.8 (3.3)	4.9 (3.4)

*One mother in the home-based sample did not fill out the background questionnaire' **p<.01, ttest

Table 5.2 shows the basic background and anthropometric characteristics of the study sample by infant sex. The infants in this sample had normal birth weight and length and grew at rates similar to the international reference for breastfed infants (WHO, 2006). Birth weight and length varied between the groups with male infants being significantly heavier and longer at birth than female infants.[5] There were no other significant differences in other maternal or infant characteristics between the groups. No mothers in either group reported a history of gestational diabetes or pre-eclampsia or smoking during pregnancy and/or the study interval.

[5] The possible effects of these differences in birth size on hormonal values are a focus of analysis in the next chapter.

Sex differences in length, weight and ponderal index

Table 5.3: Sex Differences in Size

	Boys			*Girls*		
Age (mo)	**Length (cm) Median (range)**	**Weight (kg) Median (range)**	**PI Median (range)**	**Length (cm) Median (range)**	**Weight (kg) Median (range)**	**PI Median (range)**
0-2	59.5 (55.0-62.4)**	4.7 (3.8-6.7)***	2.2 (2.0-3.0)	55.1 (48.9-63.5)	4.0 (2.3-6.4)	2.5 (1.8-2.9)
2-4	65.2 (62.7-69.0)***	6.6 (4.9-7.9)***	2.3 (1.7-2.8)	61.1 (56.1-67.3)	5.4 (4.2-8.3)	2.4 (2.0-2.8)
4-6	67.9 (60.4-74.6)*	6.4 (5.4-8.1)	2.1 (1.8-2.8)	66.8 (61.4-70.7)	6.9 (5.4-9.5)	2.4 (1.9-2.8) †††
6-8	72.0 (64.9-77.0)***	8.2 (6.6-9.6)	2.2 (1.8-2.7)	69.6 (64.0-73.8)	7.8 (5.6-10.6)	2.5 (1.8-2.9) †††
8-10	74.5 (70.0-78.5)***	9.2 (7.2-10.1)***	2.1 (1.7-2.9)	71.2 (62.3-78.1)	8.1 (6.9-11.5)	2.2 (2.0-3.4) ††
10-12	75.6 (72.0-78.9)	9.7 (7.9-10.6)*	2.2 (1.6-2.6)	74.6 (68.8-80.5)	8.7 (7.1-12.4)	2.1 (1.9-2.5)

Mann-Whitney rank sum
*Denotes significant sex differences with males larger, p=<.10
** Denotes significant sex differences with males larger, p<.05 †† Denotes significant sex differences with females larger, p<.05
*** Denotes significant sex differences with males larger, p<.01 ††† Denotes significant sex differences with females larger, p<.01

As stated above, boys in this sample were born longer than girls, with a mean length 53.2 cm versus 50.8 cm (ttest, p=0.03), and remained longer than girls through the first year of life. These differences were significant for the first 10 months of life when the median male length was 1.1-4.1 cm longer than the median female length. The lack of significant differences after 10 months was probably due to both the smaller number of boys in the sample at this age and the presence of a female infant who was much taller than many other girls of the same age.

Male infants also weighed more than females at birth, with a mean of 3.69 kg vs. 3.38 kg, and remained heavier for the first 4 months of life, when the median male weigh was 1.2 kg heavier than the median female weight at the same age. Unlike, length, where boys remained longer throughout infancy, male infants were no longer significantly heavier than female infants between 4 and 8 months. Interestingly, during this same time period,

female infants were significantly plumper for their length, as indicated by their higher ponderal indices. Female infants had significantly higher ponderal indices from 4 months until 10 months, after which the differences were no longer significant. Beginning around 8 months, male infants again had higher weights and ended the first year with moderately higher weights than female infants (1.0 kg, p=0.07).

Sex Differences in Circumferences and Skinfolds

Table 5.4 documents median trunk and limb circumferences from birth through 12 months of life. These data document sex differences in trunk and limb circumferences during much of the first year. Prior to four months of age, male infants had significantly larger abdominal and calf circumferences compared to female infants. After 4 months of age, however, females had larger trunk and limb circumferences despite their smaller body size. This was particularly evident for supra-iliac circumference which was larger in females in the first two months of life, though this difference did not reach statistical significance, and significantly larger from 4 months through 12 months age. Other circumferences showed more limited associations with sex. From 4 to 6 months of age, girls had significantly larger upper arm circumference, suprailiac circumference, and calf circumference when compared to male infants. At 6 to 8 months of age, they had greater abdominal circumference, suprailiac and quadriceps circumferences. Significant differences in suprailiac and quadriceps circumferences were documented through 10 months of age.

Similarly, **Table 5.5** depicts median trunk and limb skinfolds from birth to 12 months of age. Unlike the circumferential measures, the skinfold measures of infant girls were larger at almost every age measured. With the exception of suprailiac skinfold, which

was significantly larger at 0-2 months of age, the greater female skinfold thicknesses were significant only after 4 months of age. At 4 months, females had significantly larger triceps, abdominal, suprailiac, and quadriceps skinfold thickness. These larger skinfolds persisted through the end of the first year for the abdominal, suprailiac and quadriceps depots and through 10 months for the triceps. Additionally, female infants had greater sub-scapular skinfold thickness from 6 to10 months of age. The only skinfold which did not show sex differences was the calf skinfold, which was similar in male and female infants throughout the first year. These differences in skinfolds and circumferences are depicted graphically in the appendix to this chapter.

Sex Differences in Adipose Tissue Distribution

Table 5.6: Sex Differences in Adipose Tissue Distribution

	Boys			***Girls***		
Age (mo)	**Trunk Median (range)**	**Leg Median (range)**	**Trunk:Leg Median (range)**	**Trunk Median (range)**	**Leg Median (range)**	**Trunk:Leg Median (range)**
0-2	9.6 (7.4-18.5)	19.9 (15.5-33.9)	.48 (.40-.60)	14.9 (8-22)	25.7 (14-34.2)	.66*** (.51-.73)
2-4	18 (7.1-24.2)	30.5 (15.8-38.5)	.60 (.36-.75)	15.5 (10-22.6)	26.4 (21.8-38)	.57 (.42-.72)
4-6	12 (8-17.8)	27.2 (18.8-37.5)	.44 (.28-.65)	13.4*** (10-21.2)	29.8** (23.4-37.5)	.46** (.35-.65)
6-8	13.4 (7.2-18.9)	29.8 (23-34.4)	.50 (.23-.57)	17.2*** (11.5-27.8)	31.8*** (24.4-36.2)	.54** (.38-.77)
8-10	11.8 (9.4-18.8)	27 (23.2-36)	.42 (.34-.58)	16.8*** (11-22.8)	30.2*** (24.6-37)	.56*** (.38-.66)
10-12	11.2 (8.2-19.8)	26.6 (21-33.6)	.46 (.27-.61)	16*** (10.6-22.2)	28.4* (22-33.8)	.54* (.40-.77)

Mann-Whitney rank sum
*Denotes significant sex differences with females larger, p=<.10
** Denotes significant sex differences with females larger, p<.05
*** Denotes significant sex differences with females larger, p<.01

Following previous literature, sex differences in adipose tissue distribution were also tested. Comparison of the composite measures of trunk and leg skinfold thicknesses and the ratio of the two measures indicated that female infants had significantly larger trunk

and leg adipose tissue depots from 4 months through 12 months. Female infants not only had greater depots in both of these regions; they also had significantly greater ratios of trunk to leg skinfolds, indicating a greater central distribution of adipose tissue. Mixed model analysis confirmed the sample median results in the table above; female sex predicted greater trunk (xtreg, β=1.71, p=0.049) and trunk:leg ratio (xtreg, β=0.05, p=0.02), controlling for age, weight, and subject. The sex differences in leg skinfolds did not remain significant, suggesting that, for their weight, female infants do not have proportionally greater leg adipose tissue.

Finally, waist-to-hip ratio (WHR) also differed significantly by sex over the whole first year, such that female sex predicted a lower WHR (xtreg, sex β =-0.02, p=0.01), controlling for age and weight. The circumference analysis above showed abdominal circumference was not statistically lower in female infants over the first year. Thus, the lower waist-to-hip ratio in female infants was likely explained by their greater suprailiac circumference.

Body size and hormone levels

Table 5.7: Relationship between Body Size and Testosterone (by Sex) – 2 Month Intervals

	0-2		2-4		4-6		6-8		8-10		10-12	
Outcome	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)
Length cm	.02 (.66)	-.49 (.01)	-.01 (.63)	.01 (.82)	.04 (.45)	-.01 (.67)	.06 (.69)	-.02 (.72)	-.003 (.91)	.11 (.34)	-.29 (.02)	-.16 (.11)
Weight gm	-.01 (.73)	-.20 (.0/1)	-.01 (.001)	.0001 (.98)	.01 (.01)	-.005 (.28)	-.03 (.40)	-.02 (.36)	.006 (.91)	.01 (.41)	.04 (.12)	-.006 (.70)
PI	-.007 (.73)	-.05 (.002)	-.02 (.18)	-.001 (.74)	-.003 (.53)	-.002 (.55)	-.02 (.13)	-.004 (.58)	-.0001 (.96)	.01 (.30)	.03 (.005)	.01 (.20)

Sex stratified mixed model for outcome (length, weight or ponderal index) with testosterone as a main effect controlling for age and the random effect of subject

Having established that infant body size and composition differ by sex in this sample of infants, we next tested whether these differences in size and composition were associated with sex steroid production. Testosterone in male infants was associated with lower body weight between 2 and 4 months, but increased body weight from 4 to 6 months. At 12 months, testosterone was associated with reduced length and increased PI, something that was not supported in analysis of larger intervals (i.e. stratified by 6 months rather than 2 months). These results may be due to the effects of smaller sample size at 12 months. In female infants, testosterone only predicted body size variables before 2 months of age, when it was associated with reduced length, weight and PI.

Table 5.8: Relationship between Body Size and Estradiol (by Sex) – 2 Month Intervals

	0-2		**2-4**		**4-6**		**6-8**		**8-10**		**10-12**	
Outcome	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)
Length cm	-.25 (.72)	-2.47 (.03)	.34 (.57)	.33 (.39)	-.05 (.95)	1.12 (.02)	-.06 (.91)	-.06 (.71)	-.56 (.18)	-.10 (.87)	-.55 (.46)	-.50 (.14)
Weight kg	.31 (.67)	-1.03 (.01)	-.14 (.43)	.006 (.89)	.07 (.33)	.05 (.50)	-.10 (.53)	-.18 (.001)	-.10 (.23)	-.07 (.27)	4.75 (.97)	-.03 (.61)
PI	.15 (.70)	-.33 (.003)	-.07 (.44)	-.02 (.69)	.02 (.80)	-.12 (.04)	-.02 (.81)	-.05 (.02)	.01 (.79)	.02 (.83)	.002 (.96)	.04 (.16)

Sex stratified mixed model for outcome (length, weight or ponderal index) with estradiol dose as a main effect controlling for age and the random effect of subject

When stratified by 2-month intervals, estradiol was only significantly associated with growth measures in female infants. As in the testosterone analysis in female infants, estradiol in the first 2 months of life also had a negative effect on length, weight and ponderal index. The similarity between the estradiol and testosterone results at this time may indicate that the increased level of hormones is associated with smaller size in female infants, rather than testosterone per se. Conversely, between 4 and 6 months,

increased estradiol was associated with longer length in female infants and reduced ponderal index. There was no effect of estradiol on length after 6 months of age in the 2-month analysis, but estradiol continued to predict lower weight and ponderal index from 6 to 8 months. There was no significant effect of estradiol on weight or ponderal index after 8 months in the 2-month analysis.

Six month analysis of the relationship between size and sex steroids

Table 5.9: Sex differences in the relationship between size and sex steroids- 6 month analysis

	Testosterone		**Estradiol**	
	Male β (p)	Female β (p)	Male β (p)	Female β (p)
< 6 months				
Weight (kg)	-.01 (.02)	.009 (.21)	-.04 (.56)	-.15 (.04)
Length (cm)	-.02 (.43)	.01 (.64)	-.91 (.003)	-.39 (.46)
PI	.000 (.99)	.001 (.79)	.09 (.01)	-.09 (.002)
> 6months				
Weight (kg)	-.001(.80)	-.001(.86)	-.04 (.44)	-.12 (.001)
Length (cm)	-.01(.73)	.01(.49)	-.28 (.29)	-.24 (.057)
PI	-.001 (.77)	-.003 (.39)	.01 (.85)	-.01(.64)

Sex stratified mixed model for outcome (length, weight or ponderal index) with testosterone or estradiol dose as a main effect controlling for age and the random effect of subject, repeated at 6-month intervals

In contrast to the above results, stratification by 6 months, the timeframe corresponding to the sex differences in testosterone and estradiol presented in the previous chapter, identified somewhat contradictory results to the 2-month analysis. During the first 6 months of life, the time during which testosterone was significantly higher in male infants, testosterone was only associated with lower weight in male infants. While these results correspond to the 2 monthly associations documented between weight and testosterone in male infants, they do not capture the association between testosterone and smaller overall size in girls' weight, length and ponderal index. This difference in results

suggests that the effect of higher testosterone in girls is limited to the period closest to birth and does not continue to have an effect later in infancy.

In the 6-month analysis, estradiol levels were associated with growth measures in infant boys. Estradiol levels were higher among shorter boys and those with a higher ponderal index during the first six months of life. These results taken together suggest that estradiol was higher among shorter, plumper boys.

The 6-month analysis also documented that the association between size and estradiol in infant girls extended beyond six months of age, as would be expected given the higher estradiol levels seen in infant girls at this time. Prior to 6 months, higher estradiol levels were found in girls who weighed less and who weighed less for their length (i.e. had a lower ponderal index). After 6 months, higher estradiol levels were found in girls who were shorter and weighed less.

The discrepancies between the 2-month analyses and 6-month analyses may be due to the greater statistical power offered by the 6-month level analysis, which necessarily includes a greater number of observations. The 6-month analysis may further be buffered from the influential effects of individual infants. Because of the rolling enrollment of infants into this longitudinal sample, the 6-month intervals are more homogeneous in terms of the subjects included than are the 2-month samples.

Body composition and hormone levels

Trunk and Limb Circumferences

Examination of the relationship between sex steroid levels and body composition, measured through circumferences and skinfold thickness, also revealed sex-specific

associations between hormonal levels and body composition measures. In the 2- month analysis, testosterone was associated only with circumferential measures in boys between 2 and 4 months of age when testosterone negatively predicted suprailiac and calf circumference. In the six month analysis (see Table 5.10), testosterone only predicted hip circumference in boys prior to six months, when testosterone was associated with narrower hip circumference.

Table 5.11: Relationship between Testosterone and Trunk and Limb Circumferences (by sex)

	0-2		**2-4**		**4-6**		**6-8**		**8-10**		**10-12**	
Outcome cm	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)
UAC	.-.04 (.59)	-.38 (.002)	-.01 (.59)	.02 (.47)	.01 (.74)	.01 (.74)	.02 (.87)	-.02 (.66)	-.01 (.60)	-.10 (.20)	.02 (.91)	.12 (.09)
ABDC	.07 (.71)	-1.07 (.000)	.01 (.81)	-.05 (.27)	-.03 (.61)	.001 (.98)	-.16 (.52)	.01 (.94)	-.09 (.18)	-.07 (.65)	-.18 (.63)	.40 (.20)
SILC	.11 (.60)	-.82 (.04)	-.12 (.006)	-.03 (.60)	-.06 (.31)	-.02 (.78)	.05 (.83)	.05 (.66)	-.05 (.43)	-.04 (.84)	.58 (.22)	-.28 (.16)
QC	-.02 (.85)	-.58 (.001)	-.03 (.43)	-.01 (.59)	.05 (.45)	.04 (.34)	-.01 (.92)	-.18 (.07)	-.03 (.69)	.05 (.75)	.08 .80	.10 (.67)
CC	.002 (.97)	-.34 (.001)	-.03 (.04)	.01 (.42)	.01 (.67)	.01 (.73)	-.03 (.76)	-.001 (.99)	.008 (.86)	-.03 (.75)	.-.003 (.99)	.02 (.72)

Sex stratified mixed model for outcome (circumference measures) with testosterone dose as a main effect controlling for age and the random effect of subject

The 2-month analysis documented a negative association between testosterone and all measures of circumference (upper arm, abdominal, hip, thigh and calf) in the first 2 months of life in infant girls. After that time period, testosterone was only negatively associated with quadriceps between 6 and 8 months and positively associated with upper arm circumference between 10 and 12 months. Neither of these associations reached the 0.05 significance level and may be due to chance. Over the first 6 months of life, testosterone was associated with greater upper arm circumference only in infant girls.

Table 5.12: Relationship between Estradiol and Trunk and Limb Circumferences (by Sex)

	0-2		2-4		4-6		6-8		8-10		10-12	
Outcome **cm**	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)
UAC	1.71 (.14)	-1.50 (.12)	-.39 (.58)	.43 (.15)	-.10 (.82)	.32 (.45)	.10 (.72)	.03 (.81)	.45 (.41)	.12 (.80)	.41 (.45)	.07 (.75)
ABDC	.12 (.97)	-3.80 (.06)	-2.56 (.10)	-.32 (.50)	-.32 (.68)	.74 (.28)	-.99 (.40)	.08 (.80)	-2.60 (.007)	2.60 (.03)	.91 (.53)	.14 (.88)
SILC	2.02 (.69)	-5.80 (.13)	-.86 (.70)	-.17 (.80)	-.77 (.39)	-1.24 (.40)	-1.29 (.32)	.03 (.95)	-2.62 (.02)	1.83 (.09)	-3.56 (.22)	-.20 (.72)
QC	1.09 (.59)	-2.05 (.15)	-.13 (.93)	.09 (.80)	.13 (.89)	.03 (.97)	-.26 (.62)	.19 (.55)	.70 (.62)	-1.08 (.17)	.97 (.42)	.28 (.70)
CC	1.05 (.41)	-1.28 (.12)	.15 (.84)	.19 (.16)	.37 (.37)	-.53 (.33)	-.55 (.20)	.14 (.32)	.24 (.72)	-.19 (.75)	.86 (.27)	.05 (.74)

Sex stratified mixed model for outcome (circumference measures) with testosterone dose as a main effect controlling for age and the random effect of subject

Estradiol showed little association with circumferential measures in male or female infants. Between 2 and 4 months of life, estradiol was negatively associated with abdominal circumference, a result that is corroborated by the 6 month analysis (Table 5.10) which documented narrower abdominal circumference in males with higher estradiol levels. Additionally, higher estradiol was associated with narrower hip circumference in boys between 8 and 10 months of age, a result not found in the 6 month analysis. In female infants, higher estradiol levels were associated with reduced abdominal circumference prior to 2 months of age and increased abdominal and suprailiac circumference from 8 and 10 months of age. Neither of these associations was found in the 6-month analysis.

Limb and Trunk Skinfold Thicknesses

Table 5.13: Relationship between Testosterone and Trunk and Limb Skinfolds (by sex)

	0-2		2-4		4-6		6-8		8-10		10-12	
Outcome	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)
SSF	.03 (.83)	-.60 (.01)	.02 (.42)	.-.001 (.81)	.10 (.06)	.01 (.71)	.04 (.67)	-.02 (.72)	-.07 (.07)	-.17 (.02)	-.08 (.71)	.10 (.45)
TSF	-.01 (.95)	-.40 (.11)	.01 (.79)	-.02 (.68)	.03 (.58)	-.001 (.97)	-.21 (.14)	.08 (.36)	-.06 (.30)	-.26 (.03)	.03 (.90)	.16 (.36)
ABDSF	-.09 (.60)	-.32 (.001)	.006 (.82)	-.001 (.98)	-.05 (.05)	-.03 (.38)	-.08 (.57)	-.02 (.77)	.001 (.85)	-.11 (.33)	.05 (.84)	.11 (.25)
SILSF	-.02 (.83)	-.40 (.07)	-.04 (.35)	.04 (.29)	-.04 (.26)	.04 (.27)	-.06 (.54)	.14 (.19)	.03 (.43)	.05 (.62)	.19 (.18)	.24 (.09)
QSF	-.05 (.82)	-.77 (.001)	-.05 (.06)	.03 (.64)	.07 (.26)	.04 (.50)	-.18 (.22)	-.02 (.88)	-.002 (.96)	-.18 (.18)	-.29 .37	-.08 (.61)
CSF	.06 (.60)	-.19 (.28)	-.08 (.15)	-.01 (.81)	.01 (.93)	.03 (.50)	-.01 (.96)	-.12 (.23)	-.08 (.46)	-.22 (.10)	.-.01 (.98)	.02 (.93)

Sex stratified mixed model for outcome (skinfold measures) with testosterone dose as a main effect controlling for age and the random effect of subject

In boys, testosterone showed little association with skinfold thickness. Testosterone was negatively associated with quadriceps skinfold thickness between 2 and 4 months of life, abdominal skinfold thickness between 4 and 6 months of life and subscapular skinfold thickness between 4 and 6 months. None of these associations, however, reach the .05-level of significance and none are supported by the 6-month analysis.

In female infants, skinfold thickness in the first 2 months of life, like circumferences in the first 2 months of life, are negatively associated with testosterone. During this time period, higher testosterone levels are associated with significantly lower subscapular, abdominal, suprailiac and quadriceps skinfold thicknesses in female infants. Similar results were found between 8 and 10 months, when subscapular, triceps and calf skinfolds were negatively associated with testosterone in girls.

None of these associations were documented by the 6 month analyses. However, the fact that both higher estradiol and testosterone levels were associated with reduced circumferences and skinfold thickness in all measures from 0-2 months, suggests that there is something unique about this time period and that perhaps this period may be important in shaping female body morphology.

Table 5.14: Relationship between Estradiol and Trunk and Limb Skinfolds (by sex)

	0-2		2-4		4-6		6-8		8-10		10-12	
Outcome	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)
SSF	-.57 (.86)	-.77 (.65)	.05 (.95)	-.03 (.07)	.1.67 (.01)	-.27 (.68)	.17 (.67)	-.06 (.75)	-.37 (.56)	.07 (.88)	.31 (.76)	-.26 (.53)
TSF	1.34 (.67)	-.40 (.80)	.79 (.67)	-.48 (.45)	-1.09 (.09)	-.70 (.43)	-.55 (.32)	-.40 (.13)	.38 (.66)	.34 (.66)	1.65 (.05)	-.42 (.40)
ABDSF	.58 (.87)	-1.75 (.02)	-.47 (.72)	.56 (.19)	-.32 (.37)	-.65 (.33)	.-.19 (.75)	.14 (.45)	-.23 (.67)	-.17 (.80)	.28 (.77)	.09 (.77)
SILSF	.48 (.86)	-.14 (.92)	-.84 (.65)	1.09 (.01)	-.30 (.53)	.37 (.58)	-.59 (.27)	-.31 (.37)	-.51 (.30)	.15 (.82)	-.97 (.08)	.23 (56)
QSF	2.34 (.61)	-3.44 (.09)	.15 (.93)	.01 (.98)	.12 (.90)	-1.53 (.15)	-1.35 (.08)	-.05 (.89)	.33 (.73)	1.95 (.02)	.56 .67	-.09 (.85)
CSF	-.80 (.71)	-1.78 (.27)	-1.72 (.54)	-.01 (.98)	.10 (.90)	-.93 (.17)	-.59 (.72)	-.74 (.22)	1.85 (.21)	-.16 (.87)	4.89 (.01)	.25 (.69)

Sex stratified mixed model for outcome (skinfold measures) with testosterone dose as a main effect controlling for age and the random effect of subject

Like testosterone, estradiol also had sex-specific associations with skinfold thickness measures. In male infants, higher estradiol levels were associated with increased subscapular, but decreased triceps, skinfold thickness between 4 and 6 months of life and a trend towards decreased quadriceps skinfold between 6 and 8 months of life. After 10 months, higher estradiol levels were associated with increased triceps and calf skinfold thicknesses. The 6-month analyses provided further support for an association between higher estradiol levels and greater subscapular and thigh skinfold thickness.

In girls, higher estradiol was associated with decreased abdominal and quadriceps skinfold thickness before 2 months of age and decreased subscapular thickness between 2 and 4 months of age. Conversely, higher estradiol levels were associated with greater suprailiac skinfold thickness between 2 and 4 months. These results were supported by the 6 month analysis which documented a significant, positive relationship between estradiol level and hip skinfold in infant girls before 6 months.

Adipose tissue distribution was also associated with hormone levels in 6-month analysis (data not shown). Boys with higher estradiol levels had greater leg skinfold thickness both over the entire first year (β=1.93, p=.03) and during the first 6 months of life (β=1.81, p=.06). Girls with higher testosterone levels, on the other hand, had higher trunk skinfold measures both over the whole first year (β=.08, p=.02) and before 6 months of age (β=.10, p=.045). Waist to hip ratio, WHR, did not appear to relate directly to hormone levels, which may indicate differential relationships between the adipose tissue deposition areas and hormones.

Summary: sex differences in the association between testosterone, estradiol and body composition measures

In summary, over the first 6 months of life, higher testosterone was associated with narrower hips in boys and increased upper arm circumference in girls. Testosterone showed no significant associations with skinfold measures in either male or female infants during this period. Higher estradiol, on the sample level, predicted greater fat on the back and thigh and reduced abdominal and thigh circumference in male infants. In girls, higher estradiol predicted greater suprailiac fat and larger circumferences of the abdomen and upper arm.

Growth and sex steroids

The preceding analysis identified sex differences in the association between body size and composition and sex steroid levels. However, the question of the direction of this association remains unanswered; it is not clear from the above analysis whether hormonal levels are associated with body size and tissue depot or whether hormonal levels co-vary contemporaneously with the growth of the tissues. The latter, if documented, would provide evidence for a mechanistic linkage between hormonal levels and changes in length, weight and adipose tissue/muscle accrual and utilization. Consequently, this analysis asked whether, on the individual level, changes in sex steroid levels predicted linear growth, weight gain or changes in body composition in the concomitant weekly interval.

Length growth

Table 5.15: Sex Steroids and Significant Weekly Length Growth

Predictor	**Model A (unadjusted) OR (CI)**	**Model B (age)* OR (CI)**	**Model C (age+sex)** OR (CI)**
>median estradiol dose	1.45 (1.00-2.10) p=.05	1.46 (1.004-2.11) p=.046	1.46 (1.002-2.11) P=.049
>median testosterone dose	.95 (.66-1.37) p=.78	.93 (.64-1.35) p=.70	.93 (.64-1.35) p=.70

*age does not significantly predict significant weekly length growth
** age and sex do not significantly predict significant weekly length growth

In logistic regression models for correlated data (STATA, *xtlogit*), estradiol levels above the median for an individual (e.g. a bivariate variable) were associated with significant length growth within the concomitant weekly interval, with infants having a greater than

the median estradiol level being 45% more likely to have grown in length during the weekly interval. This relationship is largely independent of age and sex, as shown in the above table. Testosterone levels above the individual median had no significant association with weekly growth in length.

Weight

Table 5.16: Sex Steroids and Significant Weekly Weight Gain

Predictor	**Model A (unadjusted) OR (CI)**	**Model B (age)* OR (CI)**	**Model C (age+sex)** OR (CI)**
>median estradiol dose	1.13 (.78-1.64) p=.51	1.14 (.78-1.66) p=.49	1.14 (.78-1.66) p=.49
>median testosterone dose	1.37 (.94-1.98) p=.10	1.29 (.89-1.88) p=.19	1.29 (.88-1.88) p=.19

*age does significantly predict significant weight gain (OR= .996, CI: .994- .998)
** sex does not significantly predict significant weekly weight gain

Neither estradiol nor testosterone levels above the median for an individual were associated with significant weekly weight gain. When the data are stratified by sex, greater than the median testosterone levels show a moderate association with weekly weight gain in female infants (OR: 1.61, CI: .98-2.64, p=.06).

Changes in Body Composition

Both estradiol and testosterone levels above the median for an individual were associated with positive gains in weekly circumferential and skinfold measures before and after 6 months. This cut-off was chosen for analysis first to capture sex differences in hormonal values and, second, to provide sufficient statistical power for the logistic models. In

these models, having an estradiol level higher than the individual median predicted a greater likelihood of gaining abdominal circumference, suprailiac circumference and calf circumference in boys during the weekly interval before 6 months of age. During this same period, an elevated estradiol level only predicted an increased likelihood of a gain in abdominal skinfold in girls.

Table 5.17: Sex Steroids and Change in Body Circumferences by 6-month intervals

<180 days				
Outcome	> Median E2 OR (CI)		>Median T OR (CI)	
	Male	Female	Male	Female
Abdc gain	2.44 * (.92-6.48)			
Silc gain	2.81 * (.98-8.10)			
Calf c gain	3.98 ** (1.38-11.45)			
SSF gain				2.31* (.96-5.56)
ABDSF gain		2.61** (1.14-5.98)		
SILSF gain	7.78** (2.21-27.40)			
CSF gain	3.10* (1.01-9.53)			
>180 days				
	> Median E2 OR (CI)		>Median T OR (CI)	
	Male	Female	Male	Female
UAC gain				2.15** (1.13-4.07)
Silc gain	4.76* (1.19-19.14)			
Calf c gain			2.00* (.88-4.54)	
ABDSF gain	2.25* (.97-5.22)	.59* (.31-1.12)		
SILSF gain				2.09* (.96-4.55)
CSF gain	4.7** (1.28-17.21)			

Repeated-measures logistic model measuring the probability of a positive gain in body/limb circumference/skinfold given a greater than the median level of estradiol or testosterone (xtlogit); only significant results are shown; *p<.10, **p<.05.

After 6 months of age, elevated estradiol continued to be associated with a greater likelihood of an increase in hip circumference and calf skinfold in male infants and abdominal skinfold in female infants. Additionally at this time, elevated estradiol was associated with an increased likelihood of abdominal skinfold accrual in male infants. Testosterone was only associated with subscapular skinfold prior to six months, when elevated testosterone was associated with a greater likelihood of subscapular skinfold expansion in female infants. After 6 months of age, elevated testosterone was associated with an increased likelihood of growth in upper arm circumference and hip skinfold in female infants and calf circumference in male infants.

Over the whole year, elevated estradiol predicted gains in hip circumference (OR=2.96, p=0.02) and hip (OR=2.61, p=0.009) and calf (OR=3.38, p=0.002) skinfold thickness in male infants. Elevated estradiol did not consistently predict the growth of any circumference or skinfold thickness in female infants over the entire year. Elevated testosterone, on the other hand, predicted increased weekly upper arm circumference in both male (OR=1.76, p=0.02) and female (OR=3.38, p=0.002) infants.

Discussion

The above analyses document both sex differences in infant growth and body composition as well as numerous sex-specific associations between linear growth, weight and body composition and sex steroid levels. These results point to, already in infancy, a hormonal association with body morphologies that are known to reflect sex-specific hormonal profiles in adolescents and adults. Interestingly, these results also document a stronger association between sex steroid levels and changes in size and body composition than between absolute size and body composition.

Table 5.18: Summary of Findings

	Body Size and Composition	Testosterone	Estradiol
Boys	- Longer than girls from birth – 10 mo - Heavier than girls from birth- 4 mo and after 8 mo - Decrease in weight relative to girls from 4- 8 mo - Greater abdominal c and calf circ to 4 mo - Lower skinfolds at all ages	- Higher T↔ lower wt before 6mo - Higher T ↔ narrower hips before 6 mo - Elevated individual T ↔ growth in upper arm circumference	- Higher E2 ↔ reduced length and increased PI before 6 months - Higher E2 ↔ reduced ABD circ and thigh circ before 6 months - Higher E2 ↔ increased SSF and QSF - Higher E2 ↔ greater leg skinfold thickness - Elevated E2 ↔ weekly length growth - Elevated E2 ↔ growth in hip and calf circ and sf before 6 months
Girls	- Higher ponderal index 4-10 mo - Larger circumferences and skinfolds from 4 mo onward (except for calf) - Greater trunk:leg skinfold ratio indicating more central fat distribution - Lower WHR due to larger hip circumference	- Higher T in first 2 months ↔ reduced body and limb circumferences and skinfolds - Higher T from 8-10 months ↔ lower SSF and TSF - Higher T ↔ greater truncal distribution of adipose tissue - Elevated individual T ↔ growth in SSF and UAC	- Higher E2 in first 6 months ↔ lower weight and ponderal index - Higher E2 ↔ decreased length and weight from 6-12 mo - Higher E2 ↔ increased hip skinfold from 0-6 mo - Elevated individual E2 ↔ weekly length growth - Elevated E2 ↔abdominal skinfold accrual from 0-12 mo

Sex differences in growth results

Male infants in this sample of healthy, American infants were born longer and heavier than female infants and remained this way for much of infancy, results similar to the sexual dimorphic body size documented in previous studies of neonates (Copper et al

1993; Guihard-Costa et al 1997) and young infants (Frisancho et al 1977; Hamill et al 1979). Despite their longer length and greater weight, male infants in this sample were not larger in trunk or limb circumferences or ponderal index after 4 months of age. These results conflict with two large studies of newborn infants which found both (1) no sex differences in ponderal index and (2) greater male trunk and limb circumferences (Copper et al 1993; Guihard-Costa et al 1997). This departure from previous research may be an artifact of small sample size or may be due to the weekly, longitudinal measurement protocol which was able to capture developmental trends missed with measurements timed close to birth. The size of infants in the current sample was near the median of the newly developed, WHO international reference standard for the growth of breastfed infants (WHO 2006), suggesting that, despite the small sample size, the sample is composed of infants that are of a representative size.

The results of the present study also suggest that male and female infants differ in both their levels of subcutaneous adiposity and adipose tissue distribution. Female infants had larger limb and trunk skinfold thickness at all ages measured and significantly greater skinfold thickness from 4 months of age through the end of the first year. These results indicate that female infants have greater subcutaneous fat depots despite their smaller body size and length. The presence of such sex differences in adiposity in infants remains controversial in the existing literature (Harrington et al 2004), partly because of the different ages of infants studied (neonates vs. toddlers, for example) and the different methods (skinfolds vs. DEXA vs. MRI) used to assess body composition. However, the results derived from anthropometric measures in the current study are in line with both skinfold-based and DEXA-based observations of the greater subcutaneous adiposity of

female infants (Copper et al 1993; Koo et al 2000; Rodriguez et al 2004). Skinfold thickness measurements have been validated against DXA-derived assessments of fat mass in newborns and young infants and have been shown to correlate at a R^2 of 0.94 (Schmelzle and Fusch 2002). Consequently, the skinfold measures attained in this study can be considered likely to represent real physiological differences in both fat mass and adipose tissue distribution.

Interestingly, female infants in this study had a greater amount of trunk fat than similarly size male infants and a greater trunk:leg ratio, a pattern, that in adults, is typically associated with an android adipose distribution (Bjorntorp 1996). This observation is not without precedent in infant or adolescent body composition research, where a greater central to peripheral ratio of adipose tissue was found in both preterm and term female newborns (Rodriguez et al 2004) and with increasing pubertal stage in adolescent girls (Goulding et al 1996). The greater central fat distribution seen in female infants and adolescents has been independently hypothesized to relate to increased androgenic steroid production during these times (Rodriguez et al 2004). Further, this greater level of adiposity in female infants and, in particular, greater truncal adiposity in female infants has been suggested to be related to their greater survival in the neonatal period (Copper et al 1993; Rodriguez et al 2004).

Sex differences in relationship between hormones and linear growth, weight and body composition

Adolescent growth studies have documented an increase in sex steroid and somatic hormone levels prior to, and as a necessary condition for, an increase in height velocity and body composition changes (Roemmich et al 2002; Spelsberg et al 1999; Styne 2003;

Suter et al 2000; Veldhuis et al 1997). For example, studies in adolescents have shown that the GH, IGF-I, and IGBF-3 all rise during puberty under the influence of sex steroids (Kerrigan and Rogol 1992) and have suggested that this concurrent rise in GH and gonadal steroid hormones, acts both independently and synergistically, to organize increased linear growth and bone density, muscle mass and adipose tissue accrual (Roemmich et al 2002).

Sex steroid levels are associated with increases in length and changes in body composition in the infants in this sample as well. An above the individual median level of estradiol, but not testosterone, was associated with linear growth within the weekly measurement interval for both male and female infants. Estradiol at low levels has been shown to promote linear bone growth in children and adolescents through both indirect effects on GH and through direct stimulation of chondrocyte proliferation (Juul 2001). The association between higher than usual estradiol levels and concurrent linear growth in the infants of this sample provides preliminary evidence that estradiol production in infants may be linked mechanistically to the process of linear growth.

Bivariate analysis of infant weight gain and sex steroids did not reveal an association between greater than the median levels of estradiol or testosterone and weight gain, suggesting that sex steroids are not directly promoting weight gain or loss. This result is not unexpected as infant weight gain is a summary measure, dependent on multiple factors, including feeding style (Butte et al 2000b; Dewey 1998), illness (Cohen et al 1995) and activity level (Wells et al 1997). Sex steroids, however, are related to absolute weight in this sample such that boys with higher testosterone have lower weights in the first six months of life and, conversely, girls with higher estradiol have lower weights

during the same time period. As weight is a summary measure of numerous types of body tissues including fat, muscle and bone, these results may be explained by the differential effects of testosterone and estradiol on different body tissues in male and female infants. The negative relationship between testosterone and weight in male infants, who have greater levels of testosterone than female infants in the first 6 months, may be due to the lipolytic and anabolic effects of testosterone. Testosterone administration to growth-hormone deficient prepubertal boys has been shown to decrease fat mass while increasing whole body protein synthesis (Mauras et al 2003). Similarly, in vitro studies of the effects of testosterone on adipocytes and pre-adipocytes indicate that testosterone inhibits lipid uptake and lipoprotein-lipase (LPL), the enzyme that regulates lipid uptake by the cell, activity in mature adipocytes, stimulates lipolysis by increasing the number of lipolytic receptors in the adipocyte, inhibits leptin production and inhibits the differentiation of pre-adipocytes into mature fat cells (Ahdjoudj et al 2001; De Pergola 2000; Rosenbaum and Leibel 1999). This lipolytic effect may explain the negative associations seen between all measures of body composition and testosterone levels in the first 2 months of life in female infants, a time when testosterone tends to be higher in female infants. This association is supported by logistic regression analysis of the dichotomous variable "younger than 2 months," which predicted higher testosterone in female, but not male, infants (β=1.32, p=.10).

While this study is unable to measure fat free mass (FFM) directly, fat free mass has been documented to increase dramatically during infancy, particularly from birth to 3 months of age, with larger values in male infants (Butte et al 2000a; Fomon et al 1982; Koo et al 2000). It is unclear whether this increase in FFM is related to muscle accumulation

and/or organ growth (Roche and Sun 2003b), but the low, relatively unchanging production of creatinine, a measure of muscle metabolism, in infants before 12 months of age suggests that the greater FFM in male infants may be associated more with organ growth than muscle accumulation (Fomon 1993). The current results provide indirect support for an association between testosterone and non-muscle FFM gain earlier in infancy and with muscle growth in later infancy. Male infants in this sample had abdominal circumferences that were the same size or larger than those of female infants before 6 months of age, despite having significantly smaller abdominal skinfolds. This result suggests that male infants had either a larger amount of visceral fat or larger vital organs than female infants. As most of the fat in infants is subcutaneous (~80%) rather than visceral (Bernstein 2005), larger organ size seems a more likely explanation of the greater abdominal size in male infants.

Elevated testosterone levels above the individual median, on the other hand, were associated with increases in upper arm circumference in both male and female infants after 6 months of age. This suggests that testosterone is mechanistically related to increased arm muscle accrual in later infancy. Both *in vivo* and *in vitro* research have documented that testosterone increases skeletal muscle mass by increasing muscle protein synthesis (Griggs et al 1989). Administration of testosterone to hypogonadal or elderly males has been shown to restore muscle mass and grip strength (Bhasin et al 2001a; Bhasin et al 2001b). On the tissue level, injections of testosterone led to a reutilization of intracellular amino acids and increased muscular protein synthesis (Tipton 2001). This positive effect of testosterone on whole body protein synthesis has been theorized to explain the differences in muscularity, particularly upper body muscularity, in men and

women (Tipton 2001). Conversely, the relative lack of sex differences in muscularity between boys and girls prior to puberty has been explained by the similar levels of testosterone in prepubertal children (Ramos et al 1998). Testosterone in adolescents of both sexes, however, was related to muscle mass. Thus, testosterone levels in this sample may reflect, and may even be mechanistically involved in, the differentiation of muscle tissue in the second half of infancy.

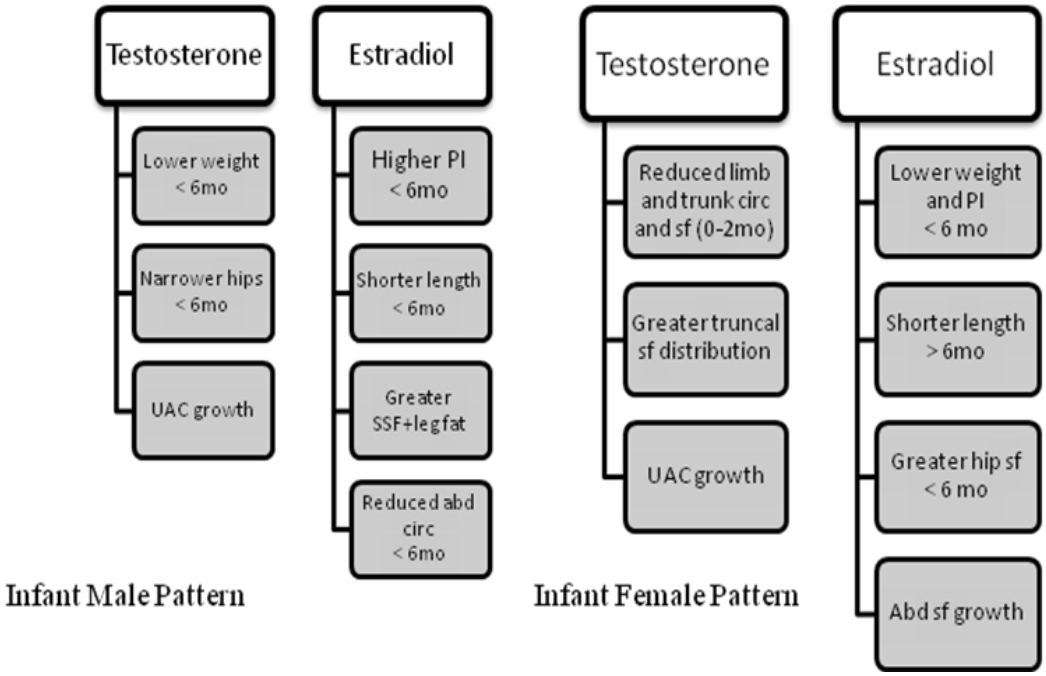
Much less information exists about the role of ovarian steroids on muscle development, particularly during development. However, in animal models, the accumulation of fat-free mass was greater in ovariectomized rats than those given estrogen or progesterone replacements (Toth et al 2001), suggesting to researchers that ovarian hormones inhibit protein synthesis. In vitro studies further support the inhibitory effect of ovarian hormones on muscle synthesis and imply that estradiol may attenuate muscle growth in females (Toth et al 2001). Such an effect could explain the current finding that higher levels of estradiol in infant boys was associated with lower abdominal and quadriceps circumferences.

The inhibitory effects of estradiol on fat free mass may also explain the somewhat counter-intuitive result that higher estradiol levels in female infants in this sample are associated with lower body weight. Differences in infant body weight between populations, such as those seen between Indian and Western newborns, and intra-population, such as those between infants born small-for-gestational-age (SGA) versus those born appropriate-for-gestational-age (AGA), tend to be explained by differences in fat free mass rather than fat mass, which tends to be more similar (Hediger et al 1998; Yajnik et al 2003). Thus, if estradiol has an inhibitory effect on fat free mass, then female

infants should weigh less than male infants, which they do in this sample. This explanation is further supported by the lower weight seen among the female infants with higher estradiol levels, which supports a dose-dependent relationship between estradiol and weight. Alternatively, the association between elevated individual estradiol and linear growth may indicate that the lower weight seen in girls with higher estradiol is associated with a greater frequency of linear growth.

In this sample of infants, estradiol was associated with increased skinfold thickness, albeit in sex-specific ways. In boys, higher estradiol levels were associated with greater subscapular, quadriceps, and overall leg skinfold thickness, depots that are typically associated with a more peripheral, gynoid pattern of fat deposition (Malina 1996). Paired with the higher ponderal index seen in boys with higher estradiol levels, these results suggest that boys with higher estradiol levels have plumper, more typically feminine body morphologies. This morphology may be particularly beneficial for male survival in energetically-challenging circumstances since femoral adipose tissue is not as active metabolically as more central depots (Bjorntorp 1996) and, thus, may provide a better site for long-term adipose storage. Conversely, among girls in this sample, higher estradiol is associated with a lighter, leaner body morphology, but greater abdominal and suprailiac skinfold thicknesses, a measure associated with increasing estradiol levels during puberty (Lassek and Gaulin 2007). Indeed, these sex differences in both total adiposity and skinfold thickness, which show site-specific sex differences, correspond to many of the body composition changes seen during adolescence when increasing estradiol levels are thought to contribute to sex-specific body morphologies.

Unlike testosterone, estradiol seems to have both lipogenic and lipolytic effects in both males and females one effect of which is to promote a subcutaneous, stereotypically female pattern of fat distribution (Cooke and Naaz 2004). Estrogens play an important role in adipogenesis in both males and females, by regulating the number of adipocytes that differentiate (Cooke and Naaz 2004). In mature adipocytes, estradiol attenuates lipolysis by up-regulating the number of anti-lipolytic adrenergic receptors in subcutaneous, but not visceral fat depots, thus shifting the assimilation of dietary fat from visceral to subcutaneous depots (Pedersen et al 2004). On the other hand, estrogens can also directly inhibit adipose deposition by decreasing lipogenesis and limiting the activity of LPL (Hamosh and Hamosh 1975). Because estradiol receptors are differentially distributed in subcutaneous, and particularly gluteofemoral subcutaneous, adipose tissue (Price et al 1998), estradiol has the ability to dually regulate, through deposition and lipolysis, the pattern of adipose tissue distribution in both men and women. Further this dual control of adipose tissue deposition and mobilization is influenced by energetic balance, exercise and other markers of stress (Perreault et al 2004; Wade et al 1985), indicating that the greater adiposity of infants with higher estradiol levels may provide some preliminary evidence that estradiol is a marker of energetic sufficiency.



These sex-specific patterns of growth in length weight and body composition in response to sex steroid production are summarized in the figures above.

Growth and sex steroids: Mechanisms and Physiological Implications

In both infancy and adolescence, then, somatic growth rates are rapid, body composition changes in sexually dimorphic ways, and the HPG hormonal axis undergoes organization and increased levels of activation. During this period of rapid growth in length, weight, and adiposity, the somatotrophic axis, composed of growth hormone (GH) and insulin-like growth factor-I (IGF-I) among other growth regulating hormones, also undergoes developmental organization with GH, under the control of insulin, stimulating local and hepatic production of trophic hormones, such as IGF-I, in concert with other metabolic hormones (Veldhuis et al 2006). While the role of GH in infant growth continues to be debated and many researchers believe that GH is only active in the later half of infancy (Karlberg et al 1994; Tse et al 1989), recent research using ultrasensitive assays for GH,

IGF-I and their binding proteins suggest that, not only is GH produced from birth, it also shows characteristic pulsatility and sex differences (Geary et al 2003; Ghigo et al 2000; Pirazzoli et al 1997), indicating functionality of the axis. The similarities between infancy and adolescence in growth rates and hormonal activity provide preliminary support for the idea that similar physiological mechanisms also underlie the two periods with that inter-axis communication between the somatic and sex hormone axes acting synergistically to drive and regulate somatic growth.

As discussed in the previous chapter, the postnatal gonadotropin and sex steroids surge is theorized to have organizational effects on sexual development and differentiation in infancy (Main et al 2000; Schmidt et al 2002). Animal models, however, highlight the potential for sex steroids to organize the somatotrophic axis during this period as well (Jansson et al 1985; Jansson et al 1984; Jansson and Frohman 1987). Animal research documents the entraining effects of sex steroids on sex-specific growth hormone pulsatility patterns during infancy. These investigations have demonstrated that gonadal steroids affect both GH secretion patterns and hepatic steroid metabolism (Gustafsson et al 1983), with neonatal testosterone being necessary for adult male GH pulsatility to develop and neonatal estrogen contributing to the high tonic levels of GH seen in adult females (Pincus et al 1996; Robinson et al 1998). Similar sex specific patterns of GH release are seen in human infants (Geary et al 2003), adolescents (Veldhuis et al 2000), and adults (Veldhuis 1998), suggesting that sex steroids regulate GH secretion in humans as well. While the role of GH and sex steroids in promoting growth and body compositional changes in infancy remain unelucidated, these sex-specific patterns of GH release also have sex-specific affects on linear growth and body composition during

puberty (Albertsson-Wikland et al 1994; Juul et al 2000). Given the similarly high rates of growth in infancy and sex differences in body composition, the postnatal sex steroid surge may also serve to enhance growth during early infancy, providing a common mechanism through which the linear growth and adipose tissue development are coordinated during both infancy and adolescence.

Conclusions and Epidemiological Implications

During infancy, the somatic and hormonal axes are being organized at rates of growth not matched again until puberty. Given the similarities between sex steroid levels, growth hormone levels, and somatic growth rates in infancy and puberty, it is surprising that this association between infant growth and adolescent growth has not been more fully described. Although the gonadotropin surge of early infancy was termed a "mini-puberty" (Forest et al 1974) when testicular activity in infancy was first documented, the similarities between infancy and adolescence have escaped mainstream attention. While preliminary, the results of this chapter suggest that sex steroid levels are related more to changes in body size and morphology in infancy than to absolute size, a finding that suggests that HPG activation in infancy may have the potential to influence the physiology underlying normal growth and development.

As birth weight and first year growth rates have been associated with the timing of maturation (Adair 2001), adult size (Dietz 1994), reproductive function (Ibanez et al 2000; Main et al 2006a) and morbidity risk (Barker and Clark 1997; Samaras et al 2003), infancy serves as an important developmental window proximally and evolutionarily linking infant growth and later physiology. In this way, the infant surge in somatic growth and endocrine activity may not just be the "mini-puberty" described in textbooks

but may actually prime the future tempo of growth, development, and reproduction, entraining adolescent and adult physiology and morbidity.

Table 5.4: Sex Differences in Trunk and Limb Circumferences

	0-2			*2-4*			*4-6*			*6-8*			*8-10*			*10-12*		
Measure	M	F	Sex Dif	M	F	Sex Dif	M	F	Sex Dif	M	F	Sex Dif	M	F	Sex Dif	M	F	Sex Dif
UAC	12.9 (11-15.1)	11.7 (9.4-16.7)		14.7 (11.5-16.6)	13.2 (10.6-17.3)		13.7 (12-16.5)	14.2 (12.4-17.9)	***	14.4 (13.2-17.1)	14.7 (12.1-19.6)		15.1 (13.5-18.8)	14.5 (12.5-20.3)		15.3 (14.2-18.2)	14.7 (12.4-20.2)	
ABDC	38.5 (34-45.4)	37.4 (31.5-44.1)		42 (35.2-45.6)	39.3 (35.7-48.6)	**	40.2 (36.8-44.8)	40.5 (31.2-48.2)		42.2 (37.5-49.5)	44.4 (36.5-50.1)	***	45.1 (38.1-50)	44.3 (33.5-50.4)		44.8 (39-52)	44.2 (32.8-52)	
SILC	33.1(29.4-44.3)	36.4 (29.6-44.3)		38.7 (30.2-45.8)	37 (33.6-44.7)		36.1 (33-43.2)	38.2 (31.4-46.1)	***	40.1 (32.1-45.1)	41.8 (35.7-49.4)	**	41.7 (34.9-46)	42.2 (38.4-49.8)	*	42 (37-46)	42.6 (37.5-51.4)	*
QC	17.8 (15.3-23.6)	16.9 (13.3-24.9)		22.7 (16.9-27.4)	20.1 (16.5-25.9)		22 (17.4-27.4)	23.4 (19.6-27.4)		23.7 (20.8-27.7)	25 (19.2-31)	**	23.7 (19.6-30.1)	24.2 (22.1-33.1)	**	24.3 (21-31.4)	25.3 (21-33.6)	
CC	14.4 (12.2-18.0)	12.7 (10.5-17.2)	**	17.1 (14-19.9)	15 (12.8-19.4)	**	16.4 (14.3-19.3)	17.6 (14-20.4)	**	18.4 (15.4-22.1)	17.7 (16-21.4)		18.4 (15.9-26)	17.9 (16.5-21.6)	*	18.4 (12.4-21.6)	18 (16-22.8)	

Median (range) * p<.10 ** p<.05, *** p<.01; Mann-Whitney U test

Table 5.5: Sex Differences in Limb and Trunk Skinfolds

	0-2			2-4			4-6			6-8			8-10			10-12		
Measure	*M*	*F*	*Sex Dif*	*M*	*F*	*Sex Dif*	*M*	*F*	*Sex Dif*	*M*	*F*	*Sex Dif*	*M*	*F*	*Sex Dif*	*M*	*F*	*Sex Dif*
SSF	6 (4.8-11.2)	8.3 (4.2-11)		7.8 (4.8-11.4)	7 (5-10.8)		6.8 (4.4-10.4)	7.2 (5-10.2)		7 (5.4--10.2)	8.2 (5-11.4)	***	7.2 (5-10.8)	8 (6.2-11)	***	8 (5-13)	7.9 (5-11.2)	
TSF	6.8 (5-13.6)	9.2 (6-14)		9.4 (4-14.2)	8.8 (4.8-13.4)		8.8 (5.8-10.8)	9.6 (6-14.2)	**	8.4 (6-12.4)	10.2 (5.1-14)	***	9.2 (6.2-14)	10 (6.2-14.2)	*	10 (7-15.2)	9.8 (6.6-13.8)	
ABDSF	4.9 (4-14.2)	8 (3.4-11)		9.2 (3.8-14)	8.8 (4.8-11.8)		6.8 (4.2-9.3)	7.9 (4.4-12)	***	8 (3.8-12.1)	10.2 (6-13)	***	7 (4.6-13.4)	10.2 (5.4-14)	***	7.8 (4.4-8.4)	9.4 (5-13.4)	*
SILSF	4.2(3.4-9)	6.8 (4-12)	***	7.8 (3-12.8)	6.8 (3.8-11.4)		5 (3.4-9.4)	6 (3.8-10.4)	***	5.6 (3.4-9.2)	7.2 (4.7-16)	***	5 (1.2-6.4)	7.2 (3.4-9.8)	***	4.4 (3.2-8.4)	6 (3.4-10.4)	***
QSF	10.6 (7.5-18.8)	11.8 (6.8-19.8)		18.1 (8.4-23.4)	14.4 (12.2-23)		15.2 (10-20)	16.9 (12-22.4)	***	16.2 (13.2-22)	18.3 (13-22.1)	***	14.6 (10-22)	18 (14-22.1)	***	16 (11.2-21.4)	16.4 (13-20.8)	**
CSF	10.1 (8-15.1)	10.1 (6.2-15.4)		12.6 (7-19.6)	11.8 (8.2-15.2)		12 (8.8-15.4)	13 (9.8-16)		12.8 (6.8-15)	13.1 (9.8-17.8)		12.8 (9-16)	12.1 (9.2-15.4)		12.8 (9.2-16.8)	12 (8.8-14.6)	

Median (range)

* p<.10 ** p<.05, *** p<.01; Mann-Whitney U test

TABLE 5.10: 6-month interval analysis of Sex Steroids and skinfold and circumferences

T and Circumferences- 6 mo intervals

	0-6		6-12	
Outcome	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)
UAC	-.01 (.27)	.03 (.10)	-.02 (.32)	-.003 (.89)
ABDC	.04 (.17)	.004 (.91)	-.03 (.66)	.05 (.44)
SILC	-.07 (.04)	.05 (.31)	-.007 (.93)	.09 (.13)
QC	-.004 (.90)	.03 (.31)	-.005 (.93)	-.02 (.73)
CC	-.02 (.19)	.02 (.27)	.03 (.43)	.10 (.000)

E2 and Circumferences- 6 mo intervals

	0-6		6-12	
Outcome	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)
UAC	.27 (.25)	.09 (.64)	.21 (.32)	-.13 (.24)
ABDC	-.83 (.08)	-.47 (.23)	-1.13 (.13)	.46 (.12)
SILC	-.78 (.81)	-.17 (.84)	-.94 (.23)	.10 (.70)
QC	-1.83 (.01)	-.21 (.50)	.32 (..48)	-.04 (.84)
CC	.15 (.56)	-.14 (.42)	-.05 (.87)	.07 (.57)

T and Skinfolds- 6 mo intervals

	0-6		6-12	
Measure	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)
SSF	.04 (.09)	.01 (.66)	-.05 (.17)	-.03 (.25)
TSF	.03 (.26)	.023 (.57)	-.06 (.17)	.02 (.72)
ABDSF	-.01 (.58)	.02 (.47)	-.03 (.48)	.05 (.09)
SILSF	-.01 (.72)	.05 (.06)	.02 (.58)	.01 (.87)
QSF	.005 (.89)	.04 (.39)	-.01 (.84)	-.02 (.76)
CSF	-.006 (.88)	-.01 (.75)	-.02 (.77)	-.05 (.32)

E2 and Skinfolds- 6 mo intervals

	0-6		6-12	
Measure	*M* β(p)	*F* β(p)	*M* β(p)	*F* β(p)
SSF	1.21 (.003)	-.08 (.77)	.17 (.59)	-.003 (.98)
TSF	.20 (.69)	-.27 (.53)	.14 (.74)	-.04 (.84)
ABDSF	-.05 (.89)	-.38 (.22)	-.07 (.84)	.07 (.63)
SILSF	-.59 (.34)	1.10 (.02)	-.07 (.84)	-.35 (.10)
QSF	1.16 (.04)	.247 (.70)	.20 (.69)	-.03 (.88)
CSF	.59 (.30)	.21 (.68)	1.19 (.17)	.02 (.96)

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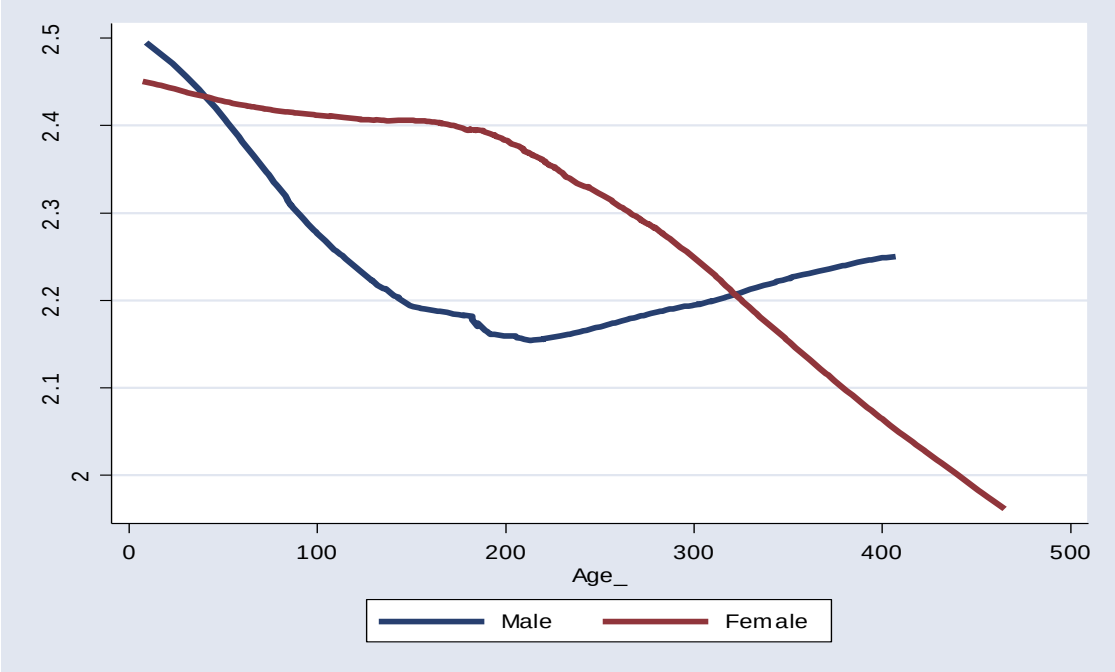
SUPPLEMENTAL FIGURES:

Graphs of Sex Differences in Ponderal Index, Circumferences and Skinfold Thickness

Ponderal Index



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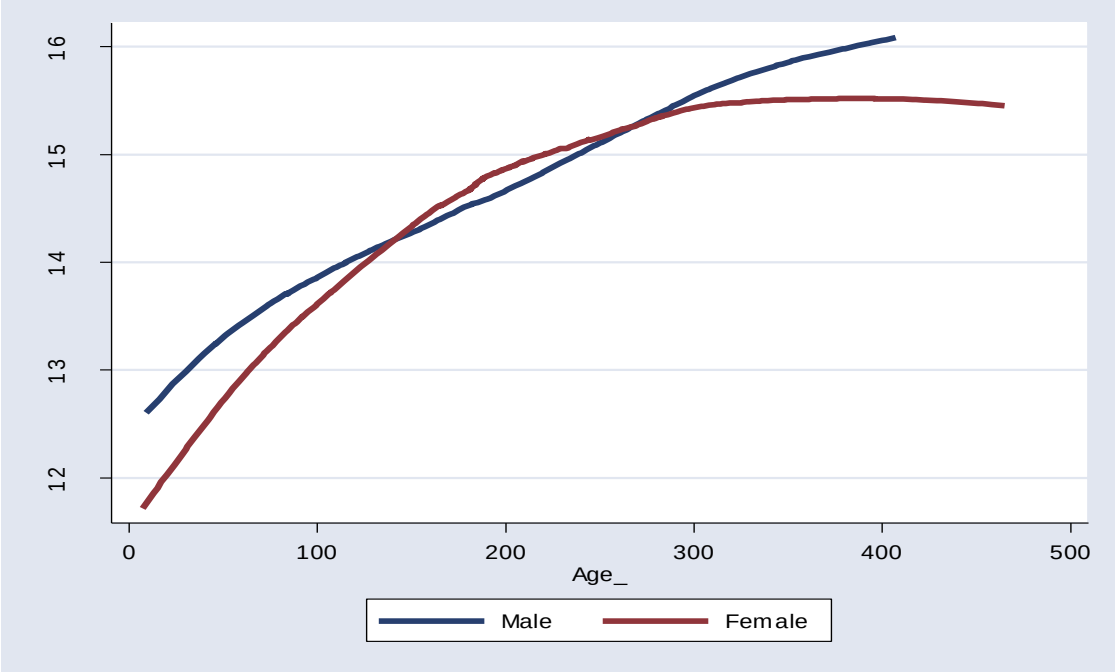

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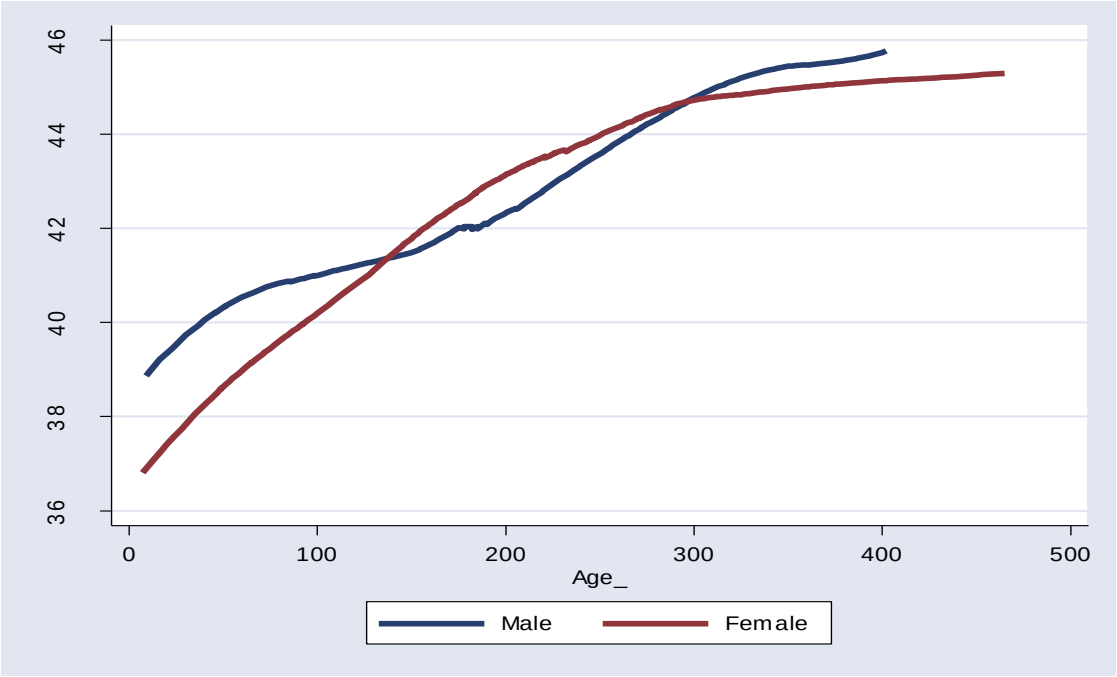
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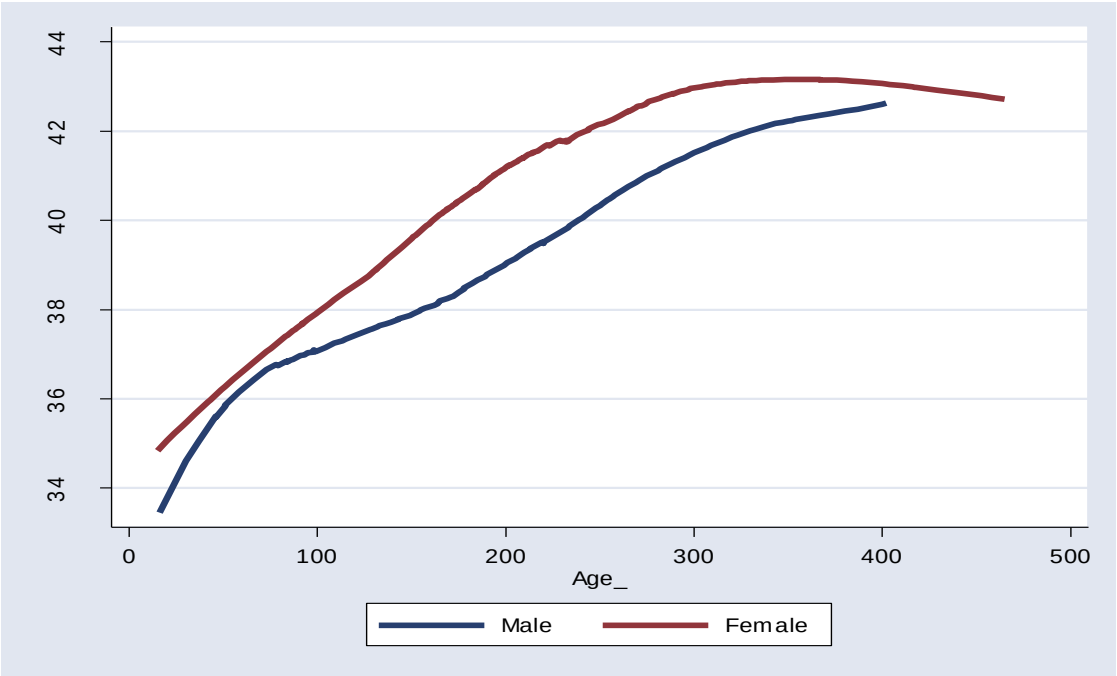
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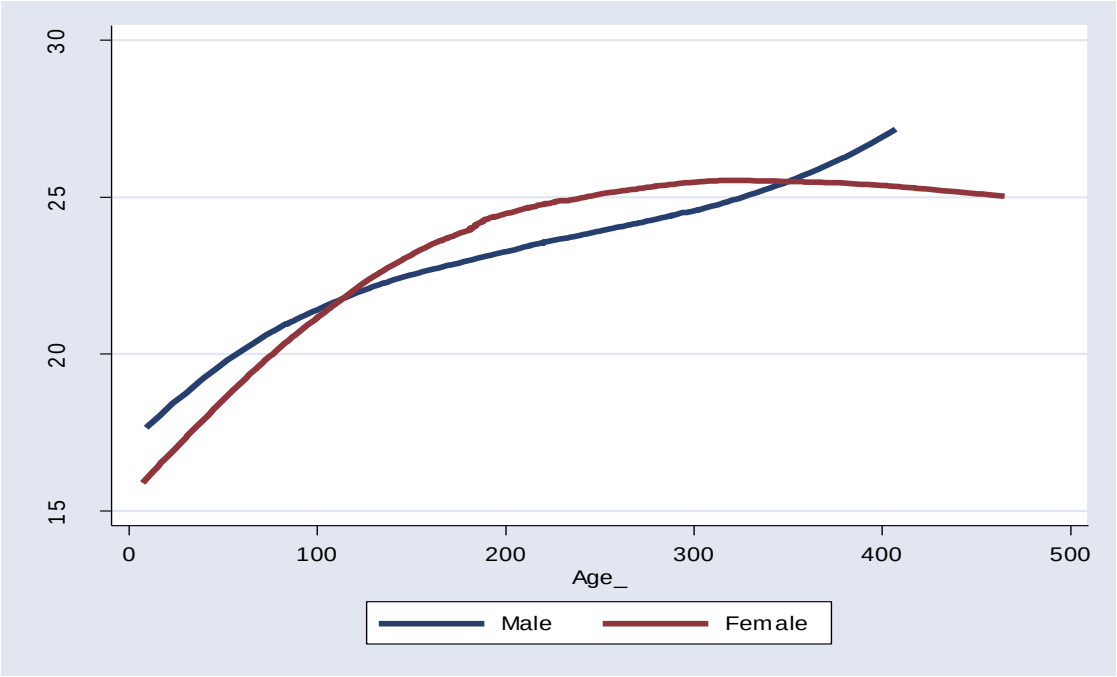
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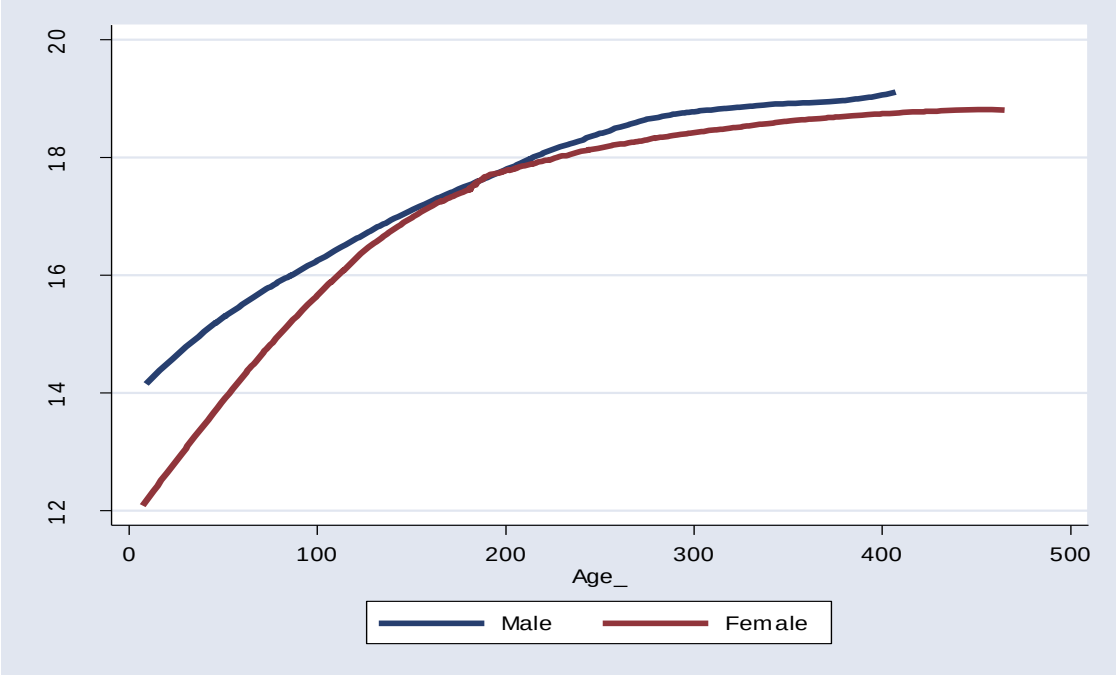
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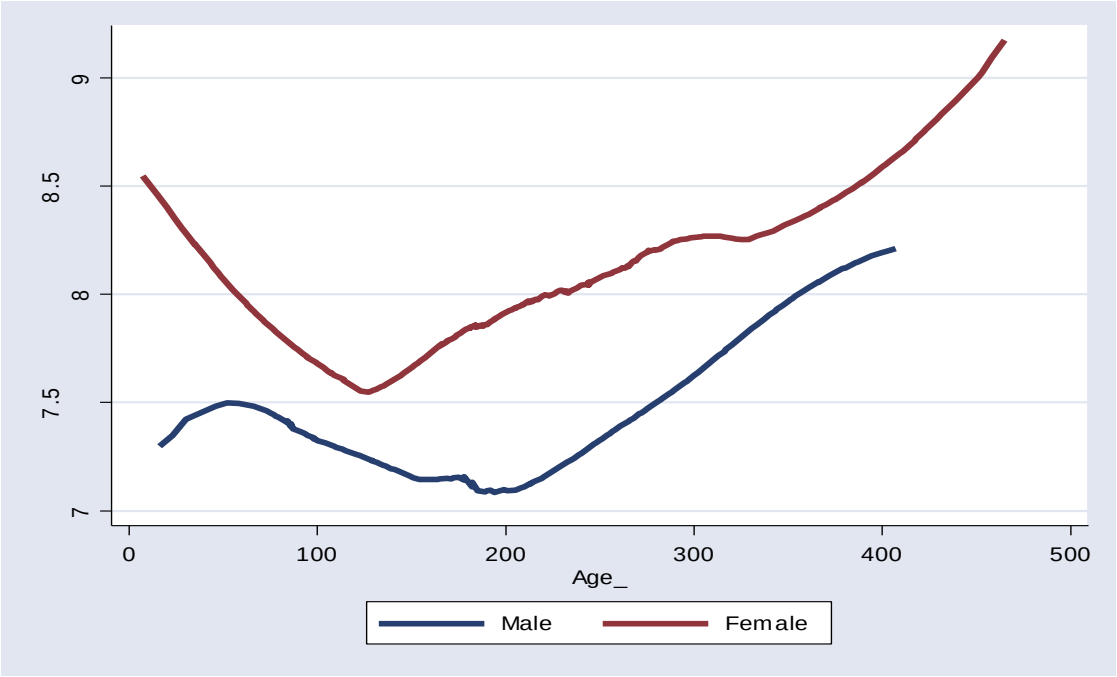
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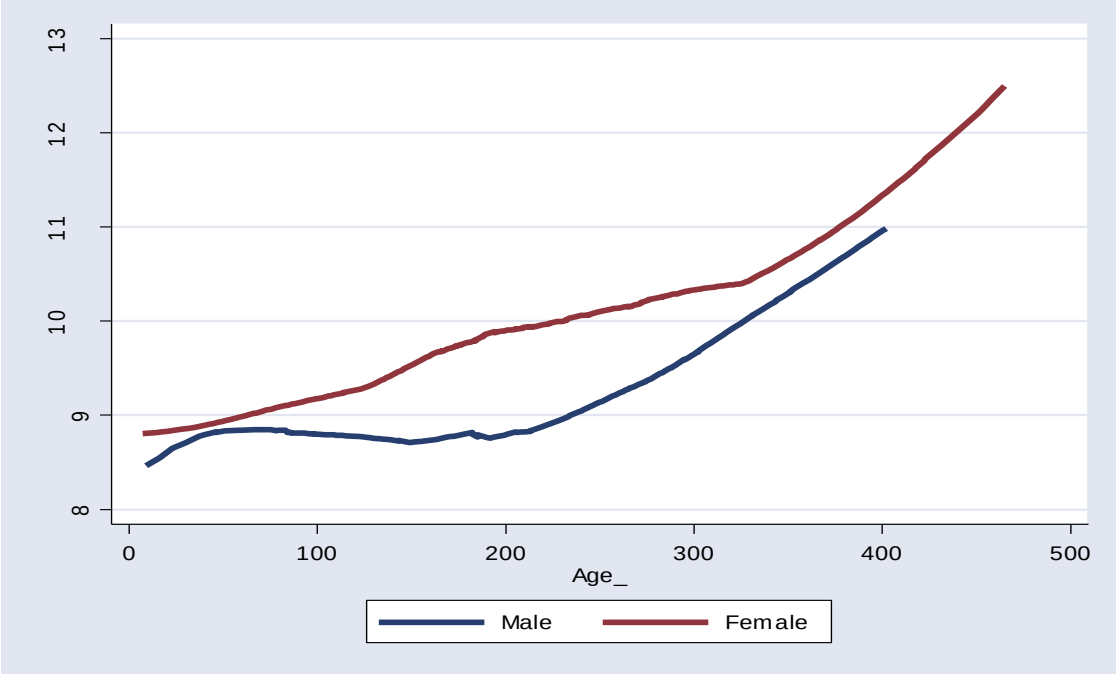
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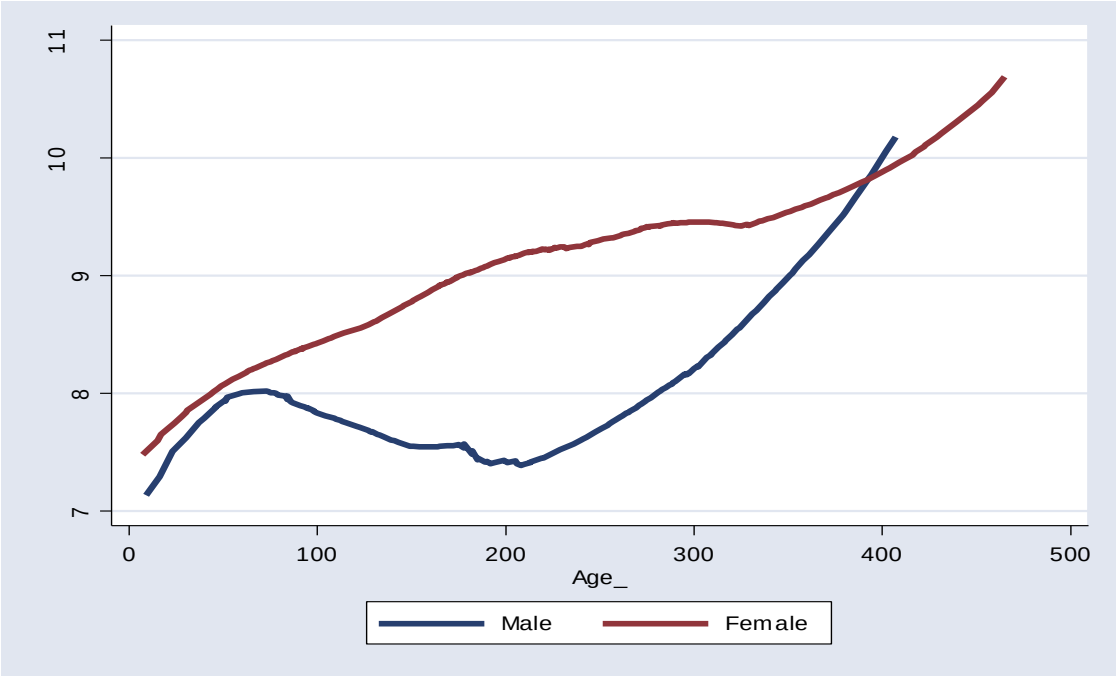
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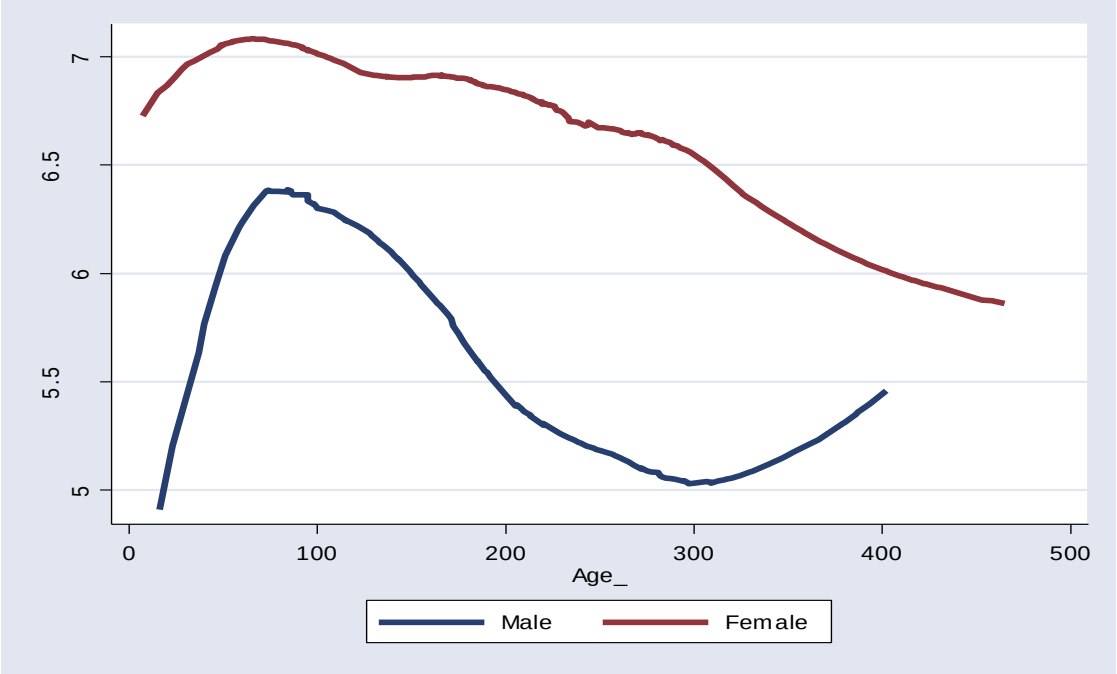
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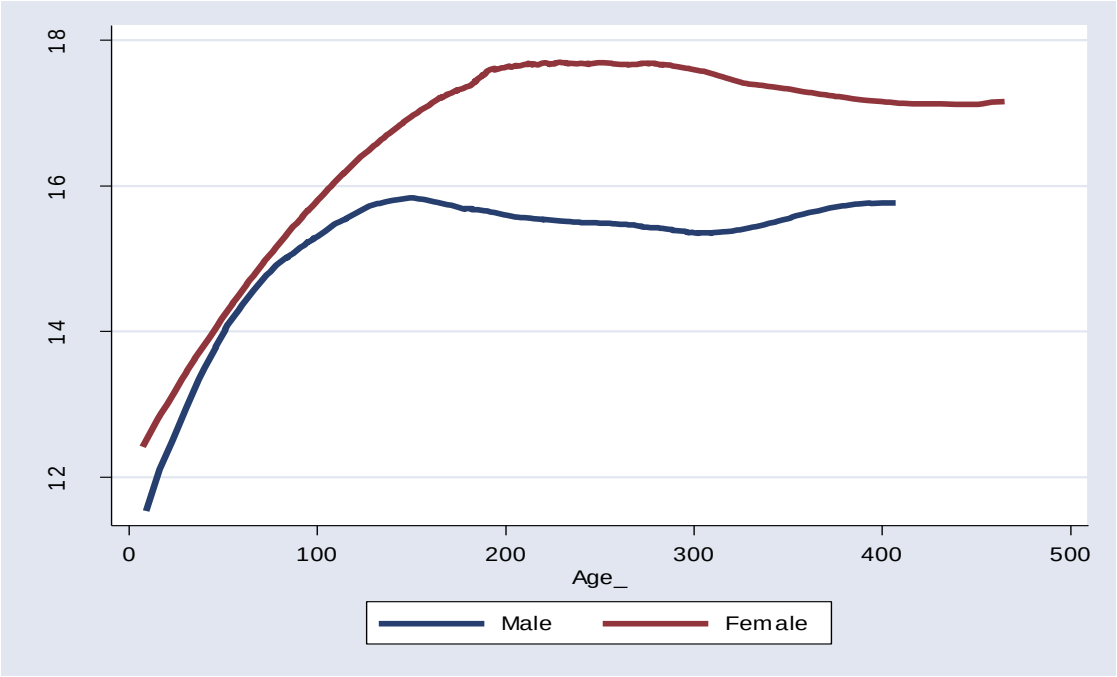
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Chapter 6: Feeding, hormones and body composition

Introduction

Within the field of anthropology, reproductive ecology examines reproductive effort from an evolutionary perspective with the underlying assumption that the allocation of reproductive effort has been shaped by natural selection (Ellison 2001). Since reproductive effort can be modified both physiologically and through behavioral and cultural adaptations, the study of reproductive ecology is inherently biocultural in nature. From this perspective, reproductive ecological models suggest that variations in the onset and maintenance of reproductive cycling are adaptive responses to environmental constraints and reflect "economizing' strategies to limitations in time and energy (Bentley 1999; Ellison 1994; Ellison et al 1993; Vitzhum 2001). In these models, childhood growth represents a "bioassay" of environmental conditions such that poor childhood conditions result in slowed rates of growth, lowered reproductive hormone set points, and delayed maturation (Ellison 1996). The idea that chronically-poor or chronically-enriched environmental conditions can result in population-wide variability in the timing of maturation and the tempo of growth, links reproductive ecology to a more evolutionary, developmental perspective as well.

This relationship between somatic growth and reproductive development has long been of interest to biological anthropologists, reproductive ecologists and human biologists, who have demonstrated that, on the individual and population levels, growth and maturation are related to developmental and environmental conditions, particularly energy intake and utilization. An example of the effects of the developmental environment on reproductive maturation is seen among girls in the Philippines, where

birth size was found to predict menarcheal age. In this population, girls who were long and light at birth matured earliest, particularly if they were fast growers in infancy (Adair 2001). Earlier ages at menarche have also been reported for British girls who were heavy at birth (dos Santos Silva et al 2002). Interestingly, growth rate during infancy and childhood appears to modify this relationship (Cooper et al 1996), suggesting that the postnatal environment is also important. The effects of the postnatal environment are also documented in Guatemalan girls, where those who were severely stunted at age three reached menarche later than those who were moderately or not stunted (Khan et al 1995). Since both somatic growth and reproductive function depend not only on the structural development of the HPG axis and somatic axis components, but also on the quantity and quality of communication between them, numerous opportunities exist for the local ecology to shape endocrine production and regulation (Lummaa 2003). Recently, size at birth has also been documented to affect adult responses to ecological conditions, with women born with lower ponderal indices, presumably as a response to energetic constraint *in utero*, having suppressed estradiol levels in response to moderate physical activity in adulthood (Jasienska et al 2006a; Jasienska et al 2006b). Together these results suggest that the energetic environments of early life may play an important role in shaping later reproductive physiology.

While the effects of local ecologies on the development of the hormonal axis during the period of infant development have not been described previously, reproductive ecological studies of the effects of diet, energetic expenditure, and body composition on HPG function in adults demonstrates that local ecologies can affect both reproductive development (reviewed in: Lipson 2001) and function. Differences in energetic intake

have been linked to differences in the production of sex steroids, particularly with higher levels of estradiol related to increased caloric consumption , but also of testosterone in extreme energetic conditions such as severe caloric restriction or starvation (Bribiescas 2001; Campbell and Leslie 1995; Ellison 2003). Having documented that sex steroid levels are linked to infant growth and body composition in sex specific way in the previous chapter, we explored whether the documented relationships between growth, energetic availability and the reproductive axis can also be evidenced in infancy. Infant energy intake, rather than output, is thought to be the major determinant of both energetic requirements and body size in infancy (Stunkard et al 1999). Differences in intake have been linked to the type of infant feeding with exclusively breastfed infants having lower intakes of energy, protein, fat and carbohydrates when assessed at 3 and 6 months compared to formula fed infants (Butte et al 2000). These lower levels of energy and protein have been associated with both the slower rates of growth and weight gain documented in breastfed infants (Dewey 1998) and are thought to contribute to the greater fat free mass seen in breastfed infants (Butte et al 2000). The use of formula as a supplement to, rather than as a substitute for, breastmilk also alters energy intake; infants receiving both substances have 20-27% higher caloric intake than exclusively breastfed infants at 4 months of age (Haisma et al 2003).

Although concern has been issued as to whether the lower protein content of human milk is adequate for linear growth (reviewed in Kramer et al 2002), studies of the energy intakes of breast- and formula fed infants indicate that, at least in the developed world, breastmilk intakes more than adequately fulfills energy requirements for growth (de Bruin et al 1998). Along with its nutritional content, breastmilk also provides infants with

a host of bioactive factors including growth factors and cytokines (Donnet-Hughes et al 2000; Garofalo and Goldman 1998; Grosvenor et al 1993; Hamosh 2001), which can directly affect linear growth in breastfed infants. Further, the lower protein levels of human milk may be protective against the development of overweight (Grummer-Strawn and Mei 2004) and insulin resistance (Singhal et al 2003). Due to these direct and indirect benefits of breastfeeding, exclusive breast-feeding (EBF), the intake of breast milk and no other liquids or solids except for small quantities of medicines, is now universally recommended for normal, healthy infants during the first 6 mo of life (WHO 2001). Given the rarity of exclusive breastfeeding worldwide (Lauer et al 2004), full breastfeeding (FBF), when breast milk is the predominant source of nourishment but is supplemented with limited quantities of liquids and juices, is often considered a more useful indicator for distinguishing relatively healthy and unhealthy feeding practices in early infancy. Recent studies suggest that exclusive and full breastfeeding for 6 months of life are associated with similar benefits in terms of reduced infant morbidity and mortality (Raisler et al 1999). At approximately 6 months of age, the introduction of complementary foods is recommended (WHO 2002) and much clinical and epidemiological research indicates that nutritional needs of growing infants may exceed maternal milk production at this time (reviewed in: Sellen 2007). However, the frequency of feeding and volume of breastmilk do not necessarily diminish after 6 months and breastmilk may remain an important source of nutrients and immune factors through the third year of life even in infants receiving complementary foods (Dettwyler 1992; Sellen 2007).

Given the public health and adaptive foci on infant feeding categories as important nutritive and developmental transitions (Sellen 2006), we focused on infant feeding style as a key marker of energetic intake. Specifically, we asked whether variations in sex steroids are linked to feeding characteristics in infancy. Secondarily, we explored whether other dimensions of the local ecology of infancy, such as maternal anthropometric characteristics, birth size and other sociobehavioral measures further contributed to variation in estradiol and testosterone levels.

Sample and Methods

Sample

Thirty-two infants (15 male, 17 female) participated in a prospective, longitudinal hormonal and growth research study. Subjects were recruited from an Emory University affiliated daycare center and opportunistically from university academic departments, Atlanta-area mothers' groups and lactation support groups. Infants entered the study between the ages of 7 days and 11 months and were followed for a median of 29 weeks (range: 4 days-15 months). All participating infants had a full term birth (birthweight >2500g and gestational age>37 weeks), were singletons and were born following uncomplicated pregnancies. While the infants had common respiratory and intestinal illnesses during the course of the study, there were no infants with known endocrine, metabolic or other significant pathologies.

The study site varied by sample sub-group. Sixteen of the infants were measured during home visits with their mother and/or caretaker present at the time of measurement. The remaining 16 infants were measured weekly at a daycare center. These study protocols

were approved by the Emory University Social/Humanist and Behavioral Institutional Review Board.

Anthropometry

Recumbent length, weight and head, body and limb circumferences, and trunk and limb skinfold thickness were measured following standard techniques (Cameron, 1986). Total recumbent length was measured to the nearest 0.1 cm by two observers using an infant measuring board equipped with fixed headboard and a mobile footboard (Precision Enterprises; Portage, MI). Length measurement protocol differed depending on the subsample of the study and the location of measurement. At the daily home and weekly day-care visits, length was measured using the maximal stretch technique by two trained observers (Lampl et al 2001). At the weekly home visits, mothers and/or caretakers acted as assistants in holding the infant's head during the measurement. Length was measured in duplicate in the majority (~80%) of measurements, unless the state of the infant precluded additional measurement.

During the home visits, unclothed infants' weight was measured using a portable, digital scale accurate to the nearest 10gm, calibrated before measurement (MedWeigh, MS-2410). At the daycare center site, infants were weighed clothed in a diaper. The average diaper weight for the infant was subtracted from the weight measurement in analysis. Five limb and trunk circumferences (mid-upper arm, abdominal, supra-iliac, mid-thigh and calf) were measured using a soft, non-stretchable fiberglass tape to the nearest 0.1 cm. Six trunk and limb skinfolds were measured with Holtain skinfold calipers (subscapular, supra-iliac, abdominal, triceps, quadruceps, and calf) to the nearest 0.1mm, according to standard techniques (Frisancho 1990; Roche and Sun 2003).

Fecal Sample Collection and Hormone Analysis

Parents were asked to retain all diapers from the evening prior to through the morning of the day of measurement in the case of the weekly samples and all daytime and evening diapers in the case of the daily sample. Soiled diapers were placed in zip-top bags, labeled with the date and time of collection and stored in portable coolers and chilled with freezer blocks frozen to -80^{o}C until collected. Upon collection, samples were stored at -80^{o}C until processed. This protocol yielded 487 useable, weekly fecal samples. Fecal samples were excised from the diapers, extracted using methanol and filtered through microfilter centrifuge tubes (Centrex, Whatman Laboratory; for a detailed description of this method, see chapter 2).

Extracted samples were then assayed using validated modifications (see chapters 2 and 3) of commercially-available RIA kits for estradiol and testosterone (DSL labs, Weber, TX). All samples were assayed in duplicate and the mean value of these duplicate samples was used for analysis. Samples were standardized for weight and values are expressed as pg/gm. These modified microassays were able to detect steroid concentrations of 0.35pg/ml and intra- and inter-assay coefficients of variation (CVs) were within the accepted range for RIA assays (<10% and <15%, respectively). Steroid recovery was high and samples were diluted to reduce potential competitive binding from other estradiol or testosterone metabolites.

Infant Feeding

The mothers of each infant (n=32) filled out pre-printed forms detailing her infants diet, sleep, and activity in the 24 hours preceding the growth measurement. Parents were instructed on how to fill-out these pre-printed forms upon enrollment in the study and

were provided with sample forms to guide their entries. The types and quantities of foods and liquids consumed were recorded on these forms to assess dietary intake in the 24 hours preceding measurement. Breastfeeding duration was recorded to the nearest 5 minutes. These forms were then coded for feeding type by the investigator (A.L.T.) using WHO feeding (2001) criteria to determine breast-feeding status (described in greater detail below).

Quantitative feeding questionnaires were provided at study entry and after the introduction of solid foods. This questionnaire was used to collect information about the timing of and rationale behind the introduction of complementary foods and liquids, a food frequency questionnaire, and an assessment of parental feeding beliefs and behaviors, adapted from the Infant Feeding Questionnaire (Baughcum et al 2001). These questions used a 5 point Likert-style response scale and assessed parental concern about their infant's weight, concern over infant eating, control over eating, nonnutritive uses of food, establishment of a feeding schedule and awareness of infant hunger and satiety cues. Additional qualitative data was derived from observational and open-ended interviews with parents and care-takers about infant feeding.

Infant behavior

Infant behavior, including sleep, activity, temperament and illness, were assessed with weekly activity records and behavior scales (see appendix). Parents recorded their infant's sleeping, awake and active, awake and quiet and crying/fussy behavior to the nearest 30 minutes, on a pre-printed, grid labeled by hour. Infant temperament in the preceding weekly interval was assessed on a 5-point Likert-type scale. With these scales, parents were asked to assess their infant's activity level, hunger, contentedness,

sleepiness, fussiness and crying in comparison to their usual behavior, with a range of much less to much more. Parents were then asked to record any illness symptoms their infants exhibited. These symptoms were provided in a pre-printed list with a space for parents to record any other symptoms. Parents were also asked whether they had visited a doctor for these symptoms, whether a diagnosis had been made and if any medications had been prescribed. Additionally, parents were asked if their infants had shown any developmental milestones in the past week and were asked to note any other changes in sleeping, eating, diapers, behaviors and health in the past week. These responses were coded by the investigator (ALT).

Maternal and family characteristics

At study entry, parents completed a questionnaire detailing maternal and paternal background and pregnancy and delivery information. These self-report data included: maternal and paternal age, maternal and paternal height and weight, maternal and paternal education, family income, parity, smoking, current medical conditions and/or medications, pregnancy weight gain and breastfeeding history. This questionnaire also assessed infant characteristics such as birth weight, birth length, gestational age, and ethnicity.

Data Analysis:

SAS version 9.0 (SAS Institute, North Carolina) and STATA/SE version 8 (Stata Corporation, College Station) statistical software were used for all statistical analyses. Steroid fecal values were corrected for fecal weight and are represented in pg/gm. The steroid hormone values were not normally distributed due to a right skew of the values. However, given the large sample size of hormonal values (n=487), results did not differ

whether the values or the log-transformation of the variables were included in analyses. Consequently, the untransformed variables were used for ease of interpretation. Descriptive statistics of hormonal measures are given as median values and reference ranges (minimum to maximum). If a measured hormone was below the detection limit, the value was expressed as 0 pg/gm for subsequent analyses.

Sex differences in growth and feeding measures were assessed using Mann-Whitney two-sample rank sum non-parametric tests and Kruskal-Wallis rank sum tests for measures with more than 2 groups. Repeated-measures mixed models were used to assess significant effects of feeding and other energetic measures on testosterone and estradiol levels. To test for sex differences in the associations, models were stratified by sex to control for confounding by sex and age was included as a covariate. Subject was included as a random effect in all repeated measures models to control for subject-specific effects such as measurement dependability and to account for the within-subject correlation. Statistical significance was defined as $p < 0.05$. Statistical significance of $p < 0.10$ was taken as evidence of a trend in association.

Derived Variables

Ponderal Index: Infant weight and length data were used to calculate ponderal index [PI= (weight in g/length in cm^3) *100].

Maternal BMI: Maternal body mass index (BMI) was calculated based on the self-reported height and weight information and was calculated as weight in kilograms divided by height in centimeters squared [wt(kg)/length $(m)^2$]. Maternal BMI was categorized according to WHO criteria and mothers with a BMI greater than 25 kg/m^2

were categorized as overweight and those with a BMI of greater than 30 kg/m^2 were categorized as obese.

Infant feeding: The type of infant feeding was classified on a weekly-basis using the 24hr food recalls. Feeding type was coded according to the WHO classification schema (WHO 2001), such that infants were classified as exclusively breastfed (EBF) if they only received breastmilk and small amounts of medicine or vitamins. Infants were classified as complimentary fed (CF) if they received supplementary solids and liquids (including non-human milk) in addition to breastmilk. This definition requires that infants receive solid foods. Infants who received formula in addition to breastmilk, but did not receive solid foods, were classified as mixed fed (MF).

For infants fed formula, infants fed only formula and no complimentary foods or liquids were defined as exclusively formula-fed (EFF). Infants receiving formula and other foods and liquids (except breastmilk) were classified as complimentary formula fed (CFF).

A series of dichotomous feeding classifications were created to test the specific effects of breastmilk versus formula versus solid foods on hormonal levels. The derived variables included:

AnyBM: This variable measures whether infants receive some breastmilk, either singularly or in combination with any other complementary food or liquid.

Formula: Like the *anybm* variable, this variable measures whether infants receive some formula, either singularly or with any other complementary food or liquid.

Solids: This variable measures whether infants are given solid foods, including cereals, pureed baby foods, and all other semi-solid and solid foods.

EBFat4mo/EBFat6mo: Infants were retrospectively and/or prospectively classified as EBF at 4 or 6 months based on information provided on the feeding questionnaire (if entering the study after those ages) or based on weekly records (if studied at those ages). *Early solids* was defined dichotomously to assess whether infants received solids prior to the 6 months recommended by the American Association of Pediatrics (Pediatrics 1997) and World Health Organization (WHO 2001).

Results

Description of the sample

This sample of mothers and infants is generally healthy with normal ranges of infant birth weights and maternal BMI's. Slightly less than half the sample was male and more than half the sample attended daycare, either at the day care center study site or in another daycare setting. The mothers tend to be highly educated with the majority having both undergraduate and graduate degrees and family income is relatively high.

Table 6.1: Sample Characteristics by Infant Feeding

	EBF<4 months	**EBF >4 months**
Maternal Characteristics	*n=15†* Mean (SD)	*n=16* Mean (SD)
Age	33.1 (4.5)	33.9 (3.4)
Primiparity %	87.0%	62.5%
Education (≥ college)	79.0%	87.0%
Income (≥ $75,000)	53.0%	75.0%
Pre-pregnancy Weight *(kg)*	63.3 (8.1)	63.0 (6.1)
Height *(cm)*	163.8 (6.4)	167.7 (7.0)
Pre-pregnancy BMI	23.5 (2.1)	22.5 (2.5)
Pregnancy weight gain *(kg)*	14.4 (4.8)	16.8 (6.2)
Infants		
Male sex	33.0%*	63.0%
Gestational Age *(weeks)*	39.8 (1.4)	39.5 (1.5)

Birth weight *(kg)*	3.22 (0.4)**	3.62(.35)
Birth length *(cm)*	53.2 (2.3)**	53.3 (2.2)
BMI	12.7 (1.3)	12.8 (1.5)
% Daycare	40%	68%

† One mother did not fill out the background questionnaire
*p<.10 **p<.01, Mann-Whitney two-sample rank test

Approximately half the sample was exclusively breastfed to 4 months. The infants not exclusively breastfed to four months differed in several characteristics from those exclusively breastfed longer than 4 months. Those who were not breastfed to four months included a larger number of female infants and, for this reason, had lower mean birth weights and lengths than those infants breastfed for at least 4 months. There were no significant differences in the background or anthropometric characteristics of mothers who exclusively breastfed to 4 months versus those that did not. No mother in either group reported a history of gestational diabetes or smoking during pregnancy. No mothers smoked during the study period.

Feeding Characteristics

There was a universal initiation of breastfeeding in this sample. Rates of exclusive breastfeeding were lower, with 41% of infants exclusively breastfed by one month of age and 27% exclusively breastfed to 6 months. Breastfeeding was not completed in all infants by the end of the study; infant age at the end of the study was used in place of age at weaning for infants who had not been weaned at study end. Using this truncation, the minimum median duration of breastfeeding was 7.5 months with a range of 2 weeks to 15 months.

60% of infants received formula either solely or as a supplement to breastmilk. The median age at the introduction of formula was 5 months and ranged from right after birth to 11 months. Similarly, the median age at introduction of solid foods was 5.25 months with a range of 4 months to 6.5 months. 62% of infants received solids before 6 months of age.

Feeding and Sex Steroid Levels

Estradiol

As illustrated in **Figure 6.1a** and **b** and **Table 6.2**, differences in feeding style were associated with differences in estradiol levels. Infants who received breastmilk exclusively had lower estradiol levels than both infants who received only formula (Mann-Whitney rank sum, $Z=-1.74$, $p=0.08$) and infants who received breastmilk and formula (Mann-Whitney $Z=-3.23$, $p=0.001$). Among infants who received breastmilk, infants who were fed breastmilk and solids had lower estradiol levels than those who received breastmilk, formula and solids (Mann-Whitney $Z=-3.37$, $p<0.001$). Interestingly, infants who received formula and solids had the lowest estradiol levels of any of the groups (median 0.16). These levels were significantly lower than infants receiving breastmilk and solids (Mann-Whitney $Z=2.71$, $p=0.007$) and infants receiving formula exclusively (Mann-Whitney $Z=2.69$, $p=0.007$).

Figure 6.1a: Estradiol Levels and Feeding Categories (No Solids)

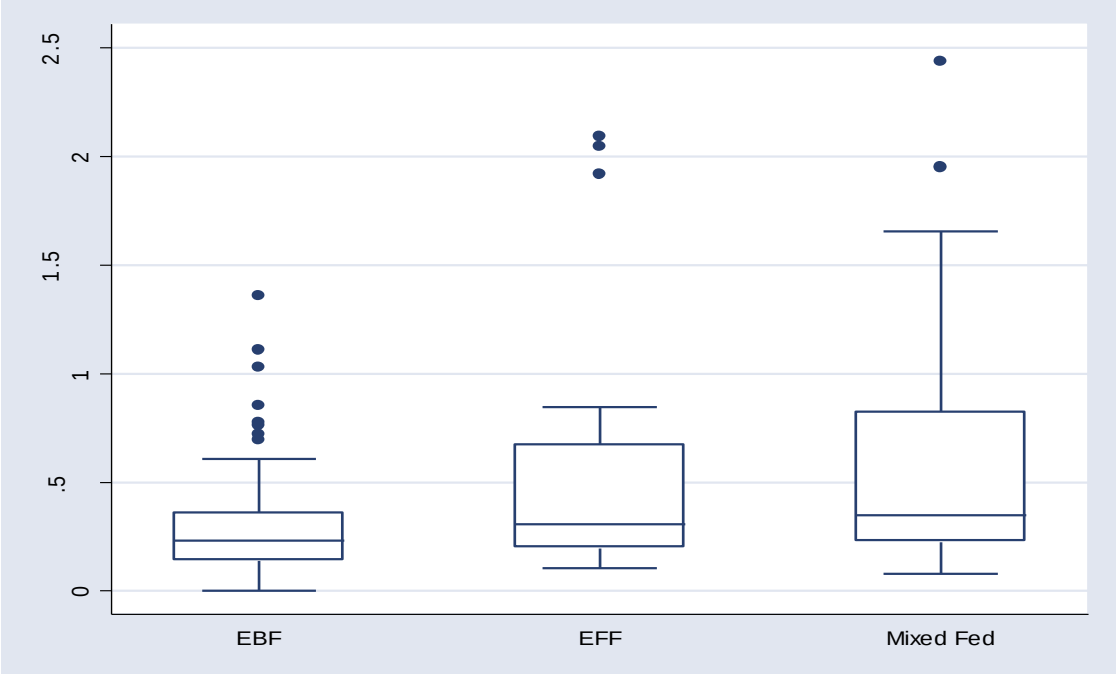
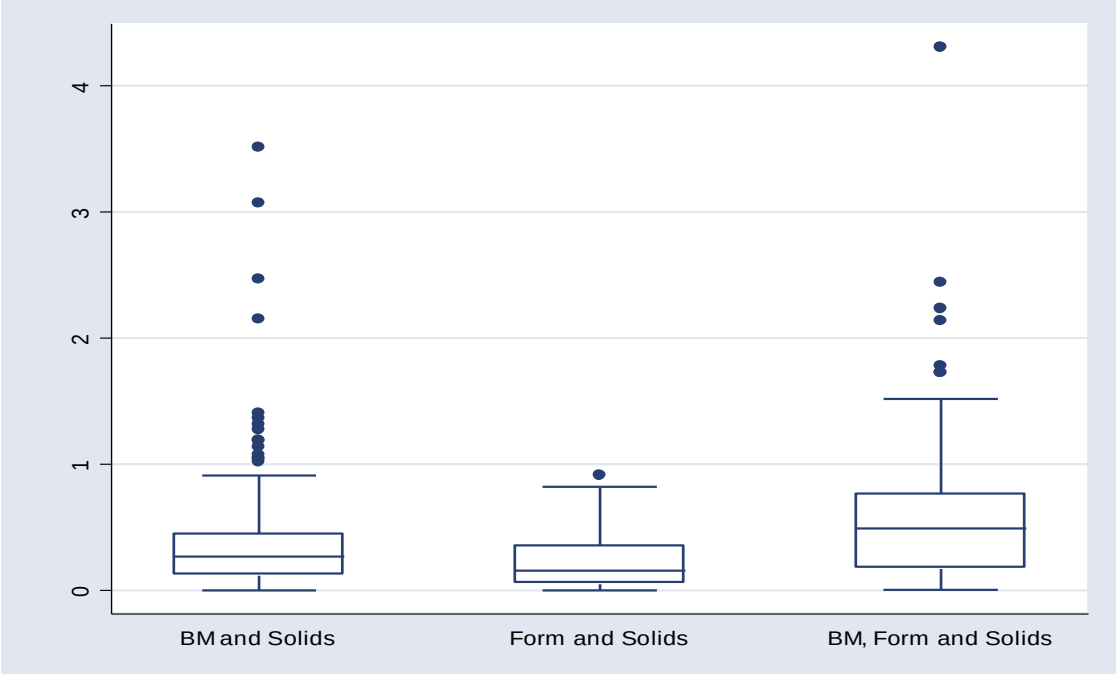


Figure 6.1b: Estradiol Levels and Feeding Categories (Solids)



These trends towards higher estradiol levels in infants receiving formula and/or solids (shown in **Table 6.2**) may be partly explained by age, since children receiving breastmilk exclusively tend to transition into a diet composed of breastmilk and solids while infants receiving breastmilk and formula tend to transition to a diet composed of breastmilk, formula and solids. To test for the potentially confounding effects of age, mixed model regression analysis was used to test whether sex steroid levels were directly associated with feeding style between infants and within individual infants as feeding type transitioned over the course of the study, controlling for age and sex. The results of these models indicated that infants who received formula exclusively or as a supplement have higher estradiol levels than EBF infants (xtreg, β= 0.21 p=0.002). Further, among infants receiving breastmilk, the addition of formula and/or solids is associated with higher estradiol levels (xtreg, β= 0.17 p=0.04).

Table 6.2: Median Estradiol Levels by Feeding Type

	Feeding Type	**Estradiol (pg/gm)** ***median(range)***	**Age (mo)** ***median(range)***	**Significance**
No Solids	EBF	.23(0-1.4)	3.8 (0.5-7.2)	X^2=12.17 p=.002*
	EFF	.31 (0.1-2.1)	6.4 (.2-11.9)	
	BM and Form	.35 (0.1-2.4)	2.5 (0.7-3.9)	
Solids	BM and Solids	.27 (0-3.1)	8.5 (4.9-13.9)	X^2=25.61 p<.001*
	Form and Solids	.16 (0-0.9)	8.9 (4.3-14.2)	
	BM, Form and Solids	.50 (.01-4.3)	9.4 (4.1-14.9)	

*K-Wallis for trend in estradiol values by feeding style

Testosterone

The results for testosterone are shown in **Figures 6.2 a** and **b** and **Table 6.3**. As with estradiol, differences in feeding style were associated with differences in testosterone levels. Infants who received breastmilk exclusively had lower testosterone levels than those infants who received both formula and breastmilk (Mann-Whitney rank sum, Z=-3.30, p=0.001). However, unlike the estradiol results, there was no significant difference in testosterone between infants who received breastmilk exclusively or formula exclusively (Mann-Whitney rank sum, Z=0.92, p=0.36). Further unlike the estradiol results where the addition of solids was associated with higher hormonal values, the addition of solids to the diet was associated with a reduction in testosterone values.

Figure 6.2a: Testosterone Levels and Feeding Categories (No Solids)

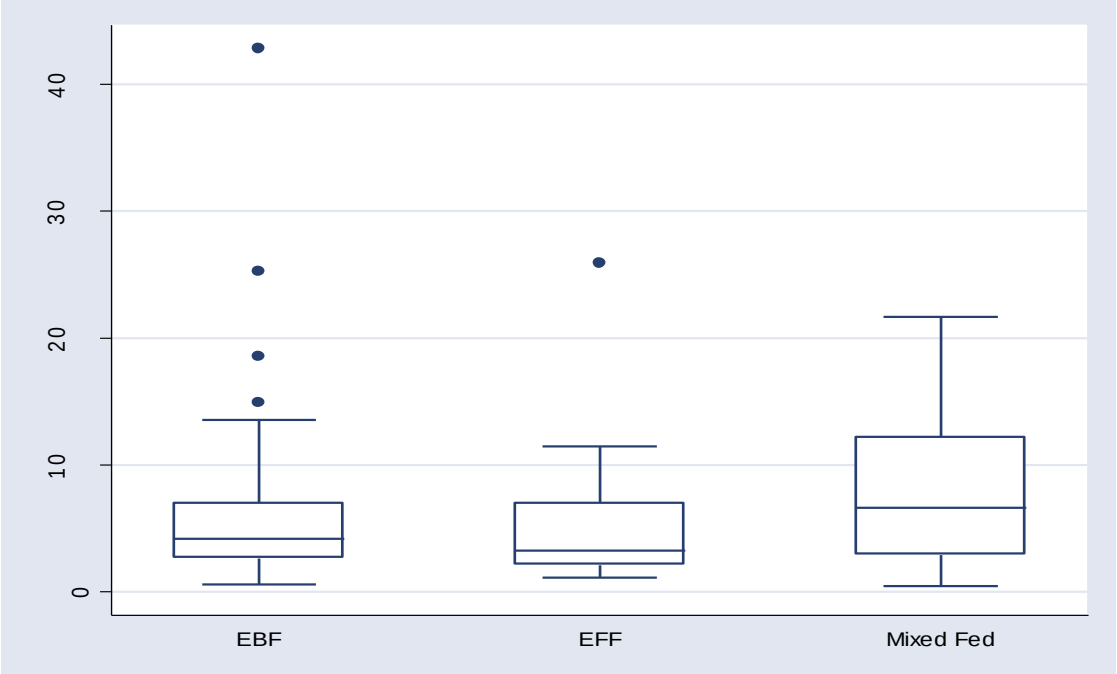
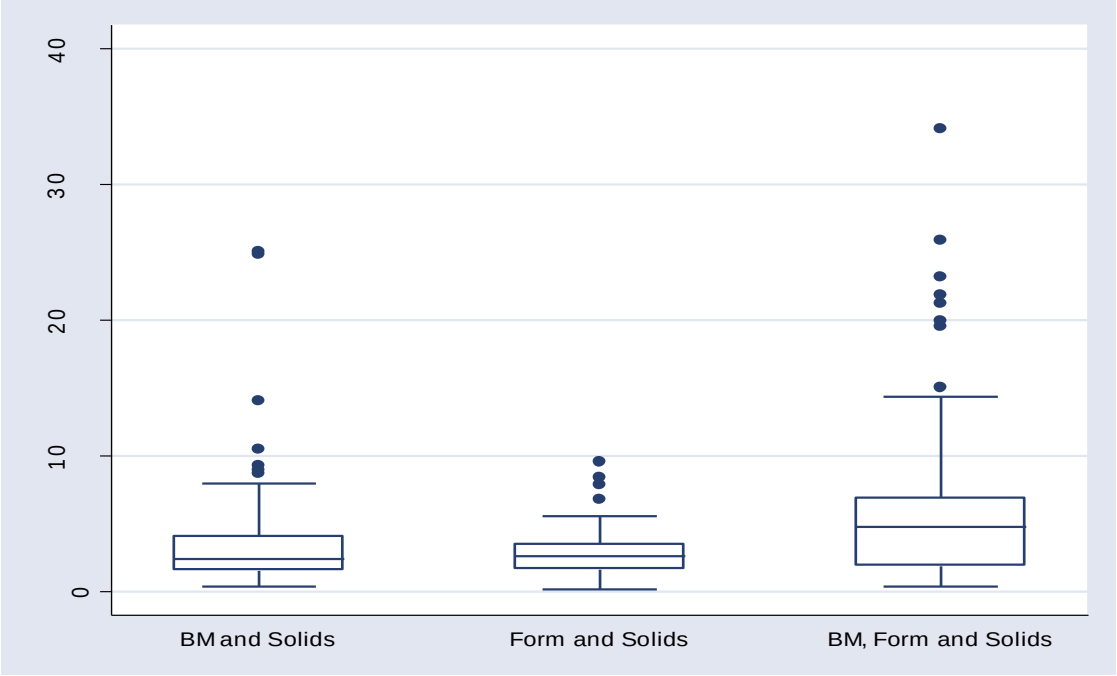


Figure 6.2b: Testosterone Levels and Feeding Categories (Solids)



Testosterone values did not differ significant between infants receiving breastmilk and solids and those receiving formula and solids (Mann-Whitney rank sum, Z=0.12, p=0.90). Higher testosterone levels were seen in infants receiving breastmilk, formula and solids than either of the other two solid groups (compared to BM and solids: Mann-Whitney rank sum, Z=-3.37, p=0.001; compared to formula and solids: Mann-Whitney rank sum, Z=-3.01, p=0.003).

Because testosterone declines with age and feeding styles change with age, the effect of age on these associations between testosterone and feeding type were also tested using mixed model analysis controlling for age and sex. The results of these models indicated that infants who received breastmilk exclusively or breastmilk and formula had higher testosterone levels than EFF infants (xtreg, β= 2.08 p=0.04).

Table 6.3: Median Testosterone Levels by Feeding Type

	Feeding Type	Testosterone (pg/gm) *median(range)*	Age (mo) *median(range)*	Significance
No Solids	EBF	4.19(1.2-25.2)	3.8 (0.5-7.2)	X^2=15.52 p<.001*
	EFF	3.27 (1.1-25.9)	6.4 (.2-11.9)	
	BM and Form	6.63 (.46-21.7)	2.5 (0.7-3.9)	
Solids	BM and Solids	2.44 (.42-24.8)	8.5 (4.9-13.9)	X^2=6.17 p=.046*
	Form and Solids	2.62 (.19-9.6)	8.9 (4.3-14.2)	
	BM, Form and Solids	4.78 (.41-34.1)	9.4 (4.1-14.9)	

*K-Wallis for trend in testosterone values by feeding style

The consumption of solid foods was not associated with lower testosterone levels among infants receiving breastmilk and/or formula when age and sex where controlled for in the model (xtreg, β= -0.24, p=0.74).

Feeding, hormones and sex differences

Mixed-models stratified by sex were employed to test for sex specific associations between feeding style and hormone levels, controlling for age. Over all age ranges, EBF was associated with lower estradiol levels in both male and female infants. There were no significant effects of exclusive breastfeeding (EBF) on testosterone levels in male or female infants or for exclusive formula feeding (EFF) on estradiol or testosterone in either sex.

Table 6.4: Sex-specific associations between hormone levels and feeding style

	Feeding Type	Estradiol (pg/gm) β(p)		Testosterone (pg/gm) β(p)	
		Male	Female	Male	Female
No Solids	EBF	-.22(.003)	-.20(04)	-.83(.52)	-.37 (.61)
	EFF	.20 (.13)	.22(.24)	.94 (.61)	.15 (.91)
	Mixed BF	.09(.23)	.32 (<.001)	.33 (.82)	1.71 (.009)
Solids	Comp BF	.57 (<.001)	.01 (.96)	.82 (.61)	-.55 (.48)
	Comp FF	-.06 (.65)	.17 (.25)	.53 (.66)	-.01 (.99)

Xtreg model stratified by sex and controlling for age; results are for the effect of feeding style on hormonal outcome variable

Mixed breastfeeding, the use of both breastmilk and formula, was associated with higher estradiol and testosterone levels in female, but not male, infants. Finally, complementary feeding, the use of solids either with breastmilk alone or with breastmilk and formula, was associated with higher estradiol levels in male infants. There were no other significant associations between feeding and hormonal levels over the whole study period.

Table 6.5: Association between formula and solid food use and hormonal levels before 6 months of age

	Estradiol β(p)	Testosterone β(p)
Mixed BF		
Males	-.10 (.13)	-1.69 (.56)
Females	.32 (<.001)*	2.87 (<.001)*
Comp Feeding		
Males	.36 (.002)*	3.85 (.14)
Females	.35 (<.001)*	4.79 (<.001)*

Xtreg model stratified by sex and controlling for age; results are for the effect of feeding on hormonal outcome variable before 6 months of age in infants who receive breastmilk

By contrast, when the type of complementary liquid or food was examined by sex before six months, the time when these items were added to the diet, the inclusion of formula in the diet of breastfed girls was associated with both higher estradiol and testosterone compared to girls who received only breastmilk. There was no association between formula use and hormonal level in boys before 6 months of age. Complementary feeding, the addition of solids to a diet or breastmilk or breastmilk and formula, before 6 months was associated with higher estradiol in boys and higher estradiol and testosterone in girls when compared to infants receiving only breastmilk.

Confounding effects of infant size and feeding

Since hormonal levels were shown to be associated with size differences in this sample (Chapter 5) and infant size has been shown to be related to feeding style among infants in the United States (Butte et al 2000; Dewey 1998), we next explored whether feeding style was associated with size differences in this sample since such a relationship may confound the results for feeding style and hormonal levels documented above.

Table 6.6: Relationship between EBF and Infant Size

Age	Length (cm) β(p)	Weight (kg) β(p)	PI (kg/cm^3) β(p)
< 4 months	.99 (.15)	.75 (.004)	.09(.44)
< 6 months	1.04 (.001)	.40 (<.001)	.03 (.50)

Xtreg model controlling for age and sex; results are for the effect of EBF on size

The association between infant size and feeding practices in this sample were assessed separately for three age categories, before 4 months, before 6 months and during the entire study period. The four and six month periods were chosen since preliminary

analyses of this data indicated that parental feeding decisions were influenced by infant size at these ages. The present data document that infants who were exclusively breastfed were 0.75 kg heavier before 4 months and 0.40 kg heavier before 6 months than infants who were not EBF, in mixed model analysis controlling for age and sex. EBF infants were also longer before 6 months (1.04 cm) than those who were not.

To test the potential interaction between feeding and size on hormonal levels, mixed model analysis was used to assess the effects of estradiol or testosterone level and feeding style and the interaction between them on length, weight and skinfold thickness measures. Complementary feeding (CF) and estradiol level interacted significantly in predicting body size and composition, controlling for age, such that in CF girls, estradiol levels positively predicted length (β=1.97, p=0.02) and thigh skinfold (β=2.15, p=0.01). In CF boys, estradiol levels positively predicted length (β=3.94, p<.0001), weight (β=0.32, p=0.045), and thigh skinfold (β=3.15, p=0.006) and negatively predicted PI (β=-0.19 p=.01). Similar analysis with testosterone indicated that among CF girls, testosterone negatively predicted weight (β=-0.07, p=0.02) and PI (β=-0.02, p=0.002). There were no significant effects on body composition measures for either sex.

Other energetic markers

Table 6.7: Other energetic markers and hormone levels

Characteristic	Estradiol (pg/gm) β(p)	Testosterone (pg/gm) β(p)
Maternal age	-.004(.61)	.003 (.98)
Maternal height	-.01(.05)	-.03 (.62)
Maternal BMI	.01(.58)	-.01 (.94)
Preg weight gain	-.002(.74)	.03 (.65)
Primiparity	.15(.05)	1.01 (.42)
Maternal education	-.004 (.89)	-.34 (.37)
Family income (SES)	-.13 (.004)	-2.01(.006)
Paternal height	-.01 (.28)	-.03 (.75)
Paternal BMI	-	.12 (.33)
Infant ethnicity	-.03 (.22)	-.15 (.64)
Gestational age	.02 (.53)	.28 (.38)
Birthweight	-.11 (.16)	.23 (.82)
Birth length	-.04 (.004)	-.21 (.23)
BMI at birth	.01 (.62)	.41 (.20)
Daycare	-.12 (.05)	-1.68 (.04)
Illness	-.01 (.84)	.06 (.88)
Active	.01 (.83)	-.20 (.45)
Hungry	-.02 (.50)	.24 (.26)
Content	-.02 (.58)	.22 (.37)
Sleepy	-.04 (.37)	.32 (.56)
Fussy	.02 (.57)	-.09 (.67)
Cried	-.0002 (.99)	-.001 (.99)

Xtreg models controlling for age and sex; results are effect of characteristic on hormonal outcome

The relationship between sex steroids and other energetic markers were tested in a series of mixed models, controlling for age and sex, shown in **Table 6.7** above. These results indicated that estradiol levels were positively associated with maternal primiparity and negatively associated with daycare attendance, birth length and family income. Testosterone levels were negatively associated with daycare attendance and family income. There were no significant associations between estradiol or testosterone levels and other energetic markers such as maternal or paternal size, infant health, or behavioral measures.

Discussion

Table 6.8: Summary of Findings

	Estradiol	Testosterone
Feeding Style and Hormones	- -EBF infants had the lowest E_2 levels - Infants receiving breastmilk, formula and solids had highest E_2 - EFF and Mixed BF infants have ↑E_2 - Addition of solids ↔ ↑E_2 levels in breastfed infants	- EFF infants had lowest T levels - EBF infants had ↓T than Mixed BF infants - No significant difference in EBF and EFF infants - No effect of solids
Feeding Style and Hormones in Girls	- EBF ↔ ↓E_2 - No effect of EFF - Mixed BF ↔ ↑ E_2 - No effect of CF - Formula < 6 months ↔ ↑ E_2 - CF < 6 months ↔ ↑ E_2	- No effect of EFF - Mixed BF ↔ ↑T - Formula< 6 months ↔ ↑T - CF < 6 months ↔ ↑T
Feeding Style and Hormones in Boys	- EBF ↔ ↓ E_2 - No effect of EFF - No effect of Mixed BF - CF ↔ ↑ E_2 (< 6 months and at all ages)	- No effect of EFF, Mixed BF or CF
Interaction between Size, Feeding and Hormones	- Among CF girls, ↑E_2 ↔ ↑ length and ↑ qsf - Among CF boys, ↑E_2↔↑length, ↑weight, ↑qsf and ↓PI	- Among CF girls, ↑T↔↓weight and ↓PI
Hormones and Other Energetic Markers	- ↑E2↔ primiparity - ↓E2↔ daycare, birth length and family SES	- ↓ T↔ daycare and family SES

The results of this analysis document a relationship between sex steroid levels in infants and measures of feeding style that differ by infant sex and are confounded by size differences between breastfed and non-breastfed infants. Further, sex steroid levels were found to be associated with birth order, daycare attendance and family income, suggesting that the local ecology of infancy may also be important in shaping hormone levels. As with the growth results in the last chapter, these results suggest that the association between energetic markers and sex hormones seen in adults are present already in infancy.

Feeding Style

In this sample of infants, there is a universal initiation of breastfeeding, much higher than the 73% national average estimated from the 2002 CDC National Immunization Study (Li et al 2005). The rates of exclusive breastfeeding, while much lower in this sample, are nonetheless 3 times higher than the rate seen in the national study, with 27% exclusively breastfed to 6 months (Li et al 2005). Formula is used by 60% of the parents, initially as a supplement to breastfeeding and later as a replacement. Solids are introduced at a median age of 5.25 months and over 60% of infants received solids before the 6 months of age recommended by the Nutrition Section of the American Academy of Pediatrics (Pediatrics 1997) and the World Health Organization (WHO 2001).

There were no significant differences in the background or anthropometric characteristics of mothers who EBF for fewer than four months and those who continued to EBF past four months. Infants who were EBF for less than four months, however, were more likely to be female and to have lower weights and lengths at birth. These results are in contrast to other studies examining the determinants of early cessation of exclusive

breastfeeding and the introduction of solid foods. Among upper-class mothers in Brazil, for example, both maternal age and BMI were found to differ between mothers of 4-month-old infants who were EBF and those who were partially BF (infants received waters, teas and juices) (Haisma et al 2003). Maternal age was lower and BMI higher among mothers who were partially BF their infants compared to EBF mothers, who were both older and leaner. Similarly, lower rates of exclusive breastfeeding have been found with increasing maternal BMI in developed nation settings (Baker et al 2004; Donath and Amir 2000; Hilson et al 1997). These discrepancies between the results of the current study and previous research may be due to the particular characteristics of this study sample. While maternal age did vary within the sample (range: 26-42), there was relatively little variation in BMI values (mean=22.96; SD=2.34). Only 16% of mothers had a BMI of over 25, the cut-off for overweight, and none were classified as obese. Additionally, the majority of mothers was affluent, highly educated, employed in academics and health-related fields and professed a strong motivation to breastfeed. Thus, this sample may represent something of a "best case scenario" for the initiation and maintenance of breastfeeding.

Other energetic markers

We found few associations between the local ecologies of the sample infants and hormonal levels most likely due to the relative homogeneity of the sample in terms of infant size at birth, breastfeeding exposure and other background characteristics. However, even within this relatively homogeneous sample, higher estradiol levels were seen in infants who were firstborn, had lower family income, were shorter at birth or who attended daycare, controlling for age and sex effects. Similarly, higher testosterone

levels were seen among infants who had lower family income and attended daycare. Neither family income nor daycare attendance significantly predicted the duration of breastfeeding (for SES: β=0.03, p=0.97; for daycare: β=-0.06, p=0.96) or the odds of being exclusively breastfed to 4 (for SES: OR:0.45 p=0.19; for daycare: OR: 0.30, p=.011) or 6 months (for SES: OR:0.90, p=0.87; for daycare: OR: 2.22, p=0.35) in this sample. Thus, the association between these variables and levels of estradiol and testosterone are most likely explained by an unaccounted for confounding with other aspects of growth that have been documented to be associated with estradiol and testosterone levels in this sample. Family income, however, is significantly associated with other characteristics in this sample, including maternal age (ordered logit with robust SE, OR: 1.67, p=0.007), paternal age (xtlogit, OR: 1.56, p=0.03), daycare attendance (xtlogit, OR: 5.11, p=0.06) and maternal BMI (xtlogit OR: .58, p=0.01), suggesting that family income is a summary variable that indicates something about the healthfulness of the family lifestyle.

Effects of feeding style on sex steroid levels

Infant feeding variables were strongly associated with hormonal levels in this study in both male and female infants. In exploring the relationship between weekly measures of infant feeding type and hormonal levels, we found that infants who are EBF have significantly lower median estradiol and testosterone levels than those who received formula exclusively (EFF) or received both formula and breastmilk (Mixed BF). This association between formula intake and higher estradiol and testosterone level suggests that breastmilk is not the source of circulating sex steroids in infants (Sahlberg and

Axelson 1986); rather, a reasonable hypothesis is that formula intake either directly or indirectly augments hormonal levels.

One potential explanation for this observation that higher estradiol levels are found with formula feeding is that the formula acts as an exogenous source of hormones. However, soy formula, which contains phytoestrogens that could potentially modify endogenous estradiol production and have an anti-androgenic effect (Irvine et al 1998; Setchell et al 1998), was regularly ingested by only one infant in this study and is not thought to strongly contribute to the above results. If this infant is removed from the analysis, the results of the study do not change. Further, cow's milk formula, used by the other parents and at the daycare, has been found to have negligible levels of phytoestrogens (Irvine et al 1998; Setchell et al 1997). This suggests that the hormonal content of formula does not directly contribute to the higher estradiol and testosterone levels seen in formula fed and mixed infants and instead suggests that formula's effects are more likely to be indirect.

Among the possible indirect effects are the greater energetic intakes found in formula fed infants (Butte et al 2000; de Bruin et al 1998) and those who receive both breastmilk and formula (Haisma et al 2003). Research measuring infant energy intake and utilization have documented that the energy intake of mixed fed infants can be as much as 20% higher than the energy intake of similarly-aged EBF infants (Haisma et al 2003). While the effects of these higher intakes on sex steroid levels in infants have not been previously studied, greater caloric intake has been associated with higher circulating estrogen levels in adolescent girls and adult women (Dorgan et al 2003; Dorgan et al 1996). An effect of energy intake on testosterone levels in adolescent and adult men is

more equivocal. In a dietary intervention study, adolescent boys showed no evidence of an effect of caloric intake on serum testosterone levels (Dorgan et al 2006) whereas comparisons of the serum testosterone levels of vegetarian and non-vegetarian men have found higher testosterone levels in omnivores (Howie and Shultz 1985), higher testosterone levels in vegetarians (Key et al 1990) and no difference between the groups (Deslypere and Vermeulen 1984). That greater energetic intake may influence hormonal levels in the present sample of infants is supported by the finding that among infants receiving breastmilk, infants who are fed solids or formula and solids have higher estradiol levels, with the highest levels seen among those who receive all three substances.

Along with modest effects of energetic intake on hormonal differences, dietary content differences may also influence hormone levels. In adults, dietary fat intake, in particular, has been shown to influence both estradiol and testosterone levels (reviewed in: Adlercreutz et al 1994; Allen and Key 2000; Goldin et al 1982). In adolescent girls undergoing a dietary intervention aimed at reducing dietary fat intake, even modest reductions in fat intake during puberty was associated with lower estradiol levels (Dorgan et al 2003). Similarly, a recent meta-analysis of studies exploring the links between dietary fiber and plasma estradiol found that a diet with <20% of calories obtained from fat was associated with a 2.9%-11.7% reduction in circulating estradiol in adult women (Wu et al 1999). Dietary fat also seems to have a relatively large impact on serum testosterone. Men provide with a diet with <25% of calories from fat, for example, had 12% lower circulating testosterone levels than men with a higher fat diet, despite a identical caloric value (Wang et al 2005). In a study of the nutritional factors on sex

hormone levels in adult male twins, Bishop and colleagues found that dietary fat was the only macro- or micronutrient that significantly predicted testosterone levels (Bishop et al 1988).

While the effects of macronutrients, such as dietary fat, on infant sex steroid levels has not been explored, differences in dietary content have been documented in breast and formula fed infants. In a sample of American infants, for example, intakes of energy, protein, fat, and carbohydrates were lower in infants who had been EBF for at least 4 months than formula fed infants when measured at 3 and 6 months (Butte et al 2000). These differences in macronutrient intake provide the possibility that sex steroid levels differ as a result of diet as well. Whether the relationship between feeding type and hormonal levels documented here is related to the higher energy content of a diet that contains breastmilk in addition to other supplementary foods or to differences in the nutritional components of different feeding styles requires further research as this study did not measure caloric or macronutrient intake directly. However, if the results of the adolescent and adult studies are applicable to infants, the higher estradiol, but not testosterone levels, seen in infants given solid foods may indicate that increased energetic intake, rather than increased dietary fat, is responsible for some of the associations between feeding style and hormonal levels documented here.

Alternatively, age could be a confounding factor in this relationship, since infants who are exclusively breastfed tend to transition to breastmilk and solid consumption while infants who are mixed breast fed tend to transition to breastmilk, formula and solids. The inclusion of age into mixed model analysis did not change the significance or effect size of the results for estradiol levels. The consumption of solid foods, on the other hand, was

not associated with lower testosterone levels among infants receiving breastmilk and/or formula when age and sex where controlled for in the model (xtreg, $\beta = 0.17$ $p=0.04$), indicating that the lower testosterone levels associated with solids are a function of the older ages of infants receiving solids, not the solid foods themselves.

Sex differences in the relationship between feeding and steroids

When formula use and complementary feeding are examined by sex before six months, the time when these items are added to the diet, sex differences are also seen in the relationship between feeding and hormonal levels. In both male and female infants, EBF is associated with lower estradiol levels throughout the ages measured. The inclusion of formula, but not solids, before 6 months in the diet of breastfed girls is associated with both higher estradiol and testosterone compared to girls who receive only breastmilk. Conversely, the introduction of solid foods, but not formula, is associated with higher estradiol levels in male infants. Feeding style has no significant effect on testosterone levels when mixed fed boys are compared to EBF boys or between boys given complementary solids and EBF boys. This lack of effect of feeding style on male testosterone levels suggests that diet is not a major direct contributor to testosterone levels in infant boys.

The greater sensitivity of female hormonal levels to dietary changes is documented in the previously described studies of adolescents where reductions in dietary energy and fat were associated with reduced sex steroid production in female (Dorgan et al 2003), but not male (Dorgan et al 2006), adolescents. This greater sensitivity of the female hypothalamic-pituitary-gonadal axis to energetic availability is well-documented in adolescents and adults (described in chapter 1 and Bribiescas 2001; Ellison 2003; Ellison

et al 1993; Jasienska and Ellison 1998; Worthman 1993) and the present results suggest that these differences may be seen as early as infancy.

Interaction between feeding and sex steroids and growth

A further confounding factor between feeding style and hormonal levels is the sex-specific associations between hormonal levels and growth documented in the previous chapter. In this sample, sex steroid levels are associated with differences in size, such that shorter, plumper boys with greater leg skinfold thickness or longer, leaner girls with greater hip skinfold thickness have higher estradiol levels and lighter boys with leaner hips and girls with greater truncal fat have higher testosterone levels.

Differences in the infant size and growth rate related to breast versus formula feeding and the introduction of solid foods has been extensively documented in both the developed and developing worlds (Dewey 1998; Haschke and van't Hof 2000; Hediger et al 2000; Heinig et al 1993; Kramer et al 2002; Nielsen et al 1998). Breastfed babies differ in their growth and body composition during the first year of life, tending to end the first year of life both longer and leaner than formula fed infants (Butte et al 2000; Dewey 1998). To explore whether this relationship between size and feeding was seen in this sample as well, we asked whether the exclusively breastfed infants of this sample differed in their body composition from infants receiving complementary formula and solids. We found that, in this sample as in the larger epidemiological samples of American infants, EBF infants were heavier and longer than non-EBF infants in the first half of infancy.

These results document a complex interaction between sex steroids, body composition and feeding style in infancy and suggest that the associations between hormone levels

and feeding style may be inherently and biologically confounded by differences in growth. Investigating the combined effects of estradiol and complementary feeding through mixed model techniques accounting for feeding style, hormonal level and the interaction between them identified that complementary feeding positively predicted length in both sexes and thigh skinfold thickness in girls and had a negative effect on ponderal index in boys. In other words, girls who are longer with a greater thigh fat reserve and boys who are long and lean both have higher estradiol levels when given complementary foods before 6 months of life.

Setting up a metabolic package?

Previous research on the relationship between weight, skinfold and pulsatile growth suggests that these long, lean body morphologies may be associated with infants who are rapid growers (Lampl et al 2005). In this study, these are precisely the infants who are receiving the complementary foods and liquids. It is interesting to consider whether these results, that leaner, more rapidly growing infants receive supplementary foods, may be priming metabolic function in infancy by driving a body morphology that has been linked to metabolic disorders in later life. This interpretation is supported by epidemiological research that suggests that, at least among boys, fast growers with low PI are at increased risk of developing metabolic disorders in later life (Barker et al 2002; Desai and Hales 1997; Gluckman et al 2005; Godfrey and Barker 2000; Kuzawa and Adair 2004; Singhal and Lucas 2004).

Research into the blood cholesterol levels associated with different feeding styles in infants suggests that early feeding may program cholesterol synthesis. In their analysis of

the blood cholesterol in breast-fed and formula-fed infants, Bayley and colleagues (1998) found increased cholesterogenesis and higher plasma cholesterol levels in formula fed infants compared to breastfed infants. Theoretically, such differences have the potential to shape cholesterol metabolism in later life since the smaller central pools of cholesterol seen in breastfed infants may contribute to reduced low-density lipoprotein-cholesterol concentrations in the plasma, reducing the risk of cardiovascular disease (Bayley et al 2002). Altered cholesterol synthesis also has the potential to affect sex steroid production. Although the mechanisms by which dietary fat intake modulates steroid production is unclear, it has been suggested that lower levels of cholesterol in the blood may inhibit the utilization of cholesterol by the acute steroidogenic regulatory protein (STAR) for steroidogenesis, resulting in decreased total serum testosterone and estradiol production (Wang et al 2005). If supported, such a mechanism would link infant diet to metabolism and sex steroid levels in the long term, providing a mechanism behind the associations between feeding and hormonal levels documented here.

Conclusion and Implications

In summary, we found that a complex interaction exists between estradiol, body composition, and feeding style in infancy. Specifically, higher estradiol levels in infants were associated with a diet that included breastmilk in addition to other complementary liquids and foods. This result is supported by the growth data which documented that higher estradiol levels were associated with lower weight and leaner trunk and lower limb circumferences and skinfold thickness and that the infants who were not EBF were precisely those infants who weighed less and had lower skinfold thicknesses at 4 and 6

months, the times chosen because they represent the time immediately before and just after most parents began to include solid foods into their infant's diets.

Whether this association between size and feeding style is due to the nutritive and growth-promoting factors in human breastmilk or, conversely, due to maternal perception of infant size as a marker of infant well-being that in turn influences feeding practices, remains unresolved. However, the later explanation is supported by the observation that, in this sample, infants who weighed less than the median of 6.9kg at 4 months, a time just prior to the introduction of solids, received solid foods a full month earlier than larger infants, suggesting that among these parents, infant size did influence feeding behaviors. Although few parents expressed that their feeding decisions were based on infant size, larger infant size and perceptible plumpness were associated with a longer duration of exclusive breastfeeding and later introduction of formula and/or solids.

These results suggest that energy availability in infancy, represented by both diet and body composition, along with parental feeding practices may have an important role in shaping hormone levels in early development. This observation may have important functional and evolutionary consequences for later growth and reproductive fitness and may hint towards a mechanism underlying the epidemiological associations between size in the first year of life and later life morbidity.

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Summary and Conclusion

Summary of Main Findings

These results of this study, described in **Table 7.1**, document: (1) measureable and variable inter- and intra-individual differences in sex steroid levels, (2) novel sex-specific patterns of testosterone and estradiol activity during infancy, (3) sex-specific associations between length, weight and body composition and sex steroid hormones and (4) sex differences in hormonal response to feeding style in early infancy.

Table 7.1: Summary of Main Findings by Chapter

Chapter 4	Findings
HPG Activation: Developmental Trends and Sex	*Overall results* - Sex steroids detectable through 15 months of age - Significant inter- and *intra-individual* variability - *Overlap with adult fecal steroid range for both testosterone and estradiol* *Testosterone* - No single peak in testosterone - Testosterone declines with age - *Testosterone is higher before 6 months in male infants* *Estradiol* - Estradiol shows no decline with age - *Estradiol is higher after 6 months in female infants*

Italics indicate results that are novel to this study

Results of the analyses in chapter 4 indicate that sex steroids were detectable in infants from 1 week through 15 months of life. Testosterone was detectable in all samples and estradiol in approximately 90% of samples regardless of infant sex. A wide range of variation in testosterone and estradiol levels was detected both within and between individuals. Individual developmental trends, age and sex were all found to contribute to

this variability. Testosterone levels declined significantly with age while estradiol showed no statistically significant association with age. On the sample level, fecal testosterone levels were highest closest to birth, declined to 6 months, and then remained detectable throughout the first 14 months of life. Fecal estradiol declined within the first month and remained variable over the first 14 months. These results further documented sex-specific developmental patterns. Male infants had significantly higher testosterone levels than female infants before 6 months while female infants had significantly higher estradiol levels than male infants from 6 months through 12 months.

Chapter 5	**Findings**
HPG Activation: Body Size	
Boys	
Testosterone < 6 months	– associated with lower weight – associated with narrower hips
Testosterone (entire age range)	– no significant results
Estradiol < 6 months	– higher among shorter boys and those with higher ponderal index – higher in boys with reduced abdominal and quad circumferences
Estradiol (entire age range)	– associated with greater subscapular and thigh skinfold thickness – associated with higher leg skinfold thickness
Girls	
Testosterone < 6 months	– associated with greater UAC in girls – associated with higher truncal skinfold thickness
Testosterone (entire age range)	– no significant results
Estradiol < 6 months	- higher in girls who weighed less or who weighed less for their length before 6 months – higher in girls who weighed less or who were shorter after 6 months

Estradiol (entire age range)	– associated with greater hip skinfold thickness
HPG Activation: Growth	*Overall results* - Infants, of any age or sex, with greater than the median estradiol levels were 1.45 times more likely to grow in length during the weekly interval
Boys	
Testosterone level greater than the median < 6 months	- no significant effects
Testosterone level greater than the median 6-12 months	- predicted a greater likelihood of gaining calf circumference
Estradiol level greater than the median < 6 months	- predicted a greater likelihood of gaining abdominal, suprailiac and calf circumferences and hip and calf skinfold thickness during the weekly interval
Estradiol level greater than the median 6-12 months	- predicted a greater likelihood of gaining suprailiac and calf circumferences and abdominal and calf skinfold thickness during the weekly interval
Girls *Testosterone level greater than the median < 6 months*	- associated with gain in SSF
Testosterone level greater than the median 6-12 months	- associated with a greater likelihood of upper arm circumference gain and hip skinfold gain
Estradiol level greater than the median < 6 months	- predicted a greater likelihood of gaining abdominal skinfold thickness during the weekly interval
Estradiol level greater than the median 6-12 months	- predicted a greater likelihood of gaining abdominal skinfold thickness during the weekly interval

The analyses in Chapter 5 document sex-specific associations between length, weight and body composition and sex steroid levels. Although significant associations were seen between both hormones and size variables, when the data were stratified by age greater than or less than 6 months, estradiol appeared to be more strongly associated with size than was testosterone. The only significant relationship between testosterone and whole body size was between testosterone and weight in boys before 6 months of age, with higher testosterone levels documented in boys who were lighter. In boys younger than 6 months of age, estradiol levels were higher among those who were shorter or had a higher ponderal index. In girls, higher estradiol levels were seen in those who weighed less and who had lower ponderal indices. After 6 months, higher estradiol levels were documented in girls who are shorter and lighter.

Unlike the results for body size, the only significant relationships between hormones and body composition measures in 6-month analysis were found before 6 months of age. Before 6 months of age, testosterone was only associated with hip circumference in boys such that higher testosterone levels were associated with narrower hips. In boys, higher estradiol, on the other hand, predicted greater fat on the back, thigh and calf and greater calf circumference. In girls, higher estradiol predicted greater suprailiac skinfold thickness and larger circumferences of the abdomen and upper arm.

Thus, among boys, higher testosterone was associated with narrower hips while higher estradiol was associated with greater overall plumpness and a more typically feminine pattern of fat deposition, which in adult women is associated with greater back and lower limb fat depots. Among girls, higher estradiol was associated with a lighter, leaner body

morphology, larger abdominal and arm circumferences and greater supra-iliac skinfold thicknesses.

While these results documented interesting sex-specific associations between hormone levels and measures of body size and composition that are consistent with those previously documented in adolescents, these results also documented a stronger association between sex steroid levels and changes in size and body composition than between absolute size and body composition. Elevated individual estradiol levels were associated with a greater likelihood of length growth in the weekly interval regardless of infant age or sex.

Among boys, elevated estradiol levels were associated with increased likelihood of gaining abdominal, suprailiac and calf circumferences and hip and calf skinfold thickness during the weekly interval before 6 months and a greater likelihood of gaining suprailiac calf circumferences and hip and abdominal and calf skinfold thickness during the weekly interval after 6 months Elevated individual testosterone levels, on the other hand, were associated only with an increased likelihood of a gain in calf circumference after 6 months of age. In girls, elevated individual testosterone was associated with a greater likelihood of subscapular skinfold gain in the weekly interval before 6 months of age and with a greater likelihood of upper arm circumferential growth and suprailiac skinfold gain in the weekly interval after 6 months of age. While not proving causation, these results indicate a potential mechanistic association between increased sex steroid levels and growth in body length and changes in body composition.

Chapter 6	Findings
Hormones and feeding	*Overall Results* - Infants receiving formula exclusively or as a supplement have higher estradiol levels than EBF infants - Among BF infants, the addition of solids or formula and solids is associated with higher estradiol levels - BM consumption, either exclusively or with formula, was associated with higher testosterone - Solids were not associated with testosterone levels over the whole year *Boys* - Over the entire study period, EBF was associated with lower estradiol levels and, conversely, complementary feeding was associated with higher estradiol levels - The introduction of solid foods before 6 months of age was associated with higher estradiol levels - There was no association between feeding style and testosterone *Girls* - Over the entire study period, EBF was associated with lower estradiol levels - Mixed BF was associated with higher estradiol and testosterone levels - Solid intake was not associated with estradiol or testosterone levels over the whole year - The introduction of formula and/or solids before 6 months was associated with higher estradiol and testosterone
Hormones, feeding and size	*Interaction effects* - Interaction between estradiol and complementary feeding on size and body composition - In CF girls, ↑estradiol levels predicted greater length and thigh skinfold thickness

	- In CF girls, ↑testosterone levels predicted lower weight and ponderal index - In CF boys, ↑estradiol levels predicted greater length, weight, and thigh skinfold and negatively predicted PI
Other energetic markers	*Overall results* - Estradiol levels were positively associated with maternal primiparity and negatively associated with daycare attendance and family income - Testosterone levels were negatively associated with daycare attendance and family income

The results of Chapter 6 documented sex-specific relationships between sex steroid levels and measures of feeding style. While estradiol levels were lower in exclusively breastfed infants of both sexes, formula and solids were associated with different hormonal responses in male and female infants. In boys, complementary feeding and the introduction of these complementary solid foods before 6 months of age were associated with higher estradiol levels. There were no significant associations between feeding and testosterone levels in boys. In girls, on the other hand, the use of formula, but not solids, was associated with higher estradiol and testosterone levels. The introduction of formula or solids before 6 months of age was similarly associated with higher estradiol and testosterone levels.

These relationships between feeding style and hormonal levels were confounded statistically and, most likely, biologically by the size differences seen between breastfed and non-breastfed infants. In models testing specifically for an interaction between feeding style and hormone level in predicting body size, complimentary feeding and

estradiol were found to significantly interact in predicting body size measures, such that, among CF girls, higher estradiol levels predicted greater length and thigh skinfold thickness and, among CF boys, higher estradiol levels predicted greater length, weight, and thigh skinfold and reduced ponderal index.

Finally, these analyses documented that sex steroid levels were also associated with other aspects of the local family ecology. Estradiol levels were positively predicted by birth order and negatively predicted by daycare attendance and family income. Similarly, testosterone levels were negatively predicted by daycare attendance and family income.

Best Statistical Models

Stepwise model selection (STATA, *stepwise regression*), with a $p<0.20$ criteria for entrance into the model, was used to find the "best model" for explaining the variance in estradiol and testosterone by sex. This selection was done in 4 steps: (1) background characteristics were entered into the model, (2) significant background characteristics were retained for each sex and re-entered into the model selection process with additional birth characteristic values, (3) significant characteristics were retained for each sex and entered into the model selection process with current size and feeding characteristics and (4) significant characteristics were entered into mixed models to control for potential confounding effects of individual. The "best model" for predicting estradiol and testosterone for each sex are presented in **Table 7.2**.

Table 7.2: Variance Explained by Final Models Predicting Hormone Levels

	Male	Model R^2			Female	Model R^2		
	Variable	Between	Within	Overall	**Variable**	Between	Within	Overall
Estradiol		.55	.09	.33		.87	.10	.25
Birth size	↑ PI birth				↓ Length birth			
Current size	↓ Length ↓ PI				↓ Length ↓ PI ↑Weight			
Feeding	↓ EBF				↑ Mixed BF			
Parental characteristics					↓ SES			
Confounders	↑ Age							
Testosterone		.71	.06	.47		.75	.0002	.28
Prenatal characteristics	↑ Gestational age at birth				↓ Gestational age at birth			
					↑ Pregnancy weight gain			
Birth size	↑ PI birth							
Current size	↓ Weight				↓PI			
Feeding					↑ Mixed BF			
Parental characteristics	↑ SES				↓ SES			
	↑ Maternal education							
	↓Daycare							
	↓ Paternal height							
Confounders					↓ Age			

The results of this model selection identify that in each sex hormone levels are associated with both current size and maternal/birth characteristics and that these characteristics explain a large proportion of the inter-individual variance in sex steroid levels. In both males and females, current length and ponderal index negatively predict estradiol levels, supporting the previous analysis that estradiol levels are related to current body size. What is more interesting is where the models diverge by sex. Among boys, ponderal

index at birth and current age are significant positive predictors and exclusive breastfeeding is a negative predictor of estradiol levels. Among girls, on the other hand, mixed breastfeeding and current weight are positive predictors and family income and length at birth are negative predictors of estradiol levels. These results indicate that while both male and female estradiol levels are associated with conditions experienced during fetal development (as indicated by size at birth), current body size and feeding regime, female estradiol levels are further influenced by aspects of the family ecology, such as family income.

Like the models for estradiol, family characteristics, birth size and current size predicted testosterone levels in both sexes. Unlike the estradiol models, however, the factors included in the selection model for testosterone differed substantially between the sexes. In boys, daycare attendance, paternal height, and weight negatively predicted testosterone while maternal education, family income, ponderal index at birth and gestational age positively predicted testosterone levels. In girls, present ponderal index, gestational age at birth, current age and family income negatively predicted testosterone levels while mixed breastfeeding and maternal pregnancy weight gain positively predicted testosterone levels. These differences in the best statistical models indicate that female, but not male, testosterone levels are predicted by feeding regime and that male, but not female, levels are predicted by paternal characteristics. Interestingly, maternal weight gain during pregnancy, a marker of fetal energetic availability, predicts higher testosterone levels in girls. Further, gestational age and family income, two factors present in both models, have opposite effects in male and female infants. In male infants, increasing gestational

age at birth and family income predict higher testosterone levels whereas, in female infants, these factors predict lower testosterone levels.

Model: Sex differences in hormonal production during infant development shape infant physiology, priming maturation and life-long reproductive function in response to the energetic environment

The statistical 'best models' presented above explain over 70% of the variance in testosterone levels and between 55-87% of the variance in estradiol between individuals and suggest that many aspects of the fetal and developmental environment influence infant hormone levels. Further, the differences in models predicting sex steroids in male and female infants indeed support the proposed hypothesis that sex-specific differences in response to the energetic availability of the fetal and infant environment may be mediated by sex steroid signaling and may prime rates of growth and HPG function in sex-specific ways.

As the results of the present investigation have shown, sex differences characterize testosterone and estradiol production over the first 12 months of life. Testosterone is higher in male infants prior to 6 months and, conversely, estradiol is higher in female infants after 6 months. These sex differences may point to gonadal function differences in male and female infants, with testicular production of sex steroids being higher closer to birth and ovarian production continuing either for a longer time and/or at relatively greater levels in the second half of infancy. The results of the growth analysis further imply that this sexual dimorphism in steroid production may be linked to sex-specific patterns of growth and body composition changes, patterns that may form part of an adaptive strategy enhancing infant survival. Overall, the results documented that estradiol levels, more so than testosterone levels, were linked to measures of body size and

composition, potentially promoting fat deposition and/or inhibiting muscle synthesis, with implications for the metabolic cost of growth.

Estradiol promotes increased adiposity

The female infants in this sample had greater subcutaneous adipose tissue, as measured by skinfold thickness, at nearly every depot measured throughout the first 12 months of life, despite their lower body weights. Over this same period, female infants also tended to have higher estradiol levels than male infants and these higher estradiol levels may underlie some of this greater adiposity seen in female infants. Greater subcutaneous adiposity, particularly in the gluteofemoral region, has been associated with the higher estradiol levels seen female adolescents and adults (Veldhuis et al 2005), where the greater adiposity in these areas has been thought to provide efficient storage for periods of energetic stress, such as pregnancy and lactation (O'Sullivan et al 2001). Interestingly, in this sample, estradiol levels were also higher in boys with larger subscapular, quadriceps, and overall leg skinfold thickness, depots that are typically associated with a more peripheral, gynoid pattern of fat deposition (Bjorntorp 1996; Malina 1996). Along with the higher ponderal index documented in boys with higher estradiol levels (chapter 5), these results suggest that boys with higher estradiol levels have plumper, more typically feminine body morphologies. This stereotypically female body morphology may be particularly beneficial for survival of infants of both sexes in energetically-challenging circumstances since gluteofemoral adipose tissue is not as metabolically active as more central depots (Bjorntorp 1996; Gooren 2006) and, thus, may provide a better site for longer-term adipose storage. This ability to store more adipose tissue and greater adiposity, more generally, have been previously hypothesized to underlie the

greater survivorship of female infants (Copper et al 1993; Rodriguez et al 2004). The results of the present study suggest that this ability to store extra energy as adipose tissue may also be seen in male infants with higher estradiol levels as well, potentially underlying an adaptive strategy for survival in infants of both sexes.

The accumulation of body fat in the periods of fetal and infant development, when the infant is largely dependent on his or her mother for nourishment, has been previously hypothesized to be critical for survival given the costly energetic expense of brain growth (Kuzawa 1998). Greater adiposity has also been linked to infant survival in poor environmental conditions (Wiley 1994; Yajnik et al 2003), such that infants with higher skinfold measures tend to have greater survival even if their with weight is low. What is novel about the present study results is that they point to a mechanism; estradiol produced during the period of HPG activation may act to promote infant survival in both sexes by enhancing body fat accrual during early infancy.

The contradictory roles of estradiol and testosterone on lean body mass

Beyond its effect on adipose tissue deposition, estradiol may also promote infant survival through its inhibition of muscle synthesis (Toth et al 2001). In vitro studies have documented that ovarian hormones suppress protein metabolism and muscle synthesis (Tipton 2001), results that have been hypothesized to document an attenuating effect of estradiol on muscle growth in females (Toth et al 2001). Although the effects of estradiol on muscle mass in human males has received less attention (Tipton 2001), a similar inhibitory effect is suggested by the findings of the current study describing higher levels of estradiol in infant boys associated with lower abdominal and quadriceps circumferences before 6 months of age. The larger abdominal circumferences, but

smaller abdominal skinfold thickness, of males infants before 6 months of age compared to female infants is likely related to their larger organ size, a component of fat free mass that is also metabolically active (Weinsier et al 1992). These results, along with the lower quadriceps circumference and greater adiposity seen in male infants, may indicate that estradiol promotes thrifty energy utilization in male infants by increasing adipose storage at the same time as inhibiting the development of fat free mass.

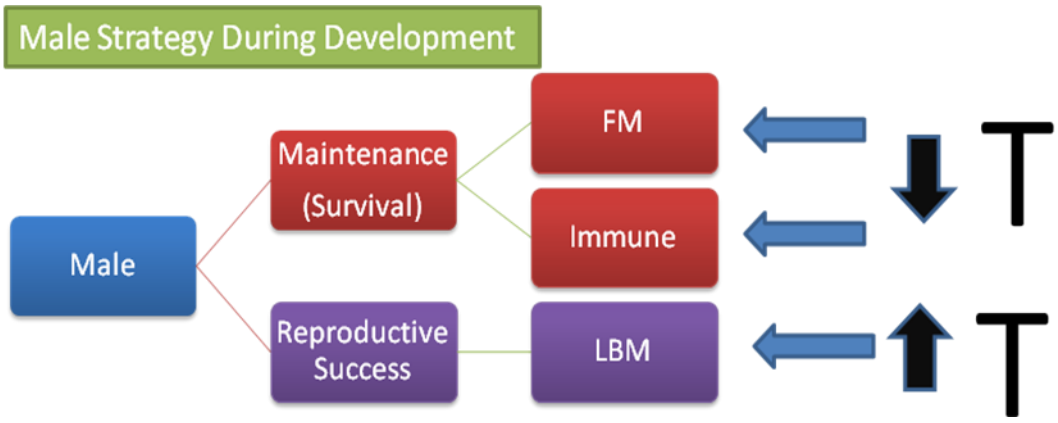
Testosterone, on the other hand, increases muscle protein synthesis and muscle protein balance, resulting in increased muscle mass (Tipton 2001). In this sample, elevated testosterone levels, or those above the individual median, were associated with increases in upper arm circumference in both male and female infants after 6 months of age. Testosterone levels in this sample may reflect, and may even be mechanistically involved in, the differentiation of muscle tissue in the second half of infancy. Muscle tissue mass, however, is costly, accounting for nearly 20% of basal metabolic rate in adult men, a figure that is similar to that of the brain and other metabolically active organs (Weinsier et al 1992). Although the energetic costs of muscle development in infants are less understood, fat free mass appears to contribute significantly to basal metabolic rate and energy expenditure in infancy (Cole et al 1999). If testosterone levels are indeed associated with increased fat free mass, as is preliminarily suggested by these analyses, the deposition and/or differentiation of muscle tissue in infancy may be an energetically costly strategy, particularly during the time of rapid brain growth.

The energetic costs of growing: building male v. female infants

Thus, the results of the present study document sex differences in both body size and also in the relationship between body size and sex steroids in infants. These results provide

support for the hypothesis that differences in body size may be mediated by sex steroid levels that serve to modulate the energetic costs of growth and development. Most simplistically, estradiol appears to be associated with both the mechanisms of linear growth and also the deposition of body fat that may be used to fuel that growth in both male and female infants (Lampl et al 2005). Conversely, testosterone shows little association with body size or growth in early infancy and may be more closely linked to the development of muscle mass in later infancy.

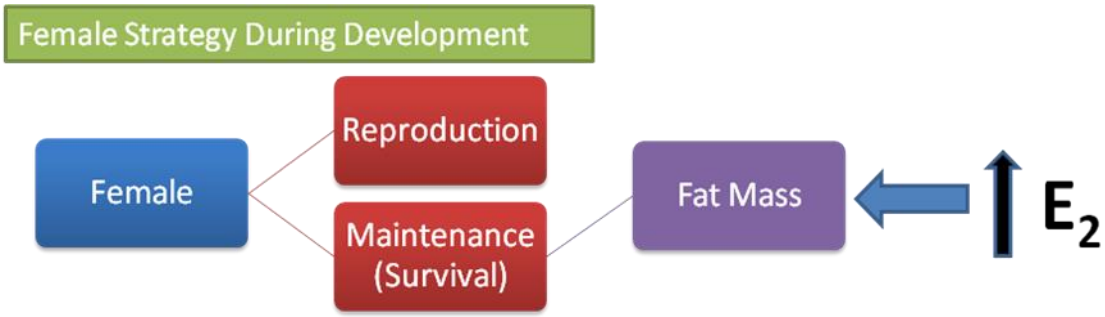
Given the sexually dimorphic patterns of sex steroid production in infancy, it is possible that these associations between growth and sex steroid levels represent adaptive strategies that differ both between and within the sexes. The larger body size of male infants both *in utero* and postnatally may make being a male more energetically costly for both infants and their mothers (Elsmén et al 2004; Rosenfeld and Roberts 2004; Sidebottom et al 2001), since larger body sizes are associated with greater daily energy expenditure and basal metabolic rate (Cole et al 1999; Wells et al 1996). Indeed, the higher body weight of male infants in comparison to female infants, like that of Western vs. Indian infants or between infants born small-for-gestational-age (SGA) versus those born appropriate-for-gestational-age (AGA), tend to be linked to differences in fat free mass rather than fat mass, which tends to be more similar (Hediger et al 1998; Yajnik et al 2003). Fat free mass, however, is more energetically costly than fat mass, both in its deposition and its maintenance (Butte et al 1989; Wells et al 1996). Thus, not only do male infants have greater energetic needs, larger males may also have greater energetic needs than smaller male infants if their larger size is due to increased fat free mass.



These increased metabolic costs of larger size sets up a tradeoff, depicted in the figure above, and may lead to at least two contrasting male strategies for surviving infancy. The first, which may be selected by an environment with sufficient energy availability, is to grow large both *in utero* and postnatally when being provisioned adequately by mom. Such a strategy could favor both the deposition of a greater proportion of lean body mass and higher testosterone levels. These two factors could further influence the future tempo of growth and reproductive function, by determining both the number of muscle fibers (Yliharsila et al 2007) and the function of Sertoli cells in the testes (Chemes 2001; Main et al 2006). While risky in environments with limited energetic availability, due to the higher metabolic costs imposed by larger body size, this strategy may provide long term reproductive success if this larger size is related to enhanced spermatogenic, steroidogenic and somatic potential in later life. Differences in testosterone production have been documented in populations (Bentley et al 1993; Bribiescas 2001; Ellison et al 2002) and have been linked to somatic condition, particularly body weight and muscle mass (Bribiescas 2001), suggesting that conditions experienced during development, at

least theoretically, have the potential to affect adult size and possibly reproductive function.

Alternatively, male infants may follow a more typically female strategy, depositing greater subcutaneous tissue and proportionally less lean body mass in response to higher estradiol levels. This strategy may represent an alternative pathway dually promoting survival when the energetic environment is challenging, since it both promotes energy storage and prevents the development of energetically costly tissue. Perhaps, then, the relatively elevated estradiol levels early in infancy, the period when infants are traditionally nursed (Dettwyler 1995), allows for enhanced fat deposition in smaller male infants. This enhanced fat deposition may, in turn, be beneficial for survival during weaning (Kuzawa 1998). However, this strategy could impose a long term cost if relatively higher estradiol levels suppress testosterone levels and/or alter testicular function postnatally, reducing spermatogenic or steroidogenic production in later life. It has been suggested that, in adult males, investing in greater adiposity may lead to greater aromatization of circulating testosterone to estrogen and inhibit hypothalamic and pituitary gonadotropin release (Bribiescas 2001). While the effects of estrogens on male development in infancy are unknown, research on the effects of phyto- and xeno-estrogens suggests that exposure in infancy could theoretically contribute to subfertility and reproductive dysfunction (Irvine et al 1998; Setchell et al 1998; Sharpe 2003; Toppari et al 1996).



The relatively higher estradiol levels of females in later infancy, when compared to males, may explain their greater fat deposition throughout this second half of infancy. Their ability to maintain adipose tissue and their greater tissue mass may underlie the reduced morbidity and mortality documented in infant girls during this period (reviewed in: Rosenfeld and Roberts 2004; Stinson 1985). Interestingly, in female infants, higher estradiol levels do not promote greater fat deposition as they do in male infants. Rather, female infants with the highest estradiol levels tend to be leaner and lighter for their length. Given the association of greater than the median estradiol levels with a greater likelihood of linear growth within the weekly measurement interval, higher estradiol levels in these leaner, lighter girls may indicate that they, like the longer, leaner boys, are the more rapid growers. Indeed, the highest levels of estradiol and testosterone are seen in the first two months of life in girls and are negatively associated with length, weight, ponderal index, trunk and limb circumferences and trunk and limb skinfolds, generating the hypothesis that perhaps at their highest levels, sex steroids are associated with catch-up growth. Unfortunately, sample size is too small at these young ages (n=5 infants) to test this hypothesis but the data nonetheless suggest that elevated sex steroids may at least

be associated with the costs of growth. If substantiated with further data, this association between higher estradiol levels in girls and more rapid growth may underlie the documented associations between rate of infant growth and earlier menarcheal age in girls (Adair 2001).

Feeding and sex steroids: Setting up a metabolic package?

Inherently confounded in these associations between infant size, energetic needs and hormonal levels are the effects of feeding style. As the present analyses documented, both estradiol and testosterone levels were lower among EBF infants than among infants receiving formula. Conversely, higher estradiol levels were seen among girls being supplemented with formula and boys receiving solids before 6 months of age. Interestingly, the infants who were not EBF were those infants who weighed less, had lower skinfold thickness at 4 and 6 months, and had higher steroid levels. Further, an interaction between estradiol, particularly, and complementary feeding was evidenced, such that the higher estradiol levels in infants receiving supplementary foods and liquids were associated with greater length and higher thigh skinfold in girls and greater predicted length, weight and thigh skinfold in boys.

Thus, these results suggest that, in a formula feeding environment, infants with long, lean body morphologies, a physiology associated with more rapid growth (Lampl et al 2005), are precisely the infants who are receiving the complementary foods and liquids. It is interesting to consider whether these results, that leaner, more rapidly growing infants receive supplementary foods may be driving metabolic organization in infancy, a result indicated by the synergetic interaction between feeding and estradiol in driving length and thigh skinfold deposition in complementary fed infants. That this may be a risky

strategy in an environment of energetic abundance is supported by the epidemiological evidence that, at least among boys, fast growers with low PI are at increased risk of developing metabolic disorders in later life (Barker et al 2002; Desai and Hales 1997; Gluckman et al 2005; Godfrey and Barker 2000; Kuzawa and Adair 2004; Singhal and Lucas 2004). On the individual level, then, hormonal mediation of the energetic availability on the environment may alter growth physiology, resulting both in the morphological patterns described above and also altered lifelong metabolism. As described by Kuzawa and Adair (2004), the potential exists for such a mismatch in fetal development, in this case the mismatch between a high fetal demand for nutrients and poor energy availability due to maternal under-nutrition during pregnancy, to program altered adolescent physiology, resulting in higher lipid levels in adolescence and greater CVD risk. Similarly, the increased adiposity of otherwise small Indian neonates is thought to alter metabolism in ways predisposing Indian adults to early onset type 2 diabetes (Yajnik et al 2003).

Possible Mechanisms: Body Composition, Energetics and HPG function

That early postnatal experiences *could* influence both growth and reproductive function was discussed in the introduction to this study; the current results, however, *suggest* that sex steroids may play an organizing role, a role that is supported mechanistically by the interconnectedness of the gonadotrophic and somatotrophic axes and their dual responsiveness to energetic availability. While the long-term effects on the infant energetic environment and the role of sex steroids in the interface between the energetic environment and infant growth and development remain to be elucidated, sex steroid production has the potential to alter, in a long-term manner, body composition and energetic function.

Adipose Tissue as an Endocrine Organ

Since Rose Frisch's proposal that the timing of menarche and the maintenance of menstrual cycling depends on amount of fat mass in women, the role of adipose tissue in the development and function of the reproductive system has received much attention (Frisch and Revelle 1970). How peripheral estrogen metabolism occurring in adipose tissue could influence the onset and maintenance of GnRH pulsatility, the neurophysiological mechanisms of puberty and reproductive cycling, remains unexplained. Adipose tissue, however, has been recognized to be an endocrinologically active tissue, actively secreting cytokines and other hormones, such as leptin, that influence peripheral fuel storage, mobilization and combustion, and energy homeostasis (Fruhbeck et al 2001; Wade et al 1996). Even more importantly, the adipocyte is recognized to be part of a large network of signaling pathways that enable organisms to adapt to metabolic challenges, pathways that, among other things, link fertility and energetic condition.

As adipocyte number in humans appears to be determined *in utero* and postnatally (Knittle et al 1979; Pettersson et al 1984), conditions experienced during infant development have the potential to influence future reproductive function through an alteration in metabolic responses to body composition. One potential mechanism through which infant developmental conditions may alter both body composition and future reproductive function is through modifications in leptin production. Leptin, a peptide hormone produced by adipose tissue and secreted into general circulation, has been theorized to be a key metabolic mediator for the neuroendocrine axis, perhaps providing the "missing link" between adipose stores and the brain (Chan and Mantzoros 2001;

Terasawa and Fernandez 2001; Wauters et al 2000). Leptin receptors have been found at all levels of the HPG axis and leptin and its receptors reciprocally regulate gonadal steroids and their receptors (Brann et al 2002). Thus, it has been suggested that, by modulating the hypothalamic-pituitary-gonadal axis both directly and indirectly, leptin may serve as the signal from the periphery to the brain about the adequacy of fat stores for both pubertal development and later reproductive function (Wauters et al 2000).

That leptin may play a sex-specific role in linking growth to reproductive function is suggested by the marked sexual dimorphism seen in leptin concentrations. Leptin levels are higher in females of every age, even after correcting for fat mass. These higher levels in females have led to the proposition that the interaction between adipose tissue and the HPG axis is modulated in a different way in males and females by androgenic and estrogenic hormones (Casabiell et al 2001). Sex differences in leptin have been documented in newborns and infants when female levels are already higher (Ertl et al 1999; Lonnerdal and Havel 2000; Yang and Kim 2000). As with adults, these higher levels of leptin in female infants are thought to be due to both their greater adiposity and also the inhibitory effects of testosterone on leptin (Ertl et al 1999). More recently, leptin has been documented to differ between breastfed and formula fed infants (Lonnerdal and Havel 2000; Petridou et al 2005), such that leptin levels are higher among newborns fed formula exclusively or in combination with breastmilk when compared to exclusively breastfed infants. These results, that leptin levels differ with feeding style, form an interesting parallel to the findings of the current study that sex steroids differ by feeding style and further lend support to the influence of energetic intake on the development of regulatory hormonal systems.

Energetics and the HPG Axis

The various ecological conditions known to affect both growth and reproductive function, such as dietary insufficiency and exercise, are, for the most part, energetic ones, concerned with energy intake, expenditure, storage, or the balance between the various components (reviewed in: Ellison 2003). Consequently, the proposed physiological mechanisms linking these energetic stresses to variations in reproductive function are similar. The effect of energetic imbalance seems to be hypothalamic, interfering with GnRH release and having disruptive effects downstream on LH pulsatility. LH pulsatility in adults, for example, is disrupted by both acute energetic imbalance, such as five day dietary restriction (Loucks and Thuma 2003), and longer-term energetic deficiencies, such as yearlong marathon training (Laughlin and Yen 1996). While it has long been assumed that the body's energetic status is centrally relayed to the brain (Frisch 1984; Frisch 1987), these more recent studies suggest a possible circuit through which energetic status can effect HPG function (Aubert et al 1998; Magni et al 2000; Schneider et al 2002; Wade et al 1996).

These studies propose interplay between leptin, a hormone secreted by adipose tissue, and the neurotransmitter NPY. Acting as a metabolic signal of satiety, in the short term, or of adequate adipose stores, in the longer term, leptin down-regulates the expression of NPY in the brain, reduces food intake, and increases energy expenditure (Magni et al 2000). NPY, on the other hand, stimulates appetite and controls such metabolic functions as thermogenesis and GnRH secretion (Aubert et al 1998). Episodic NPY stimulates the release of GnRH, while high tonic levels due to continuous activation of NPY inhibits

GnRH release (Kalra and Kalra 1996), possibly linking the effects of malnutrition to LH disruption and impaired fecundity. Thus, leptin and NPY act to counter-regulate the secretion of GnRH, with high leptin levels suppressing tonic levels of NPY, but permitting episodic NPY pulsatility and gonadotropin secretion (Kalra and Kalra 1996). Interestingly, the effect of NPY on LH release appears to be mediated by the sex steroid milieu further linking energetic signals to reproductive function (Aubert et al 1998).

Although leptin's role as an acute or longer-term signal of energy availability has been questioned (Brann et al 2002), consistent with its purported action as a mediator of the metabolic signal, leptin synthesis and secretion are influenced acutely by changes in metabolic fuel availability (Schneider et al 2000). These acute changes may link energetics and nutrition more immediately to changes in sex steroid production and reproductive function, not relying on intermediate changes in the amount of adipose tissue. In fact, a series of studies conducted on estrous receptivity in hamsters (Schneider et al 2002; Schneider et al 2000; Wade et al 1996) indicate that reproductive physiology and behavior in female mammals can respond to minute-to-minute and hour-to-hour changes in metabolic fuel oxidation and glucose availability. Such a response has also been documented in male monkeys where a single day of fasting led to suppression of pulsatile LH and testosterone secretion within four hours of the missed meal (Cameron et al 1993). LH disruption, a common finding in studies of human reproductive dysfunction due to diet or exercise (Judd 1998; Laughlin and Yen 1996), has also been documented in adolescent sheep given glucose antagonists (Bucholtz et al 1996).

This metabolic fuel hypothesis proposes that, rather than act as a direct modulator of reproductive function, leptin may actually be a messenger of energetic status, detecting as

yet unknown signals of glycoprivation and lipoprivation and transmitting them to the hypothalamus (Schneider et al 2002; Schneider et al 2000; Wade et al 1996). Although the mechanisms linking the availability of oxidizable glucose and/or fatty acids to GnRH suppression remain, as yet, hypothetical, the existence of complex and overlapping neural networks regulating energy and reproduction in the hypothalamus, such as the GABA, glutamate, NPY, POMC networks among others, make such a scenario increasingly likely. Further, that a stomach to brain messenger can exist has been demonstrated by the recent discovery of ghrelin and orexin, hunger and satiety signals secreted by the stomach and received in the hypothalamus through NPY receptors (Lawrence et al 2002). As with leptin, ghrelin levels also appear to differ in response to infant feeding style, being higher in formula fed infants (Savino et al 2005) and levels in the postnatal period have been hypothesized to play an important role in organizing GH secretion (Soriano-Guillen et al 2004), food intake and, perhaps, formation of the hypothalamic pathway that conveys leptin and other metabolic signals to the brain (Bouret et al 2004; Bouret and Simerly 2004).

This hypothesized mechanism would link feeding style in infancy to the production of ghrelin and leptin, providing a putative link between energy intake and the neural circuits regulating food intake, energy consumption, growth hormone and HPG function. Further, this hypothesis allows for short term energy availability to influence the development of reproductive physiology and further suggests that energy flux may be the more important component affecting reproductive function. This hypothesis fits well with reproductive ecological observations that large energy expenditure can nonetheless suppress ovarian function in women who are consuming enough food to ensure a stable

weight (Jasienska 2001; Jasienska and Ellison 1998). This suggests that reproductive processes are inhibited or delayed when energy expenditure outstrips energy intake and mobilization from storage, but does not require a threshold of adipose stores. That this metabolic response can occur even in conditions of adequate dietary intake has an important implication for the present study, which documents wide variability in sex steroid levels in infants across a normal, healthy range of weights, body compositions and energetic intakes.

Somatotrophic Hormones and the HPG Axis

While the reviewed mechanistic studies suggest that the energetic modulation is predominantly hypothalamic, leptin and other metabolic hormones such as GH and IGF-I also have more localized pituitary and gonadal effects on steroidogenesis, gametogenesis, gonadal differentiation and gonadotropin secretion and responsiveness (Hull and Harvey 2000). *In vitro* studies of human granulosa cells have demonstrated that leptin can directly suppresses estradiol secretion, playing a direct regulatory role on steroidogenesis (Ghizzoni et al 2001). GH also has direct and indirect effects, including those on IGF-I during follicular development in females (Magni et al 2000; Smith and Grove 2002). GH stimulates growth and prevents atresia in small follicles and promotes estradiol production (Hull and Harvey 2001). Similarly, GH is thought to play an important role in the differentiation of the Leydig and Sertoli cells, differentiation that may be necessary for both testosterone synthesis and spermatogenesis (Hull and Harvey 2000). A focus on this interplay between metabolism and reproductive function also indicates that hormones normally considered "growth" hormones also influence reproductive development and function.

IGF-I, for example, has been shown to have effects on HPG function at multiple levels including promoting the growth of hypothalamic glial cells controlling GnRH release (Terasawa and Fernandez 2001), enhancing LH secretion in pituitary cells (Lackey et al 1999) and regulating gene expression in steroidogenic cells (Lackey et al 1999). In fact, the IGF-I and steroid hormone systems participate in extensive cross-talk and comprise a complex regulatory network that also influences the relationship between metabolic signals and fertility. Additionally, IGF-I interacts with both leptin and NPY and its effect is modified depending on local sex steroid concentrations. These interactions potentiate the effects of estradiol on hypothalamic feedback and may explain why, in primate models, IGF-I plays a role in the onset and maintenance of menstrual cycling (Wilson 1995; Wilson 2001). Consequently, energetic constraints may act on multiple levels of the HPG axis to modify reproductive function in response to local ecological challenges. On the inter-individual level, these mechanisms may contribute to differences in reproductive outcomes, such as differential reproductive success.

Sex differences in Somatotrophic-HPG Axes Crosstalk

The postnatal surge in gonadotropin activity and sex steroid production is theorized to have organizational effects on sexual differentiation in infancy (Main et al 2000; Schmidt et al 2002), but animal models have also demonstrated the potential for sex steroids to organize the somatotrophic axis during this time (Jansson et al 1985; Jansson et al 1984; Jansson and Frohman 1987). This research documents the entraining effects of sex steroids on sex-specific growth hormone pulsatility patterns during infancy. Gonadal steroids have been shown to affect both GH secretion patterns and hepatic steroid metabolism (Gustafsson et al 1983), with neonatal testosterone being necessary for adult

male GH pulsatility to develop and neonatal estrogen contributing to the high tonic levels of GH seen in adult females (Pincus et al 1996; Robinson et al 1998). Similar sex specific patterns of GH release are seen in human infants (Geary et al 2003), adolescents (Veldhuis et al 2000), and adults (Veldhuis 1998), suggesting that sex steroids regulate GH secretion in humans as well.

Sex-specific patterns of GH release, acting synergistically with sex steroids, may coordinate growth and body compositional changes. Differences in growth rates between males and females (Udy et al 1997) and between children with normal and abnormal growth (Achermann et al 1999) have been linked to patterns of GH pulsatility, with the more regular, male peak and trough pattern linked to higher growth rates (Veldhuis 1998). In contrast, females have higher tonic levels of GH secretion, a longer GH half-life, and more irregular pulse profiles than males (Vahl et al 1997). This female pattern, thought to be partially explained by the stimulatory effects of estradiol on GH (Gatford et al 1998), has been linked to lower rates of growth as a result of its lower pulse amplitude (Achermann et al 1999).

Body composition also determines the pattern of GH in sex-specific ways. For example, sex differences in adiposity, measured as BMI or percent body fat, alters both the amplitude of GH pulses and also the number of pulses within a 24 hour period (Abdenur et al 1994). Furthermore, site-specific sex differences in adipose tissue may also be involved in the regulation of GH. In males, nocturnal rises in GH appear to be mechanistically related to site-specific lipolysis in abdominal subcutaneous fat mass and circulating leptin levels (Samra et al 1999). This rise is characteristic of the GH profile in

males, but not females, and may be further evidence of a differential adipose utilization in males.

Such sex differences in the relationship between GH and body composition may be the result of sex differences in leptin patterning and concentration. Analysis of leptin production in healthy human adults at frequent intervals (i.e. 7 minute) documented that the strongest distinction between the sexes in the level of organization of leptin concentration appears to be the amount of leptin released or removed per unit time. The overall amount of leptin produced was much higher in women, suggesting that women might be more resistant to the effects of leptin than men (Licinio et al 1998). If women are more resistant to the effects of leptin than men, then leptin pulsatility may be a stronger signal for GH in men than in women.

These differences in GH and leptin regulation highlight an important sex difference between males and females, a difference in both the sensitivity to and the organization of metabolic signaling at the hypothalamic and pituitary levels. Sex-related differences in GH response to negative feedback by IGF-I have been documented and these differences are thought to at least partially explain the differences in GH secretion profile in males and females (Jaffe et al 1998). In contrast to males, the GH response to growth hormone releasing hormone (GHRH) at the pituitary level is not suppressed by the administration of IGF-I in women, suggesting to researchers that negative feedback is necessary at both the pituitary and hypothalamic levels in females (Jaffe et al 1998). This difference in regulation at the hypothalamus has been shown in animal studies to be dependent on sex steroid levels with estradiol and testosterone promoting sex-specific release of GHRH (Hassan et al 2001). Thus, these studies suggest that GH is dually-regulated at the

hypothalamus and pituitary in females; pituitary regulation, on the other hand, appears more important in males. Such differences in responsivity may act adaptively, providing females with the ability to integrate a greater number of signals and respond only to peaks that are above the level of physiological "noise." These differences would, on the other hand, allow males to grow larger and faster in environments of energetic sufficiency as maximal area under the GH curve elevates both IGF-I (Ghigo et al 2000) and leptin production (Lissett et al 2001).

Female buffering?

Returning to the theoretical framework presented in the beginning of this work, these results document sex differences in growth and body compositional changes in response to steroid levels and suggest that the environment is being read differently by male and female infants, something originally proposed in the arguments for female buffering reviewed in the introductory chapter (Greulich 1951; Greulich et al 1953; Stini 1969; Stinson 1985). In the present study, female hormonal values were predicted by feeding style and, in turn, influenced body size and composition. Male hormonal levels, on the other hand, were predicted by maternal and paternal characteristics, summary variables which may represent the energetic availability of the *in utero* environment and also genetic potential, and current size. The differential importance of current feeding regime in girls and fetal environment in boys suggest that sex steroid differences are associated with sex-specific differences in energy utilization during growth.

However, rather than suggesting that female infants are less affected by the environment, the current results document a different response in female infants. This differential response is highlighted in the analyses of the growth in body circumferences and skinfold

thickness and elevated sex steroid levels. In girls, there was less response of circumferences and skinfold thickness measures to estradiol levels elevated above the individual median; however, boys responded to rises in estradiol with changes in many body tissues. Such differences in response suggest that, rather than being less sensitive to environmental conditions, female infants show greater integration, only responding to "significant" signals above the higher background of noise. This explanation is supported by the analogous evidence presented above that the GH response in females requires a larger peak in hormone production due to higher tonic hormone levels. The question therefore of whether females are less affected by their environment in infancy appears to be no, but more importantly, the close associations between body size and hormonal levels during infancy suggest that the concept of canalization relies on a trade-off model that is too simplistic.

Life History and Infant Growth: Plasticity and Population Level Adaptation

Returning to a life history perspective, these individual variations in phenotype, resulting from the developmental interaction of genotype and environment, also contribute to population-level differences and, consequently, can provide the variation on which natural selection may act, linking infant developmental experience to evolutionary processes. Given the importance of environmental inputs in organizing development from the molecular to the behavioral levels, it is likely that variations in life history parameters between populations and species reflect such facultative adjustments to environmental conditions, filtered through the on-the-ground experience of individual-level development (Worthman 1999). On the species-level scale, changes in parameters such as growth rate can act as reliable signals of environmental change. Consequently, on an

evolutionary scale, infant growth and hormonal development and the ability to facultatively adjust these energetic costs over the course of development represent adaptations to environmental constraints in energy availability and suggest that these growth rates will vary between populations with different environmental risks of inadequate energy availability or mortality (Hill and Kaplan 1999).

Limitations of the Current Study

The proposed model of sex steroid involvement in the development of sex differences in growth and energy utilization is supported by the evidence that sex steroid production varies between the sexes in infancy and that these hormonal levels are related to growth and body composition changes in sex specific ways. The model is further supported by the finding that male and female sex steroid levels are differentially affected by feeding style in infancy, supporting a role for energetic intake and dietary components in modulating both growth and hormonal production. However, the present study has several methodological limitations that could affect the generalizability of this model.

Fecal sampling

First, the use of fecal samples to measure steroid levels introduces challenges not seen in serum studies. Among these challenges are potential interference from the cotton diaper matrix, bias due to uneven time between samples and heterogeneity of steroid levels through the fecal sample. As discussed in greater detail in chapter 2, the effects of these potential sources of variability appear relatively minor in comparison to the enhanced detectablility of steroids in fecal samples. The consistent associations between hormone

levels and measures of size, particularly the association between higher than median estradiol levels and the greater likelihood of linear growth within the weekly interval, serve as a physiological validation that these fecal levels of sex steroids are linked to hormone production.

Small sample size

The development of the novel methodology utilized in this study necessitated both intensive sample collection and proximity to the lab. Consequently, the number of subjects that were enrolled is relatively small (N=32), which could be another potentially important limitation of this study. However, the longitudinal nature of the study led to a large number of fecal samples: 1374 samples for methods development and 487 weekly samples for analysis. The use of mixed-model analyses for correlated data (Stata, *xtreg*) allowed for the detection of significant associations between hormone levels and size and sex-specific differences in these associations. Further, the median length, weight and BMI of this sample were similar to those of the newly developed WHO growth standards for breastfed infants (WHO 2006), which suggests that this sample is unremarkable in these measures of body size. Thus, while the effects of small sample size cannot be ruled out, the results of this analysis can be considered hypothesis generating for larger, less intensive study protocols.

Limited to sex steroids, not metabolic hormones

Although GH and IGF-I have been measured in the urine of infants and young children (Fall et al 2000; Quattrin et al 1993) , the urinary analysis of protein hormones is not

without methodological difficulties. The source of urinary IGF-I is unknown and has been suggested to be more representative of local renal production than systemic levels (Hizuka et al 1988). Further, IGF-I is found in urine at 1/1000th of serum levels (Ratcliffe et al 1995), which may make its detection in young infants difficult and may require the use of multi-step, separation techniques (Hall et al 1999). Urinary GH, on the other hand, may be confounded, in a non-linear fashion, by urinary volume leading to overestimation at larger volumes (Gelander et al 1998). These methodological difficulties have led researchers to question the clinical utility of urinary IGF-I and GH and the ability of urinary assays to detect deficiencies in production (Georges et al 1997; Hall et al 1999; Leger et al 1995). Despite these limitations, urinary IGF-I and GH have been correlated to serum levels (Hall et al 1999) as well as measures of length, weight and body composition (Fall et al 2000), suggesting that this may be a valuable future path of inquiry if appropriate laboratory methods can be developed for use with diaper samples.

Leptin has been previously measured in the urine of children (Zaman et al 2003), at levels that correlate with serum leptin and physiological measures such as body size and sex. However, repeated attempts to measure leptin in the urine of infants following previously developed protocols (Clayton, unpublished manuscript) were unsuccessful in this study to date. Leptin was detected in fewer than 20% of tested samples and assay performance was poor. The range of binding was very low and there was little differentiation at the low end of the curve, the area where most of the samples fell. Urinary cortisol has been independently measured in these samples (Rikin, Thompson, and Lampl, unpublished data) and there was no evidence that breakdown of the hormone over time was responsible for the low levels of leptin detected. These results suggest that low

endogenous levels, rather than storage conditions, are responsible. Although problems with assay performance cannot be ruled out, leptin levels in the urine of these infants appear to be below the level of detection of even a modified, microassay procedure.

Building a New Model: Predictive Reproductive Ecology

Despite these limitations, the results of the present analysis generate the hypothesis that physiologically sex differences in growth are mediated by differential responses to environmental stimuli and are at least partially coordinated by sex steroids. The results of the present analyses linking sex steroid levels and growth and body compositional changes suggest that growth and reproduction are coordinated processes, regulated by the same hormonal systems and responsive to the external environment. Perhaps even more importantly, the results hint towards a mechanism linking postnatal hormonal production with a metabolic process that may alter the sensitivity and developmental response when hormonal levels rise again during puberty, indicating that postnatal experiences modifying hormonal levels may also influence the tempo of maturation and future reproductive function.

This conceptualization of growth and reproduction as coordinated processes makes evolutionary and physiological sense, linking sex differences in growth to future reproductive requirements. Further, these results suggest that arguments about greater female canalization and male sensitivity rely on a false dichotomy between growth and reproductive development; instead, growth and reproductive development may be more coordinated processes regulated by the same hormonal systems and responsive to the external environment. These results also point to the need for a new conceptualization of

infant growth in which sex differences are mediated by differential hormonal responses to environmental conditions in such a way as to link growth to future reproductive function.

While preliminary, these results suggest that the sex steroid activation in infancy has the potential to alter the physiology of growth and development. Previous epidemiological observations have linked birth weight and first year growth rates to the timing of maturation (Adair 2001), adult size (Eriksson et al 2001; Samaras et al 2003), reproductive function (de Bruin et al 1998; Francois et al 1997) and morbidity risk (Barker et al 1989), supporting the view of infancy as an important developmental window proximally and evolutionarily linking infant growth and later physiology. In this way, the infant surge in somatic growth and endocrine activity may not just be the "mini-puberty" described in textbooks but may actually prime the future tempo of growth, development, and reproduction, entraining adolescent and adult physiology and morbidity. Given these associations between body size, feeding and hormone levels in infancy, cultural values and beliefs about infant body size and feeding, particularly in a culture that values large infant size and views formula feeding as the norm, may play a powerful role in driving growth biology, having functional and evolutionary consequences for both growth and later reproductive fitness.

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